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Original Article

Inhibition of Pro-Inflammatory Cytokine TNF-α by *Boerhaavia diffusa* (L.) in Liposaccharide stimulated Human THP-1 cells

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ABSTRACT: The production and release of proinflammatory cytokines such as Tumor necrotic factor-α (TNF- α), Interleukin-1β and Iinterleukin-6 from monocytes/macrophages play critical role in the pathogenesis of chronic inflammatory diseases and immune response. *Boerhaavia diffusa* L is a medicinal plant in the Indian traditional system known as *Rasayana* (Indigenous plants with immunomodulatory activity) and an important source of immunomodulators. In present study, the anti-inflammatory activity of herbal immune modulator *Boerhaavia diffusa* L was studied by evaluating the inhibition of Lipopolysaccharide stimulated TNF-α secretion using human Monocytic THP-1 cells. Differentiation of the human monocytic cell THP-1 into macrophage-like cells was induced by exposure of the cells to Phorbol Myristate Acetate. When cells were pre-incubated with Methanolic extract of *Boerhaavia diffusa* L for 4 hrs and then stimulated with lipopolysaccharide for 12 hrs, secretion of tumor necrosis factor- α was significantly decreased compared to control. The present study has shown that the *Boerhaavia diffusa* L inhibits the production and release of proinflammatory cytokines, TNF-α from monocytes/macrophages which play critical role in the pathogenesis of chronic inflammatory diseases and hence this could be the probable mechanism, by which this immunomodulatory herbal plant extracts possess anti-inflammatory activity.

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INTRODUCTION

Boerhaavia diffusa L, belonging to the family of the Nyctaginaceae, is mainly a diffused perennial herbaceous creeping weed of India (known also under its traditional name as Punarnava). The root, leaves, aerial parts or the whole plant of *Boerhaavia diffusa* have been employed for the treatment of various disorders in the Ayurvedic herbal medicine. It is scientifically documented for its immunomodulatory [1] and immunosuppressive [2, 3] activity. Cytokines are biologically highly potent peptides endogenously synthesized upon stimulation and are involved in numerous cellular processes including inflammation and various immunological responses. The production and release of proinflammatory cytokines such as Tumor necrotic factor-α (TNF-α), Interleukins (IL-1β and IL-6) from monocytes and macrophages play critical role in the pathogenesis of chronic inflammatory diseases [4].

TNF-α is the most widely studied pleiotropic cytokine of the TNF superfamily. It is a 17 kDa protein that belongs to cytokine family of proteins is and it mediates variable numbers of immune-inflammatory reactions ranging from activation and differentiation of immune-inflammatory cells to expression of various adhesion molecules on various cell types and finally involved in expression of major histocompatibility complex [5].

The TNF-α secretion is thought to act via several nuclear factors and nuclear factor kB (NF-kB) was found to have an important role in the regulation of TNF-α gene transcription. NF-kB is an inducible eukaryotic transcription factor which exists in the cytoplasm of most cell types and plays a key role in immune and inflammatory response. Many stimuli activate NF-kB, including pro-inflammatory cytokines, oxidants, viruses and activators of protein kinase C [6].

The THP-1 cell line, a human monocytic leukaemia cells, has been widely used in *in vitro* mechanistic studies evaluating anti-inflammatory activities [7, 8]. Activated macrophages elicit many of their effects via the secretion of soluble inflammatory mediators.

Lipopolysaccharide (LPS) derived from gram-negative bacteria is the most potent activator of the macrophage secretory response. TNF-α is one of the earliest major proinflammatory mediators secreted by macrophages when stimulated with LPS *in vivo and in vitro* [9]. TNF-α has been implicated in the pathogenesis of several inflammatory diseases, including asthma [10] and its production has been suggested as a possible target for therapy in many diseases.

We undertook a study to evaluate the anti-inflammatory potential of Methanolic extract of *Boerhaavia diffusa* L (MEBD) in human monocytic THP-1 cells using a Phorbol Myristate Acetate (PMA)-induced cell differentiation model.

