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Research Article

Immunosuppressive activity of flavonoids isolated from *Terminalia arjuna, Prosopis spicigera* and *Mimusops elengi*

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ABSTRACT

In the present work, three Indian medicinal plants (*Terminalia arjuna*, *Prosopis spicigera and Mimusops elengi*) were evaluated for their flavonoid content from fresh harvested leaves and analyze its immunosuppressive activity. The plant material was extracted individually in same solvents using methanol and ethyl acetate. Amongst the flavonoids of variable doses of three medicinal plants (6.25 mg, 12.5 mg, 25 mg, 50 µl) were screened for immunopharmacological activity in human whole blood using hepatitis B vaccine as specific antigen (20 µg/ml, 10 µl) in order to estimate the total blood counts (lymphocytes, monocytes and granulocytes), CD14 FITC monocyte surface marker and cytotoxicity assay. The results showed that these three medicinal plants showed dose dependent decrease in blood counts, CD14 surface marker and cytotoxic effect. These flavonoids from three medicinal plants should be considered as a medicinal source for the treatment and prevention of many human related diseases.

Key words: *Terminalia arjuna, Prosopis spicigera, Mimusops elengi, immunosuppressive*

INTRODUCTION

According to the World Health Organization (WHO) estimates that 80 % of the world's population including India still relies on medicinal plant products. The majority of new drugs (70 %) which are available in the market or still under clinical trials introduced in all over the world are derived from natural products, primarily plants[1,2]. These medicinal plant products are widely used as therapeutic tool for eliminating or preventing the growth of micro-organisms/pathogens[2]. These plant products contained various metabolites (primary and secondary) and showed various medicinal properties e.g. anti-inflammatory[3], anti-viral[4], anti-diabetic[5] etc. One of the members of secondary metabolites i.e. flavonoids, present abundantly in almost every part of medicinal plant products and can be easily degraded by enzyme action only when fresh plant materials were collected. For flavonoid extraction, the criteria for selecting the solvent on the basis of polarity i.e. less polar flavonoids (e.g., isoflavones/flavanones/methylated flavones/flavonols) are extracted with chloroform, dichloromethane, diethyl ether or ethyl acetate[6]. As per the literature survey, there are many valuable drugs (e.g. taxol, comptothecin, vincristine and vinblastine) are derived from various medicinal plants. In the present work, three medicinal plants *Terminalia arjuna* (Arjun, family *Combretaceae*); *Prosopis spicigera* (Shami, family *Fabaceae*) and *Mimusops elengi* (bakul, family *Sapotaceae*), were selected for these studies. These plants are commonly found in Maharashtra (Baramati region) and parts of Andhra Pradesh[3,7,8]. As per the literature survey of these three medicinal plants, showed number of medicinal properties such as anti-bacterial, anti-inflammatory, anti-hyperglycemic and anti-oxidative activities[3,7,8]. In the present study, our group focused on these three medicinal plant products especially crude flavonoids and observed its effect on human whole blood using hepatitis B vaccine (HBsAg) as specific antigen.

MATERIALS AND METHODS

Collection and Processing of Plant Samples

Plant samples of *Terminalia arjuna*, *Prosopis spicigera and Mimusops elengi* were collected from the udyan of vidya pratishthan's School of Biotechnology (college

affiliated to Savitribai Phule University Pune), Baramati, Maharashtra, India in the month of March-April 2015. Mature leaves of the selected plants were washed thoroughly with tap water and air dried in the shade. They were ground to a fine powder form using electric grinder (high capacity), which were then stored or packed individually in air-tight containers and kept in cool, dark and dry place for further use.

Qualitative screening of flavonoid estimation

Different plant extracts i.e. aqueous/methanolic/chloroform of *Terminalia arjuna, Prosopis spicigera and Mimusops elengi* were screened for the presence of alkaloids, terpenoids, saponin and flavonoids by using standard protocols. For thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) analysis, solvents (purchased from Merck, analytical grade) are generally used for extraction of secondary metabolites. In this study, our group focused on total flavonoid (crude) content extracted from these medicinal plants.For flavonoid estimation, 10 g of fresh plant leaves macerated in liquid nitrogen using mortar and pestle to prepare grounded plant leaves powder and reflux with 50 ml of 80 % methanol for 3 h at 90 - 100 ºC. Thereafter, cool down the solution containing plant leaves powder and proceed for filtration using whatman filter paper. Collect the filtrate and then suddenly add in the ratio of 2:1 of distilled water and ethyl acetate. Incubate the samples for 4-6 h at room temperature, collect only upper layer (ethyl acetate present, evaporate it) and then dried the plant leaves extract. Afterwards, the extracts settled at the bottom and observed qualitatively the flavonoid content using lead acetate solution, yellow precipitation appears[6]. This yellow color precipitation indicates the presence of flavonoid content.

