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Immunosuppressive and Cytotoxic Potential of Flavonoids from *Mitragyna parvifolia, Mangifera indica* and *Aegle marmelos*

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Research Article

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ABSTRACT

The objective of our study is to isolate the flavonoids from the leaves of these three medicinal plants and evaluated *in vitro* effect on human peripheral blood mononuclear cells (PBMC) using hepatitis B vaccine containing surface antigen (HBsAg; 20 µg/ml, 10 µl) and determined its HBsAg proliferation, nitric oxide production and CD14 monocyte surface marker. The results showed that flavonoids at higher doses (25 mg/ml; 50 µl) inhibited HBsAg stimulated proliferation of human PBMC cells as well as nitric oxide production and also blocked the activation of CD14 monocyte surface marker, which are necessary for T cell activation. Our results reporting the immunosuppressive activity of *Mitragyna parvifolia*, *Mangifera indica* and *Aegle marmelos* against HBsAg, specific protein antigen. Thus, these crude flavonoids isolated from medicinal plants could be a candidate for development as an immunosuppressive and cytotoxic agent.

INTRODUCTION

Medicinal plants have been continuously used as a traditional medicine from ancient times and are generally used for the treatment of bacterial, viral and parasitic diseases [1,2]. In these medicinal plant products, exhibit a variety of phytochemicals, which has indispensable approach in the fields of medicine especially for veterinary animals and human which played a major role in expanding as well as flourish new drugs for the treatment and prevention of animal and human diseases [3,4]. Natural compounds isolated as well as purified from medicinal plant products and other life forms (bacteria, fungi, marine organisms) represent a major source of molecules with traditional medicinal properties [5]. Among them, immunosuppressant substances are of particular interest. Generally, these medicinal plant products or parts are commonly rich in phenolic compounds (flavonoids, phenolic acids, tannins, coumarins, lignans and lignins) [6,7]. These phenolic compounds especially flavonoids have multiple immunopharmacological effects including immunomodulatory activity [8]. Therefore it is very important to have sufficient knowledge regarding flavonoids extracted from medicinal plant products not only because of their widespread uses, but also because they have the prospective to cause toxic reactions or interact with other drugs [9,10].

Flavonoids (ubiquitous in nature) are one of the members of secondary metabolites and are categorized according to chemical structure, i.e., flavonols/flavones/flavanones/isoflavones/catechins/anthocyanidins/chalcones etc. [7,9,10]. These flavonoids are polyphenolic compounds and are generally found in citrus fruits, berries, onions, parsley, legumes, green tea, and red wine. In addition, naturally benzo-g-pyrone derivatives of flavonoids, have been shown to possess several immunopharmacological properties, (i.e., hepatoprotective, anti-thrombotic, anti-inflammatory, and antiviral activities), many of which may be related, partially at least, to their antioxidant and free-radical-scavenging ability [7,10].

Mitragyna parvifolia (Family Rubiaceae) popularly known as *Kadamb* and is found emerging companionable throughout the drier parts of India, Pakistan and Sri Lanka [11,12]. This medicinal plant showed diverse immunopharmacological properties such

as analgesic, anti-pyretic, anti-inflammatory, anti-arthritic, anti-helmentic, anti-oxidant etc. [12-14] which have been reported as well as recognizable scientifically.

Mangifera indica (family Anacardiaceae), medicinal plant showed number of polyphenolic compounds and displayed number of immunopharmacological activities such as wound healing properties; anti-diabetic; immunomodulation; anti-oxidant etc. [15-17]. In addition, leaves (burning) of this medicinal plant provide some relief from hiccups and infections of the throat.

Aegle marmelos (family Rutacae), most commonly phytoconstituents isolated as well as purified from leaves (citral, lupeol, cineol etc.) stem bark (Fagarine, marmin etc) and fruits (tannin, marmelide). Mostly, the leaves of this medicinal plant are generally used to treat inflammation, asthma, hypoglycemia, febrifuge, hepatitis and analgesic [18-21].

The objective of our study is to isolate the flavonoids from three different medicinal plants and determined its activity against specific protein antigen (HBsAg) in order to examine immunosuppressive activity using various immunopharmacological techniques.

