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The Effects of *Phyllanthus niruri* Aqueous Extract on the Activation of Murine Lymphocytes and Bone Marrow-Derived Macrophages

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Phyllanthus niruri L. (Euphorbiaceae) is acclaimed world-wide for its versatile ethnomedicinal uses. It features in recipes used by some herbalists to manage different diseases, including claims of efficacy against many life-threatening infections, such as HIV/AIDS and hepatitis. In order to understand the mechanisms and the involvement of the immune system in mediating these activities, the effects of the aqueous extract of *P. niruri* on the activation of murine lymphocytes and macrophages were investigated. The study showed that the extract of *P. niruri* is a potent murine lymphocytes mitogen, inducing significant ($p < 0.01$) increases in the expression of surface activation maker (CD69) and proliferation of B and T lymphocytes. The production of interferon-γ (IFN- γ) and interleukine-4 (IL-4) by *P. niruri extract*-stimulated naïve splenocytes cultures was also significantly ($p < 0.05$) increased in a concentration-dependent manner. Various indices of activation and functions murine bone marrow-derived macrophages were significantly ($p < 0.05$) enhanced by pre-treatment with *the extract*, including phagocytosis, lysosomal enzymes activity, and TNF-α release. *Phyllanthus niruri extract* was also shown to modulate nitric oxide release by macrophages. These activities suggest that stimulation of the immune system by the extracts of

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P. niruri could be partly responsible for the ethnomedicinal applications in the management of infectious diseases.

Keywords Immunostimulation, Lymphoproliferation, Bone marrow-derived macrophages, Phagocytosis, *Pyllanthus niruri*.

INTRODUCTION

Phyllanthus niruri (Euphorbiaceae) is reputed globally for its versatile application in traditional medicine of many Asian, African, and South American countries. In many preclinical and clinical studies, extracts of *P. niruri* have been associated with many beneficial pharmacological and therapeutic effects (Calixto et al., 1998; Thyagarajan et al., 1988). Some of the most outstanding medicinal values of the herb include anti-hepatotoxic (Prakash et al., 1995), anti-lithic (Whole World Botanical, 2009), anti-tumour (Rajeshkumar et al., 2002), anti-HIV (Ogata et al., 1992; Quian-Cutrone, 1996), and anti-hepatitis B (Liu et al., 2001; Shead et al., 1992; Thyagarajan et al., 1988). It has also been reported that *Phyllanthus niruri* extract could improves specific and non-specific immune responses to infections (Sarisetyaningtyas et al., 2006, Nworu et al., 2010) and is generally believed to be beneficial in immunodeficiency conditions. Traditionally, aqueous or aqueous alcoholic decoctions of the whole aerial parts of the plant are taken for different ailments.

Phyllanthus niruri is a small, erect, annual herb that grows 30–50 cm in height. It is indigenous to the rainforests of the Amazon and other tropical areas throughout the world, including the Bahamas, southern India, and China. *P. niruri* is prevalent in the Amazon and other wet rainforests, growing and spreading freely. *Phyllanthus amarus* and *Phyllanthus sellowianus* are closely related to *Phyllanthus niruri* in appearance, phytochemical constituents, and application, but are typically found in the drier tropical climates of India, Brazil, Florida, and Texas (Rain Tree data base, 2009). *Phyllanthus niruri* is known in many languages; as Stonebreaker (English), Chanca Piedra (Spanish), and Quebra Pedra (Portuguese). It is a widespread tropical plant commonly found in coastal areas. Extracts of this herb have shown promise in treating a wide range of human diseases (Bagalkotkar et al., 2006).

Many active constituents, responsible for these pharmacological activities of *P. niruri*, have also been identified in phytochemical investigations. Interestingly, some of the isolated bioactive constituents have been found only in the *Phyllanthus* genus. Generally, biologically active lignans, glycosides, flavonoids, alkaloids, ellagitannins, and phenylpropanoids have been identified in the leaf, stem, and root of the plant. Specifically, the main phytoconstituents isolated from *Phyllantus niruri* include niruretin, nirurin, nirurine, niruriside, norsecurinines, phyllanthin, phyllanthine, phyllanthenol, phyllochrysine,

phyltetralin, repandusinic acids, quercetin, quercetol, quercitrin, astragalin, brevifolin, carboxylic acids, corilagin, cymene, ellagic acid, ellagitannins, gallocatechins, geraniin, hypophyllanthin, lignans, lintetralins, lupeols, methyl salicylate, niranthin, nirtetralin, rutin, saponins, triacontanal, and tricontanol (Colombo et al., 2009; Rain Tree data base, 2009).

In south eastern Nigeria, *P. niruri* is popularly called "Enyikwonwa" and features in herbal recipes used by herbalist to treat a variety of infections, including claims of effectiveness in the management of HIV/AIDS and hepatitis. Although, it is believed that most of these beneficial effects of *P. niruri* could be related to the immunomodulatory activities, there is paucity of information on the effects of *Phyllanthus niruri* on the various immune cells. This stimulated the interest in the study of the mechanisms of immunostimulant activities of *P. niruri*.

The effect of the aqueous extract of *P. niruri* on the activation of murine lymphocytes was studied by the analysis of CD69 expression and cell proliferation. The effect of the extract on the production of IL-4 and IFN-γ by splenocytes was also studied. Macrophages are versatile cells that play many roles in both innate and specific immune responses. As scavengers, they rid the body of worn-out cells and other debris. They are important antigen presenting cells and also secrete important immuno-regulatory substances. It was, therefore, necessary to determine the effect of the extract on the functions and activation status of macrophages. The study is expected to establish the modulatory activities of *P. niruri* extract on immune cells, which could explain the many applications of the herb in complementary therapy. The knowledge of these mechanisms could be used to harness the potentials of this versatile herb, recognised in ethnomedical practice of many countries of the world.

MATERIALS AND METHODS

Collection and Preparation of Plant Material

The fresh whole aerial parts of *Phyllanthus niruri* was collected in the month of February, 2008 with assistance of a plant taxonomist, Alfred O. Ozioko, of the Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka, Nigeria; who also performed the botanical authentication. The plant material was air-dried, pulverized, and the herb powder (200 g) macerated for 2 h in warm sterile distilled water with intermittent agitations. The extract was filtered and lyophilised to give 21.64 g of the dried extract (PN), which was stored in aliquots in the refrigerator until needed for the experiments. Preliminary phytochemical tests were carried out on samples of PN using standard procedures (Harborne, 1998). The endotoxin level of the PN was tested by Limulus Amoebocytes Lysate kit (Endosafe®, Charles River, Sulzfeld, Germany).

Animals

Female C57BL/6 mice obtained from Janvier (Le Genest-ST-Isle, France) were used in the study. The animals were maintained on standard livestock pellets (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and were allowed unrestricted access to drinking water. The use and care of laboratory animals in the study were in accordance with ethical guidelines as contained in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC) of 1986.

Preparation of Mouse Spleen Cells for Experiments

Mice (C57BL/6) were euthanized and the spleens removed aseptically into ice-cold Hanks' Balance Salt Solution (HBSS, Gibco). Single cell suspension was prepared by gentle dispersion of the cells and straining through BD Falcon™ nylon cell strainer (70 μM). Red blood cells were lysed by the addition of 3 ml of ACK lysing buffer (Lonza, Walkersville) per mouse spleen for 5 min. The cells were washed and suspended in R-10, consisting of RPMI 1640 medium (Gibco, Germany) supplemented with 10% heat-inactivated foetal calf serum (FBS), 50 μM 2-mercaptoethanol (Gibco, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Determination of Cytotoxicity and Lymphotoxicity of PN

The cytotoxicity of the PN was evaluated on human embryonic kidney cells expressing SV40 large T-antigen (293 T). The cells were cultured in D-10 medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Germany) with glucose (4.5 g/L), L-glutamine (2 mM), and supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. A modification of the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay procedure, as originally described by Mosmann (1983) was employed.

