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ESTIMATION OF BIOCHEMICAL STATUS AND IMMUNOSTIMULANT POTENTIAL OF CHOSEN FRUITS

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ABSTRACT

In the present study investigates biochemical status and immuno-stimulant potential of different fruits such as Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota. The amount of carbohydrate was high in Custard apple followed by Paneer guava, Pomegranate, Carambola, Muskmelon and Sapota. The amount of protein was high in Paneer guava followed by Custard apple, Sapota, Pomegranate, Muskelon and Carambola. The Percentage of T cells were varied remarkable due to exposure of antigens (*Staphylococcus aureus* was 64*, Streptococcus pyogenes* was 67*, Bacillus subtilis* was 62*, Salmonella typhi* was 64 and *Shigella sonnei* was 67). Percentage of T cells in fruits extract administered mice showed in the following order Paneer guava (54), Sapota (56%), Muskmelon (57), Custard apple (58), Pomegranate (59) and Carambola (59). The Percentage of B cells composition was 38, 43, 37, 40 and 35 in animals administered with antigens of *Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus subtilis, Salmonella typhi* and *Shigella sonnei* respectively. B cells in fruits extract administered animals showed 45, 44, 43, 40, 39 and 37 percentages in Muskmelon, Paneer guava, Custard apple, Carambola, Pomegranate and Sapota respectively. The results showed injection of antigen in paw, after 3hrs paw volume is increased about 8mm than the normal. The paw volume was increased about 10mm at 24hrs and after 24hrs the paw volume was increased more than 10mm in all the five antigens. The immunoglobin concentration of Ig G was observed in the antigen administered mice and fruit extracts administered mice. From the results the chosen fruits have effective immunostimulant potential.

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KEYWORDS

Fruits, biochemicals, immuostimulant potential and mice.

INTRODUCTION

Nature provides enough food, nutrition and environmental security for every living being. The presence of various nutrients is essential in human diet for healthy and active life. There have been several other terms used for functional foods such as pharma foods, designer foods, smart foods, health foods etc (Khan and Siddiqui, 2007). In human nutrition, fruits play an important role for balanced diet. They provide not only energy with foods, but also vital protective nutrients like vitamins and minerals. Comparatively fruits are the cheapest source of natural nutritive foods which help in building resistance against diseases (Ravindran *et al.*, 2004). In human diet, fruits and vegetables in general are considered to be the primary source of carbohydrate, protein, and fat. They can be used as stable food. Most of fruits are rich in minerals, vitamin A and C. Besides there are some trace elements required by the body like copper, manganese and zinc which act as enzyme co-factor (Yunfeng *et al.,* 2006). As per the specifications of the National Institute of nutrition at least 300g of fruits and receive less than 50mg of vitamin C daily, to be consumed by an individual for a balanced diet (Anju puri *et al.*, 2000). Fruits are abundant in nutrients, such as fibre, potassium, folate and vitamin C. Moreover, they also contain carotenoids and polyphenol and which act as antioxidants within the body.

In recent days, lot of medicine, chemicals as well as natural products have been introduced in order to stimulate the non-specific defense mechanism as well as specific immune responses if the treatment is followed by infection or vaccination (Mitra *et al.*, 1999). Traditional Indian system of medicine like Siddha and Ayurveda has suggested means to increase the body's natural resistance to disease. A number of fruits used in the traditional medical system of remedies in India. They have been shown to possess immunostimulating activity acting at different levels of the immune system (Dhasarathan *et al.,* 2010a). *Punica granatum* is a medicinal fruit claimed to possess number of therapeutic uses Yenfeng *et al*., (2006) reported that *Punica granatum* is an important source of compounds and has been used for folk medicine for many centuries. Hence, in the present study investigates biochemical status and immunostimulant potential of chosen fruits.

MATERIALS AND METHODS

Collection of sample: The six fruits Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota were used for the study. The collection of five fruits Paneer guava, Custard apple, Pomegranate, Muskmelon and Sapota from Pazhamuthirsolai, Palayamkottai, Tamilnadu, India and the carambola was collected from Courtalam, Tamilnadu, India.

