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RESEARCH ARTICLE

INHIBITION OF NITRIC OXIDE AND PROINFLAMMATORY CYTOKINES BY AQUEOUS EXTRACT OF *TERMINALIA ARJUNA* IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Despite its beneficial role in host defense mechanisms, enhancement of nitric oxide (NO) production and pro-inflammatory cytokines from human peripheral blood mononuclear cells has been implicated in several inflammatory diseases. To clarify the mechanisms of the inhibition of NO as well as pro-inflammatory cytokines from the aqueous extract of stem bark of *Terminalia arjuna*, we evaluated whether the aqueous extract could modulate the production of NO and pro-inflammatory cytokines from human peripheral blood mononuclear cells. The results indicate that the aqueous extract of *Terminalia arjuna* were effective inhibitors of lipolysaccharide (LPS)-induced NO and pro-inflammatory cytokines in human peripheral blood mononuclear cells. To test the inhibitory effects of aqueous extract of *Terminalia arjuna* on Th1 type of cytokines, we performed ELISA assays for TNF-alpha and IFN-gamma in LPS-stimulated human peripheral blood mononuclear cells. In these assays, aqueous extract of *Terminalia arjuna* produced dose-dependent decreases in the production of TNF-alpha and IFN-gamma.

Key words: *Terminalia arjuna*, nitric oxide, PBMC, lipopolysaccharide

INTRODUCTION

The use of natural products as anti-inflammatory, anti-cancer as well as anti-oxidant agents has a long history that began with folk medicine. Now a day, researchers focused as well as attention on medicinal plants used by indigenous people is the most efficient way to identify as well as synthesized or purified the bioactive substance from the medicinal plants [1, 2]. Although the exact mechanisms of this protective immune response have not been determined, a number of natural product candidate responses at the transcriptional and protein level have been identified. During a search for the medicinal plants to showed the inhibition of nitric oxide production as well as pro-inflammatory cytokines from human peripheral blood mononuclear cells [2, 3].

Terminalia arjuna also known as Arjuna (deciduous tree found throughout India growing to a height of 60-90 feet and it belongs to the family *Combretaceae*) is one of the species of *Terminalia* [4]; other two species are *Terminalia Billerica* and *Terminalia chebula* [5]. It is naturally found on the banks of the lakes, rivers and streams [6]. The main constituents which are present in the *Terminalia* plant are tannins, cardenolide, triterpenoid saponins (arjunic acid; arjunolic acid; arjungenin; arjunglycosides), flavonoids (arjunone; arjunolone; luteolin), gallic acid, ellagic acid, oligomeric proanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc, and copper. Generally, the bark of *Terminalia arjuna* has been used in India for more than 3000 years ago primarily and has been commonly used for disorders of the heart and blood vessels including high blood pressure and cholesterol [6, 7, 8, 9]. In this plant, it contains the antioxidant properties which is similar to Vitamin E and is able to strengthen the muscles of the heart and is able to maintain the heart functioning properly. Generally, the plant Arjuna is used for the treatment of coronary artery disease, heart failure, edema, angina pectoris etc. Similarly, the bark of the plant possesses diuretic, increased in the level of prostaglandin and also be able to showed coronary risk factor modulating properties and also considered as beneficial in the treatment of Asthma [10, 11]. Now a days in India, the number of heart patient cases are increasing and a number of medications starting from aspirin to sophiscated open heart surgeries are now common in developed countries but most of them believed on Ayurveda traditional system of medicine and our ancient medical scientists have already mentioned the cardio protective and heart muscle strengthening properties of Arjuna plant [12]. In Hindu religion, the

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leaves and flowers of this tree are offered to the Lord Vishnu and Lord Ganpati on the several religious occasions [12, 13]. Therefore, numbers of pharmacological studies are available on the various activities and medicinal importance of this leaves, stem and root of this plant worldwide. Therefore, this study was designed to investigate the inhibition of nitric oxide and pro-inflammatory cytokines by using aqueous extract of bark stem of *Terminalia arjuna* plant.

MATERIALS AND METHODS:

Plant material and preparation of aqueous extract

Terminalia arjuna was collected in July 2014 from the garden of Vidya Pratishthan's School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. The materials used for the aqueous extraction of leaves were properly washed and dried at room temperature and macerated with liquid nitrogen and finely powdered into a fine powder. The plant leaves powder was grinded in phosphate buffered saline by continuously stirring. The aqueous extract of plants was centrifuged at 5000 rpm for 15 minutes. The supernatants were filtered through Whatman filter paper and the supernatant was collected and was used for various immunological assays.

