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Original Article

EVALUATION OF IMMUNOMODULATORY AND ANTIOXIDANT ACTIVITIES OF POLYSACCHARIDES ISOLATED FROM *CALLICARPA MACROPHYLLA VAHL.*

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ABSTRACT

Objective: To evaluate the immunomodulatory and antioxidant activities of polysaccharides from *Callicarpa macrophylla Vahl.* leaves.

Methods: Three different fractions of polysaccharides (PC, PH, PA) were isolated. The percentage of sugar in the extracted samples was checked by phenol-sulphuric acid method. Lymphocyte proliferation assay was done to evaluate its immunomodulatory activity. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay and total antioxidant assay was done to evaluate its antioxidant property.

Results: The total polysaccharide content in PC, PH and PA was found to be 88%, 14% and 87% by the phenol-sulphuric acid method. All the three polysaccharides showed *in vitro* growth stimulatory effect on isolated normal lymphocytes. The proliferative index of PC, PH, PA was found to be 1.28±0.03, 1.71±0.04 and 1.12±0.01 at a concentration of 500 µg/ml indicating immunomodulatory activity. PC, PH, PA showed a % inhibition of DPPH radical at 40.11±0.005 %, 29.18±0.01 % and 16.82±0.007 %. A dose-dependent activity was shown for the DPPH assay. Total antioxidant activity was found to be higher in PA. Both PC and PH showed almost equal antioxidant activity.

Conclusion: The results of the present study indicates that polysaccharides from *Callicarpa macrophylla Vahl.* boosts the immune system and help overcome the negative effects of oxidative stress, thus, contributing to the development of new drugs.

Keywords: *Callicarpa macrophylla*, Lymphocyte proliferation assay, DPPH assay, Total antioxidant assay.

INTRODUCTION

Indian traditional systems of medicines like Siddha and Ayurveda have emphasized on increasing the body's natural resistance to disease [1]. Plant polysaccharides have traditionally been used as folk remedy for various diseases due to their multiple biological properties including anti-inflammation, anti-hepathitis, anti-ulcer etc. They have attracted researchers because of their advantages as: (I) the renewable character, (II) biodegradation, (III) the relatively low cost and (IV) the possibility of conversion into various derivatives due to their reactivity with many organic molecules [2].

Among the macromolecules, polysaccharides offer the highest capacity for carrying biological information because they have a great potential for structural variability [3]. Although research on polysaccharides, has been limited, due to the cumbersome isolation and purification procedures the fact that they possess multiple properties initiated this study. Polysaccharides act as immunomodulators and have profound effects in the regulation of immune responses during the progression of infectious diseases. They have been shown to act on the innate and cell-mediated immunity through interactions with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes. Even though many polysaccharide immuno modulators have been identified, relatively few polysaccharides have been examined in detail where both structure-function and mechanism of action studies have been performed [4].

Immune dysfunction is responsible for disease like allergy, asthma, arthritis, cancer and other infectious diseases. Modulation of immune response helps to control various infectious diseases [5]. It has been recognized that immunomodulation could provide an alternative to conventional chemotherapy in the treatment of various diseases [6]. Immunomodulation can improve the host's immune response to infections which can augment the current treatment regimens such as antimicrobial therapy that are becoming less efficacious with the advent of antibiotic resistance. Thus, the characterization of polysaccharides could lead to development of drugs with a potential for clinical use [4].

Callicarpa macrophylla Vahl. (Family-Verbenaceae) commonly known as Priyangu or Daya is globally distributed across India, Nepal, Bhutan, Myanmar, South East Asia, and China [7]. It is a perennial, deciduous shrub attaining 2.5 m in height [8]. The plant is used alone and in combination with other plants in Ayurvedic Siddha and Unani medicine and other folk medicines for the treatment of different diseases and disorders such as polydipsia, diarrhoea, diabetes, dysentery, fever and acts as blood purifier. The Ayurvedic Pharmacopeia of India describes the fruits of *Callicarpa macrophylla Vahl* as an essential component of several ayurvedic formulations [9]. The plant has been reported to have various medicinal properties. The bark is used to heal cuts and wounds. Seeds and roots are used for digestion and leaves are used for rheumatism. The fruits are used for blisters and boils. The antimicrobial and anti-inflammatory activities of this plant have been already proved [10]. As many as 20 species from *Callicarpa* have reported ethnomedical uses, and several members among these are well known in the traditional medical systems of China and South Asia. Ethnomedical reports indicate their use in the treatment disorders like hepatitis, rheumatism, fever, headache, indigestion, and other ailments [11]. The plant is already reported to have Antibacterial, Antidiabetic, Analgesic and antipyretic, Antifungal, Anti-inflammatory and Anti-arthritic activity [12].

