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Phytochemical Screening and Immunomodulator Activity of Some Green Vegetable Drugs

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Abstract

The present study includes phytochemical screening and immunomodulator activity of green vegetable drugs (*Artocarpus heterophyllus*, *Cichorium intybus*, *Brassica oleracea*) that can be useful in terms of reducing side effect and used in daily diet. The green vegetables were extracted and evaluated for their immunomodulatory activity. The immunomodulatory & phagocytic activity were ascertained by in-vivo (male wistar rats) and in-vitro (*Blood PMN cells*) respectively. In addition, antioxidant activity also performed by DPPH Method & Free radical scavenging assays.

Introduction

Traditional and folklore medicines play an important role in health services around the globe. About three quarters of the world's population relies on plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity. Several plants have been used in folklore medicine[1]. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Ayurveda, the traditional medicinal system in India, describes certain plants which strengthen the host immune system. All parts of the plant have medicinal properties. Precedence exists in literatures, the root of *Artocarpus heterophyllus* is a remedy for skin diseases and asthma and the extract is taken in cases of fever and diarrhea[2]. the various biological activity of *Cichorium intybus* may help humans with weight loss, constipation, improving bowel function, and general health.[3-5] the leaves of *Brassica oleracea* is used for its anticancer properties[6]. As potential biological activities, it has been hypothesized that these novel plant extract will

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be worth investigating for its immunomodulator and antioxidant activity. Hence in this communication, the plant material were extracted and assessed for their biological properties (in vivo & in vitro).

Experimental

Preparation of Extract:

The vegetables were shade dried and the coarse powders were extracted in soxhlet apparatus. The extract was then concentrated to dryness under reduced pressure by using rotary evaporator and preserved in a dessicator for further use.

The extract was fractionated into alcohol soluble, petroleum ether soluble, chloroform soluble and insoluble fraction.

1. Extraction of *Artocarpus heterophyllus* (Outer covering):

Authenticated outer covering of *Artocarpus heterophyllus* was shade dried at room temperature, pulverized, and 100 g of the powder was extracted exhaustively with 95% ethanol at temperature 60 °C, in a Soxhlet extractor. The extract was concentrated in a rotary flash evaporator, residue was dried in a dessicator over sodium sulfite. Another 100 g of the powder was extracted exhaustively & successively with petroleum ether (40-60 °C) and chloroform. Each extract was concentrated to a small volume and allowed to dry. After drying, the respective extracts were weighed and evaluated for biological activity.

2. Extraction of *Cichorium intybus* (Leaves):

Leaves of *Cichorium intybus* was shade dried at room temperature, pulverized, and 200 g of the powder was extracted exhaustively with 95% ethanol at temperature 60 °C, in a Soxhlet extractor. The extract was concentrated in a rotary flash evaporator, residue was dried in a dessicator over sodium sulfite. Another 200 g of the powder was extracted exhaustively & successively with petroleum ether (40-60 °C) and chloroform. Each extract was concentrated to a small volume and allowed to dry. After drying, the respective extracts were weighed and evaluated for biological activity.

3. Extraction of *Brassica oleracea* (Leaves):

Authenticated leaves of *Brassica oleracea* were shade dried at room temperature, pulverized, and 300 g of the powder was extracted exhaustively with 95% ethanol at temperature 60 °C, in a Soxhlet extractor. The extract was concentrated in a rotary flash evaporator,and residue was dried in a dessicator over sodium sulfite. Another 300 g of the powder was extracted exhaustively & successively with petroleum ether (40-60°C) and chloroform. Each extract was concentrated to a small volume and allowed to dry. After drying, the respective extracts were weighed and evaluated for biological activity.

Biological activity :

***1. In vivo* immunomodulator activity**

Male Wister rats weighing 150-180 g were procured from Laboratory Animals Resources and all rats were treated in accordance with the guideline for the care and use of

laboratory animals with the permission of Institute Animal Ethical Committee.

Animal Grouping:

For experimental procedure, Male Wister rats were divided in the following four groups containing six rats in each group. Group I (n=6): Negative control: Rats treated with 2 ml of 1% gum acacia solution in distilled water. Group II (n=6): Positive control: Sensitized rats (by administrating $1x10_8$ SRBCs, i.p.) treated with 1% gum acacia solution orally. Group III (n=6): Rats treated with cyclophosphamide 100 mg/kg/p. o. Group IV (n=6): Sensitized rats treated with alcoholic extracted 100 mg/kg/p. o. in the following regimens.

a) 4 days prior to sensitization (days -3, -2, - 1, 0).
b) 7 days after sensitization (days +1, +2, +3, +4, +5, +6, +7).