High performance thin layer chromatography (HPTLC) profile of the stem bark aqueous extract of Terminalia arjuna

The metabolites of the aqueous extract were determined through HPTLC. The plates used for HPTLC (10 x 10 cm) were purchased from Merck and the solvents (used in mobile phase) from Qualigens and detect its wavelength at 366 nm. The stock solution of aqueous extract of stem bark of *Terminalia arjuna* was prepared for HPTLC studies and dissolved the 2 g of weighed stem bark in phosphate buffered saline in a final volume of 20 ml. The aqueous extract showed the presence of saponin, terpenoids and flavonoids in the phytochemical profile of *Terminalia arjuna*. The retardation factor (Rf) values of terpenoids (0.96) and saponin (0.36, 0.48 and 0.79).

Human blood samples and determined the cytotoxicity assay of PBMC

Informed consent letter was obtained from all subjects or their guardians prior to blood collection only if the participants are healthy and does not show any signs or symptoms of asthma exacerbation or respiratory infection or any other illness. PBMC (10^5 cells/ml) were separated by Ficoll–Hypaque gradient centrifugation and plated in 96-well plates were pre-incubated for 24 h and then treated with LPS (1 µg/ml) plus serial dilutions of aqueous extract at 37°C for 24 h. The plates were centrifuged at 1800 rpm for 10 minutes and then the supernatant (50 µl) was collected for the estimation of nitric oxide and cytokine profile and then add equal volume of fresh medium. Add 10 µl of MTT solution (5 mg/ml) were added to each well and then incubated for 4 h. Again, the plates were centrifuged at 1800 rpm for 5 minutes and the supernatant was discarded. Add 100 µl of DMSO solution to the formazon crystals and the absorbance was evaluated in an ELISA reader at 570 nm [14]. All experiments were performed in triplicate.

Determination of nitric oxide (NO) production

After preincubation of PBMC (10^5 cells/ml) with or without LPS (1 µg/ml) for 24 h, the quantity of nitrite accumulated in the culture medium (RPMI containing 10 % fetal bovine serum) was measured as an indicator of nitric oxide (NO) production. Briefly, 50 µl of PBMC cell culture medium was mixed with 50 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated the 96 well plates at room temperature for 10 minutes, and the absorbance at 540 nm was measured in a microplate reader. The fresh culture medium (RPMI containing 10 % fetal bovine serum) was used as a blank. The nitrite quantity was determined from a sodium nitrite standard curve. All experiments were performed in triplicates [15].

Measurement of pro-inflammatory cytokines (TNF-gamma TNF alpha) production

The aqueous extract was diluted with cell culture medium containing PBMC and incubates the plate for 48 h. The plates were centrifuged at 2500 rpm for 10 minutes and the supernatant was collected for the estimation of Th1 type of cytokines. The effect of the aqueous extract on pro-inflammatory cytokine (TNF alpha and IFN-gamma) production in LPS-treated PBMC and was determined by ELISA as described in the manufacturer's instructions (BD Optia kits). All experiments were performed in triplicate [16, 17].

Statistical analysis

All values are mentioned as Mean ± S.E. Data is represented by One way ANOVA test (Boniferroni multiple comparison test).

RESULTS:

Cytotoxicity assay

The effect of aqueous extract of *Terminalia arjuna* on cytotoxicity assay as shown in **Figure 1**. PBMC were cultured with different concentration of aqueous extract and incubated the plate for 48 h and determined the proliferation assay using MTT. The results showed that the aqueous extract showed dose dependent decrease in the proliferation assay.

Effect of aqueous extract on nitric oxide (NO) estimation

The effect of NO was observed in the cell culture medium of PBMC containing different concentration of aqueous extract as shown in **Figure 2**. PBMC were cultured with or

without with LPS (10 µg/ml) and the contents of the indicated nitric oxide (NO) were measured in the supernatants as a function of macrophage activation. There was a significant decreased in NO production elicited by the aqueous extract.

Effect of aqueous extract on Th1 (IFN-gamma and TNF alpha) cytokines

The effect of aqueous extract of *Terminalia arjuna* on PBMC in Th1 type of cytokines (IFN-gamma and TNF alpha) as shown in **Figure 3.** At low concentration of aqueous extract, there is moderate increase in IFN-gamma and TNF alpha but at high doses, there is sudden decline in the level of Th1 type of cytokines.