The cells were seeded in triplicate into 96-well culture plates at a density of 2×10^4cells/well in 150 μl volume. After 24 h incubation at 37°C under 5% CO_2, the cells were then treated with graded concentrations (10-1280 μg/ml) of the PN. The D-10 medium was used as the 'no-drug' control. After additional incubation for 48 h at 37°C under 5% CO_2, a solution of MTT (5 mg/ml, 20 μl per well) was added and further incubated at 37°C in 5% CO_2 for 2 h to allow formazan formation in viable cells.

Thereafter, the medium was removed and 150 μl DMSO was added to dissolve the blue formazan crystals formed in viable cells. The plate was shaken for 30 min. on a plate shaker and the optical density (OD) determined at 550 nm using a multi-well microtitre plate reader (Tecan, Austria). Each single value of the triplicate determination was expressed as a percentage of the

mean of triplicates of the 'no-drug' control cultures and the mean calculated for each measurement. The concentration of PN producing 50% cellular toxicity (TC_{50}) was calculated by regression analysis.

Similarly, Lymphotoxic effect of PN was evaluated on naïve murine splenocytes using a modification of the trypan blue exclusion and viability protocol (Freshney, 1987). Splenocytes of C57BL/6 mice were prepared, seeded at a density of 1×10^6 cells/ml into 96-well plates and then treated with increasing concentrations of PN (0, 50, 100, 250, 500, and 1000 μg/ml) in triplicates. The treated plates were incubated at 37°C under 5% CO_2 and cultured for 24 h. Samples of the splenocytes in each treatment well were taken and diluted 1: 4 with trypan blue to assess the viability of the cells before and after incubation. Dead, necrotic or apoptotic cells, which have lost viability were stained by the trypan blue while viable cells appeared bright under a light microscope.

The viable cells and non-viable cells were enumerated and the viable cells expressed as a fraction of the total cells. For each treatment well, four different samples were enumerated and the mean values recorded and calculated as a percentage of the mean viability for the 'no drug' control. A plot of mean viability (%) versus Log. concentration values was made and the TC50, which is the concentration of PN producing 50% reduction in cell viability, was calculated by regression analysis.

Lymphoproliferation Studies on Mouse Splenocytes

Splenocytes from C57BL/6 mice were prepared as previously described, washed twice in cold HBSS, and adjusted to 8×10^7cells/ml in PBS. The splenocytes were stained by the addition of equal volume of 6 μM Carboxyfluorescein diacetate succinimidyl ester tracer (CFSE, Molecular Probes, Göttingen, Germany) for 6 min, with gentle vortexing. Splenocytes were washed twice and resuspended (1×10^6cells/ml) in R-10 after quenching of staining with ice-cold FBS. The stained splenocytes were seeded into 96-well cell culture plates at a density of 10^5 viable cells/well. Increasing concentrations of PN (0, 12.5, 25, 50, 100, and 200 μg/ml) were added to the test wells in triplicates. Lipopolysaccharide, LPS (10 μg/ml) (*E. coli*, serotype 0128:B12; Sigma-Aldrich) and Concanavalin A, Con A (2 μg/ml) (Sigma, Germany) were used as standard mitogens. The cells were incubated at 37°C in 5% CO_2.

After 72 h incubation, the cells were washed and lymphoproliferation was determined by FACS analysis using a FACScalibur™ flow cytometer (Becton Dickinson). In order to determine the activation status of different lymphocytes sub-population after exposure to PN, similar sets of experiments were performed. In these experiments, the cultured splenocytes were, additionally, pre-incubated with FcR blockers (anti-CD16/CD32) for 25 min at 4°C and thereafter stained with anti-B220-APC and anti-CD4/anti-CD8-PerCP surface antibodies (BD Bioscience) before gating of stained lymphocytes and FACS acquisition.

Determination of CD69 Expression on Murine B Cells and T Cells

Activation of B cells and T cells were further determined on a culture of naïve C57BL/6 splenocytes, enriched for B and T cells, respectively (Krowka et al., 1996). Splenocytes were similarly prepared from mice, as described earlier, and were then enriched for either B cells or T cells by negative selection using magnetic cell sorting (B cell and T cell isolation kit, Miltenyi Biotec, Germany). The B cells or T cells cultures were seeded (100 μl) at a density of 1×10^5cells/well into a 96-well culture plate and treated with PN (0, 12.5, 25, 50, 100 and 200 μg/ml) and LPS (10 μg/ml) for B cells, or ConA (2 μg/ml) for T cells in triplicates. The plates were incubated at 37°C in 5% CO_2. After culturing for 18 h, the cells were washed with PBS/BSA/Azid and then preincubated with anti-CD16/CD32 (FcR-blockers) for 25 min at 4°C. Thereafter, the cells were stained with FITC-conjugated anti-CD69 monoclonal antibody (BD Bioscience) for 25 min at 4°C (BD) before FACS acquisition.

Determination of IL-4/IFN-γ Release by Stimulated Splenocytes

Single cell suspension of total spleen cells of C57BL/6 mice were prepared in R-10 medium containing only 2% FBS and seeded at a density of 5×10^5cells/ml into a 96-well culture plate. The splenocytes culture in the wells were treated with PN (0, 25, 50, and 100 μg/ml), LPS (10 μg/ml) and ConA (2 μg/ml) in triplicate wells. The plates were incubated at 37°C in 5% CO_2. After culturing for 48 h, the plates were centrifuged and the cell-free supernatants were aspirated and stored at –80°C until cytokines were measured. Commercial cytokine Elisa kits (PeproTech GmbH, Hamburg, Germany) were used to assess the levels of IL-4 and IFN-γ in the culture supernatants.

Generation of Bone-Marrow Derived Macrophages

Murine bone marrow-derived macrophages (BM-MØs) were generated from the bone marrow cells of the tibia and femur of C57BL/6 donor mice by a modifications of previously described protocols (Lin et al., 2001; Weischenfeldt and Porse, 2008). Bone marrow cells were harvested and cultured in R-10 medium containing 10 ng/ml of recombinant murine colony stimulating factor (rmCSF-1, Immunotools, Germany) in T-75 cell culture flasks. The cells were incubated at 37°C and 5% CO_2 for 24 h to adhere and remove stromal cells and mature bone marrow resident macrophages.

Non-adherent cells, which are mainly progenitor, were recovered after 24 h of incubation and further incubated in cell culture flasks to expand and differentiate the cells under the influence of rmCSF-1. After 7 days of culture, non-adherent cells were removed and the adherent cells were washed and harvested using cell scraper. Viability of generated macrophages was assessed by trypan blue exclusion techniques. The bone marrow-derived macrophages

(BM-MØs) so generated were plated and used for the different experiments on the effects of PN on the functions and activation of macrophages.