By using ancient wisdom and modern science, many lead compounds can be identified which can be developed into drugs without side effects. Therefore, it is essential that a fast track programme to discover new drugs by building on traditional medicines and screening diverse plants and microbial sources of the country must be initiated [13].

MATERIALS AND METHODS

Isolation of polysaccharide fractions

Callicarpa macrophylla leaves were procured locally. It was washed in tap water, rinsed with distilled water and blotted gently between the folds of filter paper. 60 g of leaves were taken and ground into a fine paste using mortar and pestle. For extraction, cold distilled water was added to the leaf paste and centrifuged. Supernatant was taken and stored at 4˚C. Hot distilled water was added to the debris obtained and agitated for 2 h in the water bath set at 70-80 ˚C. It was then centrifuged and supernatant was collected. To another 30 g of

leave paste, ethanol was added and kept in a shaker at 120 rpm for overnight. After the complete evaporation of ethanol, 1 mol/l of NaOH was added and agitated at 100 ˚C for 4 h. All the three types of solutions were dialysed separately against double distilled water using 12-14,000 MW membrane. It was then subjected to ethanol precipitation. The precipitate was dissolved in hot water. The process was repeated 3 times. Polysaccharide precipitate soluble in cold water (PC), hot water (PH) and hot NaOH (PA) was collected by centrifugation at 12,000 rpm at 4 ˚C, re dissolved in distilled water and lyophilized [14-15].

Estimation of sugar content

The sugar content in the different extracted fractions was estimated using the phenol-sulphuric acid method [16]. About 10 mg of sample was dissolved in 100 ml of distilled water. From this 1 ml was used for sugar analysis. To estimate the polysaccharide content in sample, 1 ml of 5 % phenol was added to the 1 ml of sample, followed by 5 ml of concentrated sulphuric acid. The absorbance was measured after 10 min at 488 nm against blank. The experiment was carried out in triplicates. Glucose was used as the standard.

***In vitro* lymphocyte proliferation assay**

The immunomodulatory activity was checked in isolated human lymphocytes from blood [17]. Fresh human blood samples were layered on equal volumes of Hi-Sep LSM solution and centrifuged at 800 x g for 25 min at 18˚C. The thin white middle lymphocyte layer was collected and washed with Roswell Park Memorial Institute Medium-1640 (RPMI-1640) twice by centrifugation at 100 x g for 10 min at 18˚C. The supernat ant was discarded and the cells were suspended in 10% RPMI-1640. The cell number was counted on a hemocytometer. Cells were seeded at a concentration of 20,000cells/well in a 96-well plate and 100 µl of each extract at various concentrations (10-500 µg/ml) was added to the wells.

The plates were then incubated for 72 h at 37 ˚C in a humidified atmosphere of 5 % CO_2. After incubation, 20 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-yl)—diphenyl tetra bromide (MTT) (5 mg/ml) was added to each well and incubated for 2 more hours. The insoluble formazan crystals formed were solubilized by the addition of 100 µl MTT lysis buffer (Sodium Dodecyl Sulphate, dimethyl formamide and distilled water) followed by an incubation of 4 h and the absorbance were measured at 570 nm using a microplate reader. The proliferation rate (PR) was calculated:

$$proliferation\ rate = \frac{A_{test}}{A_{control}} \times 100$$

$A_{control}$ and A_{test} represent absorbance of control and test respectively.

Total antioxidant assay (TAA)

The total antioxidant assay is based on the reduction of molybdenum VI to molybdenum V to form a green phosphate complex [18]. Briefly 0.3 ml of different extracts ranging from 0.2-1 mg/ml concentrations were mixed with 3 ml of reagent solution (0.6 mol/l sulfuric acid, 0.028 mol/l sodium phosphate and 0.004 mol/l ammonium molybdate). Reaction mixture was incubated at 95 ˚C for 90 min in water bath. Reading was taken at 695 nm after cooling to room temperature.

