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Anti-inflammatory activity of *Vitex negundo, Boswellia serrata* and *Aegle marmelos* leaf extracts in LPS treated A549 cells

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Abstract

The objective of the study is to evaluate the therapeutic properties of the medicinal plants (*Vitex negundo, Aegle marmelos* and *Boswellia serrata)* for their potential anti-inflammatory activity in LPS treated human lung adenocarcinoma A549 cells. A549 cells were treated with or without LPS and methanolic leaf extracts of the above medicinal plants and the cell viability and nitric oxide production was measured by MTT assay and Griess reaction respectively. Expression of pro-inflammatory cytokines mRNAs (IL-8 and TNF-α) was measured by semi-quantitative RT-PCR. The methanolic leaf extracts of all the 3 plants significantly decreased the LPS induced NO production and pro-inflammatory cytokines expression. Out of the 3 plants tested, *Vitex negundo and Aegle marmelos* leaf extracts at 50 and 100µg/ml show potent anti-inflammatory activity. The novel pharmacological action provide new avenue for the isolation and purification of phytochemicals from *Vitex negundo* and *Aegle marmelos* that may be of therapeutic modality for the treatment of inflammation.
Keywords: Inflammation, LPS, Cytokines, Plant extract, A549

1.Introduction

Inflammation is a complex biological response of vascular tissue to harmful stimuli, pathogens, irritants characterized by redness, warmth, swelling and pain[1,2]. Inflammation is either acute or chronic and acute inflammation may be an initial response of the body to harmful stimuli. In chronic inflammation, the inflammatory response is out of proportion resulting in causing damage to the body. Lung epithelial cells are capable of secreting chemo attractants and proinflammatory cytokines that act as important mediators in both lung defense and inflammation reactions induced by bacterial infection. Many medicinal plants are of historical importance that are used in the treatment and prevention of diseases. Now compounds derived from plants are gaining pharmacological attention as many of them show a wide range of anti-cancer, anti-inflammatory, and anti-aging properties[3-5]. Although, more than 25% of all the prescribed drugs are derived from plants with or without modifications[6,7], still several pharmacologically active plant-derived compounds remain to be explored[8,9]. Natural products of plants are the major players in traditional medicine practices in developing countries and such products, in the form of herbal medicine found their way as alternatives to pharmaceutical drugs in Western world. Many herbal-based

remedies are believed to have a range of biomedical efficacies in the treatment of inflammation, hyperglycemia, arteriosclerosis, and osteoporosis including bone desorption. Many herbal medicines are reported to have a beneficial effect in cardiovascular diseases, immune deficiency, central nervous system disorders, and cancer[10,11]. Many natural compounds are able to suppress the production of inflammatory mediators from activated macrophages and lung cells and therefore can be used as potential anti-inflammatory agents. Hence, our study is aimed to explore and evaluate the anti-inflammatory potential of extracts from *Vitex negundo, Aegle marmelos* and *Boswellia serrata* the traditionally used medicinal plants. The biological activities of the methanolic leaf extracts against inflammation were examined *in vitro* using LPS treated lung adenocarcinoma A549 cells as a model system.

2. Materials and methods

2.1 Plant sample collection

Vitex negundo, Aegle marmelos and *Boswellia serrata* plants were selected based on an ethanobotanical survey and were collected from Charakavana, Bangalore University, Jnana Bharathi campus, Bengaluru, Karnataka and then identified by the Plant taxonomist, Department of Botany, Bangalore University, Bengaluru. The leaves were separated from the main plant and washed thoroughly under the running tap water and washed thrice with sterile distilled water, shade dried and coarsely powdered using blender then used for extraction.