Macrophages Phagocytic Activity

The effect of PN on the phagocytic functions of macrophages was investigated indirectly by the nitroblue tetrazolium (NBT) dye reduction assay as previously described (Rainard, 1986). Generated bone marrow-derived macrophages, BM-MØs (1×10^5 cells/well) were treated with increasing concentrations of PN (0, 12.5, 25.0, and 50 µg/ml) and LPS (10 µg/ml) in triplicate wells for 18 h at 37°C in a 5% CO_2 humidified incubator. The cells were incubated with zymosan (Sigma; 5×10^6 particles/well) and 1.5 mg/ml NBT salt (Sigma, Germany). After incubation for 1 h, the adherent macrophages were rinsed with R-10 medium and washed three times with methanol and allowed to air dry in the laminar flow chamber. Thereafter, 80 µl of 2 M KOH and 120 µl of DMSO were added consecutively and the absorbance measured at 570 nm using a microplate reader. The index of phagocytic function was calculated by the equation:

$$Phagocytic\ activity\ index = \left[\frac{OD\,sample}{OD\,Control}\right] \quad (1)$$

Macrophages Lysosomal Phosphatase Activity

The effect of PN on cellular lysosomal enzymes activity was assessed by measuring acid phosphatase activity in BM-MØs as previously described (Suzuki et al., 1988). Briefly, BM-MØs cell suspension, generated as previously described were seeded at a density of 1×10^5 cells/well in flat bottom 96-well cell culture plates and treated with increasing concentrations of PN (0, 12.5, 25, and 50 µg/ml) and LPS (10 µg/ml). After a 18 h incubation at 37°C in humidified 5% CO_2, the medium was discarded and the macrophage monolayer in each well solubilized with 20 µl of 0.1% Triton X-100 (Applichem, Germany).

A solution of p-nitrophenyl phosphate (p-NPP, 100 µl of 10mM) (Sigma-Aldrich, Germany) and 50 µl of 0.1M citrate buffer (pH 5.0) were added and further incubated for 30 min. Thereafter 80 µl of 0.2M borate buffer (pH 9.8) was added to terminate the reaction and the OD measured at 405 nm. Lysosomal phosphatase activity index was calculated by the following equation:

$$Lysosomal\ phosphatase\,activity\ index = \left[\frac{OD\,of\ samples}{OD\,of\ Control}\right] \quad (2)$$

Nitric Oxide (NO) Release Assay

The effect of PN stimulation on nitric oxide release by BM-MØs was determined by an assay of nitrite levels in the supernatants. BM-MØs were seeded at a density of 1×10^5 cells/well in flat-bottom 96-well cell culture plates and treated with increasing concentrations of LPS (0, 5, 10, and 20 μg/ml) with or without graded concentrations of PN (0, 12.5, 25, and 50 μg/ml) and incubated for 48 h. Nitrite levels were measured in 96-well microtiter plates by mixing 100 μl of cell-free culture supernatant with equal volume of Griess reagent (Green et al., 1982).

Griess reagent (Applichem, Germany) contains equal volume of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid. After 15 min of reaction at room temperature, the OD of the pink-red colour was measured at 540 nm in ELISA plate reader. Nitric oxide concentration was quantified using a standard $NaNO_2$ curve generated using different concentrations of $NaNO_2$ dissolved in R-10 and included in each assay plate.

Assay of TNF-α Production by Stimulated Macrophages

The stimulatory effect of PN on macrophages was further determined by measuring TNF-α in culture supernatant. BM-MØs were cultured in R-10 medium containing only 2% FBS and seeded at a density of 5×10^5cells/well in a flat 96-well cell culture plate. The BM-MØs culture in the wells were treated in triplicate with PN (0, 12.5, 25, and 50 μg/ml) and LPS (10 μg/ml). The plates were incubated at 37°C in 5% CO_2 for 48 h. Thereafter, the plates were centrifuged and cell-free culture supernatants were collected and stored at –80°C until the concentration of TNF-α was measured. Commercial kits (PeproTech GmbH, Hamburg Germany) were used to assess the levels of TNF-α in the culture supernatants by cytokine capture ELISA technique. The lower limit of determination of the kit is 16 pg/ml.

STATISTICAL ANALYSES

The data are represented as mean ± SEM and analyzed by ANOVA using GraphPad Prism 5 software. Differences between test control treatments were considered significant at $p < 0.05$. Analysis of FACS data was done using the Win MDI® software (version 2.9).The data shown are from one representative experiment of at least three repetitions with similar results.

RESULTS

Phytochemistry

The extractive yield of the aqueous extract of *Phyllanthus niruri* (PN) was estimated as 10.82%, w/w of the dried herb powder. Preliminary phytochemical

investigation on PN showed the presence of flavonoids, alkaloids, terpenoids, lignans, other polyphenolics, tannins, and saponins. The endotoxin level in PN was less than 0.3 EU/mg; which is within acceptable range.

Estimation of Cytotoxicity and Lymphotoxicity of PN

The median cytotoxic concentrations (TC50) of PN on 293T cells was estimated as as 831.76 μg/ml, while the median lymphotoxic concentrations (TC50) of PN on total spleen cells was estimated as 489.78 μg/ml.

Lymphoproliferation of Mouse Splenocytes Induced by PN

Aqueous extract of PN caused profound lympho-activation that triggered very significant ($p < 0.01$) and concentration-dependent proliferation of naïve murine splenocytes (Fig. 1a and 1b). Lymphoproliferation was studied by CFSE tracer dye dilution technique and FACS analysis. The Win MDI® software was used for the analysis. Lymphocytes was gated and the percentage of proliferating cells generated as the proportion of viable cells within a marked region (M1) of reduced CSFE intensities or 'CFSE dilution', which represents region of proliferating cells.

The percentage of spleen cells in proliferation cycles ranges between 20–50-fold higher than the un-stimulated control after culturing for 72 h, exposed to increasing concentration of PN (12.5–200 μg/ml). Staining and gating on different lymphocytes subpopulation of spleen cells in different experiments show that both B cells (B220 + cells) (Fig. 2a and 2b) and T cells (CD4 + /CD8 + cells) (Fig. 3a and 3b) were both stimulated into proliferation by PN in a concentration-dependent manner.

CD69 Expression on Murine Total Spleen Cells, B cells, and T Cells

Activation of lymphocytes by PN was also determined by measuring the expression of an early activation maker, CD69, on total splenocytes, B cells, and T cells. Culturing naïve murine splenocytes, B cells, and T cells with PN (12.5–200 μg/ml) for 18 h resulted in significant ($p < 0.05$) increase in the expression of CD69 early activation maker. The increases in the mean fluorescence intensity of CD69-FITC on splenocytes (Fig. 4a), B cells (Fig. 4b), and T cells (Fig. 4c) were very significant and at 200 μg/ml of PN treatment were increased by about 4-, 6-, and 8-folds, respectively, when compared to the expression in the un-stimulated control.