Biochemical Analysis:

Estimation of carbohydrate: (Anthrone method): Weigh 100 mg of fruit sample and hydrolyze by keeping it in boiling water bath for 3 hours with 5 ml of 2.5 N Hydrochloric acids and cool to room temperature. Then neutralize it with solid sodium carbonate until effervescence ceases. The sample makes up the volume to 100 ml and centrifuge. From the sample collect the supernatant and take 0.5 and 1 ml aliquot for analysis. Then prepare the standard by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard.'0' serves as blank. Make up the volume to 1 ml in all including the sample tubes by adding distilled water. Then add 4 ml anthrone reagent, contents heat for 8 minutes in a boiling water bath. Cool rapidly and read the green to dark green color at 630 nm. Draw a

standard graph by plotting concentration of the standard on the x-axis versus absorbance on the y-axis. From the graph calculate the amount of carbohydrate present in the sample tube. The amount of carbohydrate present in 100 mg of the sample was calculated by mg of glucose divided by volume of test sample and multiplied with 100.

Estimation of protein (Lowry's method, 1951): Extraction is usually carried out with buffers used for the enzyme assay, Weigh 500 mg of the fruit sample and grind well with a mortar and pestle in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes. Pipette out 0.1 and 0.2 ml of the sample extract in two other test tubes. Make up the volume to 1 ml in all test tubes. A tube with 1 ml of water serves as the blank. Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing for 10 minutes. Then add 0.5 ml of reagent D, mix well and incubate at room temperature in the dark for 30 minutes. Blue colour is developed. Take the readings at 660nm. Draw a standard graph and calculate the amount of protein in the sample. Express the amount of protein mg/g or 100 g sample.

Preparation of fruit extracts:
The test fruits were extracted with help of a mixer grinder using aqueous solvent. The obtained extracts were filtered with Whatman no.2 filter paper and the filterate was collected. The aqueous solvent was then removed from the filterate under reduced pressure to obtain the dry extract. The dried extracts were the suspended in Dimethyl Sulfoxide (DMSO) to give final concentration of 5 mg\ml.

Immunological Assay:
The immunological assay should follow collecting blood in bacterial antigens administered and fruit extracts administered mice.

***Preparation of antigens*:** Bacterial pathogens such as *Staphylococcus aureus, Streptococcus pyogene, Salmonella typhi, Shigella sonnei* and *Bacillus subtilis* were used. The tested organisms were checked for their viability and efficacy. Cultures were re-streaked on nutrient agar slants for storage and sub cultured on nutrient broth for analysis.

Immunization of animals with bacterial antigens and fruit extracts*:* For the experimental study, mice weighing 24 ± 0.2 gm (35 days old) were recruited from the acclimatized stock. Mice were grouped into several groups with six individuals each. These animals were housed in specially designed cage with provision for systematic supply of pellets and water. Animals were trained to take water and feed from the cage provided. The test animal divided into 12 groups and treated with bacterial antigens and fruit extracts. Six groups of mice administered with fruit extracts and check fruits immunostimulant potential. Another five groups, each culture administered to each group of animals and analysed their immunomodulatory potential. One group served as control to evaluate test animals.

Blood sample of test animals were collected after 3rd weeks following antigen exposure by cardiac puncture after anaesthetizing mice with chloroform. The serum was separated for each group separately and kept at -200° C till analyses. Heparin was used in collecting whole blood.

Analysis of humoral immune response: In the present study humoral immune response was analyzed by antibody titration, B cell e-rosette assay and isolation of antibody techniques.

Isolation of Immmunoglobin: The 1 x equilibrium buffers was prepared and add serum in column and then add equal amount of equilibrium buffer and then eluted serum was collected with help of eppendorf contain 20µl of neutralizing buffer and take O.D value at 280 nm in Spectrophotometer. From the O.D value find-out concentration of immunoglobulin using standard graph.

Cell mediated immune response: In the present study delayed type hypersensitivity reaction and T cell erythrocyte rosette assay used to evaluate cell mediated immune responses.

Erythrocyte rosette assay: Blood cells collected from test animal and control mice using heparin pretreated vials. T-cell counts in the blood were carried out up to loading of lymphocyte in nylon wool column.

Resuspended lymphocytes were loaded into activated nylon wool column. Then the column was held vertically above an eppendorf tube, now hot saline about 60^0 C was slowly dripped into the column. The hot saline passing that of the column was collected in the eppendorf tube, which contain T lymphocytes, 0.2 ml of saline containing T lymphocytes (from the eppendorf tube containing T cell) was taken in a separate eppendorf tube to the 0.2 ml of 1% SRBC was added and then the mixture was centrifuged for 12 minutes at 1600 rpm. After centrifugation those samples were incubated in an ice box or refrigerator at 4^0 C for 5 minutes. After cold incubation the pellet in the eppendorf tube was resuspended by gentle flushing with a Pasteur pipette. Then a drop of it was taken in a clean dry slide. Observed and enumerated T-cells under microscope (20x\40x) for rosettes. Number of rosettes formed was observed per hundred lymphocytes observed. The same procedure was carried out to the B-cell erythrocyte, but instead of hot saline, cold saline was used.