2.2 Preparation of plant leaf extracts

Leaf powder of *V. negundo, A. marmelos* and *B. serrata* were extracted with methanol (1:10 w/v) in orbital shaker for 48 hours at room temperature. Each extract was passed through two layered cheese cloth and the solvent from the extracts were removed from rotary evaporator. The dried methanolic leaf extracts were dissolved in DMSO to make a stock solution of 100 mg/ml and then passed through a 0.2 µM filter (Sartorius) for sterilization and further dilutions were made with filter sterile media and used for cell culture assays. The final concentration of DMSO in the culture medium during the treatment was kept below 0.1% in diluted extracts.

2.3 Chemicals

Lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5, Griess reagent, oligos forward and reverse primers for different cytokines and β-actin were designed (Table 1) and were purchased from Sigma-Aldrich (St Louis, USA). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, Roswell Park Memorial Institute (RPMI 1640) medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypan blue and methanol were purchased from Himedia (Mumbai, India). Superscript reverse transcriptase for RT-PCR was purchased from Invitrogen (CA, USA).

2.4 Culturing of cells

Human lung adenocarcinoma A549 cells were purchased from NCCS (Pune, India). Cells were grown in 25 cm^2 culture flask in RPMI 1640 media with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37° C by passing 5 % CO_2 in an incubator. Flask containing 90–100 % confluent cells were sub-cultured in 96-wells plate or in 6-wells plate for the treatment.

2.5 Cytotoxicity assay

The Cytotoxicity of each of the plant leaf extract on A549 cells was determined by MTT assay as described earlier [12]. Briefly, the cells were seeded into 96-wells plate with a density of $3x10^3$cells/well and incubated in carbon dioxide incubator at 37 °C. After 24 h, the cells were washed with fresh medium and treated with or without different concentrations of plant extracts (25-500µg/ml). The incubation was continued further for 24 h. The cells were washed with PBS and 20 µl of MTT solution (5 mg/ml in PBS) was added to each well. The plates were incubated for 4 h at 37° C. Blue formazan products formed inside the cells were dissolved in DMSO (100µl) and absorbance was measured at 540 nm using PerkinElmer Multimode plate reader (MA, USA). The effect of extracts on cell viability was calculated and represented graphically as % of viable cells compared to control.

2.6 Nitric oxide (NO) assay

Nitric oxide (NO) produced by A549 cells was assayed as nitrite, a stable NO oxidation product as per the protocol described earlier [13]. Cells ($3X10^5$cells/well) were plated in 6 wells culture plate and incubated for 24 h. Cells were treated with or without LPS (1 µg/ml) or leaf extract (100µg/ml) alone or LPS with different concentrations of extract (50 or 100 µg/ml), incubated further for 24 h. Nitrite concentration present in each well in the spent medium was measured by the Griess reaction. To 100µl of culture supernatant 100µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-l-naphthylethylenediamine dihydrochloride in water) was added and the absorbance was measured at 540 nm using Multimode plate reader. Concentration of nitrite in µM of culture supernatant was calculated using standard curve developed with known concentration of sodium nitrite.

2.7 RNA isolation and semi-quantitative RT-PCR analysis

Expression of pro-inflammatory cytokine mRNAs was performed by semi-quantitative RT-PCR. Cells ($3X10^5$cells/well) were seeded into 6 wells plate and incubated in a humidified incubator with the supply of 5% CO_2 at 37 °C. After 24 h, the cells were treated with or without LPS (1µg/ml) and different concentration of plant extract (where ever extract was used the cells were pre-incubated for 1 h either with two different concentrations of extract (50 or 100µg/ml) and then LPS was added and the incubation was continued for 24 h). Total RNA was isolated from control, LPS or LPS with extract treated cells using Trizol reagent as per the manufacturer instructions. Reverse transcription of RNA and PCR analysis was carried out as per the protocol described earlier [14]. In brief, total RNA (2µg) of different samples was reverse transcribed using Oligo (dT) primers and superscript reverse transcriptase. The cDNA was subjected to 30 cycles of PCR in a gradient Eppendorf thermocycler using different forward and reverse primers of pro-inflammatory cytokines (Table 1). The β-actin was used as a positive control and for normalization. Amplified PCR products were analyzed by electrophoresis using 1 % agarose gel. Relative mRNA levels were quantified using image analysis software (ImageJ).