Measurement of IL-4 and IFN-γ in Splenocytes Culture Supernatant

The concentrations of IL-4 and IFN-γ in the supernatants of PN–stimulated splenocytes culture were measured after 48 h of incubation using

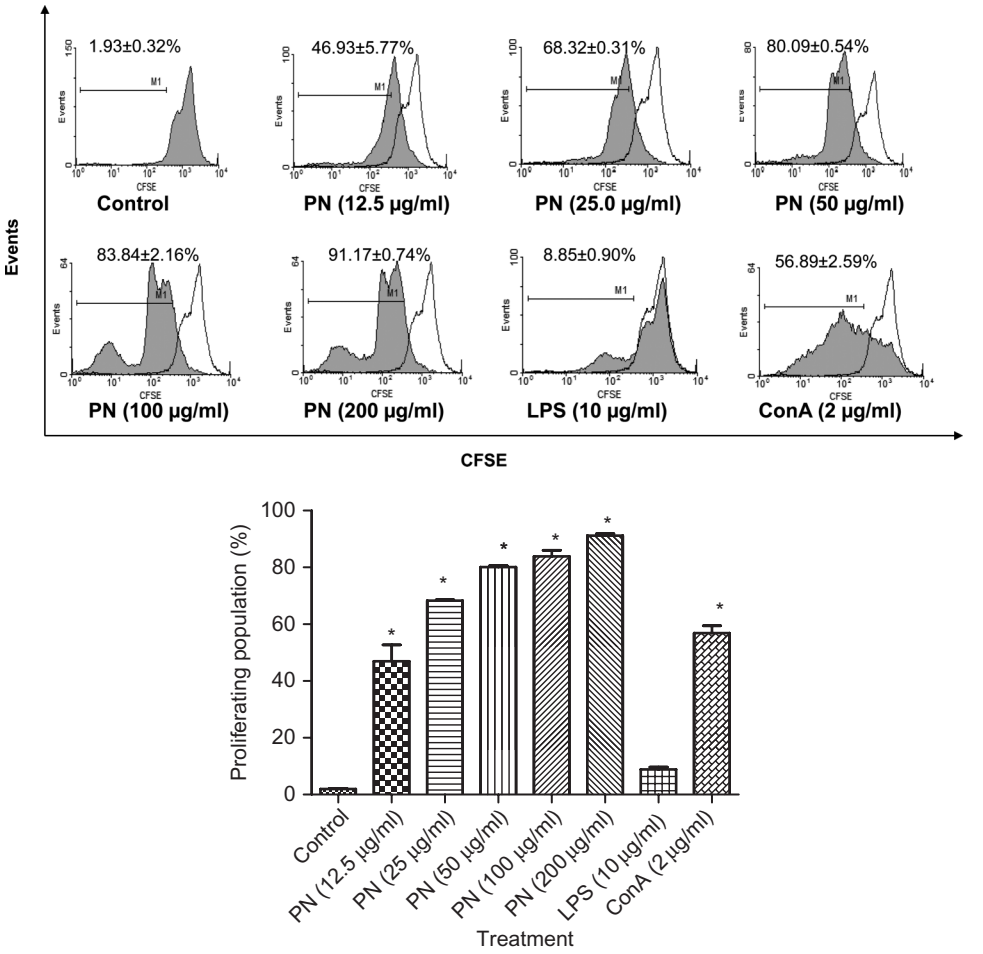

Figure 1: Proliferation of total spleen cells, T cells, and B cells by aqueous extract of *Phyllanthus niruri* (PN). Spleen cells of C57BL/6 mice stained with 3 µM Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The splenocytes (1×10^5cells/ml) were treated with PN (0, 12.5, 25, 50, 100, and 200 µg/ml), LPS (10 µg/ml), and Con A (2 µg/ml) in triplicates and incubated for 72 h. Lymphoproliferation was determined by FACS analysis of CFSE fluorescence gating on the marked (M1) proliferating lymphocytes with reduced CFSE intensities. Proliferation of lymphocytes sub-population was determined in a separate experiment by pre-incubation with FcR blockers and then staining with anti-CD4/anti-CD8-PercP and anti-B220-APC antibodies for 25 min at 4°C before FACS acquisition. The mean percentages of total spleen cells (1a and b), B lymphocytes (2a and 2b), and the T lymphocytes (3a and 3b) in the proliferation cycle are shown; $^*P < 0.01$ vs. un-stimulated control.

commercial Elisa kits. Treatments with PN caused a concentration-dependent and significant ($P < 0.01$) increases in both cytokines. At the concentration of PN (100 µg/ml), the release of IL-4 (Fig. 5a) in the supernatant of treated spleen cells was increased by as much as 13 folds and IFN-γ (Fig. 5b) by as much as 16-fold, compared to the concentrations of the cytokines in the unstimulated control wells.

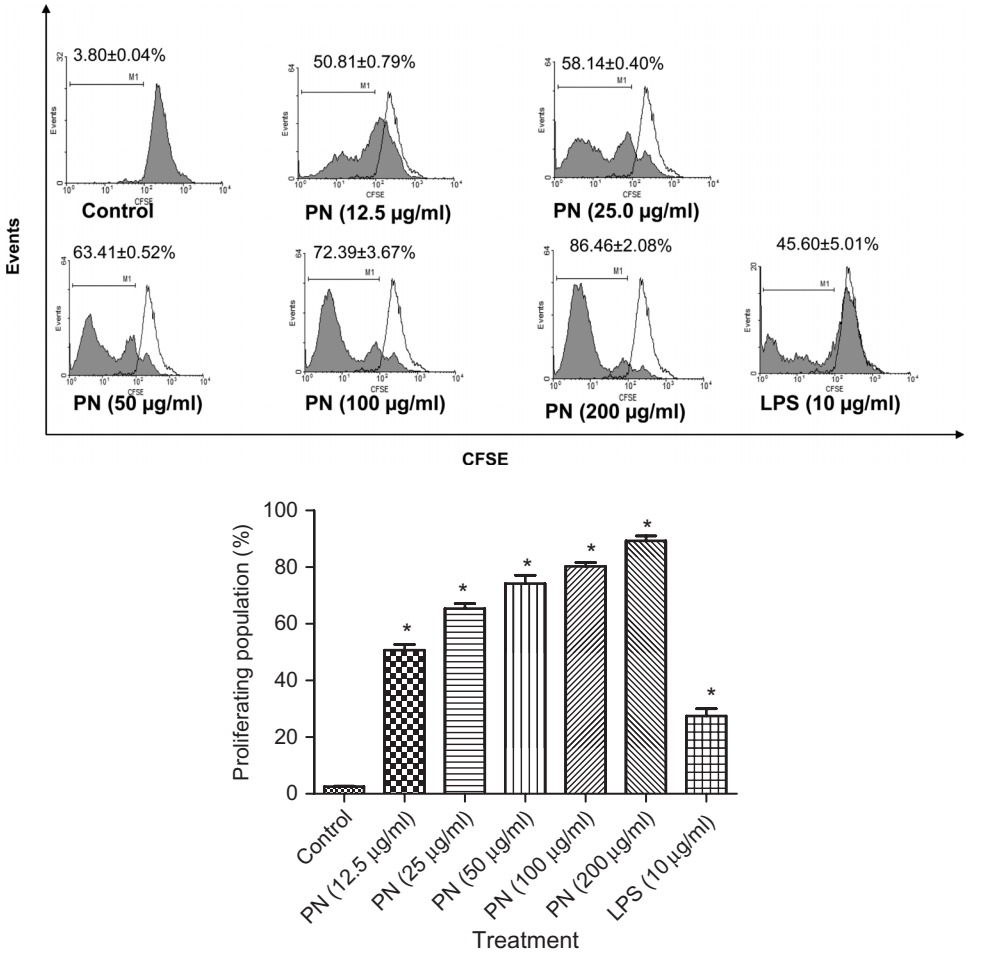

Figure 2: Proliferation of total spleen cells, T cells, and B cells by aqueous extract of *Phyllanthus niruri* (PN). Spleen cells of C57BL/6 mice stained with 3 µM Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The splenocytes (1×10^5cells/ml) were treated with PN (0, 12.5, 25, 50, 100, and 200 µg/ml), LPS (10 µg/ml), and Con A (2 µg/ml) in triplicates and incubated for 72 h. Lymphoproliferation was determined by FACS analysis of CFSE fluorescence gating on the marked (M1) proliferating lymphocytes with reduced CFSE intensities. Proliferation of lymphocytes sub-population was determined in a separate experiment by pre-incubation with FcR blockers and then staining with anti-CD4/anti-CD8-PercP and anti-B220-APC antibodies for 25 min at 4°C before FACS acquisition. The mean percentages of total spleen cells (1a and b), B lymphocytes (2a and 2b), and the T lymphocytes (3a and 3b) in the proliferation cycle are shown; $^*P < 0.01$ vs. un-stimulated control.