MATERIALS AND METHOD

Collection of plant material

Collection of fresh and mature plant leaves of these medicinal plants, i.e., *Mitragyna parvifolia, Mangifera indica* and *Aegle marmelos* from the garden of Vidya Pratishthan's School of Biotechnology (VSBT), Baramati (Pune), Maharashtra.

Extraction of flavonoid

For the extraction of crude flavonoids from the leaves of medicinal plants, i.e., *Mitragyna parvifolia, Mangifera indica* and *Aegle marmelos* were carried out. Initially, the leaves (10 g) were macerated using liquid nitrogen (-196°C) to prepared fine powder and dissolved in 100 ml of methanol (80%) for 2 h at 100ºC [6]. Thereafter, collect the filtrate using whatman filter paper and then suddenly add ethyl acetate (20 ml) along with distilled water (40 ml), shaking regularly for at least 5 minutes and then incubate the solution overnight at room temperature. After incubation, two different layers were observed, i.e., upper layer, (i.e., ethyl acetate) and lower layer (flavonoids). Finally, evaporate the upper phase i.e. ethyl acetate solution and then dried the plant extracts (flavonoids) settled at the bottom.

Qualitative analysis of flavonoid

For the estimation of flavonoid content in these three medicinal plants using lead acetate test, small volume of lead acetate solution is added and yellow precipitation appears. This yellow color precipitation showed the presence of flavonoid content.

PBMC proliferation assay and determination of nitric oxide production including CD14 monocyte surface marker

EDTA non-infected human blood samples were collected from Mangal Pathology laboratory, Baramati in order to separate the PBMC using Ficoll–Hypaque reagent. For PBMC preparation, cells were separated through Ficoll–Hypaque gradient centrifugation and plated in tissue culture plates (96 well flat bottom) and incubated in the presence of HBsAg (1 µg, 20 µg/ml) along with serial dilutions of flavonoids (6.25–25 mg/ml; 50 µl) extracted from three medicinal plants i.e. *Mitragyna parvifolia, Mangifera indica* and *Aegle marmelos* at 37°C for 24 h. After incubation, tissue culture plates were centrifuged (1500 rpm, 5 minutes) and then collect the supernatant (100 µl) from the wells of treated and non-treated PBMC with HBsAg for the determination of nitric oxide production and then add equal volume of fresh medium in 96 well plate. Afterwards, add MTT (5 mg/ml, 10 µl) solution and incubated for 4 h in carbon dioxide incubator. Again, plates were centrifuging and discard the supernatant. Add DMSO (100 µl) solution to the formazon crystals and the absorbance was evaluated in an ELISA reader at 570 nm [22,23]. All experiments were performed in triplicate.

For the determination of nitric oxide production using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in presence of 2.5% phosphoric acid), PBMC cell culture medium (50 µl) was minced with same volume of Griess reagent and incubated the plates at room temperature for 10-20 minutes, and the absorbance (540 nm) was measured in a micro plate reader [24-26]. The fresh culture medium (RPMI containing 10% fetal bovine serum) was used as a blank. The quantity of nitrite was determined from a sodium nitrite standard curve.

Similarly, CD14 monocyte surface marker was determined in human PBMC (10^5 cells/ml) were cultured for 24 h with or without HBsAg (1 µg, 20 µg/ml) and then stained with CD14 FITC surface marker. Incubate and wash the samples with phosphate buffered saline and analyzed the flavonoid samples with or without HBsAg stimulation [24-26] through flow cytometer (FACS Calibur, BD Biosciences).

Statistical analysis

All values are mentioned as Mean ± S.E. and determined the statistical analysis through One way ANOVA test (Boniferroni multiple comparison test).

RESULTS

PBMC proliferation assay

In order to find out its HBsAg proliferation on human PBMC using variable doses of flavonoid (6.25–25 mg/ml; 50 µl) as

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shown in **Figure1.** The results showed that flavonoids extracted from the leaves of *Mitragyna parvifolia*, *Mangifera indica and Aegle marmelos* showed significant inhibition in HBsAg proliferation at higher doses (25 mg/ml; 50 µl) as compared to control. HBsAg used as standard for these studies and the results showed markedly increased in proliferation as compared to control.

