

Attenuation of reactive oxygen/nitrogen species with suppression of inducible nitric oxide synthase expression in RAW 264.7 macrophages by bark extract of *Buchanania lanzan*

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ABSTRACT

Background: Oxidative stress is one of the most critical factors implicated in disease conditions. *Buchanania lanzan* Spr. (Anacardiaceae) bark powder preparation has been reported for treating an inflammatory condition in the Ayurvedic Pharmacopoeia of India. **Objective:** In the present study, we investigate the effect of the bark methanol extract (BLM) on reactive oxygen/nitrogen species (ROS/RNS), the expression of protein and mRNA of inducible nitric oxide synthase (iNOS) in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS) and sodium nitroprusside (SNP) to provide scientific validation of the above said medicinal property. **Materials and Methods:** The capacity to quench ROS and RNS was evaluated by 5-(and-6) chloromethyl-20,70-dichlorodihydrofluorescein diacetate acetyl ester fluorescence and nitrite estimations in LPS/SNP-stimulated macrophages respectively. The protein and transcript expression of iNOS was evaluated through Western Blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis respectively. **Results:** Macrophages pretreated with BLM (>100 µg/mL) for 24 h, stimulated with LPS for the last 18 h of experimental duration recorded a significantly ($P < 0.05$) reduced levels of ROS (3.45-fold) against LPS-stimulated conditions (5.7-fold). SNP-stimulation resulted in increased NO accumulation (17-fold) which was neutralized by BLM at >100 µg/ml (1.6-fold) credited to a reduced protein and mRNA expression of iNOS as recorded by Western blots and RT-PCR results respectively. The reversed-phase liquid chromatography-diode array detection analysis identified the presence of 4-hydroxybenzoic acid, quercetin and *p*-coumaric acid (Rt values 5.444, 5.569 and 9.580 respectively). **Conclusions:** The potential of BLM inhibiting ROS/RNS production validates the medical use of bark, could find beneficial application under conditions of immune stimulation and/or bacterial infection.

Key words: *Buchanania lanzan*, flow cytometer, inducible nitric oxide synthase, lipopolysaccharide/sodium nitroprusside-stimulated oxidative stress, RAW 264.7 macrophages, reactive oxygen/nitrogen species

INTRODUCTION

Oxygen partially reduced during normal metabolism yields reactive molecules termed reactive oxygen species (ROS) including hydrogen peroxide (H₂O₂), superoxide radical etc., Overload of ROS results in oxidative stress that is detrimental with a role in the etiology of several human degenerative diseases like cancer, neurodegenerative

disorders, and inflammation.^[1] Bacterial infection using *Escherichia coli* lipopolysaccharide (LPS) has been reported to cause excessive production of ROS. Upon stimulation with antigen (sodium nitroprusside; [SNP]) excessive production of nitric oxide (NO) has been reported^[2] through expression of inducible NO synthase (iNOS).^[3,4] RAW 264.7 murine macrophages have been used to study these pathological conditions *in vitro*. Upon stimulation these macrophages exhibit excessive accumulation of both NO and superoxide anion, whose interaction results in formation of toxic peroxynitrite (ONOO⁻)^[5] resulting in septic shock,^[6] cerebral injury,^[7] local or systemic inflammatory disorders.^[8] Thus, the inhibition of ROS

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production,^[9] along with iNOS expression and/or activity has been identified as a therapeutic target in screening of natural products.^[2,10,11]

Buchanania lanzan, Spr. (Anacardiaceae), commonly known as Chironji, has been used for treating ailments *viz.*, to reduce granular swelling; relieve prickly heat; bark gum for treating diarrhea and intercostals pain and leaves as anti-ophidian.^[12] Myricetin 3'-rhamnoside-3-galactoside, celidoniol, vomicine, and pinitol are reported.^[13] Immunostimulant properties for *B. lanzan* with a capacity to enhanced macrophage migration index, hemagglutinating antibody titers, plaque-forming cell counts as parameters of humoral immunity was observed and efficacy against cyclophosphamide induced genotoxicity and oxidative stress in mice has been reported.^[14] Earlier studies report on the use of methanolic extract of leaves,^[12] kernel,^[15] and roots^[16] with anti-inflammatory, antioxidant and analgesic properties using *in vivo* rat paw edema model is available.

The Ayurvedic pharmacopoeia of India describes oral intake of 5–10 g dried stem bark to cure fevers and inflammatory conditions.^[17] In this direction, earlier studies from this laboratory have reported significant antioxidant activity ($P < 0.05$) to this extract with a potential to inhibit 15-lipoxygenase and human cyclooxygenase-2, the inflammatory mediators, in a dose-dependent manner.^[18] Apart from these reports, *B. lanzan* bark extracts are not investigated. As there have been studies on antioxidant and anti-inflammatory mechanism, we became interested to look into the ROS and reactive nitrogen species (RNS) pathway. In this study, we report on the mechanism of *B. lanzan* bark methanol extract (BLM) role in LPS/SNP-stimulated macrophages, RAW 264.7 cells, providing evidence for it to be with potential therapeutic value.