Phagocytic Function of Macrophages

Treatment with PN (12.5–50 µg/ml) stimulates increase in oxidative burst and indirectly the phagocytic functions of bone marrow-derived macrophages (BM-MØs) in a concentration-dependent manner. Phagocytosis of zymosan

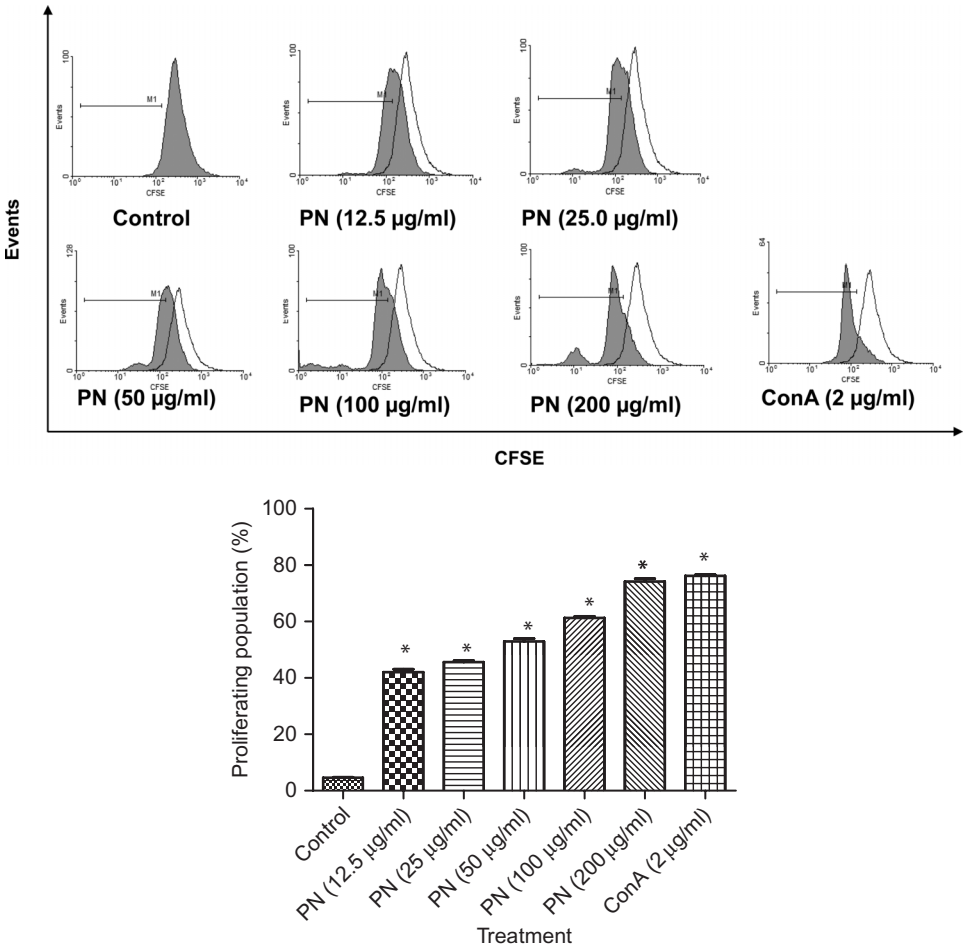

Figure 3: Proliferation of total spleen cells, T cells, and B cells by aqueous extract of *Phyllanthus niruri* (PN). Spleen cells of C57BL/6 mice stained with 3 µM Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The splenocytes (1×10^5cells/ml) were treated with PN (0, 12.5, 25, 50, 100, and 200 µg/ml), LPS (10 µg/ml), and Con A (2 µg/ml) in triplicates and incubated for 72 h. Lymphoproliferation was determined by FACS analysis of CFSE fluorescence gating on the marked (M1) proliferating lymphocytes with reduced CFSE intensities. Proliferation of lymphocytes sub-population was determined in a separate experiment by pre-incubation with FcR blockers and then staining with anti-CD4/anti-CD8-PercP and anti-B220-APC antibodies for 25 min at 4°C before FACS acquisition. The mean percentages of total spleen cells (1a and b), B lymphocytes (2a and 2b), and the T lymphocytes (3a and 3b) in the proliferation cycle are shown; $^*P < 0.01$ vs. un-stimulated control.

particles by BM-MØs was monitored by the rate of NBT reduction. BM-MØs cultures that were pre-stimulated with PN showed a significantly ($p < 0.05$) higher rate of NBT reduction and deep-blue formazan formation, when compared to the un-stimulated cells (Fig. 6). At 50 µg/ml of PN, the increase in phagocytosis was quite comparable to the effects of LPS (10 µg/ml).