MATERIALS AND METHODS

Propagation of human acute monocytic leukemia cell line (THP-1):

Human acute Monocytic leukemia cell line (THP-1) was procured from National Centre of Cell Sciences (NCCS, Pune, India). The cell line was cultured in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, Himedia) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Himedia), 2 mM glutamine (Himedia), 1 mM sodium pyruvate, 2 g/l sodium bicarbonate, 0.05 mM mercaptoethanol, 1% 100 U/ml penicillin (Himedia) and 1% 100 U/ml streptomycin (Himedia) in 25 cm^2 culture flask (Falcon, USA) and incubated at 37 °C in CO_2 (5%) incubator.

THP-1 cells were treated with Phorbol 13 Myristate 12 Acetate (PMA) for 24 hrs to convert it to THP-1 macrophage attached cells. After incubation, nonattached cells were removed by aspiration, and the adherent cells were washed with culture medium three times. After attaining 80% confluency, $1x10^6$ of cells/ml were seeded in each well of the plates with complete medium and incubated at 37° C under CO_2 (5%) incubator. The growth medium was changed every 2 days. THP-1 derived macrophage cells were cultured until complete confluency had reached.

Assessment of Viability of THP-1 cells:

Viability of THP-1 cells was assessed by trypan blue assay. Trypan blue was used to stain the cells after end of each treatment. Cells were aliquot in 0.5 ml microfuge tube and equal amount of trypan blue was added to it. Slides were prepared and observed under microscope. Number of stained (dead) vs number of unstained (live) was counted manually.

MEBD and LPS treatments to THP-1 macrophage cells:

The THP-1 derived macrophages were separately pre-challenged with different concertation of MEBD.

Cells treated with Diclofenac 250 µg/ml were used as positive control and cells without treatment were used as negative control. Cells were further incubated with 100 ng/ml LPS for 12 hrs in fresh culture medium for stimulation.

Detection of secreted TNF-α in culture supernatants:

TNF-α production was chosen as the outcome variable for studying the anti-inflammatory effect. Human TNF-α was quantified by an enzyme linked immunosorbent assay (ELISA) using TNF-α kit (GenXbio) according to the manufacturer's instructions. 96 well ELISA plate pre-coated with TNF-α monoclonal body was used. Briefly, Cell culture media centrifuged at 1500 rpm for 10 mins at 4 C. Supernatants were collected carefully. PBS was used to dilute the cell suspension to cell concentration of approximately 1 million/ml.

Cells degraded through repeated freeze-thaw cycles to release interior components. Cells were centrifuged at 2000-3000 rpm for approximately 20 mins at 4 °C. Supernatants were collected carefully. 40 µl diluted sample, 10 µl of TNF-α antibody and 50 µl of streptavidin-HRP were added in sample wells and incubated at 37 °C for 60 mins.

Plates were washed with washing buffer 5 times and 50 µl of chromogen reagent A and B were added to each well and incubated for 10 mins at 37 °C for color development. 50 µl of stop solution was added to each well to terminate the reaction and read at 450 nm. TNF-α concentration was calculated by TNF-α standard curve. The sensitivity for TNF-α detection is 1.52 ng/L and assay range is 3.1 to 899 ng/L.

Statistical analysis:

Each experiment was conducted with three independent cell cultures and was triplicated for each treatment. According to standard concentrations and corresponding absorbance (OD) values, linear regression equation of the standard curve was derived.

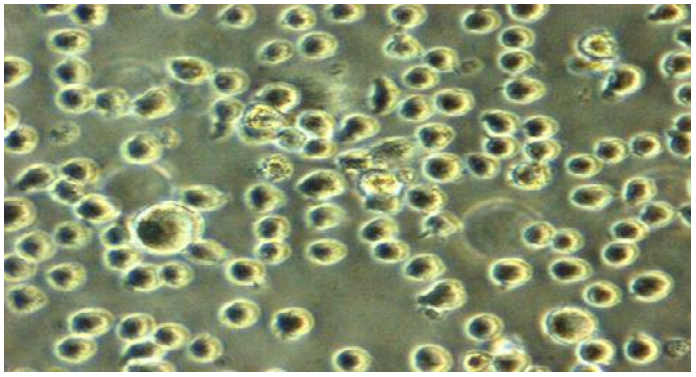
Using the standard curve, the concentration of TNF-α was calculated based on the OD of the sample. Data for supernatant cytokines were expressed as percentage of inhibition relative to the control.