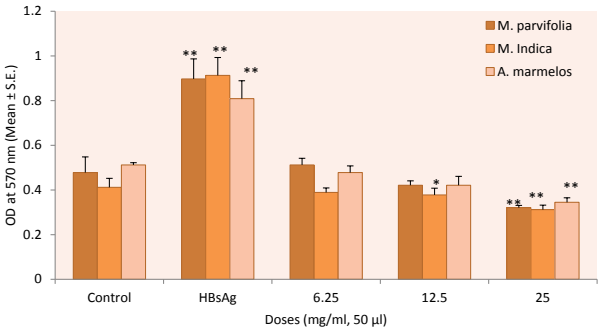

Figure 1. Effect of variable doses of flavonoid on human PBMC using HBsAg. PBMC (10^6 cells/ml, 100 µl) were cultured in triplicates in 96-well tissue culture plate along with variable doses of flavonoid (6.25–25 mg/ml; 50 µl) in complete RPMI 1640 medium in presence of HBsAg (20 µg/ml, 1 µl). Proliferation was measured by MTT assay. Values are expressed as Mean ± S.E. The absorbance was evaluated in an ELISA reader at 570 nm.

Nitric oxide production

Similarly, determination of nitric oxide was observed in the cell culture supernatant of human PBMC as shown in **Figure 2.** The results showed that flavonoids at higher doses (25 mg/ml; 50 µl) from *Mitragyna parvifolia*, *Mangifera indica and Aegle marmelos* showed significantly decline in nitric oxide production as compared to control. In contrast, HBsAg showed markedly accelerates the production of nitric oxide as compared to control.

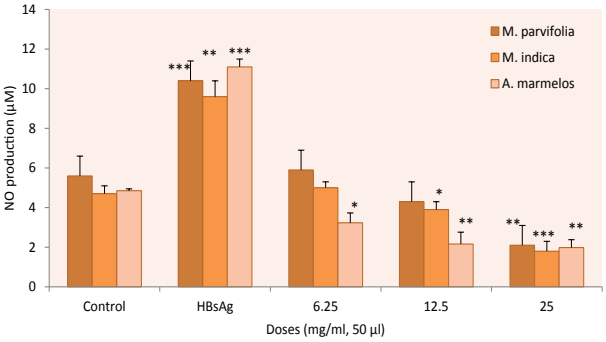

Figure 2. Effect of variable doses of flavonoid on nitric oxide production. PBMC (10^6 cells/ml, 100 µl) were cultured with variable doses of flavonoid (6.25–25 mg/ml; 50 µl) along with HBsAg (20 µg/ml, 1 µl). Incubated the plate for 24 h and centrifuging the plate and collect the supernatant for the estimation of nitric oxide. Readings are expressed in µM. Values are expressed as Mean ± S.E.

CD14 monocyte surface marker

The effect of variable doses of flavonoid (6.25–25 mg/ml; 50 µl) extracted from these medicinal plants on human CD14 monocyte surface marker using HBsAg as antigen which is determined through flow cytometry as shown in **Figure 3.** At higher doses (25 mg/ml; 50 µl), these medicinal plants showed dose dependent decrease in CD14 monocyte surface marker as compared to control.

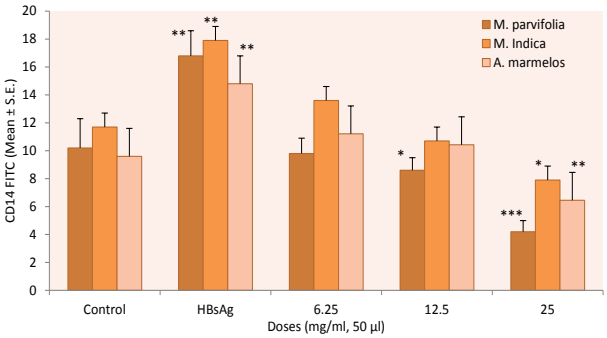

Figure 3. Effect of variable doses of flavonoid on CD14 monocyte surface marker in human PBMC using flow cytometry. PBMC's were stained with CD14 FITC surface marker and then lysed and wash the cells in PBS and analyzed in a flow cytometer (FACS Calibur). Values are expressed as Mean ± S.E. The difference between the control and treated groups of flavonoids is determined by One way ANOVA test (Bonferroni multiple comparison test). **P<0.05; **P<0.01; ***P<0.001.*