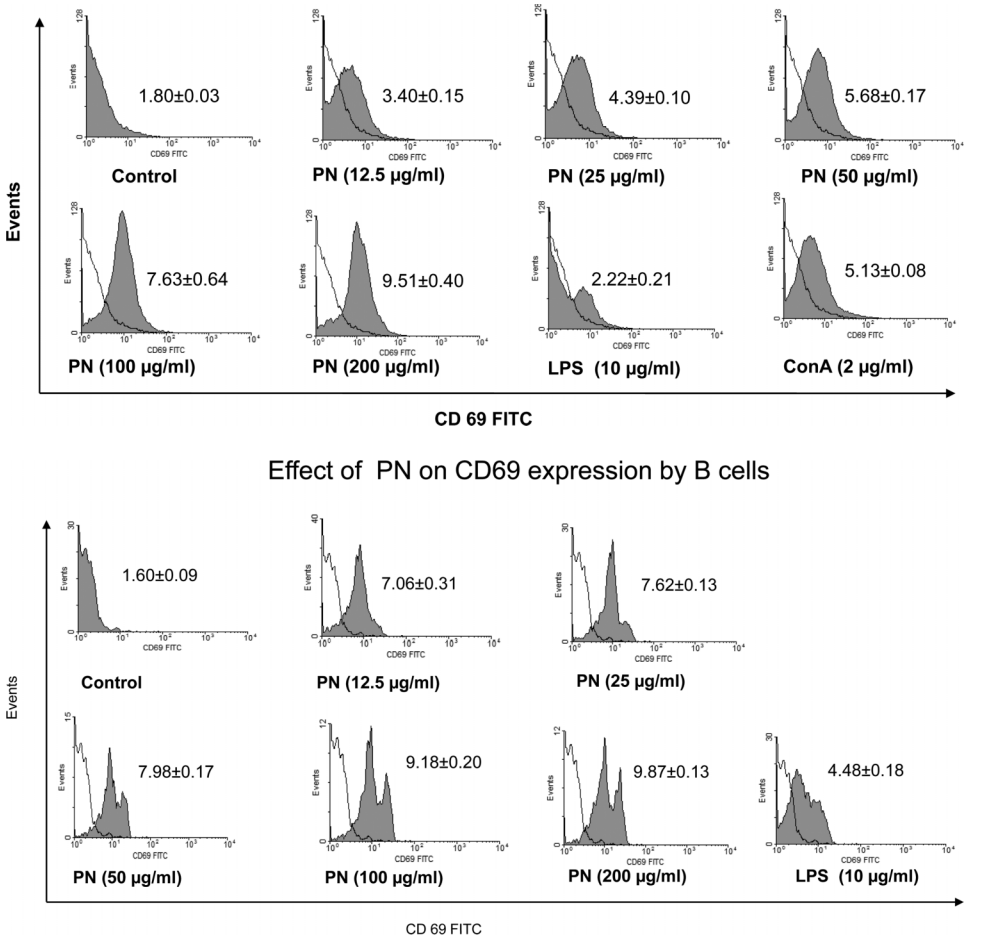

Figure 4: The effect of aqueous extract of *Phyllanthus niruri* (PN) on the expression of CD69 on total spleen cells, T cells, and B cells. C57Bl/6 mice splenocytes were prepared and in some experiments enriched for T cells and B cells using negative selection of magnetic cell sorting. The isolated cells were seeded at a density of 1×10^5cells/well into a 96-well culture plate and treated with PN (12.5–200 µg/ml), LPS (10 µg/ml) or conA (2 µg/ml) in triplicates. Negative control consisted of un-stimulated wells containing R-10 medium alone. The plates were incubated at 37°C in 5% CO_2 for 18 h. The cells were pre-incubated with anti-CD16/CD32 and stained with FITC-conjugated anti-CD69 monoclonal antibody (BD Bioscience) before FACS acquisition. The figures show mean ± SEM of mean fluorescence intensity (MFI) of CD69 expression on total spleen cells (Fig. 4a) T cells (Fig. 4b) and B cells (Fig 4c).

Macrophages Lysosomal Phosphatase Activity

Treatment of BM-MØs with PN (12.5–50 µg/ml) increased the lysosomal phosphatase enzyme activity when compared to the enzyme activity in the un-stimulated cells (Fig. 7). Lysosomal phosphatase activity was measured by the [OD] of *p*-nitrophenol, a coloured hydrolytic product of *p*-nitrophenyl

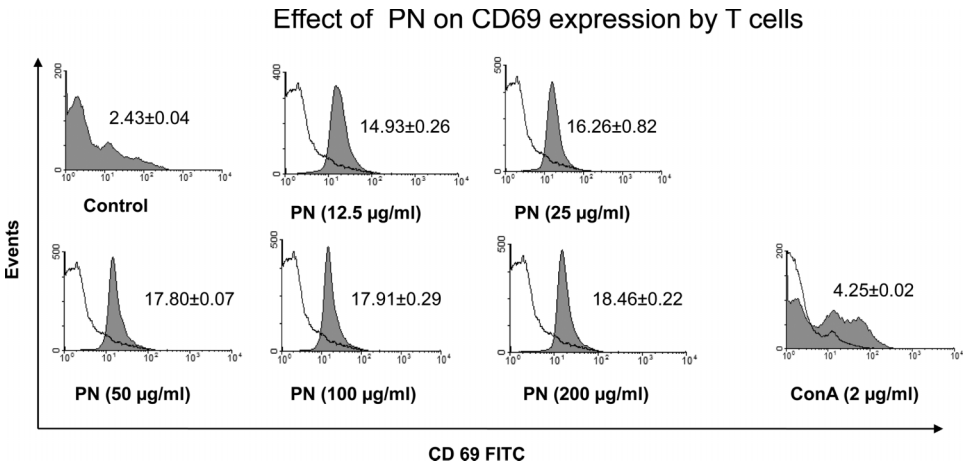

Figure 4: *(Continued).*

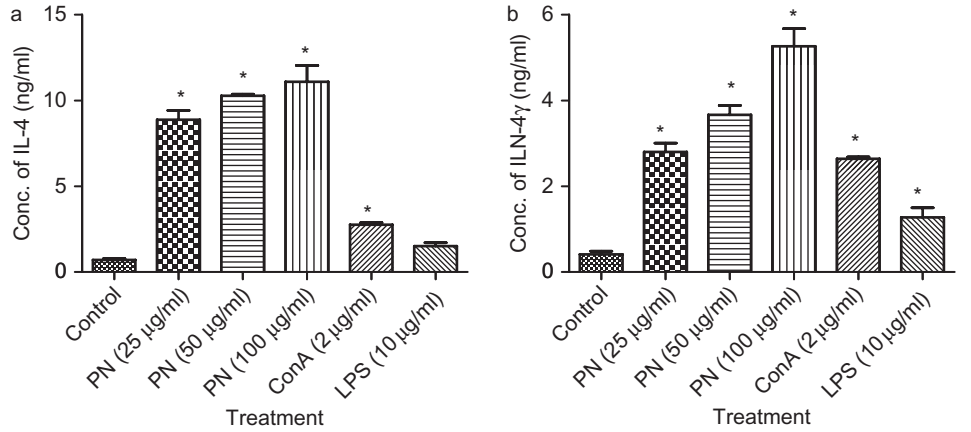

Figure 5: The effect of aqueous extract of *Phyllanthus niruri* (PN) on the levels of IL-4 and IFN-γ in naïve splenocytes culture supernatant. Single cell suspension of total spleen cells of C57Bl/6 mice were prepared and seeded at a density of 5 x 10^5cells/well into a 96-well plate. Cells were treated with PN (25-100 µg/ml), LPS (10 µg/ml) and ConA (2 µg/ml) in triplicate wells. The plates were incubated at 37°C in 5% CO_2 for 48 h. IL-4 (Fig 5a) and IFN-γ (Fig. 5b) were measured in cell-free supernatant using commercial cytokine Elisa kits. *P < 0.01 vs. un-stimulated control.

phosphate (*p*-NPP). Stimulation of lysosomal phosphatase activity increased with increase in the concentration of PN and was significant ($p < 0.05$) at 25 and 50 µg/ml.

Nitric Oxide Release by Macrophages

Slightly higher levels of NO was measured in cultures stimulated with PN alone, without additional stimulation; while LPS-induced nitric oxide (NO)

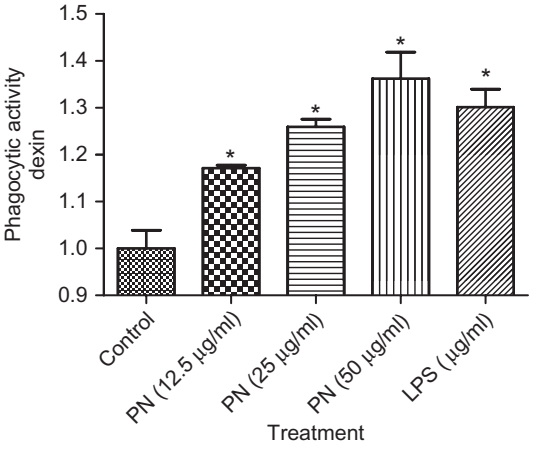

Figure 6: The effect of aqueous extract of *Phyllanthus niruri* (PN) on the phagocytic activity of murine macrophages. Bone marrow-derived macrophages, BM-MØs (1×10^5 cells/well) were treated with PN (0, 12.5, 25.0, and 50 µg/ml) and LPS (10 µg/ml) and incubated at 37°C in a 5% CO_2 for 24 h. The cells were incubated with zymosan (5×10^6 particles/well) and 1.5 mg/ml NBT salt. The index of phagocytic function was calculated as the ratio of the optical densities (OD) of dark blue formazan formed by NBT reduction. For the control treatment, the index was calculated as the ratio of the (OD) of each control sample to the mean (OD) of all controls samples. The mean ± SEM of triplicate values of a representative experiment are shown; *P < 0.05 vs. control.