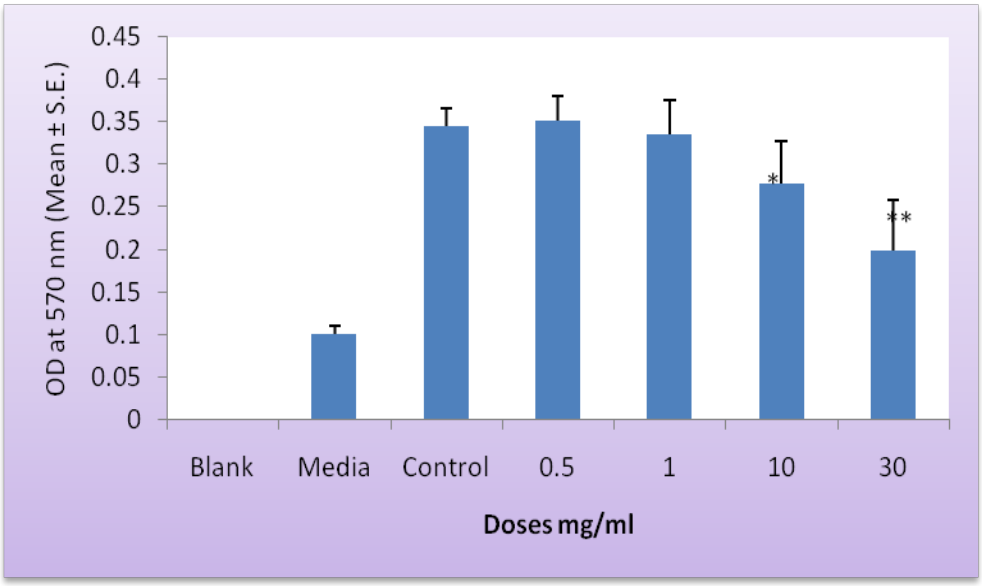

Figure 1: Cytotoxicity assay. PBMC (10^5 cells/ml) were cultured with variable concentration of aqueous extract of stem bark of *Terminalia arjuna*. Cells were incubated for 72 h and proliferation was measured by MTT assay. Values are expressed as Mean ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). **P < 0.05; **P < 0.01; ***P < 0.001*

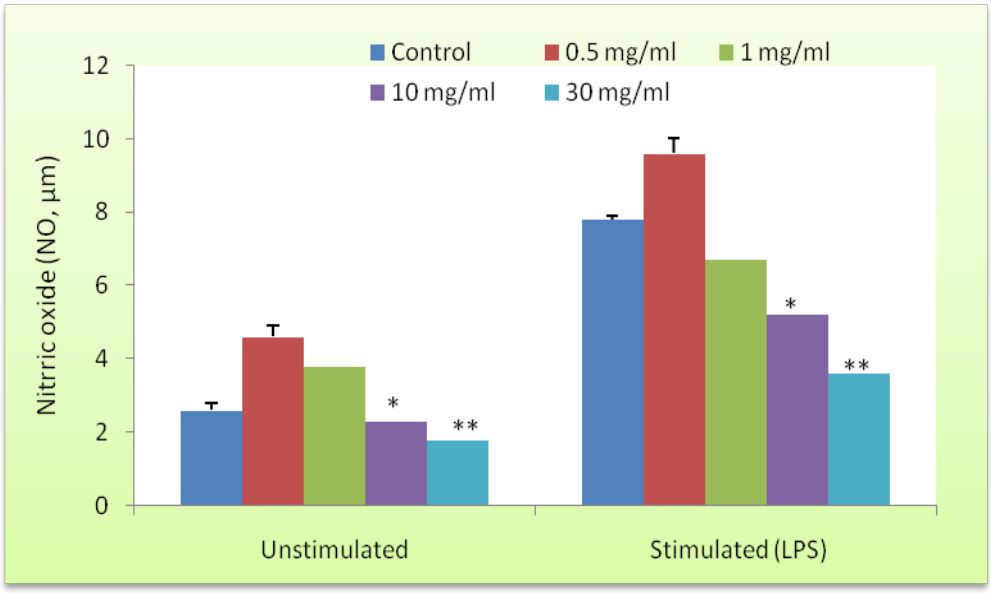

Figure 2: Production of nitric oxide (NO) by human peripheral blood mononuclear cells (PBMC). The supernatant nitrite concentration was determined by Griess reagent after the 24 h culture of cells in presence of aqueous extract of stem bark. Values are expressed as Means ± S.E. and The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). **P < 0.05; **P < 0.01; ***P < 0.001*