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DISCUSSION

Our immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases, e.g. bacteria, viruses, protozoan etc. Modulation of the immune responses using various medicinal plants in order to reduce the burden of various human diseases has been of immense interest for many researchers. In general, medicinal plants contained number of primary as well as secondary metabolites. One of the secondary metabolite, i.e., flavonoid (also called as bio-flavonoids) are naturally occurring immunobiological compounds that are often found in plants, e.g. Quercetin, kaempferol, catechin etc and provide protection to human body against various intracellular as well as extracellular pathogens. The recent explosion of interest in the bioactivity of these flavonoids from medicinal plants is to provide potential human health benefits because of these polyphenolic components that are present in major dietary constituents [7-9]. As per the literature survey, some of the flavonoids, e.g. catechin showed neuroprotective benefit against HIV mediated cognitive decline. Indeed, very high flavonoid concentrations have been associated with neurotoxic adverse effects [27].

In the present study, flavonoids from the leaves of these medicinal plants, i.e., *Mitragyna parvifolia*, *Mangifera indica and Aegle marmelos* exerts significant inhibition of HBsAg proliferation in human PBMCs as measured by cell culture based assay (MTT). Further, our group also report that flavonoids down regulates the activity of nitric oxide production in human PBMC which is directly correlated with pro-inflammatory cytokines which played a major role in the infection and pathogenesis of bacterial, viral as well as parasitic disease [28].

It is suggested that the nitric oxide assay may be regarded as an efficient, economical and relatively reliable tool in primary screening for intrinsic immunostimulatory/immunosuppressive as well as cytotoxic effect of compounds in human cell system. Most importantly, not only in animal macrophages but also in human PBMC the production of nitric oxide from flavonoids is tightly correlated with the extent of cytokine production [28]. In this study, flavonoids have the potential to inhibit the nitric oxide production with or without HBsAg stimulation. For these studies, cell culture supernatant collected from human PBMC produced significantly higher nitric oxide production level when activated *in vitro* with HBsAg at 24 h after stimulation as compared to control. The results indicate that low nitric oxide detection in our system can be related to proliferation of T cells and other immune cells.

The ultimate goal was to examine whether the flavonoid extracted from *Mitragyna parvifolia*, *Mangifera indica and Aegle marmelos* decline in the production of CD14 monocyte surface marker in human PBMC can be employed as a predictive measure for assessing the immunosuppressive and cytotoxic potential in human cell system. In addition, CD14 (55 kDa glycoprotein; multiple leucine-rich repeats) widely used as a monocyte marker in flow cytometry [23,24]. Generally, human PBMC (monocytes) show morphological heterogeneity, such as variability of size including granularity and nuclear morphology. Firstly, monocytes were identified by their expression of enormous amount of CD14 (receptor for lipopolysaccharide) present on the surface. However, the subsequent identification or recognition of differential expression of antigenic markers showed that monocytes in human PBMC are heterogeneous and this provided the first clues to the differential physiological activities of monocyte subsets. Finally, the results suggested that production of nitric oxide and CD14 monocyte surface marker may depend on the cell types and their species origin, different cells having obviously different requirements for signal transduction pathways. In particular, nitric oxide that is mainly released by human PBMC and determination of CD14 marker is believed to play a considerable role in the pathophysiology of hormonal immune system [28].

CONCLUSION

In the present study, our group found that flavonoid from *Mitragyna parvifolia*, *Mangifera indica and Aegle marmelos* significantly inhibited the HBsAg stimulated proliferation including production of nitric oxide and CD14 monocyte surface marker. Further investigations will focus on the *in vivo* assessment of the immunopharmacological activity of these flavonoids and identified the major active components responsible for immunosuppressive as well as cytotoxic effect in the efficacious extracts.

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