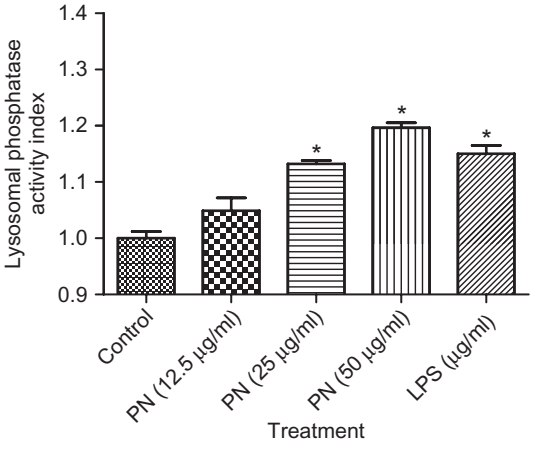

Figure 7: The effect of aqueous extract of *Phyllanthus niruri* (PN) on the activity of lysosomal phosphatase enzymes of murine macrophages. Bone marrow-derived macrophages, BM-MØs (1×10^5 cells/well) were treated with PN (0, 12.5, 25.0, and 50 µg/ml) and LPS (10 µg/ml) and incubated at 37 °C in a 5% CO_2 for 24 h. The *p*-nitrophenyl phosphate (*p*-NPP) assay protocol was used and the (OD) of *p*-nitrophenol, a coloured hydrolytic product of lysosomal phosphatase enzyme activity was measured. The index of lysosomal phosphatase activity was determined as the ratio of the optical densities (OD) of chromogenic *p*-nitrophenol formed by the treatment wells to that of the control. For the control treatment, the index was calculated as the ratio of the (OD) of each control sample to the mean (OD) of all controls samples. The mean ± SEM of triplicate values of a representative experiment are shown; *p < 0.05 vs. control.

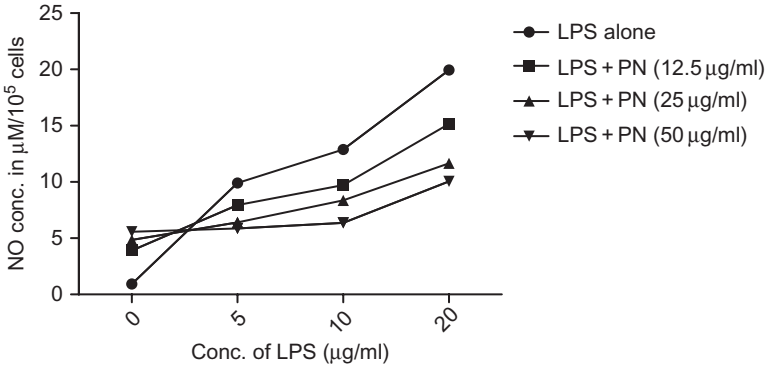

Figure 8: The effect of aqueous extract of *Phyllanthus niruri* (PN) on inducible NO production by murine macrophages. Macrophages (BM-MØs) were seeded at a density of 1×10^5 cells/well in flat bottom 96-well cell culture plates and treated with increasing concentrations of LPS (0, 5, 10, and 20 µg/ml) with or without PN (0, 12.5, 25, and 50 µg/ml) and incubated for 48 h. Nitrite levels were measured in 96-well microtiter plates by mixing 100 µl of macrophage culture supernatant with equal volume of Griess reagent. The (OD) of the developed colour was measured at 540 nm in ELISA plate reader after 10 min. Nitric oxide concentration was quantified by extrapolation on a standard $NaNO_2$ curve generated using different concentrations of $NaNO_2$.

production by BM-MØs was inhibited by PN in a concentration-dependent manner (Fig. 8). NO was estimated by the nitrite levels in culture supernatant using the Griess reagent. In the study, NO production by macrophages was stimulated with increasing concentrations of LPS (0, 5, 10, and 20 µg/ml) and PN (0, 12.5, 25, and 50 µg/ml). Increasing levels of NO were released by increasing concentrations of LPS, but these increases were lower in the presence of PN, suggesting a possible inhibition of LPS induce NO synthase activity.

Expression of TNF-α by Macrophages

Stimulation of BM-MØs with PN (12.5, 25, and 50 µg/ml) significantly ($p < 0.01$) increased the concentrations of TNF-α in the culture supernatants after incubating for 48 h (Fig. 9). At 50 µg/ml of PN, TNF-α production by the treated BM-MØs was increased by 10-fold when compared to the levels by unstimulated control cells.

DISCUSSION

Modulation of the various functions of the immune system is one approach by which medicinal plants produced their beneficial and therapeutic effects. In order to understand the mechanisms and involvement of the immune cells in mediating the effectiveness claimed in the ethnomedicinal uses of the aqueous extracts of PN in the treatment of viral infections, including

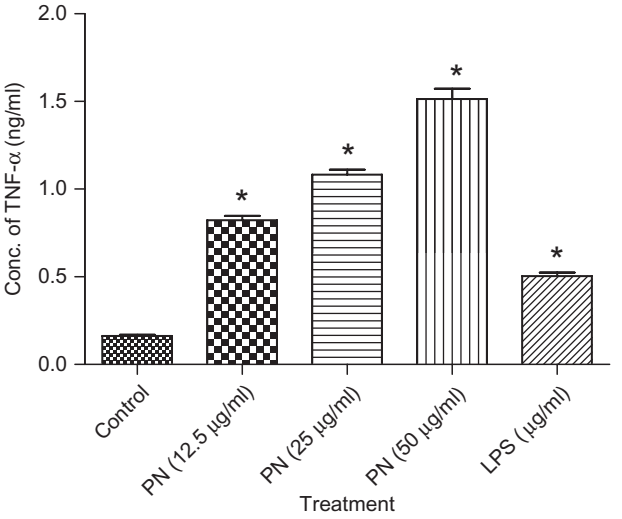

Figure 9: The effect of aqueous extract of *Phyllanthus niruri* (PN) on the release TNF-α by murine macrophages. Macrophages (BM-MØs) were seeded at a density of 5 x 10^5cells/well and cultured in R-10 medium containing only 2% FBS in flat 96-well cell culture plates. The BM-MØs culture in the wells were treated in triplicate with PN (0, 12.5, 25, and 50 µg/ml) and LPS (10 µg/ml) and then incubated at 37°C in 5% CO_2 for 48 h. TNF-α levels were measured in cell-free supernatants by a commercial ELISA kits with lower limit of determination of 16 pg/ml. The mean ± SEM of triplicate values of a representative experiment are shown; *p < 0.01 vs. control.

hepatitis B virus (HBV) and HIV/AIDS (Ogata et al., 1992; Quian-Cutrone, 1996; Shead et al., 1992b; Thyagarajan et al., 1988; Venkateswaran et al., 1987), the effects of the extract on the activation of murine lymphocytes and macrophages were investigated.

The study showed that the aqueous extract of PN produced profound activation and mitogenic activities on different subsets of murine lymphocytes. The extracts caused a significant proliferation of total naïve murine splenocytes, T lymphocytes, and B lymphocytes. This suggests that PN activates the different lymphocytes subpopulations. Activated lymphocytes often respond by entering into a cycle of mitotic division, followed by differentiation and maturation (Lane et al., 1991). The proliferation signals for cells are usually generated by mitogen activated protein kinases (MAPKs), a family of protein tyrosine kinases (PTKs) that catalyze the phosphorylation of cellular substrates, which in turn leads to B cell and T cell proliferation (Rudd, 1990; Yamanashi et al., 1991). These enzymes have been demonstrated to be intimately involved in signal transduction and cell activation processes involving cells of the immune system (Weber et al., 1997).