The intracellular ROS produced by LPS-stimulated macrophages was detected and measured using ROS-sensitive fluorescent dye, 5-(and-6) chloromethyl-20,70-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA).^[4,19] The dye enters the cells, reacts with ROS to form 2',7'-dichlorofluorescein and is trapped within the cell. The differential fluorescence was estimated by multimode plate reader and evaluated by Flow Cytometer analysis. The effect of BLM on RNS was evaluated in SNP-stimulated macrophages, by estimation of nitrite as a measure of NO, a RNS. The effect of BLM on the key enzyme for the production of iNOS was evaluated at protein and mRNA transcript levels.

MATERIALS AND METHODS

Chemicals

All chemicals used in the study were of analytical grade. β -actin, Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin, trypsin, heat inactivated fetal calf serum, *E. coli* LPS, CM-H₂DCFDA and SNP were from Sigma-Aldrich (St. Louis, MO, USA). Solvents used for chromatography were of high-performance liquid chromatography grade procured from Merck Biosciences (Bangalore, India). Whatman NYL 0.45 μ m syringe filter was used for the filtration of samples.

Plant

The *B. lanzan* tree barks was collected from Kigga region of Western Ghats in Chikmaglore District of Karnataka State, India, during monsoon period (October–November)^[18] and authenticated by taxonomist, Mr. Sampath Kumar at University of Mysore. Herbarium specimen was deposited at the Department of Studies in Biotechnology (*B. lanzan* # IOE LP0004). Fresh bark pieces were thoroughly washed under running tap water to remove debris and air-dried under shade. They were pulverized in a laboratory mechanical grinder to a coarse powder. Ground powder (250 g) was extracted sequentially using 500 ml of nonpolar and polar solvents in increasing polarity hexane < chloroform < methanol < water using Soxhlet apparatus by continuous hot percolation (boiling point, 52–62°C) until the solvent became colorless. The resultant solvent extracts were concentrated in a SpeedVac (Savant SPD 2010, Thermo Scientific, Germany) under reduced pressure. The methanol extract (24.48 g) exhibited potent antioxidant activity with a capacity to inhibit activities of human cyclooxygenase-2 and 15-lipoxygenase.^[18] Hence, this fraction was selected for further investigations in the present study and is mentioned as BLM. Before the start of the experiment, BLM was solubilized in dimethylsulfoxide (DMSO).

Cell culture

Mouse macrophage cell line RAW 264.7 was obtained from National Center for Cell Science (Pune, India) and cultured in DMEM supplemented with fetal bovine serum (10%) containing penicillin-streptomycin (10%) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were plated at a density of 1×10^4 cells/well in 25 or 75 cm² flasks, or in 6- or 96-well plate overnight, and treated with BLM (10, 50, 100 and 200 μ g/ml) for 24 h.

Reactive oxygen species measurement

With the aim to investigate the therapeutic potential of BLM, we investigated whether it could inhibit LPS-stimulated ROS generation in RAW 264.7 cells using a fluorescent dye, CM-H₂DCFDA. The differential

fluorescence in the samples: RAW 264.7 control cells, LPS-stimulated (10 ng/ml) control and BLM pretreated LPS-stimulated samples were measured.^[17,18] For testing BLM, cells were plated (1×10^4 cells/well) in a 6-well plate. Once the cells reached confluence (approximately 200,000 cells/well as visualized under inverted microscope), they were treated with BLM (10, 50, 100 and 200 $\mu\text{g/ml}$) for 24 h. Cells were stimulated with LPS (10 ng/ml) during the last 18 h of duration of the experiment. Fluorescent dye, CM-H₂DCFDA (final concentration 10 μl of 20 μM) was added to cells and incubated for 30 min in CO₂ incubator to measure the intracellular ROS produced in these cells. After incubation, the cells were washed with cold-phosphate buffered saline (phosphate buffer saline [PBS]: 50 mM PBS [pH 7.2] containing 0.8% NaCl) and the fluorescence intensity of the stained cells was measured at an excitation and emission wavelength of 485 nm and 530 nm respectively (VarioSkan Flash, Thermo Fisher Scientific, Finland). Fluorescence was measured using Cell Lab Quanta SC™ flow cytometer (Beckman Coulter) Flow Cytometer and at least 10^4 cellular events were counted on FL3. The intracellular fluorescence for all the samples was recorded using Confocal Laser Scanning Microscope, LSM 710 (Carl Zeiss, Germany).