Estimation of blood counts (lymphocytes, monocytes and granulocytes) and determination of CD14 monocyte surface marker of human whole blood using flow cytometer

Anticoagulant (EDTA) human blood samples were received from Mangal Pathology laboratory, Baramati region, District Pune, Maharashtra, India. For the estimation of blood counts, anti-coagulant human whole blood (100 µl) was taken into the eppendorf tube and stained with CD14 FITC monocyte surface marker and also treated with hepatitis B vaccine (20 µg/ml; 10 µl) antigen including variable doses of crude flavonoids (6.25, 12.5 and 25 mg; 50 µl). Afterwards, incubate all these flavonoid samples with hepatitis B vaccine antigen for 2 h in carbon dioxide (5 %) incubator at 37 °C. After incubation, lysed (using red cell lysis buffer) and wash (phosphate buffered saline, PBS) the samples and proceed for flow cytometric analysis for the estimation of total blood (lymphocytes, monocytes and granulocytes) counts using hepatitis B vaccine and CD14 monocyte surface marker in human whole blood[3,4,5].

Cytotoxicity assay

In addition, human lysed whole blood was cultured for 24-48 h in presence of variable doses of crude flavonoids extracted from *Terminalia arjuna, Prosopis spicigera and Mimusops elengi* along with or without hepatitis B vaccine antigen (10 µl) and proceeds for cytotoxicity assay. After 48 h incubation, add MTT solution (2.5 mg/ml; 10 µl) were added to 96 well flat bottom tissue culture plates and then again incubated for 4 h in carbon dioxide incubator. Again, the plates were centrifuged at 1800 rpm for 5 minutes and the supernatant was eliminated. Add DMSO (100 µl solution) to the formazon crystals and the absorbance was evaluated in an ELISA reader at 570 nm[4].

Statistical analysis

The difference between the control and treated groups of crude flavonoids extracted from Terminalia arjuna, Prosopis spicigera and Mimusops elengi. Data is represented by One way ANOVA (Boniferroni multiple comparison) test.

RESULTS

Estimation of total blood count and CD14 monocyte surface marker using flow cytometry

The effect of variable doses of crude flavonoids extracted from the leaves of *Terminalia arjuna, Prosopis spicigera and Mimusops elengi* on total blood counts and CD14 monocyte surface marker of human whole blood using hepatitis B vaccine antigen which is determined through flow cytometry. As shown in **Table 1**, the results showed that there is dose dependent decline in total blood counts of human whole blood at higher doses as compared to control. In addition, there is similar response observed in case of CD14 monocyte surface marker. The results showed that there is dose dependent decline in CD14 monocyte surface marker with treated with hepatitis B vaccine as compared to control **(Fig.1).** Over all, the data of total blood counts and CD14 monocyte surface marker at higher doses showed immunosuppressive activity.

Table 1. Effect of flavonoids on total blood counts (lymphocytes, monocytes and granulocytes count using flow cytometry

S.N.	Treatment (µg and mg)	Lymphocytes	Monocytes	Granulocytes
1	Control	9.19 ± 1.06	1.87 ± 0.15	48.08 ± 3.54
2	Hepatitis B vaccine (20 µg/ml, 10 µl)	10.31 ± 1.54	6.44 ± 0.98	52.14 ± 2.08
Terminalia arjuna				
3	6.25 mg + Hepatitis B vaccine	16.5 ± 1.98	2.01 ± 0.18	44.36 ± 3.76
4	12.5 mg + Hepatitis B vaccine	13.26 ± 2.03	1.43 ± 0.18*	45.24 ± 2.13
5	25 mg + Hepatitis B vaccine	8.16 ± 2.82	0.67 ± 0.01***	29.86 ± 2.76*
Prosopis spicigera				
6	6.25 mg + Hepatitis B vaccine	12.37 ± 1.89	1.76 ± 0.64	41.2 ± 2.89
7	12.5 mg +	9.65 ± 2.16	0.78 ±	39.47 ± 2.64

	Hepatitis B vaccine		0.02*	
8	25 mg + Hepatitis B vaccine	10.02 ± 1.81	0.34 ± 0.01***	21.08 ± 3.42**
Mimusops elengi				
9	6.25 mg + Hepatitis B vaccine	14.23 ± 1.84	1.46 ± 0.22	37.14 ± 3.21
10	12.5 mg + Hepatitis B vaccine	11.54 ± 1.87	1.02 ± 0.12	28.14 ± 1.64
11	25 mg + Hepatitis B vaccine	12.12 ± 1.43	0.45 ± 0.01***	18.14 ± 1.44***

*Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software. 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.*

