



## Anti-inflammatory and immunosuppressive activities of flavonoids from medicinal plants

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### ABSTRACT

**Introduction:** Medicinal plants have been used to treat various immunological diseases. Nitric oxide (NO) including proliferation and CD14 monocyte surface marker has played an important regulatory role in various types of inflammatory processes. This study was aimed to evaluate or scrutinize the effect of flavonoid extracted from the leaves of *Santalum album*, *Butea frondosa* and *Emblca officinalis* for determining their anti-inflammatory and immunosuppressive activity on human peripheral blood mononuclear cells (PBMC) using hepatitis B vaccine antigen (20 µg/mL; 10 µl).

**Methods:** Flavonoids extracted from these medicinal plants were prepared (0.5–10 mg/mL; 50 µl) and their effects on human PBMC proliferation were examined using HBsAg. The NO production including CD14 monocyte surface marker was also estimated.

**Results:** All these flavonoids at higher doses demonstrated a significant decrease in proliferation, NO production and CD14 surface marker. This inhibitory effect was seen after 48 hours of treatment.

**Conclusion:** These results may indicate the presence of anti-inflammatory and immunosuppressive activity in these medicinal plants.

### Implication for health policy/practice/research/medical education:

Flavonoids extracted from the leaves of *Santalum album*, *Butea frondosa* and *Emblca officinalis* demonstrated a significant decrease in proliferation, nitric oxide (NO) production and CD14 surface marker, after 48 hours of treatment which may indicate positive anti-inflammatory and immunosuppressive properties of these components.

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### Introduction

Flavonoids are inherent part of human and animal diet however, these are not synthesized within the body of animals including human. In addition, these flavonoids are of plant origin but these are found in the highest concentration in the human diet especially in the form of flavonols; flavones and isoflavones (1,2). The main function of flavonoids is responsibility for plants colour, taste, obstruction of fat oxidation and protection of vitamins and enzymes. This group of natural compounds of medicinal plant products i.e. flavonoids has phenolic structures and these are based upon fifteen-carbon skeleton, incorporating two benzene rings linked via heterocyclic pyran ring (3,4). These flavonoids are ubiquitous in nature and categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. More than 4000 flavonoids and their derivatives have already been identi-

fied out of which many of them are reported and isolated from fruits, vegetables and beverages (tea; coffee; beer; wine and fruit drinks) (5). Recently, scientists have focused on secondary metabolites especially flavonoids and have shown considerable interest because of their beneficial effects on our immune system as well as antiviral (6); anti-allergic (7); anti-inflammatory (8); anti-tumor and antioxidant (9,10) activities. According to the literature, these flavonoids including antioxidants and enzymes can provide protection of our immune system against number of diseases (9,10). As per the epidemiological studies, intake of flavonoids is inversely proportional to mortality from coronary heart disease and prevalence of heart attacks (10,11).

*Santalum album* (commonly known as chandan or Indian sandalwood, family *Santalaceae*), is a small evergreen tree and may live to one hundred years of age. According to

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the literature, oil extracted from this medicinal plant i.e. *Santalum album* is commonly used for treatment of skin disorders, fever, urinary tract infection, heart ailments etc. In addition, *Santalum album* also possessed or showed anti-microbial and anti-inflammatory properties (12-14). Medicinal plant *Butea frondosa* (also known as Palas; family *Fabaceae*), commonly found throughout India, Sri Lanka and Myanmar. It has shown the presence of phytochemicals that are effective in various diseases. The leaves glycosides are used for sore throat and diabetes mellitus. The roots components including beta-sitosterol, amyirin, stigma sterol, etc have anti-microbial property, the seeds have proteolytic and lypolytic enzymes which have anthelmintic and antiparasitic properties (15-17).

*Emblica officinalis* (also known as Amla, family *Phyllanthaceae*) is growing throughout India and has shown its medicinal properties in a number of preclinical and clinical studies to have. antipyretic, analgesic, wound healing, adjuvant, anti-anemia etc (18,19). As per these uses and properties of these three medicinal plants, this study was aimed to evaluate or scrutinize the effect of flavonoids extracted from the leaves of *Santalum album*, *Butea frondosa* and *Emblica officinalis* for determining their anti-inflammatory and immunosuppressive activity on human peripheral blood mononuclear cells (PBMC) using hepatitis B vaccine antigen (20 µg/mL; 10 µl).