Table 1: Sequence of primers (F: forward and R: reverse) used for the amplification of pro-inflammatory cytokines and β-actin gene.

Gene	Primer Sequence (5' - 3')	Annealing temp. (°C)	Product size (bp)
IL-8	F: AGATATTGCACGGGAGAA R: AACTAGGATTGTTAGTTC	55	671
TNF-α	F: CAAGCCTGTAGCCCATGTTGTAGC R: ATCCCAAAGTAGACCTGCCCAGAC	58	430
β-actin	F: TACCACTGGCATCGTGATGGACT R: TCCTTCTGCATCCTGTCGGCAAT	62	516

2.8 Statistical analysis

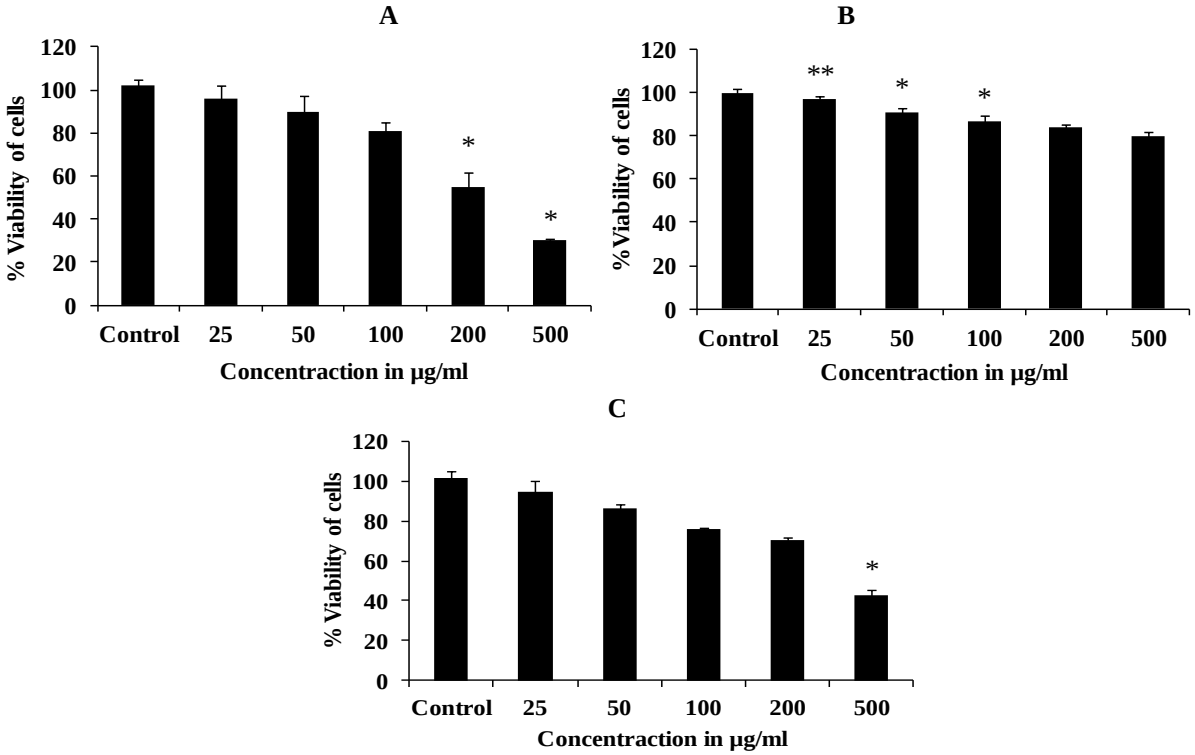
Experimental data shown as mean ± standard deviation from three independent experiments. Statistical analysis was done by Student's t test and one-way ANOVA followed by post hoc tukey test. Values were considered statistically significant if $*P < 0.05$, $**P < 0.005$ compared to control and if $^{\#}P < 0.05$ compared to LPS treated sample.