Preparation of Sheep Red Blood Cells (SRBC):

From healthy sheep blood was collected from local butcher house and mixed with sterile Alsever's solution (1:1). It was thoroughly mixed and centrifuged at 3000 rpm for 5 min. Supernatant was discarded, SRBC pellets were washed with sterilized phosphate buffer saline (pH 7.2) 2-3 times. Then the SRBC pellets were prepared in phosphate buffer saline (pH 7.2) and total SRBC was counted using Neubauer chamber, finally $1x10^8$ SRBCs (0.5ml) were injected intraperitoneally for sensitization and challenging the rats[7].

Blood Profile for Study of Immunomodulatory Activity:

Rats were divided into four groups as described earlier. After 7 days treatment, blood was collected from rats by retroorbital plexus for study of different parameters.

Determination of Differential Leukocyte Count (DLC):

A drop of blood drop was added on the centre line of the glass slide about 1 cm from one end and blood smear was prepared. Then smear was stained with diluted Leishman's stain for 30 min and washed with distilled water and dried at room temperature. For counting of DLC the slide was examined under microscope at 100x using Cedar wood oil. Finally total number of Neutrophils, Lymphocytes and Monocytes in the 100 cells were counted and results were expressed in percentage[8].

***2. In vitro* Phagocytic Activity**

Preparation of Blood PMN cells :

Separation of blood PMN cells was done as per the method described by (Daley *et al* 1991). Blood sample (1ml) was collected by retro orbital plexus in heparinised sterile tubes (20 IU heparin / ml of blood). One part of blood was diluted with two parts of sterile Tris- ammonium chloride buffer (pH 7.3) and thoroughly mixed for 1-2 minutes and was kept for 20 minutes at room temperature. Blood samples were centrifuged at 3000 rpm for 20 minutes. The supernantant was discarded and the cell pellets were removed with 5 ml sterile chilled phosphate buffer solution (PBS) (pH 7.4). Then the solution was further centrifuged for 10 minutes in the same manner twice to get PMN cell pellet. The pellet obtained was

resuspended in 1 ml of sterile cold PBS[9].

Preparation of Microorganism :

Escherichia coli (NCIM 2391) was grown and kept on a slant agar media. Before use, the microorganism was inoculated in 100 ml of 2.5% nutrient broth media for 18hrs at 37±2°C. The culture was then washed twice with sterile PBS (pH 7.2) and re-suspended in 1 ml gelatin HBSS (Hank's Buffered Salt Solution) to get a concentration of 1x107cells/ ml. During each experiment, the numbers of viable microorganisms were determined by counting colony forming units (CFU), using nutrient agar plates[10].

Viable PMN cell count :

The viable cell counts were determined by Trypan blue exclusion techniques. 20 µl each of cell suspension and 0.1 % trypan blue were mixed and kept for 2 minutes at room temperature. A drop of mixture was loaded on haemocytometer, the viable (unstained) and dead (stained) cells were counted in WBC counting chamber. The viable cell count was expressed as per the method reported earlier[11].

Microbiological Assay for the Phagocytosis Activity :

To assess phagocytosis and alcoholic extract (100µg/ml) in the final volume of 0.1ml were incubated with 2 ml of PMNCs suspension ($1x10_7$ cells/ml) and 2 ml of microorganism ($1x10_7$ cells/ml) at 37 ± 2^0C for 1hr in 5% CO_2 atmosphere in a slanting position. 1ml of the standard drug, cyclophosphamide (100 mg/ml) was incubated with fetal calf serum in the same conditions. At 30 min intervals upto 120 min, 0.5 ml aliquot of the suspension was removed and added to 1.5 ml of the ice cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 100g for 4 min. Under this condition, the non-ingested microorganisms remained in the supernatant fluid. The viable count of the microorganisms was done using the colony counter. Phagocytosis was expressed as the percentage decrease in the initial number of viable extracellular bacteria[12].

The Data of Immunomodulatory activity by *in vitro* phagocytosis test of different plant extract is shown in Table 1, Table 2 & Table 3.

Statistical analysis:

The results were expressed as mean ± S.D. and statistical evaluation of the data was done using students t-test and $P<0.05$ was considered as significant.

3. Antioxidant Activity by DPPH Method :

A simple method that has been developed to determine the antioxidant activity of foods utilizes the stable 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant are shown above. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH

Table 1. In vitro phagocytosis test of the successive extracts of *Artocarpus heterophyllus*

Test extract	Concentration (mg/ml)	% Phagocytosis (Mean ± SD)	Phagocytic index (Mean ± SD)
Petroleum ether	Control	30±1.0	1.62±0.06
	0.5	33±1.15	1.74±0.15
	1.0	29±2.52	1.77±0.15
Chloroform	Control	35±1.15	1.98±0.02
	0.5	34±2.40	1.97±0.01
	1.0	38±2.78	1.94±0.06
Alcoholic fraction	Control	39.6±1.15	2.08±0.02
	0.5	40±2.40	2.16±0.01
	1.0	39±2.78	2.24±0.06

