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Immunosuppressive activity of terpenoids from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* on human whole blood

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ABSTRACT

Background and aims: Terpenoids are one of the largest groups of natural compounds and is generally used for the treatment of human diseases e.g. taxol (anticancer drug) and artemisinin (antimalarial drug). Thus, these agents (terpenoids) derived from medicinal plant products that are effective against intracellular or extracellular infections and these are urgently required. Therefore, this research was directed to assess its immunosuppressive activity of crude terpenoids on hepatitis B vaccine containing surface antigen (HBsAg) on human whole blood.

Methods: In this study, crude terpenoids (6.25-25 mg/ml; 50 μ l) extricate from the leaves of these medicinal plants and evaluated its immunosuppressive activity on HBsAg (20 μ g/ml) using human whole blood in order to determine the analysis of blood counts (flow cytometry), cytotoxicity assay (MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and nitric oxide (NO) production from cell culture supernatant.

Results: The results showed that these terpenoids showed drastic decrease in monocytes and granulocytes count but increased in lymphocytes count and inhibited NO production at higher doses (25 mg/ml; 50 μ l).

Conclusion: These terpenoids exhibited immunosuppressive activity and could be a promising source of medicinally important natural compound.

Keywords: Immunopharmacological, *Mesua ferrea*, *Ficus benghalensis*, *Butea frondosa*.

Original Article

INTRODUCTION

Medicinal plant contained various phytochemical products that played an important role in human health and prevented of various animal and human diseases.^{1,2} Natural phytochemical compounds are present in the form of secondary metabolites i.e.

flavonoids, terpenoids, phenolics, alkaloids etc.²⁻⁴ These phytochemical compounds react with various nutrients and dietary products related to animal and human health that scavenges free radicals and is responsible for eliminating the risk of dreadful diseases

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i.e. arthritis; cancer; cardiac ailments and early aging.^{5,6} Among many phytochemical compounds found in plants, terpenoids are the most important ones. Terpenoids (also called as isoprenoids) are contained in many medicinal plant products and most of these are available for various pharmaceutical applications e.g. artemisinin and taxol used for malaria and cancer disease as medicine.^{7,8} As per the literature, more than forty thousand compounds of terpenoids are isolated and most of these compounds showed some beneficial effects related to cardiovascular diseases including type 2 diabetes, obesity induced metabolic disorders etc.^{9,10} Actually, most of the terpenoids are of plant origin and hundreds/thousands of new structures are reported every year.^{9,10}

Mesua ferrea (Nagkeshar, family *Guttifere*), *Ficus benghalensis* (Vad, family *Moraceae*), and *Butea frondosa* (Palas, family *Fabaceae*) were picked for these studies.^{3,11-14} These medicinal plants are commonly found in India (Maharashtra and parts of Andhra Pradesh), Sri Lanka, Indonesia, Pakistan, and Bangladesh and showed various medicinal properties or activities i.e. anti-inflammatory, immunomodulatory, anti-oxidant etc.¹⁵⁻¹⁷ In this study, we focused on various terpenoids isolated in crude form from these medicinal plants, i.e. *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* and determined its immunosuppressive properties in human whole blood.

METHODS

Fresh plant leaves of *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* were collected from the garden of Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra, India and properly cleaned (using distilled water and dried in a shady area) to prepare fine powder.

For extraction of crude terpenoids from these medicinal plants powder (*Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa*), it was used methanol, chloroform and sulphuric acid. In this study, weight 5 g of plant leaves powder was taken separately and soaked in alcohol for 24-48 hours. After incubation, collect the supernatant (filtrate) and then extracted with petroleum ether using separating funnel. The ether extract was treated as total terpenoids.¹⁰ For confirmation of these crude terpenoids in filtrate of medicinal plant leaves powder using chloroform and sulphuric acid were mixed together in equal proportion and the formation of reddish brown colour that indicates the presence of terpenoids in the selected plants.