3. Results

3.1 Effect of methanolic leaf extracts of medicinal plants on the viability of A549 cells

Cells were treated with different concentration of extracts (25-500µg/ml) for 24 h and cell viability was determined by MTT assay. Results show that all the leaf extracts up to 50µg/ml have no significant difference in thecell viability, while cytotoxicity was observed above 100 µg/ml concentration of extracts in a dose dependent manner compared to control (Figure 1).

Figure 1: Effect of methanolic leaf extracts of medicinal plants on the viability of A549 cells

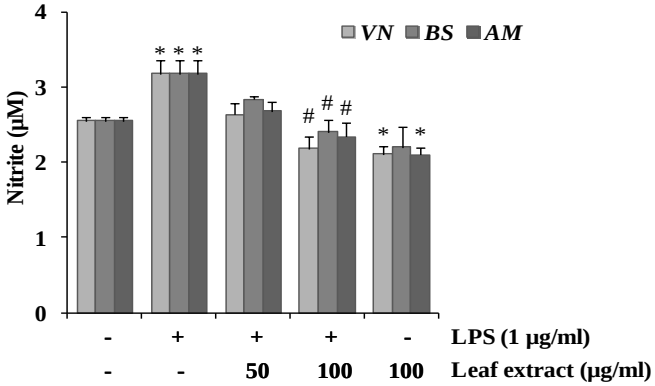


A549 cells were treated with or without different concentration of plant extracts (25-500µg/ml) (A) V. Negundo (B) B. serrata (C) A. marmelos in a 96 wells plate for 24 h and the cell viability was measured by MTT assay. Results were expressed as % of viability of cells as compared to control.

3.2 Effect of plant extracts on LPS induced NO production

Role of NO in pathophysiological conditions of various inflammatory diseases are well known. Half-life of NO is very short and hence measured as nitrite, which was an indicator of NO released from lung adenocarcinoma cells. In our study LPS induced the NO production and release by more than 24 % compared to control. While treatment of cells with LPS and the extracts both at 50 or 100 µg/ml concentrations show dose dependent decrease in nitric oxide production and all most reached the control values with 100 µg/ml of all the plant extracts. Further, the extracts alone treated samples also show significant inhibition of NO production compared to control (Figure 2). The order of anti-inflammatory activity of plant extracts in terms of decrease in the production and release of NO was found to be ***V. negundo>A. marmelos> B. serrata***

Figure 2: Inhibition of LPS induced nitric oxide production in the cell free culture supernatants



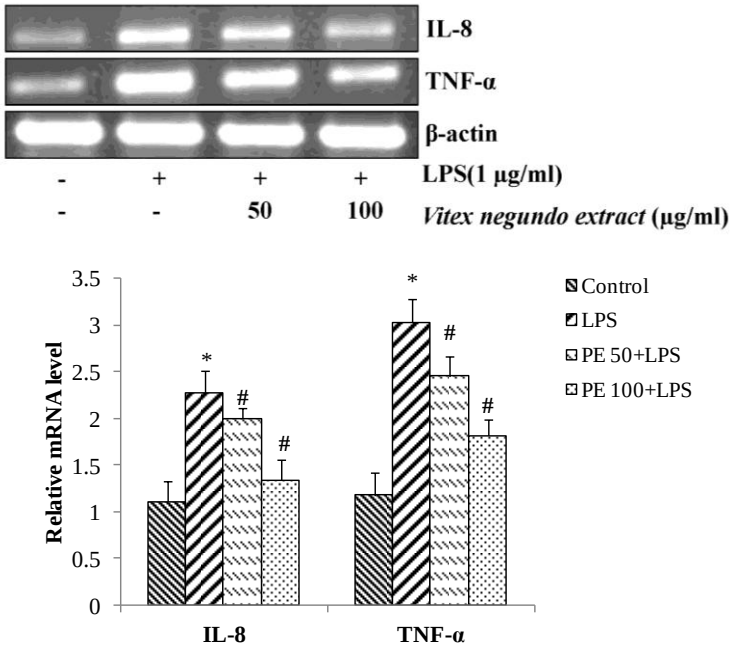
A549 cells were treated with LPS (1µg/ml) or LPS with leaf extracts (50 or 100µg/ml) or extract (100µg/ml) alone for 24 h and the concentration of nitrite in the spent medium was determined by Griess reagent using sodium nitrite