Results:

PMA-induced differentiation of THP-1 cells:

THP-1 cells are premonocytes, committed to the monocytic cell lineage. They grow in suspension and do not adhere to the plastic surfaces of the culture plates (**Figure 1a**). For the induction of terminal differentiation to macrophage-like cells, THP-1 cells were cultured in the presence of PMA for 24 hrs.

After 24 hrs of culture with 50nM PMA, the cells adhered to the dish bottom and had the morphological characteristics of macrophages (**Figure 1b**).



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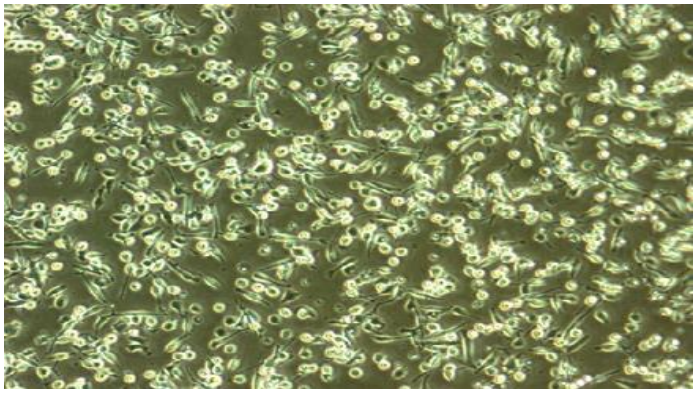

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Fig. 1: Induction of differentiation in THP-1 cells by PMA. THP-1 cells were incubated for 24 h without (a) or with (b) PMA (50 nM)

Cell viability Assay:

The results of the cell viability assay showed no significant decrease in viable cells after the treatment of the compound when compared to control. Thus, there were no detectable adverse effects of sample treatments on cell viability.

Inhibition of TNF-α by MEBD:

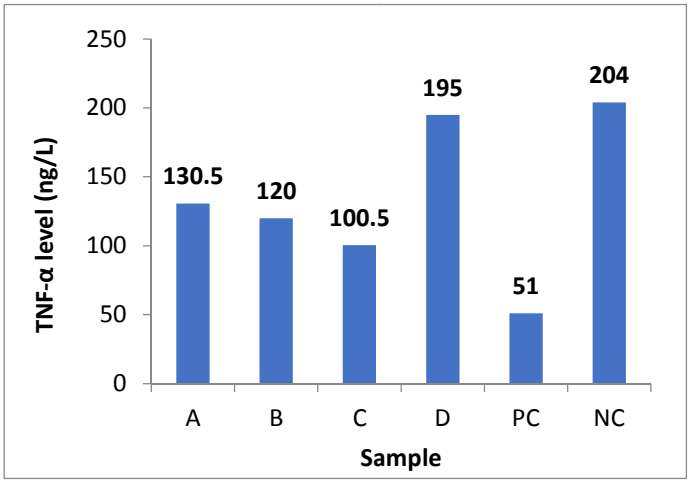
THP-1 cells were pretreated for 4 hr with various concentration of sample or vehicle control. The results for TNF-α release from THP-1 cells pretreated with various sample are shown in **table 1**.

Table 1: Effect of various concentration of MEBD on TNF-α secretion in LPS- stimulated THP-1 cells

Sample	TNF-α level (ng/L)*
MEBD 50µg	130.5
MEBD 100µg	120
MEBD 250µg	100.5
MEBD 750µg	195
Diclofenac 250µg/ml (Positive control)	51
Negative control (NC)	204

***The results are the mean (SD) of 3 different experiments run in triplicate.

The results revealed that MEBD inhibited TNF-α release significantly at all concentration used in the study (**figure 2a**). At 50 µg, 100 µg, 250 µg and 750 µg, MEBD inhibited the release of TNF-α by 36.03, 41.18, 50.74 and 4.41% respectively. TNF-α secretion was maximum inhibited at concentration 250 ug (50.74% inhibition) when compared to control (**figure 2b**).