TAA is expressed as the number of equivalents of ascorbic acid.

DPPH Radical scavenging assay

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) is a powerful free radical used to evaluate the electron donating capacity of antioxidants. DPPH is a stable free radical useful in the study of natural antioxidants [19]. The reaction mixture contained 2.8 ml of 100 µM DPPH dissolved in methanol and different concentrations ranging from 0.5-2 mg/ml of compounds in 0.2 ml DMSO. This mixture was incubated at room temperature for 30 minutes. After shaking the mixture, absorbance was measured at 517 nm. The percentage of DPPH scavenging activity was calculated as follows:

$$\%\ DPPH\ scavenging\ activity = \frac{(A_{control} - A_{test})}{(A_{control})} \times 100$$

$A_{control}$ and A_{test} represent absorbance of control and test respectively.

Statistical analysis

Data were expressed as the means±standard deviations (SD) of the triplicate values.

RESULTS AND DISCUSSION

Polysaccharide isolation

Three crude fractions of polysaccharides PC, PH and PA were obtained with a good yield of 1.944 %, 1.742 % and 1.722 % respectively. All the three fractions were of neutral pH and soluble in water. PC was light brown in colour and the other two were light yellow in colour.

Estimation of sugar content

The sugar content in different samples was determined by the Phenol-Sulphuric Acid Method. The calibration curve for different concentrations of glucose is represented in fig. 1. Using the proposed method, the calibration curve was found to be linear in the range of 0.1-0.7 mg/ml. The % Relative Standard Deviations (% RSD) lies between 0.23-0.53 indicating that the method is precise. The sugar content of PC, PH, PA was calculated using regression equation obtained from the calibration curve.

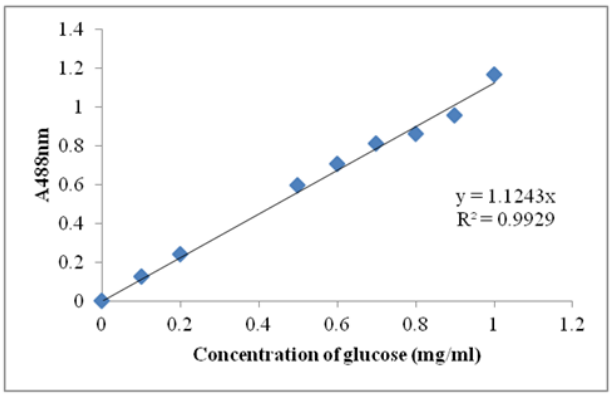

Fig. 1: Calibration curve of different concentrations of glucose estimated by phenol-sulphuric acid method

Table 1: The absorbance shown by different crude polysaccharide fractions

S. No.	Absorbance of PC	Absorbance of PH	Absorbance of PA
1	1.065	0.186	1.026
2	1.068	0.185	1.023
3	1.063	0.184	1.020
Mean	1.0633±0.002517	0.18667±0.001	1.023±0.003
% RSD	0.23	0.53	0.29

From this, the total sugar content in PC, PH, PA was found to be 88 %, 14 % and 87 % respectively.

***In vitro* lymphocyte proliferation assay**

The immunomodulatory activity of the samples was determined by the lymphocyte proliferation assay. The samples were tested in the range of 10-500 µg/ml. The highest activity was shown by PH at 500 µg/ml. The proliferative index of PC, PH, PA was found to be 1.28±0.03, 1.71±0.04 and 1.12±0.01 respectively. The proliferation rate of the samples is represented in fig. 2. Proliferation was seen to be increasing with an increasing concentration.