Delayed type hypersensitivity: Delayed type hypersensitivity was studied by Dhasarathan *et al.,* (2008) method for mice. A positive response is conventionally assessed as one giving ≤ 5 in durations. Responses can be graded with 3-4 mm = +; 5-8mm = ++, 9-11= +++; 12mm or more = ++++.

Mice were sensitized by subcutaneous injection in the intranasal region with 0.5 ml of freunds adjuvant containing 500 mg of by an intradermal injection to sterile phosphate buffer with a Vernier caliper prior to challenge, i.e. 0^{th}, 3^{rd} and 24th hour post challenge, each with three readings. The increase in skin thickness (MST) of mice was obtained after deducting the skin thickness of the same oil before challenge. Overall MST was obtained by taking the mean of individual mice with group.

RESULTS AND DISCUSSION

Biochemical Estmations: The estimation of Carbohydrate and protein of six fruits such as Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota were observed and recorded in the table1**.** The amount of carbohydrate was high in Custard apple, Paneer guava, pomegranate, Carambola, Muskmelon and Sapota. The amount of protein is high in Paneer guava, Custard apple, Sapota, Pomegranate, Muskelon and Carambola.

Table: 1

Estimation of Biochemicals (Carbohydrate & Protein) obtained in test fruits.

S.No.	Fruit sample	Volume of Sample (ml)	Carbohydrate (mg\100g)	Protein (mg\ 100g)
1.	Paneer guava	0.2	4	7.6
		0.4	20	10
		0.6	25	11.6
		0.8	28	12.4
		1.0	30	16.4
2.	Pomegranate	0.2	3	2.0
		0.4	12	2.8
		0.6	15	4.0
		0.8	16	4.8
		1.0	20	12.8
3.	Sapota	0.2	6	3.2
		0.4	10	3.6
		0.6	14	6.4
		0.8	18	7.2
		1.0	26	7.6
4.	Custard apple	0.2	6	4.4
		0.4	8	6.8
		0.6	28	8.4
		0.8	34	9.2
		1.0	60	10
5.	Muskmelon	0.2	10	1.2
		0.4	11	1.6
		0.6	12.5	2
		0.8	13	2.4
		1.0	18	4.4
6.	Carambola	0.2	12	1.6
		0.4	13	2.8
		0.6	14.5	3.2
		0.8	18	3.6
		1.0	23	4

Lymphocyte enumeration: The T cells from antigen injected mice and fruit extract injected mice were recorded in table 2 and 3. The Percentage of T cells is in antigen of *Staphylococcus aureus* was 64*, Streptococcus pyogenes* was 67*, Bacillus subtilis* was 62*, Salmonella typhi* was 64 and *Shigella sonnei* was 67. Then the Percentage of T cells is in six fruits extract injected mice blood such as Paneer guava was 54, Custard apple was 58, Pomegranate was 59, Muskmelon was 57, Carambola was 59 and Sapota was 56. The B cells from antigen and fruit extract administered mice were recorded in table 2 and 3. The Percentage of B cells composition was 38, 43, 37, 40 and 35 in antigens of *Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus subtilis, Salmonella typhi* and *Shigella sonnei* respectively. The Percentage of B cells is in six fruits extract showed 44, 43, 39, 45, 40 and 37 in Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota respectively.

Table 2

Enumeration of lymphocyte subset population using Erythrocyte rosette forming assay on test animals administered with bacterial antigens.

S.No.	Test Antigen	Lymphocyte population in test animals			
		1st week		After 3rd week	
		% of B cells	% of T cells	% of B cells	% of T cells
	Normal	24	43	36	58
1.	*Staphylococcus aureus*	24	43	36	58
2.	*Streptococcus pyogenes*	28	47	38	64
3.	*Salmonella typhi*	25	44	43	67
4.	*Shigella sonnei*	23	45	40	64
5.	*Bacillus subtilis*	24	47	37	62

Table 3

Enumeration of lymphocyte subset population using Erythrocyte rosette forming assay on test animals administered with fruit extract.

