Anti-inflammatory activity of flavonoids from medicinal plants against hepatitis B vaccine antigen on human peripheral blood mononuclear cells

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A B S T R A C T
The objective of our study is to investigate the anti-inflammatory activity of the variable doses of flavonoids (6.25 – 25 mg) extracted from the leaves of Syzygium cumini, Acacia catechu and Azadirachta indica on human peripheral blood mononuclear cells using specific antigen (i.e. hepatitis vaccine) to estimate the nitric oxide production from cell culture supernatant and also observed its cytotoxicity. In contrast, these flavonoids also measured its effect on lymphocytes, monocytes and granulocytes count on human whole blood using hepatitis vaccine as antigen which is determined through flow cytometry. The results showed that these flavonoids extracted from these medicinal plants showed maximum inhibition of nitric oxide and cytotoxicity at higher doses. Further, these flavonoids also showed its decline in the level of monocytes count at higher doses as compared to control. The data suggests that flavonoids extracted from these medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica showed anti-inflammatory activity.

Keywords: Flavonoids, Syzygium cumini, Acacia catechu, Azadirachta indica

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1. Introduction
Flavonoids, one of the members or class of secondary metabolites and it belongs to a group of polyphenolic compounds and generally classified as flavones, flavanones, catechins and anthocyanins [1, 2, 3]. The number of flavonoids of different classes extracted from various medicinal plants has several immunopharmacological activities [2] and possessed biochemical effects [3, 4]. These flavonoids are normally present in fruits especially citrus (flavonones) and also found in onions, parsley, legumes, green tea etc. In addition, number of flavonoids are also present in miscellaneous products i.e. anthocyanins (e.g. wine); flavans (e.g. apples and tea) and isoflavones (e.g. soya products). In contrast, these flavonoids can be easily degraded or break down through enzyme action only when the collected plant material is fresh or non-dried [3, 4]. For flavonoid extraction, used the dry plant material and generally ground into a fine powder. For flavonoid extraction, polarity is an important criterion for its characterization i.e. less polar flavonoids in the form of isoflavones/flavanones/methylated flavones/flavonols are extracted with chloroform/diethyl ether/ethyl acetate while flavonoid glycosides and more polar aglycones are extracted with alcohols/ alcohol–water mixtures [4, 5]. In the view of their wide immunopharmacological and several biological actions, they seem to have great therapeutic potential. Syzygium cumini (commonly known as Jamun or black plum; family Myrtaceae) important medicinal plant in various traditional systems of medicine. This medicinal plant is generally more effective and already reported for the treatment of diabetes mellitus, inflammation, ulcers and diarrhea and number of preclinical studies which is already done and reported as chemopreventive; radioprotective and antineoplastic properties [6, 7, 8, 9]. Acacia catechu (commonly known as khair; family Mimosaceae), medicinal plant and is generally used for the treatment of several diseases including fever, diarrhoea etc. Previously, Acacia catechu already reported as hypoglycaemic activity and also has hepatoprotective; antipyretic and digestive properties [10, 11, 12].

Azadirachta indica (commonly known as neem; family Meliaceae) is one of the important medicinal plant and is widely in the Indian traditional system of medicine for its diverse medicinal properties. Its extracts/fraction (methanolic/ethanolic/chloroform) extracted from plants i.e. roots, stem and leaves have vast or numerous immunopharmacological activities e.g. anti-viral [13]; immunostimulatory [14, 15]; anti-malaria [16, 17] and controlling gastric hyperacidity and ulcer [18, 19]. The present study compared the anti-inflammatory effect of these flavonoids of three most commonly used medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica against hepatitis B vaccine antigen on human peripheral blood mononuclear cells.

2. Materials and Methods
2.1. Collection of plant material
Fresh leaves of these medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica were collected from Vidya Pratishthan’s School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. Fresh harvested plant leaves were washed with tap or distilled water and then dried in a shady area.

2.2. Extraction and qualitative analysis of flavonoid
To proceed the flavonoid extraction [20] from three medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica. Weigh 5 g of fresh plant leaves macerated with liquid nitrogen to prepare the finely grounded plant powder and reflux with 50 ml of 80 % methanol for 2 h at 100 °C. Afterwards, cool down the solution containing plant material and filtered through whatman filter paper and collect the filtrate in separating funnel and then add ethyl acetate (10 ml) with distilled water (20 ml), mix it properly. After 6 - 8 h incubation, separating layers were observed i.e. collect only upper layer i.e. ethyl acetate and evaporate it and then dried the plant extracts. The dried extracts i.e. flavonoids settled at the bottom. For the estimation of flavonoid in medicinal plants using lead acetate test, small quantity of lead acetate solution is added and yellow precipitation appears. This yellow colour precipitation showed the presence of flavonoid.

2.3 Cytotoxicity assay of human peripheral blood mononuclear cells
Non-infected EDTA blood samples (2 – 3 ml) of human were collected from Mangal Pathology laboratory, Baramati for immunological set of experiments. For the preparation of human peripheral blood mononuclear cells (PBMC, 10⁵ cells/ml) using Ficoll–Hypaque gradient centrifugation and plated in 96 well flat bottom tissue culture plates and incubated in the presence of hepatitis vaccine (1 µg) along with serial dilutions of flavonoids extracted from three medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica at 37°C for 24 h. After incubation, 96 well flat bottom plates were centrifuged at 1500 rpm for 4 minutes and then the supernatant (50 µl) was collected for the estimation of nitric oxide and then add equal volume of fresh medium. Add MTT solution (5 mg/ml, 10 µl) were added to each well and then incubated for 4 h. Again, the 96 well flat bottom plates were spun or centrifuged at 1500 rpm for 4 minutes and the supernatant was discarded. Add 100 l of DMSO solution to the formazan crystals and the absorbance was evaluated in an ELISA reader at 570 nm [25]. All experiments were performed in triplicate.