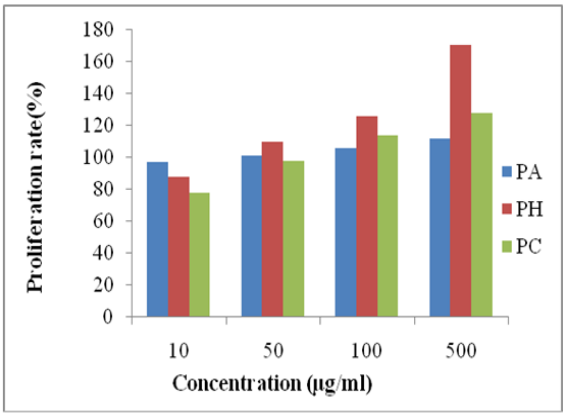

Fig. 2: The proliferation rate shown by PA, PH and PC with increasing concentrations in lymphocyte proliferation assay

From the above results it can be interpreted that all the three polysaccharides isolated are non-toxic to normal lymphocytes and have the capacity to modulate the immune system to a certain extent.

Total antioxidant assay

The total antioxidant assay is based on the reduction of molybdenum VI to molybdenum V to form a green phosphate complex. Fractions ranging from 0.5-2 mg/ml were tested for their antioxidant activity. The absorbance shown by the three fractions at different concentrations is represented in the fig. 3. All the three showed a dose-dependent increase in the antioxidant activity. PA was found to have the highest antioxidant activity. 2.8 µg/ml of ascorbic acid was found equivalent to 2 mg/ml of PA, 0.5 µg/ml of ascorbic acid was found equivalent to 2 mg/ml of PC and 0.1µg/ml of ascorbic acid was found equivalent to 2 mg/ml of PH.

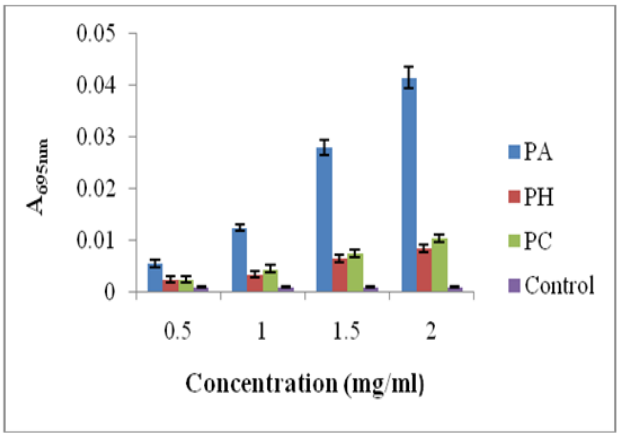

Fig. 3: The absorbance shown by different crude polysaccharide fractions and the control in the total antioxidant assay

From the above results, it can be inferred that all the three samples exhibit antioxidant property. But the highest activity was shown by PA as compared to PC and PH.

DPPH assay

DPPH is a free radical which is used to evaluate the electron donating capacity of antioxidants. The extracts were able to reduce the stable pink coloured free radical DPPH to yellow colored diphenyl picryl hydrazine. Ascorbic acid was used as standard. PC was found to have the highest value of 40.11±0.005% DPPH scavenging activity at a concentration of 2 mg/ml. 60 µg/ml, 43.5 µg/ml, 26.3 µg/ml of ascorbic acid were found to be equivalent to 2 mg/ml of PC, PH and PA respectively. The decrease in the optical density with increasing concentration is shown in fig. 4.

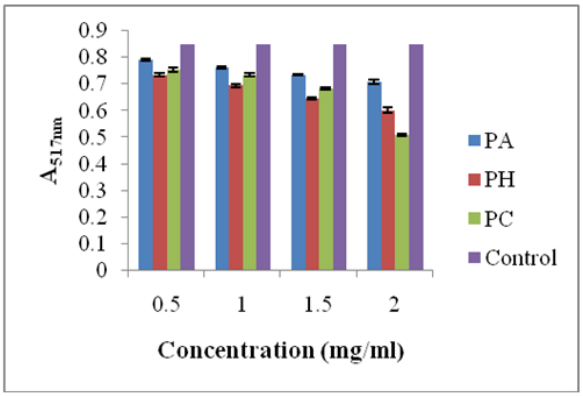

Fig. 4: A comparison of the absorbance shown by different crude fractions of polysaccharides and the control with increasing concentration in the DPPH assay

In the DPPH assay, PC was shown to have the highest activity when compared to PA and PH. PA and PH had only slight DPPH scavenging capacity.

