Evaluation of antioxidant, antitumor and immunomodulatory properties of polysaccharide isolated from fruit rind of *Punica granatum*

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Abstract. Polysaccharide (PSP001) isolated from *Punica granatum* was evaluated for its radical scavenging and antitumor activities *in vitro*. The fruit of *Punica granatum* (pomegranate) has been reported to possess several vital biological activities. This study aimed to determine the antioxidant and anticancer properties of polysaccharide PSP001 isolated from the fruit rind of pomegranate. Antioxidant activities were evaluated using various assays such as the 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical scavenging assay, ferric reducing antioxidant power assay, linoleic acid emulsion thiocyanate assay, and superoxide, hydroxyl and nitric oxide radical scavenging assays. PSP001 exhibited a dose-dependent enhancement in activity using concentrations from 10 to 1000 μg/ml except for the DPPH assay for which the highest activity was obtained at 200 μg/ml. The anticancer properties of PSP001 evaluated on MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562 (leukemia) cells by MTT assay indicate its potential as an antitumor agent. An IC50 value of 97.21±1.06 μg/ml and 52.8±0.9 μg/ml were obtained following 72 h incubation for MCF-7 and K562 cells, respectively. PSP001 showed *in vitro* growth stimulatory effect on isolated normal lymphocytes, and a proliferative index of 1.21±0.01 at a concentration of 1000 μg/ml was obtained, indicating immunomodulatory activity. The results of the present study indicate that further studies are required on PSP001 in order to use this compound as an antitumor agent.

Introduction

Polysaccharides are a structurally diverse class of macromolecules of relatively widespread occurrence in nature.

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They play major roles in the growth and development of living organisms. In the last few decades, the biological activities of polysaccharides have attracted more and more attention in medicine (1). Pomegranate (*Punica granatum*) belongs to the family of Punicaceae and is grown mainly in the Near East, India, Spain, Israel and the United States. In folk medicine, the astringent properties of this fruit have been used to treat various ailments such as cuts, sore throats, tapeworms, dysentery, and gum disease (2). Antitumor, antibacterial, antidiarrhoeal, antifungal, antiulcer, and antioxidant pharmacological properties have been reported for various extracts/constituents of different parts of this plant species (3). The medicinal properties of polysaccharides mainly depend on its chemical structure. A number of polysaccharides with β-1→3 backbones are reported for their antitumor and immunomodulatory activities. We have isolated a polysaccharide from the fruit rind of *Punica granatum*, which is a galactomannan with a molecular weight of 110 kDa with a β-1→3 galactopyranose backbone and β-D mannopyranose and α-D mannopyranose side chains (4).

Reactive oxygen species (ROS) are formed and degraded by all aerobic organisms and can readily react with the majority of biomolecules including proteins, lipids and nucleic acids. ROS is involved in various physiochemical processes and diseases such as aging, cancer, atherosclerosis, neurodegenerative diseases (Alzheimer's and Parkinson's diseases) and AIDS (5,6). Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, synthetic and natural. The uses of synthetic antioxidants are restricted by their carcinogenic properties (7). Certain plants are known to possess potent antioxidant properties and are a significant source of natural antioxidants (8,9). Therapy with free radical scavengers (antioxidants) therefore has the potential to prevent, delay or ameliorate a number of the above-mentioned disorders (10). Among various naturally occurring substances, polysaccharides have proven to be useful candidates in the search for effective, non-toxic substances with antioxidant activity (11-13). Among the most promising biopharmacological activities of polysaccharides, immunomodulatory and antitumor effects are of high priority (1). A number of the chemical compounds

identified as specific agents for killing cancer cells are also toxic to normal cells, thereby limiting their usage in therapy. Numerous antitumor polysaccharides have been isolated from plants, mushrooms, yeasts, algae and lichens and the majority of them have been found to be non-toxic to normal cells and are also found to enhance the immune system of the host (14-16). Biological activities such as the immunomodulatory and antitumor effects of polysaccharides have attracted more attention recently in medical fields (17-19) and the majority of the polysaccharides act as biological response modifiers, which enhance the immune response (20).

In view of the impact of polysaccharides to human health, the present study aimed to investigate the antioxidant, anticancer and immunomodulatory activities of the polysaccharide PSP001. Since reactive oxygen species (ROS) play a major role in the development of cancer, an antioxidant compound with anticancer properties may be a promising target of further research.

Materials and methods

Materials and chemicals. Fully ripened fruits of pomegranate were collected from the Thiruvananthapuram District of Kerala, India. The cancer cell lines MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562 (leukemia) were obtained from the National Centre for Cell Science, Pune, India. The Spectra/Por 12-14,000 MW dialysis membrane was from Spectrum Laboratories (RC membrane, Spectrum Laboratories, Rancho Dominguez, CA, USA). SephadexG-200 was from Pharmacia LKB, Biotechnology AB (Uppsala, Sweden) DMEM from Gibco (Invitrogen, Carlsbad, CA, USA) and Ficoll-Paque™ from GE Healthcare (Bio-Sciences AB, Uppsala, Sweden). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of grade AR.