Cell proliferation assay

Cells were plated at a density of 1×10^4 cells/well in 96-well plate overnight, and treated with BLM (10, 50, 100 and 200 $\mu\text{g/ml}$) for 24 h. Cells were stimulated with LPS (10 ng/ml) or SNP (10 μM) during the last 18 h of treatment with BLM. At the end of the treatment period, 50 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (0.5 mg/ml) was added to each well and the plate incubated for 3 h. The supernatant was aspirated, and the formazan crystals formed, solubilized in 200 μl DMSO for 30 min. The extent of reduction of MTT to formazan within cells was quantified by multimode plate reader at 550 nm (VarioSkan Flash, Thermo Fisher Scientific, Finland). In another experiment, designed to assess the viability, LPS-stimulated cells (1×10^4) were stained with calcein (AM) and/or propidium iodide (5 μl for 100 μl of culture medium with 1×10^4 cells) in 1 ml staining buffer and incubated for 30 min at room temperature as per manufacturers' protocol (Affymetrix, Ebioscience kit). They were washed two times with staining buffer, and the cells were read in Flow Cytometer, Cell Lab Quanta SC™ flow cytometer (Beckman Coulter) as per manufacturers' protocol.

Nitrite concentrations as a measure of reactive nitrogen species, nitric oxide levels

Nitrite production was evaluated as an indicator of NO, was measured in the supernatant of RAW 264.7 macrophages.^[2] Briefly, the macrophages were cultured in

96-well plates with 200 μl of culture medium until cells reached confluence. In order to induce iNOS, fresh culture medium (100 μl) containing SNP, 10 μM was added. Nitrite accumulation in the medium was measured after 18 h of SNP addition. To assay the effect of BLM on nitrite production, cells were pretreated with BLM (10, 50, 100 and 200 $\mu\text{g/ml}$) for 24 h and with SNP during the last 18 h of experimental duration. Nitrite was measured by adding 100 μl of cell culture extract to 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylendiamine in 5% phosphoric acid). After 10 min incubation, the optical density was measured at 550 nm in a microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Finland). The concentrations of nitrite were calculated by comparison of absorbance of standard solutions of sodium nitrite prepared in culture medium and assayed under similar conditions.

Western blot analysis of inducible nitric oxide synthase

RAW 264.7 cells in 75 cm² culture flasks were incubated for 24 h with BLM (100 and 200 $\mu\text{g/ml}$) and with SNP (10 μM) for the last 18 h of the duration of the experiment. Incubations were terminated by rapid aspiration of the cell supernatant followed by washing with ice-cold PBS. Cells were lysed in PBS containing ethylenediaminetetraacetic acid (10 mM), 1% Triton X-100, phenylmethyl sulfonyl fluoride (1 mM) and 0.1% leupeptin. Total protein in RAW 264.7 cell extracts was measured by the Bradford method using bovine serum albumin as standard. Proteins (30 μg) were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 1 mm thick 10% SDS-PAGE. For Western blot analysis, proteins separated were transferred on to nitrocellulose membranes (Millipore) using Multiphor II (LKB, Pharmacia) electrophoretic transfer apparatus according to manufacturer's protocol. The blots were blocked in 2% fat-free milk in Tris buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl). The blots were incubated for 2 h at 37°C with primary antibodies (iNOS antibodies, a polyclonal antibody against the peptide PT387 (Ac-Cys-residues 1131–1144, from the C terminus of the deduced amino acid sequence of murine iNOS),^[20] a kind gift from Prof. Mikio Nishimura, National Institute for Basic Biology, (Okazaki, Japan). The transfer conditions used were 135 mA for 90 min. After three washes with TBS, the blots were incubated with alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin G (Merck BioSciences, Bangalore, India), diluted 1:1000 for 30 min at room temperature. They were washed three times in TBS, and the blots were stained for ALP activity with nitro blue tetrazolium /5-bromo- 4-chloro-3-indolyl-phosphate (Chromos Biotech, Bangalore, India). β -actin was used as internal control.

Relative quantification of inducible nitric oxide synthase gene expression by reverse transcription-polymerase chain reaction

RAW 264.7 macrophages were cultured in 75 cm² flasks for RNA extraction. Once the cells reached confluence, they were treated with various concentration of BLM (50, 100 and 200 µg/ml) for 24 h and with SNP (10 µM) for the last 18 h of the experimental duration. Total RNA was extracted from control, SNP-stimulated and BLM with SNP-stimulated macrophages by RNA isolation kit (Sigma, St. Louis, MO, USA). RT was performed with equal amounts of RNA (2 µg) primed with oligo (dt) using hot start-reverse transcription-polymerase chain reaction (HS-RT-PCR) kit as per manufacturers' recommended protocol (Sigma). RT generated cDNA encoding iNOS and β-tubulin (internal standard) genes were amplified in a total volume of 20 µl of SYBR Green PCR Mastermix (Roche Diagnostics, Germany) with appropriate primers iNOS (5'-CCCTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3') and mouse α-tubulin (5'-ATGCCATCCTGCGTCTGGACCTGGC-3' and 5'-AGCATTTGCGGTGCACGATGGAGGG-3') in a Roche Light Cycler 480. The initial denaturation was performed (94°C for 10 min) and further, a 35 cycle of amplification was performed with these said parameters (94°C for 45 s, 65°C for 40 s and 70°C for 3 min) followed by a final 10 min extension at 72°C. A continuous monitoring of fluorescence for each PCR product was carried out. A single narrow peak was obtained for melting curve analysis at specific temperature. All the samples (*n* = 2) were analyzed, and the ΔCT method was used to quantify expression levels. Normalization was done w.r.t. housekeeping α-tubulin gene. Analysis was performed with light cycler relative quantification software.