## Materials and methods

### Collection of plant material

The leaves of medicinal plants (*Santalum album*, *Butea frondosa* and *Emblica officinalis*) were collected from the garden of Vidya Pratishthan's School of Biotechnology (VSBT), Baramati, Maharashtra, India. These medicinal plant leaves were dried in a shady area after washing under tap water and then with distilled water in order to remove the dust particles. The extraction of leaves was carried out in liquid nitrogen by simple maceration process using mortar and pestle at room temperature in order to prepare the fine powder.

### Extraction of flavonoids from medicinal plants

For flavonoid extraction from these medicinal plants (*Santalum album*, *Butea frondosa* and *Emblica officinalis*), 50 g of fresh healthy plant leaves were macerated in liquid nitrogen using mortar and pestle to prepare grounded plant leaves powder and refluxed with 250 ml of 80% methanol for 3 hours at 90-100°C. Thereafter, the solution containing plant leaves powder was cooled down and preceded for filtration using Whatman filter paper (1,2). The filtrate was collected and then added in the ratio of 2:1 of distilled water and ethyl acetate. The samples were incubated for 4-6 hours at room temperature, the upper layer (ethyl acetate present) was collected and then the plant leaves extract was dried. Afterwards, the extracts were settled at the bottom and observed qualitatively the flavonoid content using lead acetate solution, yellow precipitation appears. This yellow color precipitation indicated the presence of flavonoid content (1,2).

### PBMC proliferation assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; yellow colour dye) is a quick, precise and sensitive in vitro method to evaluate the activity of flavonoids extracted from three medicinal plants on PBMC proliferation assay using hepatitis B surface antigen (HBsAg).

In order to study the proliferation of three medicinal plants using HBsAg, PBMC ( $10^5$  cells/well) was cultured in 96 well cell culture plates along with variable concentrations of flavonoids (0.5–30 mg/ml; 50 µl). HBsAg (20 µg/mL; 10 µl) was added to culture plates and kept in an atmosphere of 5% CO<sub>2</sub> at 37°C for 24 hours. After that the media having flavonoid was removed after centrifuging and added 100µl fresh media in 96 well plates. Thereafter, MTT mixtures (2.5 mg/mL in PBS; 10 µl) were dispensed to all the wells of 96 well plates. The plates were placed in incubator (5% CO<sub>2</sub> and 37°C) for 3-4 hours (12). The media was removed cautiously and the formazan crystals in all the columns were dissolved in Dimethyl sulfoxide (DMSO; 100 µl). The formazan end product was extrapolated by measuring spectrophotometric absorbance at 570 nm by enzyme linked immunosorbent assay (ELISA) plate reader.

### Assay for nitric oxide production and CD14 monocyte surface marker

PBMC ( $10^5$  cells/well) cells were incubated for 48 hours in presence of variable doses of flavonoids (0.5–30 mg/mL; 50 µl) extracted from three medicinal plants along with or without HBsAg (20 µg/mL; 10 µl) in a final volume of 0.2 mL and then supernatant was collected after centrifuging the plate. NO production was determined by measuring the optical density (OD) of nitrite accumulated in the culture supernatant of these flavonoids along with or without HBsAg at 540 nm using the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). The concentration of nitrite was determined through serial dilutions of sodium nitrite used as standard. All experiments were plated in triplicate wells and were performed at least three times (1,2,12).

Similarly, CD14 monocyte surface marker in human PBMC was determined using flow cytometer. In this study, treated (flavonoids; 0.5–30 mg/mL; 50 µl) and non treated (control) samples of human PBMC were stained with CD14 FITC monocyte surface marker. All these samples were incubated in dark for 30 minutes at room temperature and then lysed with red cell lysis. Then it was incubated (10 minutes in dark) and washed with PBS (2500 rpm, 4°C and 10 minutes). Finally, all these samples were analyzed through FACS Calibur (BD Biosciences) (1,2,12).