Isolation and purification of polysaccharide PSP001. Ripened fruits of *Punica granatum* were obtained, the fruit rind was shade-dried and 200 g of the powdered material was used for the isolation of polysaccharide by standard protocols (21,22), with modifications. Briefly, the powdered material was treated with petroleum ether for 72 h to remove any fat present, followed by hot-water extraction using Soxhlet apparatus. The filtrate was subjected to ethanol precipitation at 4˚C, the precipitate was dissolved in distilled water and dialysis was performed against double distilled water using 12-14,000 MW membrane. The solution was treated with chloroform to remove protein, if present, in a separation funnel until the interphase became clear. The steps were repeated several times until the water chloroform interphase became clear. The aqueous phase was again dialyzed, concentrated and precipitated with ethanol. Polysaccharide precipitate was collected by centrifugation at 12,000 rpm at 4˚C, redissolved in distilled water and lyophilized.

The polysaccharides were purified by gel filtration chromatography using Sephadex G-200; 0.001 M phosphate-buffered saline (PBS) was used as the eluent and the purified product was again lyophilized. Total carbohydrate content was determined by Dubois's method (23), using D-glucose as the standard.

DPPH radical scavenging assay. The radical scavenging effects of PSP001 on a 1, 1-diphenyl-2-picryl-hydrazil (DPPH) radical were estimated through the methods of Magalhaes *et al* (24). Various concentrations of samples and standards (3 ml) were mixed with 1 ml of 0.1 mM of DPPH and the mixture was agitated vigorously and allowed to incubate at room temperature (25±3˚C) for 30 min. The scavenging activity was quantified spectrophotometrically at 507 nm against 95% ethanol as blank. Ascorbic acid was used as the standard and the percentage of the DPPH scavenging effect was expressed as the percentage of inhibition from the given formula: % inhibition of DPPH radical = $[A_0\text{-}A_1/A_0]$ x100, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample/standard.

Ferric reducing antioxidant power (FRAP) assay. This assay measures the total antioxidant capacity of the compound and the procedure was conducted according to Benzie and Strain (25), following modifications. The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripytidyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ at a 10:1:1 ratio prior to use and heated to 37˚C in a water bath. FRAP reagent (3 ml) was added to 100 μl of various concentrations of samples and standards. Absorbance was measured at 593 nm at 0 min and again after 90 min. Ascorbic acid was used as the standard and changes in the absorbance after 90 min from initial reading were compared and the percentage of inhibition was calculated as described.

Superoxide radical scavenging assay. The scavenging activity of PSP001 towards superoxide anion radicals was measured using the method of Liu *et al* (11). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nico-tinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). 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 various concentrations of samples and standards. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature (25±3˚C), the absorbance at 560 nm was measured. α tocopherol was used as the standard and the percentage of inhibition was calculated.

Total antioxidant activity in linoleic acid emulsion system. The total antioxidant activity of PSP001 was determined according to the ferric thiocyanate method described by Ilhami *et al* (26) with slight modifications. The reaction mixture contained 2.5 ml of linoleic acid emulsion system (0.28 g linoleic acid and 0.28 g Tween-20 in 50 ml, 0.2 M phosphate buffer of pH 7.0), 2.5 ml of 0.2 M phosphate buffer and 0.5 ml of test and standards at various concentrations. The mixture was incubated at 37˚C for 120 h. The absorbance was measured at 0 h and at each 24 h intervals up to 5 days. The amount of 0.1 ml of the mixture was mixed with 5 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocynate and 0.1 ml 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature for exactly 3 min. Absorbance was then read at 500 nm against a suitable blank. Ascorbic acid
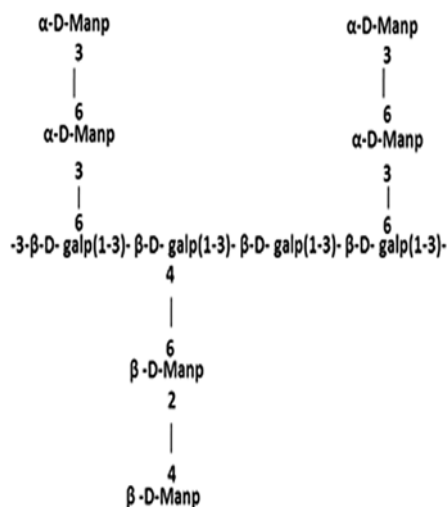
α-D-Manp
3
|
6
α-D-Manp
3
|
6
-3-β-D- galp(1-3)- β-D- galp(1-3)- β-D- galp(1-3)- β-D- galp(1-3)-
4
|
6
β -D-Manp
2
|
4
β -D-Manp

Figure 1. Proposed structure of polysaccharide PSP001.

was used as the standard and the percentage of inhibition was calculated.