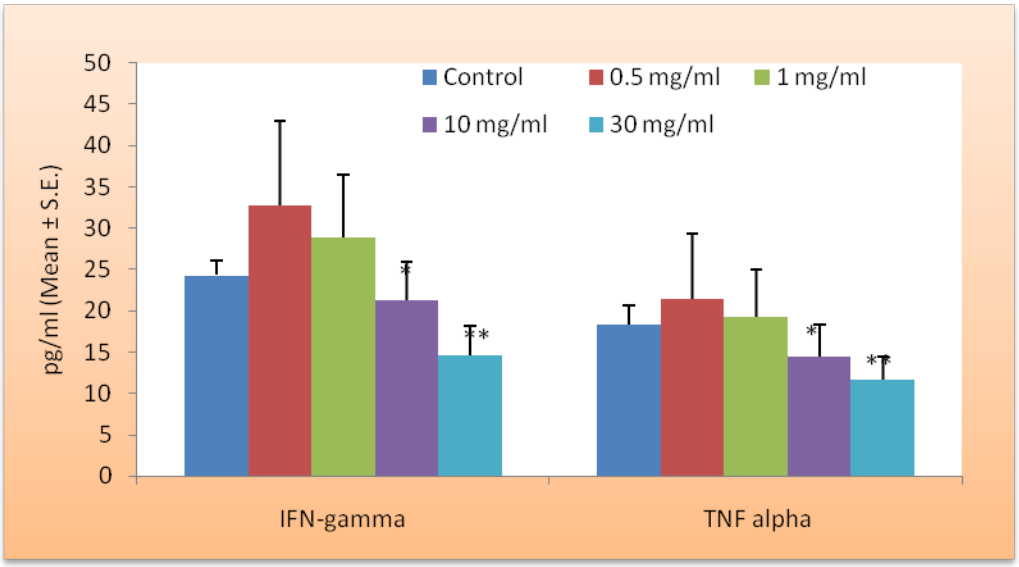

Figure 3: Effects of aqueous extract on in vitro secretion of cytokines (IFN- gamma and TNF alpha) by human peripheral blood mononuclear cells (PBMC). Concentration of cytokines was determined by ELISA in supernatants of cells cultured for 48 h. Values are expressed as Means ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). **P < 0.05; **P < 0.01; ***P < 0.001*

DISCUSSION:

The objective of this study is to investigate the *in vitro* production of NO and cytokines from human peripheral blood mononuclear cells (PBMC), in order to validate the potential use of NO assay as a tool in screening for intrinsic immunostimulatory or inhibitory effects of compounds on the immune system. The current investigation determines whether a correlation exists between the presence of altered levels of NO production by peripheral blood mononuclear cells (PBMCs) in treated aqueous extract of *Terminalia arjuna* and control samples. Venous blood of human was collected from the healthy volunteers. PBMCs were separated by means of gradient centrifugation, diluted to 10^5cells per ml and cultured with LPS, at doses of 0.5, 1, 10 and 30 mg/ml, was used to stimulate NO production. The assay was performed from the PBMC culture supernatant to determine the levels of NO produced by PBMCs at 24 h. When stimulated by LPS there was an increase in NO production in the PBMCs cultured from control but there is decline in the level of NO in aqueous extract, as compared to control. However, the data demonstrate a significant decrease in the nitric oxide production in the aqueous extract as compared to control group. It may be suggested that the NO assay using PBMC provides relatively accurate as well as reliable information regarding immunostimulatory or inhibitory activity of treated as well as control samples in human immune system with respect to cytokine activation. It should be considered as a preliminary or initial screening for stimulation of cytokine production by human PBMC. The main advantage of this initial screening is its sensitivity, general feasibility, low cost and possibility of large-scale performance. Since production of NO and cytokines from PBMC are more sensitive to the activation signal of LPS, the immunoscreening tests should always be accompanied by careful examination of the samples for possible contamination with LPS. It has been suggested that production of NO may depend on the cell types and their species origin [18,19], different cells having obviously different requirements for signal transduction pathways [20]. In NO production, there is hypothesis that there is an extensive knowledge showing that high-output NO production by cells is determined by a number of cytokines. A direct NO-stimulatory function is possessed by IFN-gamma that triggers NO production on its own [18, 19]. Although, other cytokines may occasionally stimulate NO by themselves, they usually provide an additional signal for activation of NO by IFN-gamma. It concerns mainly TNF alpha which plays a central role in regulation of hepatic iNOS activity [20]. Cytokines such as TNF alpha and IFN-gamma are pro-inflammatory both in vitro and in vivo. In particular, IFN-gamma is a major pro-inflammatory cytokine that is mainly released by macrophages and is believed to play a considerable role in the pathophysiology of hormonal immune system.

CONCLUSION:

In the present study, we found that the aqueous extract of *Terminalia arjuna* significantly inhibited the production

of the pro-inflammatory cytokines. Further investigations will focus on the in vivo assessment of the biological activity of these aqueous extracts and on the chemical identification of the major active components responsible for the anti-inflammatory activity in the efficacious extracts.

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