Activation of these lymphocytes by *P. niruri* extract was further shown by the increased expression of CD69, an immune cells growth factor receptor and early activation maker. The functional significance of CD69 is largely unknown, but its mere presence on the surface of immune cells suggests

activation. CD69 is one of the earliest cell surface antigens expressed by T cells following activation. Typically, CD69 is expressed as early as 1 h after stimulation of resting T cells (Dayer, 2002; Jung et al., 1990) and once expressed, CD69 acts as a co-stimulatory molecule for T cell activation and proliferation. In addition to mature T cells, CD69 is inducibly expressed by B cells, NK cells, immature thymocytes, monocytes, neutrophils and eosinophils, and is constitutively expressed by mature thymocytes and platelets (Ziegler et al., 1994). The increased presence of CD69 in naïve murine lymphocytes culture treated with aqueous extract of *P. niruri* compared to the control treatment shows that both T-cells and B-cells were stimulated by the extract.

The activation of murine lymphocytes by *P. niruri* extracts was also associated with very significant increase ($p < 0.01$) in the release of Interferon-γ (IFN-γ) and interleukin-4 (IL-4) in naïve murine splenocytes treated with PN. IFN-γ and IL-4 are also capable of inducing further immune cells activation. Although the order of occurrence is not clear, it is possible that the extract could be acting by first stimulating the release of these immunomodulatory cytokines which may be responsible for the profound stimulation and proliferation shown by the treated cells.

Besides the stimulation of the lymphocytes, *P. niruri* extract also activated macrophages causing enhance phagocytic functions, increased lysosomal phosphatase activity, and increased release of tumour necrosis factor-α (TNF-α). Macrophages play a critical role during the immune response and through a number of different mechanisms.

They act directly, by destroying bacteria, parasites, viruses and tumour cells; and indirectly, by releasing mediators [such as interleukin-1 (IL-1) and TNF-α], which can activate other immune cells (Xaus et al., 2001). Macrophages also act as accessory cells, by processing antigen and presenting digested peptides to T lymphocytes during immune response and are actively involved in repairing tissue damages and in wound healing processes (Wilson, 1997; Xaus et al., 2001). Bone marrow macrophages have been used as tools for elucidating different molecular processes within cells, from reactions at the cell surface to the regulation of gene expression. Bone marrow cells are non-transformed cells which can be induced by different stimuli to proliferate; to become activated, or differentiate into macrophages depending on the growth factors and stimuli encountered (Xaus et al., 2001; Celada and Nathan, 1994). In this study, bone marrow-derived macrophages (BM-MØs) were generated by treatment with recombinant murine colony stimulating factor-1 (rmCSF-1) and used as a model to study the effects of PN on the activation of macrophages.

Treatment of BM-MØs, generated from bone marrow cells, with increasing concentrations of *P. niruri* aqueous extract (12.5–50 μg/ml) and followed by a challenge with zymosan particles caused a significant and concentration-dependent increase in NBT reduction, which is a direct measure of respiratory burst and an indirect measure of phagocytosis of the zymosan. NBT reduction

is associated with respiratory burst. Respiratory burst or oxidative burst is the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide) by NADPH oxidase from different types of immune cells, especially phagocytes. The release of these free radicals and subsequent reduction of NBT to dark blue formazan in macrophages could be triggered by phagocytosis. This makes it possible to indirectly assess phagocytosis using the degree of NBT reduction.

Macrophages also carry out their non-specific defence functions at the elimination stage of the phagocytic process by activating the lysosomal phosphatases in the cytosolic vesicles. The macrophage cultures treated with *P. niruri* aqueous extract also showed enhanced lysosomal phosphatase activity, which was measured by the optical densities [OD] of *p*-nitrophenol, a coloured hydrolytic product of p-nitrophenyl phosphate.

The levels of TNF-α released in the supernatants of PN-stimulated macrophages were significantly ($p < 0.01$) higher than in the supernatant of un-stimulated control cultures. TNF-α is a proinflammatory mediator, which is released by activated macrophages, and plays a critical role in non-specific immune responses against infections and tumour (Rojas et al., 1993). Therefore, the increase in phagocytosis, increase in lysosomal enzymes activities, and the higher TNF-α release by bone marrow derived macrophages, stimulated with the aqueous extract of *P. niruri*, are very significant observations, and could be plausible explanations to the benefits claimed in the use of the extracts against so many infections and tumour.

The study also show that murine macrophages treated with *P. niruri* aqueous extract, without additional stimulation, produced slightly higher nitric oxide (NO) levels in the supernatant which were not significant; but LPS-stimulated NO release by activated macrophages was inhibited by PN in a concentration-dependent manner. NO is highly reactive and short-lived free radical synthesised by the oxidation of the guanidine nitrogen of L-arginine by an enzyme, nitric oxide synthase (NOS) (Marletta, 1994) and is converted into more stable products such as nitrite (NO_2^-) and nitrate (NO_3^-) (Stuehr and Marletta, 1987).

This enzyme is either inducible by bacterial endotoxins such as lipopolysaccharide (LPS) and by lymphokines (iNOS) or could be expressed constitutively (cNOS) in cells such as endothelial cells and neurons (Nathan, 1992). Physiologically, NO is involved in the regulation of blood vessel dilation, serves as a neurotransmitter, and play a pivotal role in innate immune responses of macrophages (Bredt and Snyder, 1994; Ignarro, 2002; Schmidt and Walter, 1994).

The inhibition of iNO by PN is very relevant in explaining some of the ethnomedicinal applications *P. niruri* extract in inflammatory disorders (Dirjomuljono et al., 2008; Kassuya et al., 2006) and disease conditions where excessive production of NO is aetiological. Excess production of NO is

associated with several diseases such as chronic inflammatory diseases, neurodegenerative disorders, septic shock, and autoimmune diseases. Although the mechanism of this inhibition is not clear from this study, it is possible that antioxidant and free radical scavenging activities of some of the phytoconstituents could also affect the levels of nitrite detected in the culture media.

The cytotoxic effects of the extract on 293T and total spleen cells were evaluated by estimating the median toxic concentrations (TC50); which were 831.76 and 489.78 μg/ml, respectively. Although, decoctions of *P. niruri* is traditionally prepared and taken for different ailments without much regard to dosing, the TC50 values obtained in this study were not very high and therefore suggest a need for careful titration of the doses to avoid the possibility of toxicity while maximising the potential benefits of the herb. *Phyllanthus niruri* and related species has been the subject of many phytochemical studies and many biologically active metabolites such as lignans, glycosides, flavonoids, alkaloids, ellagitannins, and phenylpropanoids have been identified in the leaf, stem, and root of the plant. Although the study did not associate specific metabolite(s) with the various immuno-activities, some of the phytoconstituents found in the extract have been reported to possess immunomodulatory activities in previous studies (Cho et al., 1999; Akbay et al., 2003; Chiang et al., 2003; Manu and Kuttan, 2009; Schepetkin et al., 2009).

CONCLUSION

The results of the various experiments show that the aqueous extract of *Phyllanthus niruri* stimulates both T and B lymphocytes and the indices of macrophages functions and activation. The results of the study also suggest that immunomodulation could be an important mechanism in the therapeutic activities of *P. niruri* extracts and could partly explain the efficacy of the herbal therapy in viral and other infections.

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