Chromatogram of bark methanol extract extracts by reversed-phase liquid chromatography

Chromatographic analysis of BLM was performed on Waters liquid chromatographic system equipped with Waters (Milford, MA, USA) and Waters Spherisorb column (C18; 250 × 4.6 mm, 50 mm particle size) as stationary phase with photodiode array detector. Mobile phase consisting of a mixture of acetonitrile: Water (18:82) acidified with 2% acetic acid, was delivered at a flow rate of 1 ml/min. Standards (200 ppm, injection volume 10 µl) were prepared using 4-hydroxybenzoic acid (HBA), quercetin and *p*-coumaric acid (*p*CA). The BLM sample solution was prepared in the same way and at the same concentration range. Each sample was injected three times in three independent experiments.

Statistical analysis

Each experiment was carried out at least three to six times separately. The results are presented as the mean ± standard deviation of measurements. Statistical difference between control and BLM groups for all experiments was determined using Students' *t*-test. NO production has been indicated as absolute concentrations in µM. The comparison between the groups was considered significant if *P* < 0.05.

RESULTS

Buchanania lanzan methanol extracts inhibited reactive oxygen species production

With an aim to examine BLM capacity to inhibit ROS generation in LPS-stimulated RAW-264.7 cells, they were exposed to CM-H₂DCFDA, a fluorescent dye widely used to label ROS in macrophages. Treatment with LPS (10 ng/mL) resulted in overproduction of ROS (5.7-fold) [Figure 1a]. At the lowest concentration of BLM (10 µg/mL), ROS production was not significantly different from LPS-stimulated cells. However, a dose-dependent differential intensity of fluorescence was recorded that was significantly (*P* < 0.05) less in BLM pretreated (10, 50, 100 and 200 µg/mL) cells (*n* = 6). These cells were read in the flow cytometer [Figure 1b]. It was observed that LPS essentially acted in generation of intracellular ROS (red peak). However, BLM (100 and 200 µg/mL) reduced ROS levels (blue peak - 100 µg/mL; white - 200 µg/mL) when compared to LPS-stimulated RAW 264.7 cells (*n* = 3). The differential fluorescence intensities were recorded by confocal microscopy [Figure 1c] with highest fluorescence in LPS-stimulated cells and a gradual decrease in fluorescence with increasing concentration of BLM (*n* = 3).

Based on MTT assay (*n* = 6), the concentrations of BLM (50, 100 and 200 µg/ml) during the 24 h incubation was not toxic to RAW 264.7 cells [Figure 2a]. Flow Cytometer analysis of BLM (200 µg/mL) treated cells recorded 99.57% viable cells [Figure 2b]. The result on cell viability with neutralization of ROS production due to pretreatment with BLM indicates that the inhibition of the ROS is not due to cell death (*n* = 3).

Bark methanol extract inhibited nitrite production

Nitrite production was observed to be dependent on the state of the cells. Control cells (unstimulated, minus SNP), produced basal levels of nitrite (4.34 ± 0.23 µM) even after 18 h [Figure 3a]. Addition of BLM did not significantly affect basal nitrite production even at concentrations of 200 µg/mL. When stimulated with SNP (10 µM) for 18 h, induced 17 folds increase in nitrite production