as standard. Data presented as mean ± SD n=3. VN- Vitex negundo, BS- Boswellia serrata and AM- Aegle marmelos.

3.3 Effect of *V. negundo* leaf extracts on LPS induced pro-inflammatory cytokines

A549 cells treated with LPS exhibited an appreciable increase in the mRNA levels with all most 1.93 folds in the case of IL-8 and 2.34 fold in the case of TNF-α (Figure 3). While treatment of cells with *V. negundo* leaf extracts at both 50 and 100µg/ml decrease significantly the mRNA levels of IL-8 and TNF-α in a dose dependent manner compared to LPS treated cells.

Figure 3: Effect of *V. negundo* leaf extracts on LPS induced pro-inflammatory cytokines.

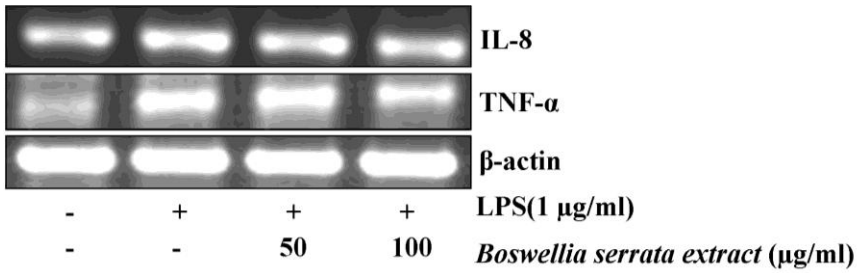


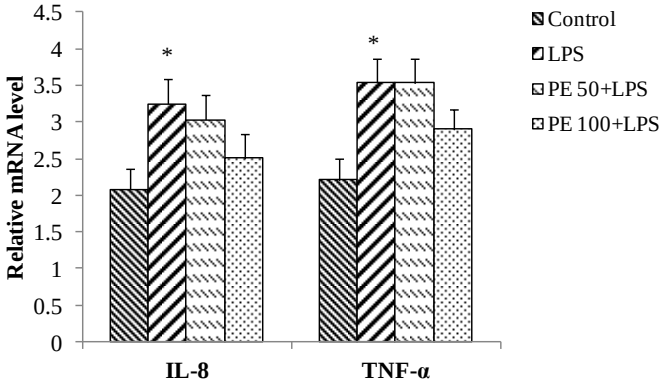
Cells were treated with LPS or LPS with V. negundo leaf extracts (50 and 100µg/ml) and incubated for 24 h. The mRNA levels of IL-8 and TNF-α in the cells were determined using semi-quantitative RT-PCR. β-actin was used as a positive control and for normalization. The bar graphs present below the respective figures are the densitometric analysis of mRNA levels.

3.4 Effect of *B. serrata* leaf extracts on pro-inflammatory cytokines

Treatment of cells with *B. serrata* leaf extracts at both 50 and 100 µg/ml decrease the LPS induced mRNA level of IL-8 in a dose dependent manner. Whereas, TNF-α mRNA decreases only at 100 µg/ml concentration of extract (Figure 4).

Figure 4: Effect of *B. serrata* leaf extracts on LPS induced pro-inflammatory cytokines.