### Statistical analysis

All statistical data analysis was carried out by using one way analysis of variance (ANOVA) test (Bnferoni multiple comparison test). Data was presented as mean ±

standard error (SE).

## Results

### PBMC proliferation assay

The effect of flavonoids extracted from these medicinal plants on the growth and viability of these activated PBMC was assessed by MTT colorimetric assay. Experimentation was done in 48 hours treatment of PBMC containing variable doses of flavonoid along with HBsAg. The results showed that flavonoids at higher concentration decreased the proliferation containing HBsAg. HBsAg was used as standard and showed enhancement of proliferation as compared to control (Figure 1).

### Nitric oxide production

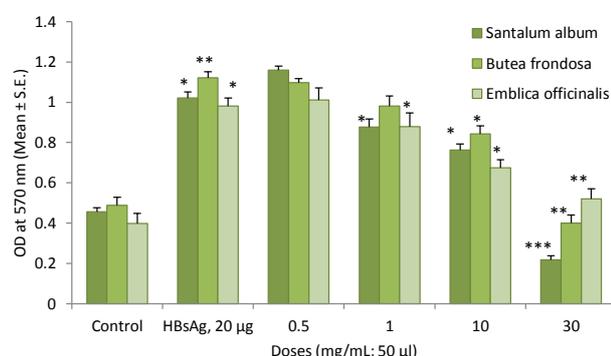
HBsAg-stimulated PBMC was studied for NO release in the presence of flavonoids extracted from three medicinal plants. The flavonoids spontaneously declined in nitrite production from PBMC cell culture supernatant at higher doses as compared to control and standard HBsAg (Figure 2).

### CD14 monocyte surface marker

The effects of flavonoids on CD14 monocyte marker are shown in Figure 3. The results showed that there was markedly decline in CD14 monocyte marker at higher doses as compared to control (Figure 3).

## Discussion

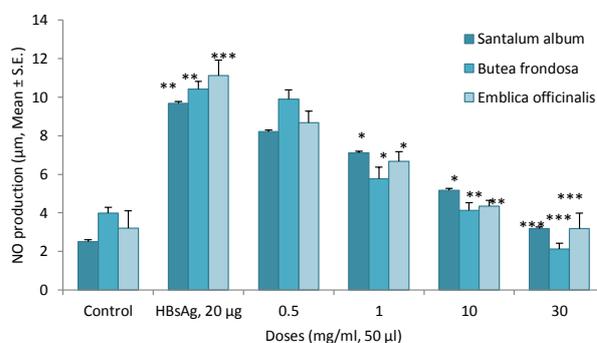
In the present study, the focus was on secondary metabolites especially flavonoids extracted from three medicinal plants (*Santalum album*, *Butea frondosa* and *Emblca officinalis*) native to India which were investigated for their anti-inflammatory and immunosuppressive effects on human PBMC using HBsAg. For these studies, firstly proliferation assay of these flavonoids against human PBMC were studied and then their effects on the NO production were evaluated including CD14 monocyte surface marker. The flavonoids of these medicinal plants showed inhibitory effects on HBsAg lymphocyte proliferation and also



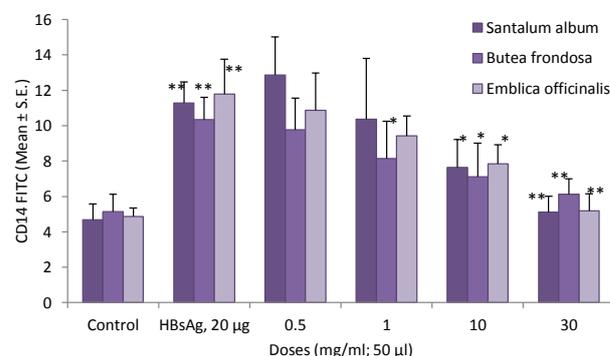
**Figure 1. Effect of variable doses of flavonoids extracted from the leaves of medicinal plants (*Santalum album*, *Butea frondosa* and *Emblca officinalis*) on human PBMC proliferation assay.** Values are expressed as mean  $\pm$  SE. The difference between the control, standard and treated samples of flavonoids is determined by one way ANOVA test (Bonferroni multiple comparison test).

sudden decline was seen in the level of NO including CD14 monocyte surface marker at higher doses compared to the control after 48 hours of treatment.