In order to determine its immunosuppressive activity of crude terpenoids, EDTA human whole blood (n=10; 3 ml) samples were collected from Mangal Pathology laboratory, Baramati. For these studies using 100 µl of human blood, samples were taken in each falcon tube and then added without or with variable concentration of crude terpenoids (6.25-25 mg, 50 µl) of these medicinal plants (*Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa*) including HBsAg (20 µg/ml, 10 µl). These blood samples were incubated for 2 h at 4°C and lysed with red cell lysis buffer and then washed with PBS (pH 7.2) and proceed for flow cytometric analysis for the estimation of total blood (lymphocytes, monocytes and granulocytes) counts in human whole blood.^{3,10,11}

In this assay, whole blood samples of human (lymphocytes) were cultured for 48 h in presence of variable concentration of crude terpenoids (6.25-25 mg/ml, 50 µl) from these medicinal plants (*Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa*) along with standard HBsAg (10 µl) in 96 well flat bottom tissue culture plates. After incubation, centrifuged the plate and collected supernatant for estimation of NO

production and then added an equal volume of fresh media in 96 well plates. Afterwards, MTT solution (2.5 mg/ml; 10 μ l) was added and incubated for 2-3 h in a carbon dioxide incubator. Thereafter, plates were centrifuging (2600 rpm for 5 minutes), supernatant was eliminated and formazon crystals were settled at the bottom.^{3,10,13} Finally, dimethyl sulphoxide (DMSO; 100 μ l) solution was added and absorbance (optical density; OD) was measured in an ELISA reader at 570 NM.

Briefly, cell culture supernatant (50 μ l) of lysed human whole blood was mixed with the Griess reagent (50 μ l containing 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Incubated the samples (crude terpenoids) in 96 well plates at room temperature for 10 minutes, and absorbance (optical density, OD) at 540 nm was measured in a microplate reader. The

fresh culture medium (RPMI containing 10% fetal bovine serum) used as blank. For standard curve using sodium nitrite and determined NO production of crude terpenoids and the experiments were performed in triplicates.¹⁸

All values are expressed in Mean \pm S.E. Comparative difference between the values of control and treated groups of terpenoids (6.25-25 mg, 50 μ l) extracted from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* containing HBsAg. Data were analysed by One way ANOVA (Boniferroni multiple comparison) test.

RESULTS

The effect of variable concentration of crude terpenoids (6.25-25 mg/ml, 50 μ l) from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* on human blood counts has shown in Table 1.

Table 1: Effect of variable doses of terpenoids on human blood counts using flow cytometry

| Plant materials | Doses (mg/ml; 50 μ l) | Lymphocytes | Monocytes | Granulocytes |
|---------------------------|----------------------------------|------------------|------------------|------------------|
| <i>Mesua ferrea</i> | Control PBS | 8.68 \pm 1.21 | 1.78 \pm 0.06 | 37.64 \pm 3.12 |
| | HBsAg, 20 μ g/ml; 10 μ l | 9.19 \pm 1.44 | 10.18 \pm 0.88 | 48.12 \pm 2.44 |
| | 6.25 | 12.34 \pm 1.98 | 7.46 \pm 0.96 | 22.45 \pm 3.12 |
| | 12.5 | 16.78 \pm 2.12 | 4.56 \pm 0.62 | 19.2 \pm 2.88 |
| | 25 | 27.12 \pm 2.82 | 2.14 \pm 0.42 | 16.6 \pm 1.72 |
| <i>Ficus benghalensis</i> | Control PBS | 8.68 \pm 1.21 | 1.78 \pm 0.06 | 37.64 \pm 3.12 |
| | HBsAg, 20 μ g/ml; 10 μ l | 9.19 \pm 1.44 | 10.18 \pm 0.88 | 48.12 \pm 2.44 |
| | 6.25 | 13.78 \pm 2.42 | 5.68 \pm 0.42 | 27.6 \pm 3.12 |
| | 12.5 | 18.46 \pm 3.12 | 4.12 \pm 0.27 | 21.2 \pm 3.02 |
| | 25 | 32.4 \pm 4.18 | 3.12 \pm 0.44 | 13.4 \pm 2.84 |
| <i>Butea frondosa</i> | Control PBS | 8.68 \pm 1.21 | 1.78 \pm 0.06 | 37.64 \pm 3.12 |
| | HBsAg, 20 μ g/ml; 10 μ l | 9.19 \pm 1.44 | 10.18 \pm 0.88 | 48.12 \pm 2.44 |
| | 6.25 | 18.8 \pm 2.08 | 6.98 \pm 0.34 | 25.2 \pm 2.16 |
| | 12.5 | 21.4 \pm 1.54 | 4.08 \pm 0.92 | 20.22 \pm 1.98 |
| | 25 | 26.14 \pm 1.68 | 2.06 \pm 0.54 | 14.6 \pm 2.12 |

The results presented here that these crude terpenoids showed their inhibition at higher doses (25 mg/ml; 50 µl) against HBsAg in the number of monocytes as well as granulocytes count. Overall, these terpenoids extracted from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* at higher doses against HBsAg showed immunosuppressive activity.