2.4. Determination of nitric oxide (NO) production
Human peripheral blood mononuclear cells were incubated for 24 h with variable doses of flavonoids in the presence of hepatitis vaccine antigen as mentioned above. After 24 h incubation using 50 l of PBMC cell culture medium was mixed with 50 l of Griess reagent (1% sulfanilamide and 0.1% naphthylethlenediamine dihydrochloride in presence of 2.5% phosphoric acid) and incubated in 96 well flat bottom 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 quantity of nitrite
Estimation of lymphocytes, monocytes and granulocytes count using flow cytometry

In this experiment, 100 ml of human whole blood was taken in each falcon tube including hepatitis vaccine (1 µl). Add serial dilutions of flavonoids of various medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica on human whole blood and then incubated the samples in dark for 2 - 3 h at 37°C carbon dioxide incubator. Subsequently, 2 ml of red cell lysis buffer/FACS lysing solution were added to each falcon tube containing whole blood at room temperature with gentle mixing followed by incubation for 10 min. The samples were spun at 4000 rpm at 4 °C and the supernatant was aspirated and washed twice with phosphate buffered saline (PBS). After ten minutes centrifugation, pellet settled at the bottom dissolved in PBS and observed the cells through flow cytometer [23, 24].

Statistical analysis

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

Results and Discussion

Cytotoxicity assay

The effect of variable doses of flavonoid on cytotoxicity assay as shown in Fig. 1. The results showed that flavonoids showed cytotoxicity at higher doses (25 mg) as compared to control.

Estimation of nitric oxide (NO) production

The effect of variable doses of flavonoid extracted from the leaves of Syzygium cumini, Acacia catechu and Azadirachta indica on nitric oxide production were observed in the cell culture supernatant of human peripheral blood mononuclear cells as shown in Fig. 2. The results showed that there was a significantly dose related decreased in NO production as compared to control.

Estimation of blood counts using flow cytometry

The effect of variable doses of flavonoid extracted from the leaves of Syzygium cumini, Acacia catechu and Azadirachta indica on human EDTA whole blood lymphocytes, monocytes and granulocytes count using hepatitis vaccine as antigen which is determined through flow cytometry as shown in Fig 3. At higher doses, there is dose dependent decrease in monocytes count at higher doses as compared to control.

Discussion

Inflammation means tissue injury induces the complex cascade of non specific events and is generally induced by physical stress, infectious agents (bacterial or viral), toxins, and other factors. Inflammation may be acute or chronic (cause tissue destruction) and this chronic inflammation may be involved in the pathogenesis of animal and human diseases [25]. In the present study, our group focused on the anti-inflammatory activity of these flavonoids extracted from the leaves of these medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica on human peripheral blood mononuclear cells using hepatitis vaccine as antigen is a matter of research interest for many students as well as scholars. Several studies related to these medicinal plants with respect to plant physiology, pharmacology, immunomodulatory activity (using specific as well as non-specific antigen on humoral and cell mediated immune response) etc have been done [26, 27].

In the present study, we explored the effect of variable doses of flavonoids (determined through qualitative based assay using lead acetate test) on human peripheral blood samples using hepatitis vaccine as antigen. The quality of flavonoids is determined through qualitative based assay which represents the immunopharmacological active components of the medicinal plants. The active component i.e. flavonoids isolated from various medicinal plants and observed its immunopharmacological parameters. One of the mediators for inducing the inflammation is nitric oxide and played an important role in mediating many aspects of inflammatory responses. It can modulate the release of various inflammatory mediators from a wide range of cells participating in inflammatory responses (e.g., leukocytes, macrophages etc). It can modulate blood flow, adhesion of leukocytes (white blood cells) to the vascular endothelium and the activity of numerous enzymes [28, 29, 30], all of which can have an impact on inflammatory responses. The results showed that these medicinal plants showed inhibitory activity of nitric oxide at higher doses in case of cell culture supernatant of human peripheral blood mononuclear cells treated with hepatitis vaccine as antigen. In addition, these medicinal plants showed cytotoxicity at higher doses. Generally, flow cytometry is commonly usage in preclinical, clinical and veterinary laboratories for the assessment of the immune status of healthy/diseased animals and human [24].

However, the main application of this flow cytometry is important in immunopharmacological studies such as hematology, toxicology etc. In this study, the results showed that the flavonoids extracted from the leaves of these medicinal plants i.e. Syzygium cumini, Acacia catechu and Azadirachta indica showed anti-inflammatory effect at higher doses, this is due to sudden loss of monocytes count. The results obtained from this study which is indicated that flavonoids extracted from these medicinal plants showed anti-inflammatory effect on human peripheral blood samples.
Figure 1: Cytotoxicity assay. Human peripheral blood mononuclear cells were cultured for 24 h in the presence of hepatitis vaccine (1 µg) for 24 h with variable doses of medicinal plants. 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

Figure 2: Estimation of nitric oxide production (NO) from 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 hepatitis vaccine (1µg) along with variable doses of medicinal plants. 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

4. Conclusion
Syzygium cumini, Acacia catechu and Azadirachta indica showed anti-inflammatory effect against hepatitis vaccine antigen with respect to inhibition of nitric oxide production from the cell culture supernatant of human peripheral blood mononuclear cells and monocytes count from human whole blood which is determined through flow cytometry. Moreover, these flavonoids extracted from these medicinal plants support the traditional as well as medicinal use as immunoprotective and could be a promising alternative for cancer therapy.

5. References