S.No.	Test Antigen	Lymphocyte subset population in test animals at different time interval			
		1st week		After 3rd week	
		% of B cells	% of T cells	% of B cells	% of T cells
1	Normal	24	43	36	58
2	Pomegranate	27	46	39	59
3	Sapota	26	46	37	56
4	Muskmelon	28	44	45	57
5	Carambola	25	46	40	59
6	Custard Apple	29	46	43	58
7	Paneer guava	28	45	44	54

Delayed type hypersensitivity: The results showed injection of antigen (table 4) in paw, after 3hrs paw volume is increased about 8mm than the normal. Then the paw volume is increased about 10mm at 24hrs and after 24hrs the paw volume is increased more than 10mm in all the five antigens *Staphylococcus aureus, Streptococcus pyogene, Salmonella typhi, Shigella sonnei* and *Bacillus subtilis* (Table 4). So it produces cell mediated immune response. So it increases in paw volume which was considered as an index of delayed type Hypersensitivity.

When the fruit extracts of Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota injected into mice paw, after 3hrs the paw volume is increased about 8mm. Then after 24hrs there is no increase in paw volume. So that fruit extracts treated group of mice produced significant decrease in paw swelling which suggests cell mediated immune response. Delayed type hypersensitivity reaction experiments in various medicinal plants showed into increase in paw volume, because in that study SRBC used as antigen (Anju puri *et al.*, 2000). The immunoglobin concentration of Ig G was observed in the antigen and fruit extracts administered mice were recorded in the table 5.

Table 4

Evaluation of Delayed type hypersensitivity response in test animal exposed to fruit extracts

S.No	Groups	Paw volume(mm) at different time intervals			
		0 hrs	3 hrs	24 hrs	After 24 hrs
1.	Pomegranate	+	+	+	+
2.	Sapota	+	+ +	+ +	+
3.	Muskmelon	+	+ +	+ +	+
4.	Carambola	+	+ +	+ +	+
5.	Custard Apple	+	+ +	+ +	+
6.	Paneer guava	+	+ +	+ +	+

Table 5

Estimation of immunoglobulin concentration in test animals administered with bacterial antigens and fruit extracts.

S.No	Organisms	Ig concentration (µg\ml)
1	*Staphylococcus aureus*	4.40
2	*Streptococcus pyogenes*	2.74
3	*Salmonella typhi*	3.94
4	*Shigella sonnei*	4.59
5	*Bacillus subtilis*	2.40
6	Pomegranate	18.6
7	Sapota	15.3
8	Muskmelon	18.6
9	Carambola	14.2
10	Custard Apple	17.5
11	Paneer guava	16.4

In the present study investigates bioactive potential of six different fruits such as Paneer guava, Custard apple, Pomegranate, Muskmelon, Carambola and Sapota. All the bacterial antigens treated mice, the reduction of Ig concentration is more in *Bacillus subtilis* than in other antigens. The degree of reduction of Ig level for the four antigens in the following order: *Streptococcus pyogene, Salmonella typhi, Staphylococcus aureus* and *Shigella sonnei*. The inhibition of Ig synthesis in bacterial antigen treated mice was significantly changed as per previous workers (Hemalatha and Dhasarathan, 2010 and Sujatha *et al.,* 2010). In fruit extracts administered mice showed significant increment of Ig. Dhasarathan (2010b) and Hu (2003) had reported that the impaired production of IFN-γ, reduction of Th-1 (T helper cell-1) mediated immune response and reduced NK cell activity affect Ig concentration drastically.

India will in the near future become the fruit and vegetable 'basket' of not just India, but a country to be reckoned with in world trade and we will also have another source of foreign exchange earnings (Van Duyn and Pivonka, 2000). The fruits and vegetables which were the corner stones of health, supplying us with a wealth of vitamins, minerals, fibers and carbohydrates have assumed utmost importance a few the discoveries of phytochemicals.

The fruit showed immunostimulant activity because in delayed hypersensitivity reaction the fruit extract thus not increase after 24hrs because B cell involved in the reaction and the fruit extract suppress the antigenic cell mediated immune response. It is interesting note that six fruits may have immunostimulator and immunosuppressant activity due to the presence of various glycosides, tannins, phenols and flavonoids. Therefore present investigation may demonstrate immunomodulator activity of six different fruit extracts. In conclusion, the finding in this study suggests that the six fruits possess antibacterial activity and immunstimulant activity in mice. Further pharmacological investigations are warranted in this direction for establishing its detailed mechanism of action and for substantiating its traditional and folk claims.

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