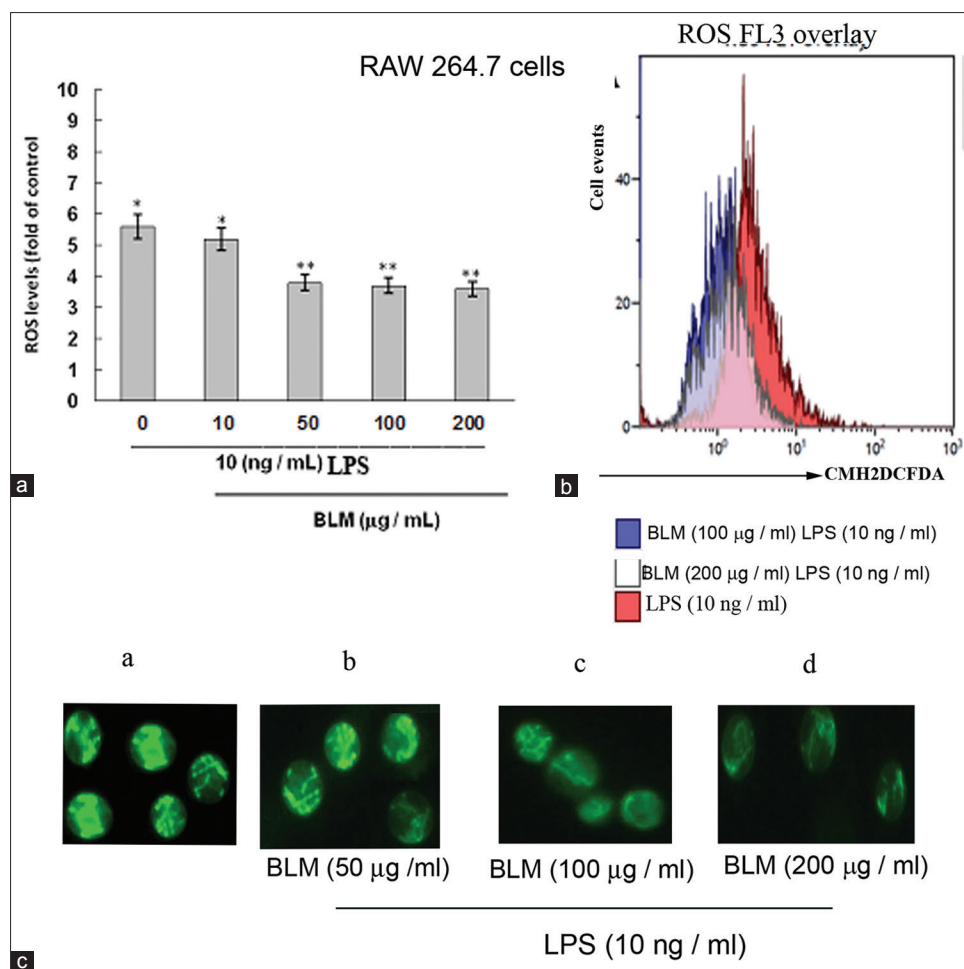


Figure 1: Effect of *Buchanania lanzan* on reactive oxygen species (ROS). RAW 264.7 cells were cultured with bark methanol extract (BLM) (10, 50, 100 and 200 µg/mL) for 24 h and exposed to lipopolysaccharide (LPS) (10 ng/mL) for the last 18 h (a) The intracellular ROS was stained with 5-(and-6) chloromethyl-20,70-dichlorodihydrofluorescein diacetate acetyl ester and BLM significantly ($P < 0.05$) reduced ROS (a) indicated (**) measured ($n = 6$). Flow Cytometer analysis of ROS (b) depicts the significantly reduced ROS upon BLM pretreatment ($n = 3$). The reduced fluorescence intensities due to BLM pretreatment (b-d) compared to LPS-stimulated RAW 264.7 cells (a) was captured ($n = 3$) by confocal laser scanning microscope (c)

to $74.56 \pm 1.23 \mu\text{M}$ [Figure 3b] in comparison to basal levels. In these experiments, addition of BLM (10, 50, 100 and 200 µg/mL), neutralized nitrite accumulation in a concentration-dependent manner to 69.8 ± 5.42 , 44.87 ± 3.24 , 12.1 ± 2.19 and 9.11 ± 1.32 respectively [Figure 3b]. The effect of BLM inhibition of nitrite production was significantly distinguished with a $> 97\%$ decrease at BLM concentration $> 100 \mu\text{g/mL}$ ($n = 6$).

Effect of bark methanol extract on inducible nitric oxide synthase protein expression

This experiment was designed to evaluate whether inhibition of production of NO due to pretreatment with BLM was due to inhibition of iNOS protein expression. In this direction, Western blot analysis was performed on whole cell lysates using monoclonal iNOS antibodies as described in materials and methods. A concentration-dependent inhibition of iNOS by BLM was recorded [Figure 4a].

Upon stimulation with SNP (10 µM), an escalated amount of iNOS (4.7 ± 0.61) was produced when compared to un-stimulated cells. However, a pretreatment with 100 and 200 µg/mL BLM, significantly inhibited the levels of iNOS protein [Figure 4b] by 2.2 ± 1.08 and 0.9 ± 0.48 respectively ($n = 3$). Cell viability, however, seemed intact in the presence of these concentrations of BLM, as β-actin protein was affected only minimally.