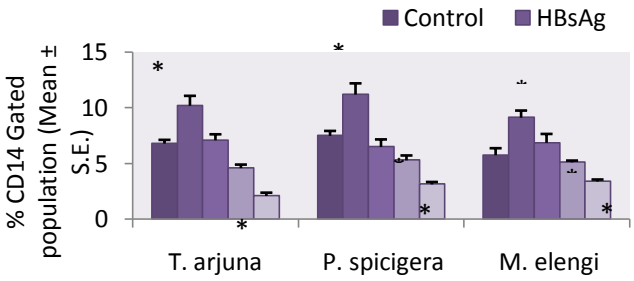

Fig.1. Effect of variable doses of flavonoids isolated from *Terminalia arjuna, Prosopis spicigera* and *Mimusops elengi* on CD14 monocyte surface marker in human whole blood. Values are expressed as Means ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). **P < 0.05; **P < 0.01; ***P < 0.001*

Cytotoxicity assay

The effect of variable doses of crude flavonoids on human lysed whole blood in order to observe its cytotoxicity as shown in **Fig. 2**. Human lysed blood cells were cultured for 48 h in the presence of variable doses of crude flavonoid along with or without hepatitis B vaccine antigen. The results showed that there was a significant cytotoxicity at higher doses as compared to control.

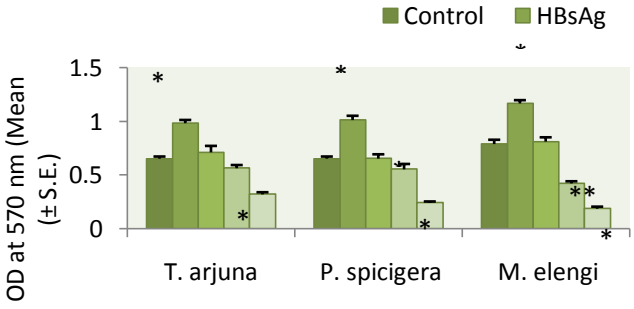

Fig.2. Cytotoxicity assay. In order to determine the effect of variable doses of flavonoids isolated from *Terminalia arjuna, Prosopis spicigera* and *Mimusops elengi* on lysed human whole blood using hepatitis B vaccine antigen**.** Values are expressed as Means ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). **P < 0.05; **P < 0.01; ***P < 0.001*

DISCUSSION

According to Ayurveda, medicinal properties of various plant products which improved the quality as well as nutritional value of these plant materials has been investigated for the last so many years. Traditionally, these medicinal plant products represent a source for discovering number of bioactive drugs including immunomodulators (stimulatory/suppressive/adjuvants), used in the pharmaceutical industry[9]. Flavonoids (secondary metabolites) are one of the medicinal plant products, derived from vegetables, fruits, herbs, flowers, seeds etc that are present in diet[10]. Generally, these flavonoids are polyphenolic compounds and display a variety of immunobiological effects such as anti-oxidant, anti-inflammatory, anti-cancer etc. In other words, these flavonoids showed so many potent compounds and have been shown to regulate immune responses[11,12].Flavonoids are the backbone of medicinal plant products and the bioactivity of these crude flavonoids isolated from medicinal plants is probably underestimated, although it has been shown in past decades to have a large spectrum of biological effects[13,14]. Thus, we showed the effect of these flavonoids isolated from three medicinal plants and verified its immunopharmacological activity in human whole blood using hepatitis B vaccine as specific antigen. The results showed that the inhibitory effect of these flavonoids from these three medicinal plants on total blood counts, CD14 monocyte surface marker and cytotoxic effect using hepatitis B vaccine as specific antigen.This study focused on flavonoids extracted from three medicinal plants on human whole blood using hepatitis B vaccine as specific antigen and this experiment will be performed by flow cytometer. This instrument is time consuming and requires experienced personnel. Therefore, flow cytometry (multi-colored) has great value as compared to traditional microscopy since it acquires as well as analyzes the number of cells (lymphocytes, monocytes and granulocytes) in a fraction of time of lysed human whole blood using forward and side scatter. The criteria for forward and side scatter in human lysed whole blood is that dead cells have higher side scatter as compared to forward scatter where as live cells have higher forward scatter as compared to dead cells[15]. As per the results related to these immunopharmacological studies showed that these flavonoids showed dose dependent decrease in total blood counts after treating with HBsAg at higher doses. Similar studies were done in case of lysed human whole blood using CD14 represents useful surface marker for monocyte (blood) or monocyte derived macrophages (tissues) surface marker and these were identified by their expression of CD14 which is a part of receptor for lipopolysaccharide[4]. The results showed similar pattern i.e. dose dependent decrease in CD14 marker at higher doses. In addition, cytotoxicity based assays are generally used for screening the flavonoids and the results indicates that these flavonoids showed immunosuppressive effect after incubating with optimized dose of HBsAg at higher doses as compared to control. Overall, the data showed immunosuppressive activity.

CONCLUSION

These flavonoids isolated from fresh harvested leaves of *Prosopis spicigera*, *Mimusops elengi* and *Terminalia arjuna* showed various medicinal properties. Hence the confirmation on the presence of these flavonoids derivatives and its immunosuppressive activity shall drive the research towards determining various immunopharmacological based studies.

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