DISCUSSION

Drug discovery has been an essential pursuit of mankind since prehistoric times. Because of the structural and biological diversity of their constituents, plants offer a unique, renewable resource for the discovery of potential new drugs and biological entities [20]. Plant polysaccharides with various biological properties especially their low toxicity and structure flexibility contribute a lot to modern medicine. Due to the arduous isolation and purification procedures, research on polysaccharides is comparatively less compared to other secondary metabolites. In this study, we have tried to isolate and evaluate the immunomodulatory and antioxidant properties of polysaccharides isolated from *Callicarpa macrophylla Vahl.* The polysaccharides isolated were water soluble, neutral in pH and had a good yield. The total sugar content in PC, PH, PA was found to be 88 %, 14 % and 87 % respectively. Phenol-sulphuric acid technique is a simple, precise and rapid spectrophotometric technique for the determination of total polysaccharides. In a previous study, the total polysaccharide content in *Cassia tora* gum was found to be 77 % by the same method [21]. Polysaccharides play a role in disease therapy by activating immune cells and the complement system; regulating the cytokines expression; promoting the production of antibodies; inhibiting tumor cell proliferation and inducing tumor cell apoptosis; inhibiting virus entering cells and replication; increasing activity of antioxidant enzyme; scavenging free radicals; and inhibiting lipid peroxidation. Currently, Lentinan polysaccharide, *Polyporus* polysaccharide, *Astragalus* poly-saccharide, *Achyranthes bidentata* polysaccharide, *etc.* are used clinically [22]. All the three polysaccharides isolated from *Callicarpa macrophylla* leaves were found to be non-toxic to normal cells and has the capacity to boost the immune system. PH has shown the highest activity as compared to others. PSK, a protein-bound polysaccharide isolated from *Coriolus vesicolor*, was proven to prolong the disease free intervals in colorectal cancer patients after surgery and can increase the life-span of patients with recurrent stomach cancer when combined with mitomycin C and 5-fluorouracil [23].

Oxidative stress, induced by the oxygen radicals is believed to be a primary factor in the development of several degenerative changes

in cells and tissues which ultimately lead to several degenerative disorders like arthritis, atherosclerosis, cancer, neuro degeneration etc [24]. Body defences are not completely capable of protecting from the negative effects of the oxygen radicals. Compounds with antioxidant properties are promising in curing such diseases [25]. The total antioxidant and DPPH assay was done to evaluate the antioxidant capacity of the polysaccharides. In the total antioxidant assay, PA was found to have the highest activity as compared to PH and PC. Increasing concentrations, lead to increasing activity. PA showed antioxidant activity near to ascorbic acid used as standard. DPPH assay was also done to evaluate the scavenging capacity of the compounds. The percentage scavenging activity of PA, PC and PH at 2 mg/ml was found to be 16.82±0.007 %, 40.11±0.005 %, 29.18±0.009 % respectively. 1 mg/ml of PC shows a DPPH scavenging activity equivalent to 30 µg/ml of ascorbic acid. It indicates that it can act as an efficient hydrogen-donor like ascorbic acid. Antioxidants can be developed from this polysaccharide fractions for the treatment of various disorders associated with free radicals.

CONCLUSION

The present study investigates the immunomodulatory and antioxidant properties of the three fractions of polysaccharides isolated from *Callicarpa macrophylla Vahl.* Polysaccharides can be used as a potent drug in the treatment of many diseases due to their non-toxicity and bio-degradability. The polysaccharides isolated from *Callicarpa macrophylla Vahl.* are water soluble and had a good yield. The immunomodulatory properties of the polysaccharides enable them to be used for many diseases like cancer, hyper-sensitivity, atheroscelorosis etc. Networking and interactions within the immune system are so complex that modulations of the immune response at will to achieve designed therapeutic success can be of great value in this modern world prone to many different diseases. All the three fractions showed a good immunomodulating activty indicating that they have the capacity to modulate our immune system positively. Further studies are required to purify the active fractions and understand the detailed mechanism involved in their immunomodulatory mechanism. The PC, PH, PA fractions isolated has shown a good antioxidant capacity and has the ability to scavenge DPPH. The combination of antioxidant and immunomodulatory properties of the plant polysaccharides can make it a good candidate as a pharmacological drug.

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CONFLICT OF INTERESTS

Declared None

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