Effect of bark methanol extract on inducible nitric oxide synthase mRNA expression

To determine the effect of BLM inhibition of NO production at protein expression or at the level of transcription, RT-PCR was performed to examine the expression profiles of iNOS gene in SNP-stimulated RAW 264.7 macrophages. The housekeeping β-tubulin gene was also amplified from each sample (control, SNP-stimulated, BLM with SNP-stimulated) of the experiment. As

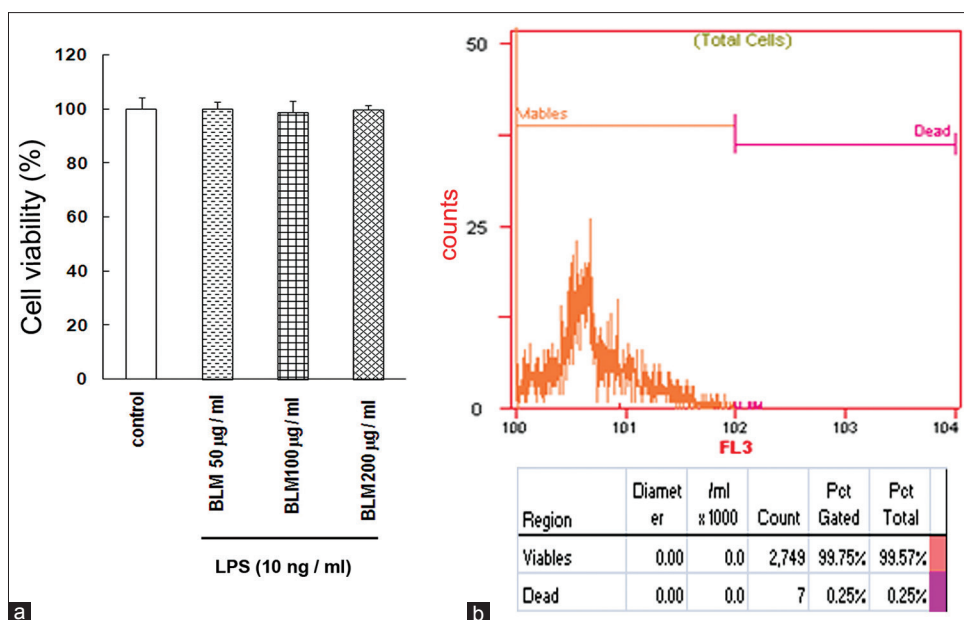


Figure 2: Effect of *Buchanania lanzan* on cell viability. Cell viability of RAW 264.7 macrophage against *B. lanzan* methanol extract (BLM) treated with lipopolysaccharide (10 ng/mL) for 18 h was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (a) and results recorded ($n = 6$). The cells were stained with AM and/or propidium iodide and subjected to analysis by flow cytometer (b) reading viable and dead cells ($n = 3$)

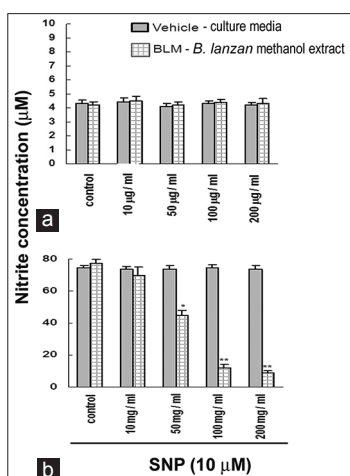


Figure 3: Effect of *Buchanania lanzan* on sodium nitroprusside (SNP)-stimulated nitrite production as a measure of nitric oxide, representative of reactive nitrogen species (RNS), in RAW 264.7 macrophages. RAW 264.7 cells were cultured alone (control) and with bark methanol extract (BLM) (10, 50, 100 and 200 µg/mL) for 24 h (a) In another group RAW 264.7 cells pretreated with BLM and exposed to SNP (10 µM) for the last 18 h (b) The nitrite produced due to SNP-stimulation and neutralized by BLM was estimated by Griess reagent. Statistically significant ($P < 0.05$) neutralization of accumulation of RNS ($n = 6$) is indicated (***) compared to the control

shown [Figure 5], RAW 264.7 macrophages do not express detectable levels of iNOS mRNA (control), however stimulation with SNP (10 µM) significantly ($P < 0.05$) increased iNOS mRNA levels 3.5 ± 0.53 fold relative to β -tubulin gene expression. BLM pretreatment of RAW 264.7 macrophages and subsequent SNP-stimulation indicated a dose-dependent inhibition of iNOS mRNA