Flow cytometric analysis of crude terpenoids (6.25-25 mg/ml; 50 µl) extracted from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* for determining its effect in human whole blood against HBsAg

containing lymphocytes, monocytes and granulocytes count. Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analysed using cell quest software.

Another study was to determine the effect of variable concentration of crude terpenoids on antigen (HBsAg) specific immune response as shown in Figure 1. The results showed that these terpenoids at higher doses (25 mg/ml; 50 µl) showed decline in HBsAg (T cell proliferation) as compared to standard and control.

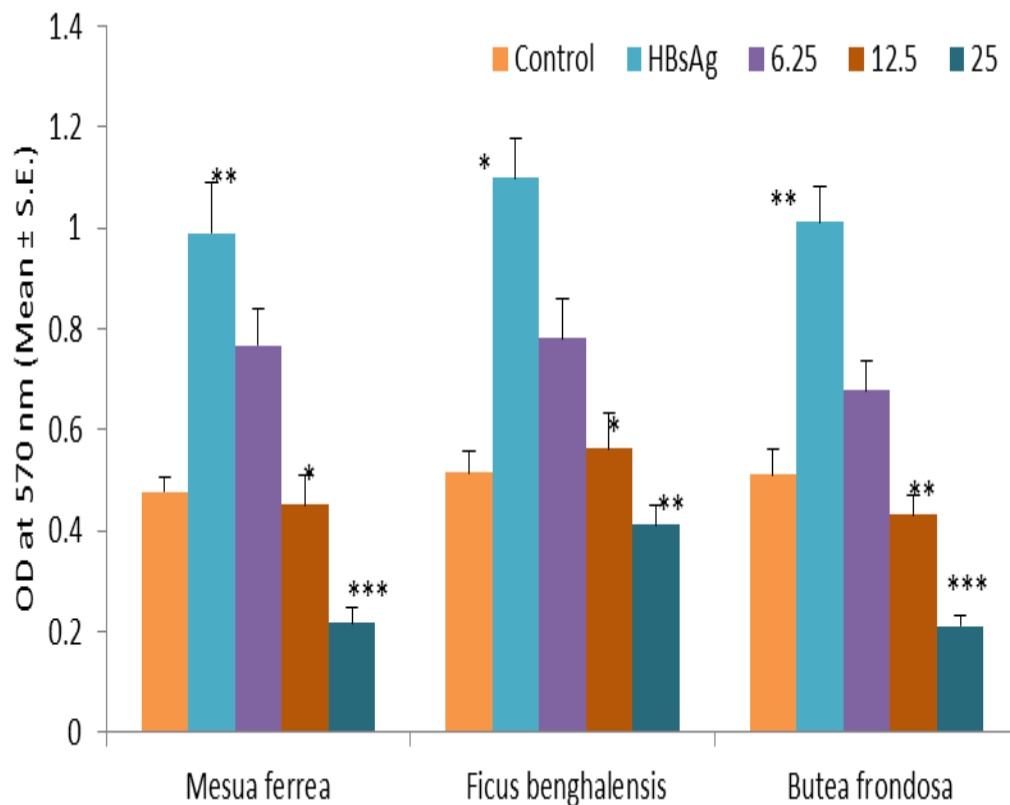


Figure 1: Cytotoxicity assay

In addition, the effect of variable concentration of crude terpenoids (6.25-25 mg/ml, 50 µl) from *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* on NO production as shown in Figure 2. The

results presented here that these terpenoids at higher doses (25 mg/ml; 50 µl) showed decline in HBsAg specific immune response as compared to standard and control.