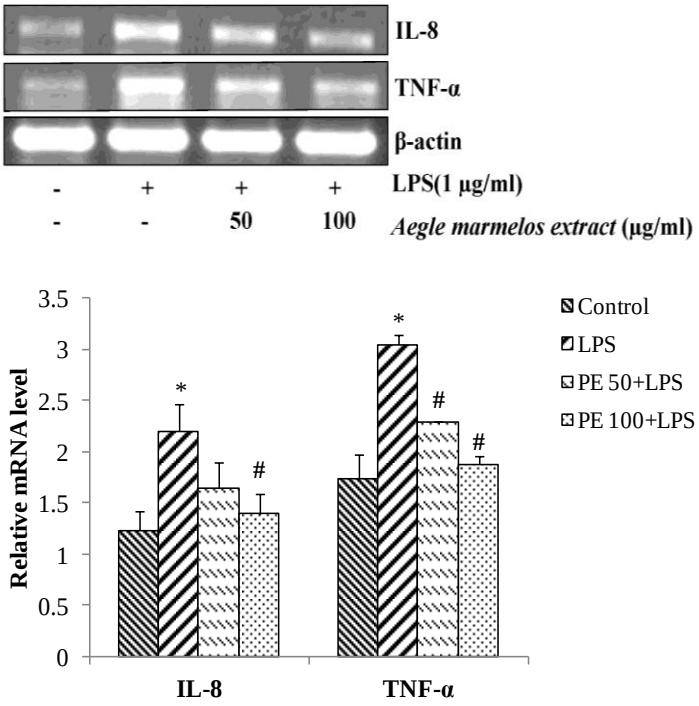


Cells were treated with LPS or LPS with B. serrata leaf extracts (50 and 100µg/ml) and incubated for 24 h. The mRNA levels of IL-8 and TNF-α in the cells were determined using semi-quantitative RT-PCR.

3.5 Effect of *A. marmelos* leaf extracts on LPS induced pro-inflammatory cytokines

The methanolic leaf extracts of *A. marmelos* at both 50 and 100 µg/ml decrease significantly the mRNA levels of IL-8 and TNF-α in a dose dependent manner compared to LPS treated cells (Figure 5).

Figure 5: Effect of *A. marmelos* leaf extracts on LPS induced pro-inflammatory cytokines.



Cells were treated with LPS or LPS with A. marmelos leaf extracts (50 and 100µg/ml) and incubated for 24 h. The mRNA levels of IL-8 and TNF-α in the cells were determined using semi-quantitative RT-PCR.

4. Discussion

Inflammation is body's immediate defensive response to injury caused due to physical or chemical or microbial agents. Inflammation plays an important role in alleviation of various prevalent global diseases, such as rheumatoid arthritis, atherosclerosis and asthma. During an inflammatory response, mediators such as pro-inflammatory cytokines, interleukin IL-1, TNF-α, INF-γ, IL-6, IL-12, IL-18 and the granulocyte–macrophage colony-stimulating factor are released; this response is antagonized by anti-inflammatory cytokines IL-4, IL-10, IL-13, IFN-γ and the transforming growth factor. The nuclear factor-kB (NF-kB), transcription factor, also plays an

important role in the inflammatory response by regulating the expression of various genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzyme cyclooxygenase-2 (COX-2)[15,16]. Use of anti-inflammatory steroids will lyse and possibly induce the redistribution of lymphocytes that cause rapid and transient decrease in peripheral blood lymphocytes. Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant-derived formulations[17-19]. The anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated. Turmeric (*Curcuma longa*), which is traditionally being used for the treatment of rheumatic disorders in ayurveda an Indian system of medicine, exerts both anti-inflammatory and antiatherosclerotic effects[20]. Ginger (*Zingiber zerumbet*) extract and its main active compound, 3-O-methyl kaempferol, inhibited the production of prostaglandin E2 (PGE2) and also production of nitric oxide (NO) by decreased iNOS mRNA expression in RAW 264.7 macrophages. In an *in vivo* model, carrageenan-induced mouse paw oedema was significantly attenuated[21]. Furthermore, ginger is effective in ameliorating arthritic knee pain[19]. Extracts of strawberry (Fragaria ananassa), loquat (Eriobotrya japonica), mulberry (Morus alba) and bitter melon (Momordica charantia) significantly decreased the secretion of IL-6 and IL-1β pro-inflammatory cytokines and up-regulated the secretion of the anti-inflammatory cytokine IL-10 in a prophylactic cell culture model [22]. Plant kingdom has a rich source of many active compounds that lead to the discovery and development of numerous photochemicals used in the treatment of rheumatoid arthritis, atherosclerosis, asthma and many inflammatory diseases [6].