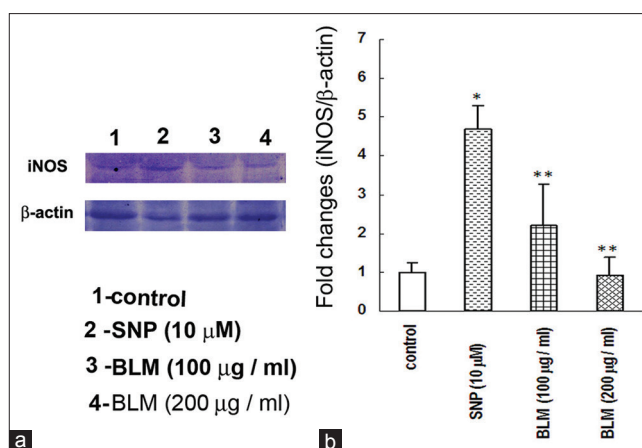


Figure 4: Effect of *Buchanania lanzan* on inducible nitric oxide synthase (iNOS) protein expression. RAW 264.7 macrophages were incubated with bark methanol extract (100 and 200 µg/mL) for 24 h and sodium nitroprusside (SNP) (10 µM) for the last 18 h of the experimental duration. Total protein was extracted for iNOS profiling and detected using a iNOS polyclonal antibody. β -actin was incorporated as an internal standard. The band intensities of Western blot (a) were recorded and quantified by densitometer (b) Statistically significant ($P < 0.05$) reduction in iNOS protein (***) compared to the SNP-stimulated sample (*) is reported $n = 3$

expression. At 50, 100 and 200 µg/mL of BLM a reduced to the absence of iNOS mRNA level was recorded.

Phytochemical screening of bark methanol extract

Phenolic compounds, a class of metabolites, with excellent antioxidant properties, significantly contributes to pharmaceutical potential of many natural product extracts. The major phenolic compounds in the methanol

fraction of BLM were identified by reversed-phase liquid chromatography-diode array detection (RPLC-DAD) screening. Quercetin, HBA and *p*CA were identified in the methanol fraction of *B. lanzan* [Figure 6a] in comparison to the retention time of standards, *p*CA [Figure 6b; 9.580], quercetin [Figure 6c; 5.569] and HBA [Figure 6d; 5.444].

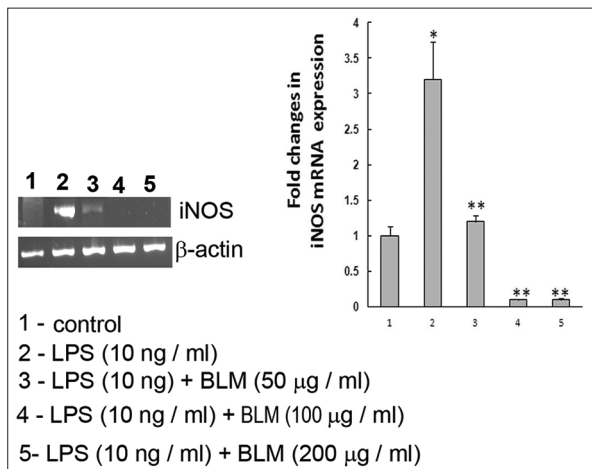


Figure 5: Effect of *Buchanania lanzan* on inducible nitric oxide synthase (iNOS) mRNA expression. RAW 264.7 cells were pretreated with bark methanol extract (BLM) (50, 100 and 200 µg/mL) for 24 h and stimulated with sodium nitroprusside (SNP 10 µM) in the last 18 h duration of the experiment. Total RNA from these samples along with control (minus BLM and SNP) was subjected to reverse transcription-polymerase chain reaction. The fold change was calculated based on normalization with β -tubulin gene expression. The analysis was performed with Light Cycler relative quantification software. Statistically significant ($P < 0.05$) reduction of iNOS mRNA expression (**) is reported in comparison to β -tubulin ($n = 3$)

DISCUSSION

Normal cellular metabolic process involving mitochondrial respiratory chain produces ROS, however, excessive production results in mitochondrial dysfunction as it is reported to inactivate enzymes of Krebs cycle including aconitase and α -ketoglutarate dehydrogenase.^[21,22] leading to disease conditions. RNS such as NO alone and with superoxide radical, produced under stress conditions, has the capacity to form ONOO⁻, inhibiting irreversibly mitochondrial respiratory enzymes.^[23,24]

Besides the investigation of single compounds, a growing interest persists on application of standardized extracts.^[25] Studies have been devoted to assess the capacity of natural products and consequently attenuating disease conditions involving inhibition of ROS and RNS generation in LPS/SNP-stimulated macrophages.^[2,4,26,27]

Buchanania lanzan, Spr. (Anacardiaceae), with reports on anti-inflammatory conditions^[12,15,17] was looked into under *in vitro* conditions in RAW 264.7 murine macrophage cells for the first time. The neutralization of ROS/RNS with inhibition of iNOS has been reported in the present study as a cause for the therapeutic effectiveness of this medicinal plant, bark extract. The therapeutic potential of *B. lanzan* methanol extract (BLM) has been credited to its capacity to neutralize ROS produced under *in vitro* conditions when RAW 264.7 cells were stimulated with bacterial LPS