The inhibitory effects of these medicinal plants on PBMC proliferation as well as NO production including CD14 marker can justify the therapeutic usefulness in rheumatoid arthritis which is an inflammatory disorder. The capacity of these medicinal plant products with respect to inhibition of B (humoral) and T (cell mediated) immune response provides useful applications in these immune mediated disorders. Both T and B lymphocytes activation including macrophages (PBMC) play critical roles in pathogenesis of these immunodeficiency's or disorders (10-12). PBMC (monocytes derived macrophages) is normally impenetrable in the inflammatory processes by generation of pro-inflammatory cytokines (IFN-gamma and TNF alpha) including proliferation and other inflammatory mediators such as NO and CD14 surface marker. These medicinal plants showed inhibition of proliferation, NO production and CD14 surface marker at higher doses



**Figure 2. Effect of variable doses of flavonoids extracted from the leaves of medicinal plants (*Santalum album*, *Butea frondosa* and *Emblca officinalis*) on NO production from human PBMC cell culture supernatant.** Values are expressed as mean  $\pm$  SE. The difference between the control, standard and treated samples of flavonoids is determined by one way ANOVA test (Bonferroni multiple comparison test).



**Figure 3. Effect of variable doses of flavonoids extracted from the leaves of medicinal plants (*Santalum album*, *Butea frondosa* and *Emblca officinalis*) on human CD14 monocyte surface marker using flow cytometry.** Values are expressed as mean  $\pm$  SE. The difference between the control, standard and treated samples of flavonoids is determined by one way ANOVA test (Bonferroni multiple comparison test).

as compared to control. NO is an important chemical mediator generated by endothelial cells, dendritic cells, macrophages, neurons, etc and also involved in the regulation of various physiological processes. Excess concentration or production of NO is associated with several diseases. Normally, animal or human diseases can be characterized through lack or excess of NO production. In some circumstances, protection against a decrease in constitutive NO production in the vasculature may intercept the development of vascular disease, while inhibition of uncontrolled NO production could also be a therapeutic target (20). These flavonoids showed drastic dwindle in NO production at higher doses as compared to control and showed its therapeutic effect.

Further confirmation of flavonoids extracted from *Santalum album*, *Butea frondosa* and *Emblica officinalis* for their anti-inflammatory and immunosuppressive activity against HBsAg in human PBMC using CD14 monocyte surface marker were determined through flow cytometry. As per literature, CD14 (55 KDa glycoprotein) identification marker on the surface of monocytes as well as macrophages and several monoclonal antibodies binding to the same epitope on human monocytes were assigned to a provisional CD14 cluster, which was categorized or designated as leucocyte differentiation antigen (21). Finally, CD14 monocyte marker production markedly decline at higher doses of these medicinal plants and this marker especially CD14 may depend on cell types and their species origin as well as different cells having different requirements for signal transduction pathways. In particular, NO production is mainly released by human PBMC and determination of CD14 surface marker is believed to play a considerable role in the pathophysiology of hormonal immune system.

### Conclusion

The present study showed that these medicinal plants down-regulated the release of NO production by human PBMC. In addition, this effect is likely to be due to inhibition of CD14 monocyte marker. The NO down-regulation may reduce pathological harms arise due to an excess in NO production in inflammatory processes. Further study is needed to complete these data and to identify the responsible compounds.

### Authors' contributions

This work was carried out in collaboration between two authors. AG designed the study, wrote the protocol and interpreted the data. AG and SRC anchored the field study, gathered the initial data and performed preliminary data analysis. AG and SRC managed the literature searches and produced the initial draft. Both the authors read and approved the final manuscript.

### Conflict of interests

The authors declared no competing interests.

### Ethical considerations

Ethical issues (including plagiarism, misconduct, data

fabrication, falsification, double publication or submission and redundancy) were completely observed by authors.

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