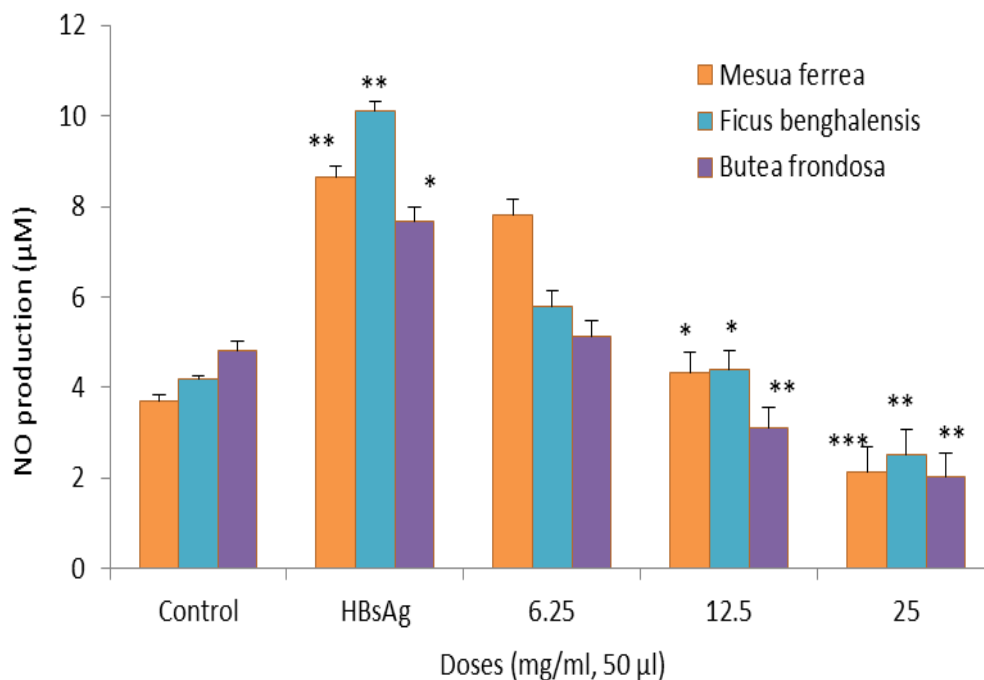


Figure 2: Estimation of nitric oxide production (NO)

DISCUSSION

Medicinal plants have been reported for various immunopharmacological activities like anti-inflammatory and immunosuppressive effects. Throughout the world, inflammatory diseases (e.g. rheumatic diseases) are reported and exist major concern in the development of inflammatory and autoimmune diseases, i.e. alteration in the T-cell responses and irregular functioning of our immune system.^{16,18} Any alteration in the immune response involving enhancement or inhibition of immune response (with respect to antibody and cell mediated immune response) referred as immunomodulation. Those agents that are responsible for modulating the immune response that are traditionally called as immunomodulators i.e. stimulatory or suppressive.¹⁶ As per literature, many different immunosuppressant drugs that are available for our new organ's survival (e.g. corticosteroids, cyclosporine, betamethasone, azathioprine etc.).¹⁹ However, many of them

come with mild to severe unwanted side effects. In view of this, efforts are being undertaken to screen those metabolites, especially crude terpenoids extracted from medicinal plant leaves that showed immunosuppressive activity and also given its benefits to human health as well. For these studies, the exact mechanism is not clear between these terpenoids from medicinal plants and cell-surface markers including growth factors involving specific protein antigen (HBsAg) activation, and it is possible that the identification and elucidation of the active constituents in this crude terpenoids may provide beneficial leads to the development of new and effective immunosuppressant drugs.

In this study, it was evaluated the immunosuppressive activity of crude terpenoids from the leaves of *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa* that can be measured by changes in the human

blood counts including HBsAg proliferation assay and nitric oxide production assay. The results of this study proved that these terpenoids showed significantly suppressed production of human blood counts (monocytes as well as granulocytes count), nitric oxide production and HBsAg proliferation assay at higher doses. In contrast, these terpenoids showed significantly dose dependent inhibition at higher doses and reported immunosuppressive activity.

As per the flow cytometry results that clearly indicates its immunosuppressive effect against HBsAg (i.e. decline in monocytes as well as granulocytes count). Generally, monocytes represent the mediator of proinflammatory cytokines after HBsAg-stimulation of human whole blood and these studies were strongly supported by these terpenoids to inhibit *in vitro* proliferation of human whole blood using HBsAg. Further confirmation of these studies, our data showed that these terpenoids significantly suppressed nitric oxide production as compared to control. These blood counts especially monocytes as well as granulocytes count including nitric oxide production displayed as a major factor in order to understand the mechanism of immunosuppression. Therefore, direct exposure of these terpenoids on human whole blood can be considered more suitable method than primary cells for evaluation purposes of immunomodulatory agents.

CONCLUSION

Studies on cell-mediated immune response with respect to HBsAg specific proliferation, estimation of blood counts, including nitric oxide production which clearly indicates the immunosuppressive effects of crude terpenoids extracted from the leaves of *Mesua ferrea*, *Ficus benghalensis* and *Butea frondosa*.

CONFLICT OF INTEREST

Authors have declared that no conflicts of interest exist.

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