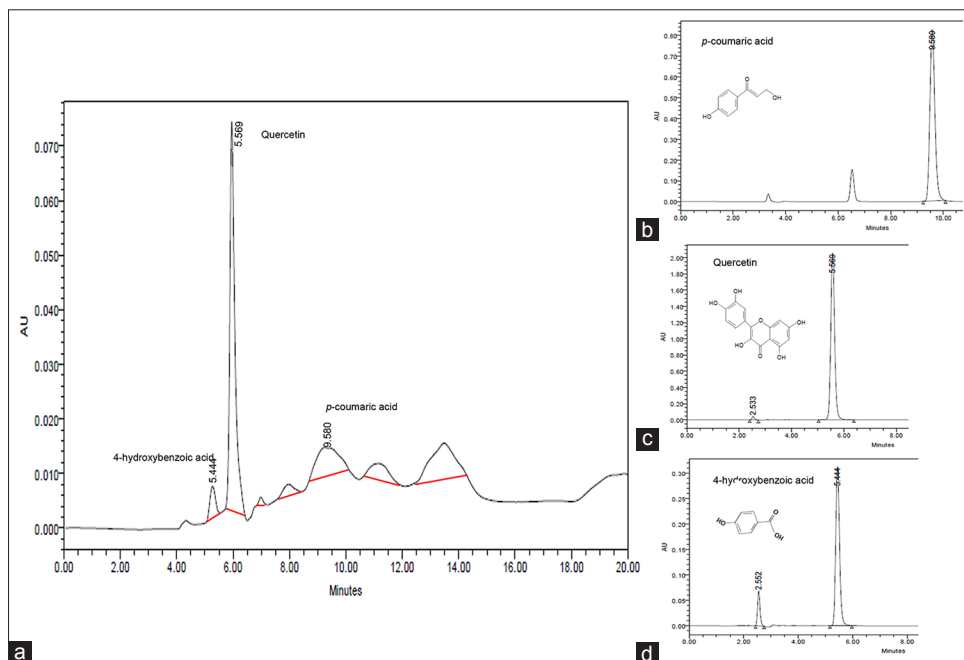


Figure 6: Reversed-phase liquid chromatography-diode array detection (RPLC-DAD) profile of *Buchanania lanzan*. The data was obtained with Waters (Milford, MA, USA) Waters Spherisorb column (C18; 250 × 4.6 mm, 50 mm particle size) using Photodiode array detector. Mobile phase (acetonitrile:Water:Acetic acid: 18:82:2) was used at a flow rate of 1 ml/min to run standards, *p*-coumaric acid (PCA), quercetin and 4-hydroxybenzoic acid (HBA) (200 ppm) with injection volume (10 µL). The RPLC-DAD profile of *B. lanzan* methanol extract (a) was analyzed to contain PCA (b) quercetin (c) and HBA (d) based on their retention time ($n = 3$)

measured using CM-H₂DCFDA, a fluorescent dye to detect intracellular ROS.^[4,19,28] BLM inhibited accumulation of RNS (NO), estimated as nitrite by Griess reagent when the RAW 264.7 cells were stimulated with SNP.^[2]

Reports on over production of NO, point to the iNOS, as a key enzyme.^[29] Minute quantities of NO produced for physiological functions (by endothelial and neuronal NOS) are reported. However, understanding the mechanism of inhibition of excessive formation of NO has been reported to aid in the development of pharmacological strategies to identify possible herbal/natural/bioactive molecule with therapeutic potential. Hence, researchers have focused on the identification of iNOS catalytic activity^[30] for screening natural products with therapeutic potential.

In the present study, the possible mechanism for reduced accumulation of NO was identified to be reduced iNOS protein expression as assessed by the data obtained with Western blot analysis. Further, data obtained from RT-PCR demonstrated the capacity of BLM to inhibit iNOS expression at the level of its transcript accumulation. As the addition of BLM at higher concentrations (200 µg/ml) did not affect cell viability, as assessed by Flow Cytometer analysis and β-actin, in Western blots experimental procedures, points to the fact that neutralization of ROS/RNS production in LPS/SNP-stimulated RAW 264.7 cells was not due to cell death, however was due to therapeutic effect of BLM.

Plant phytochemicals with health benefits have been attributed to health as they cannot be synthesized by humans^[31] and they have been linked to antioxidant activity.^[32] In the present study, RPLC-DAD identified quercetin, HBA and pCA in BLM. Quercetin is reported as one of the strongest natural anti-inflammatory agent.^[33-35] HBA and pCA were shown to exhibit anti-inflammatory and immuno-modulatory activities in rats.^[36,37] The presence of the phytochemicals in the extract could also support the therapeutic property *B. lanzan* bark for mentioned application in The Ayurvedic Pharmacopoeia of India.

CONCLUSION

The results validate the use of *B. lanzan* bark to treat disease conditions. This property was attributed to its ability to neutralize and to quench ROS/RNS produced in RAW 264.7 murine macrophages upon stimulation with LPS/SNP respectively. Further, the ability to inhibit iNOS at protein expression and also at the level of mRNA transcription, *B. lanzan* extract may represent a strong candidate for development of herbal drug.

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