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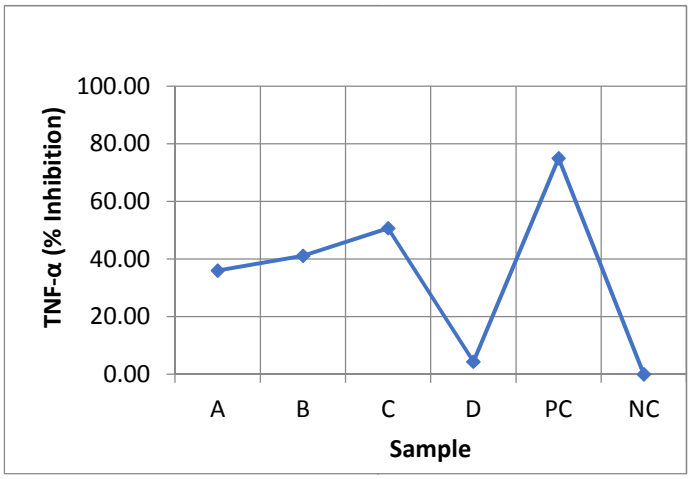

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Figure 2A: Effect of various concentrations of MEBD on TNF-α level- 50µg (A), 100µg (B), 250µg (C), 750µg (D), positive control (PC), Negative control (NC)

Figure 2B: Percentage inhibition of TNF-α by various concentration of MEBD concentrations- 50µg (A), 100µg (B), 250µg (C), 750µg (D), positive control (PC), Negative control (NC)

Data is representative of three independent experiments; Theses data suggest that the MEBD exert an anti-inflammatory effect by decreasing the inflammatory cytokine production.

DISCUSSION

The anti-inflammatory effects of many herbal drugs are now shown to be attributed to their potent suppressive effect of TNF-α, IL-1β and/or IL-6 release. Several medicinal plants used in the Indian traditional system known as Rasayana (Indigenous plants with immunomodulatory activity) and their active components have been shown to be an important source of immunomodulators [4]. In most cases the validity of these claims has not been scientifically tested. This present study demonstrates that *Boerhaavia diffusa* L, reduced the LPS stimulated production of TNF-α at low concentrations. TNF-α is particularly important for both local and systemic inflammation, and it is a potent and well-studied inducer of NF-kB. Numerous studies using gene targeting and inhibitors of NF-kB have established that NF-kB plays a causative role in inflammatory processes. The immediate targets of NF-kB-dependent pro-inflammatory cytokines, such as TNF-α, tend to be receptors that activate NF-kB. The observation that some cytokines regulated by NF-kB, such as TNF-α and IL-1 b can also activate NF-kB expression indicates that this may form the basis for the persistence of the chronic inflammatory process such as in asthma [10]. Considering that the levels of TNF-α is increased in inflammatory diseases like Asthma, in this study, a method to investigate immuno-modulating effects of *Boerhaavia diffusa* L was developed based on the THP-1 cells. THP-1 cells were selected for this study as they have been widely used as an *in vitro* model of human monocytes and macrophages in mechanistic studies of inflammatory diseases [11, 12].

Results of this current study support earlier research about the anti-inflammatory potential of *Boerhaavia diffusa* L [13]. This is important in advancing the understanding about the mechanism by which it has shown the anti-inflammatory effects which is attributed to their potent suppressive effect of TNF-α release. The results obtained in the present study have shown that MEBD inhibits TNF-α secretion in LPS stimulated in human monocytic THP-1 cells.

Hence, the probable mechanism, by which this immune-modulatory herbal plant extracts possess anti-inflammatory activity, might be attributed to their potent suppressive effect on proinflammatory cytokine release such as TNF-α and thereby NF-kB which is involved in immune system and thereby suggesting its possible use in inflammatory diseases such as Asthma. However, further detailed studies are required to establish its clinical relevance/therapeutic potential.

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