The medicinal plants *Vitex negundo, Aegle marmelos* and *Boswellia serrata* are the common plants grown widely in Western Ghats of India. Phytochemical evaluation of these plant extract reveals the presence of flavonoids, glycosides, saponins, steroids, tannins and polyphenols. In the present study anti-inflammatory activity of above medicinal plants were analyzed based on their ability to inhibit the LPS-induced NO production in A549 cells. The cytotoxic effect of the extract was evaluated on lung cell line using MTT assay to ensure that the anti-inflammatory activity was not due to cytotoxicity effect from the extract. A variety of stimuli, (like LPS, TNF-α, and IFN-γ) induced the production of massive amount of NO in activated macrophages that participate in the pathological acute and chronic inflammatory disorders [23]. Therefore, the drugs that decrease NO production by transcriptional down-regulation of iNOS gene expression, or inhibit the receptor signaling initiated by LPS or depletion of arginine substrate of arginase, have appreciable therapeutic effect in the treatment of all major inflammatory, infectious diseases and some neurological disorders [24-26]. Our study confirms that the plant extracts of *Vitex negundo, Aegle marmelos* and *Boswellia serrata* significantly inhibited LPS induced NO production in lung cells.

The approaches on the use of new herbal products for the treatment of inflammatory diseases by inhibiting inflammatory cytokines is attracting many scientists and becoming an important area of investigation because of steroidal treatment associated complications. The production of TNF-α and pro-inflammatory interleukins such as IL-1β and IL-12 is a crucial part of the immune response to many inflammatory stimuli. For instance, overproduction of these mediators were detected in both acute (septic and hemorrhagic shock) [27], as well as chronic (rheumatoid arthritis, atherosclerosis) inflammatory disorders [28-30]. Recently, new approaches on the use of herbal products for the treatment of inflammatory diseases by inhibiting TNF-α and interleukins the inflammatory cytokines, attracted many investigators because of their less associated complications [31]. IL-8, act as an important mediator of inflammation of CXC chemokine family recruits neutrophils into inflamed tissue [32]. Enhanced synthesis of IL-8 has been shown in the intestinal mucosa of patients suffering from inflammatory bowel disease (IBD) [28]. Our results show that all the plant extracts could remarkably suppress the LPS induced mRNA levels of IL-8 and TNF-α cytokines in a dose dependent manner. The *V. negundo* and *A. marmelos* extract at 100 µg/ml exhibited highest inhibitory activity towards LPS induced IL-8 and TNF-α cytokines. Based on NO inhibition assay and mRNAs expression of pro-inflammatory cytokines the phytochemicals derived from *Vitex negundo* and *Aegle marmelos* leaf extracts has a greater potential as an anti-inflammatory molecules. The present study provides an important basis for further investigation into the isolation, identification, characterization of active components responsible for anti-inflammatory property and in elucidation of possible mechanism of anti-inflammatory activity by plant extracts.

5. Conclusion

Our study demonstrated that the phytochemicals present in methanolic leaf extracts of *Vitex negundo* and *Aegle marmelos* inhibit the production of NO, IL-8 and TNF-α. The result further gives the authenticity of anti-inflammatory property of leaf extracts of above medicinal plants. Further, the extracts derived from *Vitex negundo* and *Aegle marmelos* may provide a safe and effective treatment options for a wide variety of inflammation-mediated diseases.

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