Table 2. In vitro phagocytosis test of the successive extracts of *Cichorium intybus*

Test extract	Concentration (mg/ml)	% Phagocytosis (Mean ± SD)	Phagocytic index (Mean ± SD)
Petroleum ether	Control	30±1.0	1.62±0.06
	0.5	33±1.15	1.74±0.15
	1.0	35±2.52	1.77±0.15
Chloroform	Control	36±1.15	1.98±0.02
	0.5	37±2.40	1.97±0.01
	1.0	35±2.78	1.94±0.06
Alcoholic fraction	Control	39.2±3.21	2.16±0.11
	0.5	39±1.00	2.10±0.07
	1.0	40±1.0	2.22±0.05

Table 3. In vitro phagocytosis test of the successive extracts of *Brassica oleracea*

Test extract	Concentration (mg/ml)	% Phagocytosis (Mean ± SD)	Phagocytic index (Mean ± SD)
Petroleum ether	Control	35±1.15	1.90±0.02
	0.5	39±0.75	1.94±0.01
	1.0	38±2.76	1.96±0.05
Chloroform	Control	29±3.21	1.66±0.11
	0.5	27±1.00	1.60±0.07
	1.0	30±1.0	1.61±0.05
Alcoholic fraction	Control	35±1.15	1.98±0.02
	0.5	36±2.40	1.97±0.01
	1.0	36±2.78	1.94±0.06

Table 4. 1,1-Diphenyl-2-picryl-hydazol (DPPH) free radical scavenging activity of the successive extracts of *Artocarpus heterophyllus*

Quantity of the fraction used µg	**% Inhibition**			
	R1	**R2**	**R3**	**α-tocopherol**
100	33.56±1.32	42.58±2.8	42.32±5.48	75.45±4.24
200	35.89±2.51	38.21±3.95	39.47±5.12	51.22±3.55
500	8.97±2.86	43.18±6.55	44.44±2.57	65.46±4.56

α-Tocopherol is used as standard. R^1 : Petroleum ether; R^2 : Chloroform; R^3 : Alcoholic fraction

Table 5. 1,1-Diphenyl-2-picryl-hydazol (DPPH) free radical scavenging activity of the successive extracts of *Cichorium intybus*

Quantity of the fraction used µg	**% Inhibition**			
	R1	**R2**	**R3**	**α-tocopherol**
100	52.45±3.39	35.68±5.18	48.52±4.19	76.47±5.12
200	15.56±4.17	38.68±5.16	52.04±5.11	62.45±4.89
z500	5.23±3.28	10.54±2.96	28.54±3.98	70.25±5.24

α-Tocopherol is used as standard. R^1 : Petroleum ether; R^2 : Chloroform; R^3 : Alcoholic fraction

Table 6. 1,1-Diphenyl-2-picryl-hydazol (DPPH) free radical scavenging activity of the successive extracts of *Brassica oleracea*

Quantity of the fraction used µg	**% Inhibition**			
	R1	**R2**	**R3**	**α-tocopherol**
100	27.58±2.80	50.56±1.32	52.59±4.85	60.36±3.45
200	28.18±6.50	54.97±2.86	56.19±5.22	65.32±5.48
500	10.21±3.95	58.89±2.51	60.48±3.33	68.44±2.57

α-Tocopherol is used as standard. R^1 : Petroleum ether; R^2 : Chloroform; R^3 : Alcoholic fraction

radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured. Antioxidant compounds may be water-soluble lipid soluble, insoluble, or bound to cell walls. Hence, extraction efficiency is an important factor in quantification of antioxidant activity of foods.Trolox (as the reference standard) and the sample are reacted with DPPH solution in methanol/water for four hours at 35°C in a vessel mounted on a rotary shaker and the absorbance was measured at 517 nm.

Free radical scavenging assays:

Superoxide anion scavenging activity assay

The scavenging activity of the CRRE towards superoxide anion radicals was measured by the method of Liu *et al* 23 (1997). Superoxide anions were generated in a non-enzymatidc phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 mL of NBT (300 µM) solution, 0.75 mL of NADH (936 µM) solution and 0.3 mL of different concentrations of the extract. The reaction was initiated by adding 0.75 mL of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A^0 - A_1) / A0 \times 100],$$

Where A^0 and A1 are the absorbance of the control (blank, without extract) and extract respectively.

The Data of *in vitro* antioxidant activity of different plant extract by DPPH method is shown in Table 4, Table 5 &Table 6.

Conclusion

The alcoholic extract of vegetables showed significant immunomodulatory and antioxidant activity in comparison to standard drug, petroleum ether extract and chloroform extract.Apart from the aforementioned merits, the results of the present study also emphasize the importance of the characterization and identification of active compounds. Hence, there is a need for future course of work will proceed to carry out a LC-MS/MS analysis of the active compounds.

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