Nitric oxide radical scavenging. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, as measured by Griess reaction (27). The reaction mixture (3 ml) containined 10 mM sodium nitroprusside in PBS and samples/standards at various concentrations. The mixture was incubated at 25˚C for 150 min. After the incubation, 1 ml of the mixture was removed and added to 1 ml of Griess reagent (1% sulfanil-amide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) and the absorbance was measured at 546 nm. α tocopherol was used as the standard and the percentage of inhibition was calculated.

Hydroxyl radical scavenging assay. Hydroxyl radical scavenging activity was measured comparing deoxyribose and PSP001 for hydroxyl radical generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) using the method of Kunchandy and Rao (28). The reaction mixture (1 ml) contained 100 μl 2-deoxy-2-ribose (28 mM in 20 mM phosphate buffer, pH 7.4), 500 μl of different concentrations of samples and controls, 200 μl 1.04 mM $FeCl_3$, 100 μl 1 mM H_2O_2 and 100 μl ascorbic acid, was incubated at 37˚C for 1 h. Thiobarbituric acid (1%) and trichloroacetic acid (2.8%) were added and incubated at 100˚C for 20 min. After rapid cooling the absorbance was measured at 532 nm. α tocopherol was used as the standard and the percentage of inhibition was calculated.

Antitumor activity by in vitro proliferation assay. The cell growth inhibition potential of PSP001 was measured against human cancer cell lines MCF-7 (breast cancer), KB (naso-pharyngeal carcinoma) and K562 (leukemia) with the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) assay (29), which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells. Cells were maintained in 10% DMEM/RPMI media in a CO_2 incubator with 5% CO_2 and 10% FBS at 37˚C. Quantities of 100 μl of the cell suspension of $5x10^3$ cells/well were seeded in

a 96-well plate and 100 μl of PSP001 at various concentrations (0.001-1000 μg/ml) and a positive control (doxorubicin) was similarly added to the appropriate wells. The plates were then incubated for 24, 48 and 72 h in a CO_2 incubator. After incubation, 20 μl MTT (5 mg/ml) was added to each well and incubation was continued for an additional 2 h. The insoluble formazan crystals formed were solubilised by the addition of 100 μl MTT lysis buffer (SDS and dimethyl formamide) followed by an incubation of 4 h and the absorbance was measured at 570 nm using a microplate spectrophotometer. The inhibitory rate was calculated as: Inhibitory rate (Ir) % = 100 - Proliferation rate (Pr). Pr % = [Abs sample/Abs control] x100.

In vitro lymphocyte proliferation assay. Fresh human blood samples were layered on equal volumes of Ficoll-Paque™ Plus solution and centrifuged at 800 x g for 20 min at 18˚C. The thin white middle lymphocyte layer was collected and washed with PBS twice by centrifugation at 100 x g for 10 min at 18˚C. The supernatant was discarded and the cells were suspended in 10% RPMI-1640. The cell number was counted with a hemocytometer and the viability of the cells was examined using the trypan blue exclusion assay. Samples containing cells with ≥95% viability were suspended at a concentration of $2x10^4$ cells/well. The lymphocytes were incubated at 37˚C in a humidified atmosphere of 5% CO_2. The effect of PSP001 on isolated lymphocytes was evaluated using the MTT assay as described above. The mitogen phytohae-magglutinin (PHA) was added to the wells at a concentration of 10 μg/ml. The proliferation index (proliferation rate/100) was calculated.

Statistical analysis. Data were expressed as the means ± standard deviations (SD) of three replicated determinations, and followed by analysis using Graphpad PRISM software version 5.0 (GraphPad Software, USA). One-way analysis of variance was used for repeated measurements and the differences were considered to be statistically significant if $P<0.05$. The IC50 values were calculated using EasyPlot software.

Results

Polysaccharide isolation and purification. The yield of purified polysaccharide PSP001 from the fruit rind of pomegranate was 2%. The final product was light brown in color, and was lyophilized and stored at -20˚C. It was found that, during the isolation process 4-fold volumes of ethanol and twice the volume of chloroform were optimal for the maximum yield of PSP001. The polysaccharide was found to be neutral in pH and was easily soluble in water. The total sugar content of PSP001 was determined to be 97.6% using the phenol-sulfuric acid method. Molecular weight determination and structural characterization of the polysaccharide were previously performed and it was revealed to be a galactomannan with a molecular weight of 110 kDa with a β-1→3 galactopyranose backbone and β-D mannopyranose and α-D mannopyranose side chains (Fig. 1). For structural characterization, partial hydrolysis, periodate oxidation, methylation, hydrolysis, acetylation and trimethylsilylation were carried out and analysed using ^{1}HNMR, FABMS and GC-MS (4).
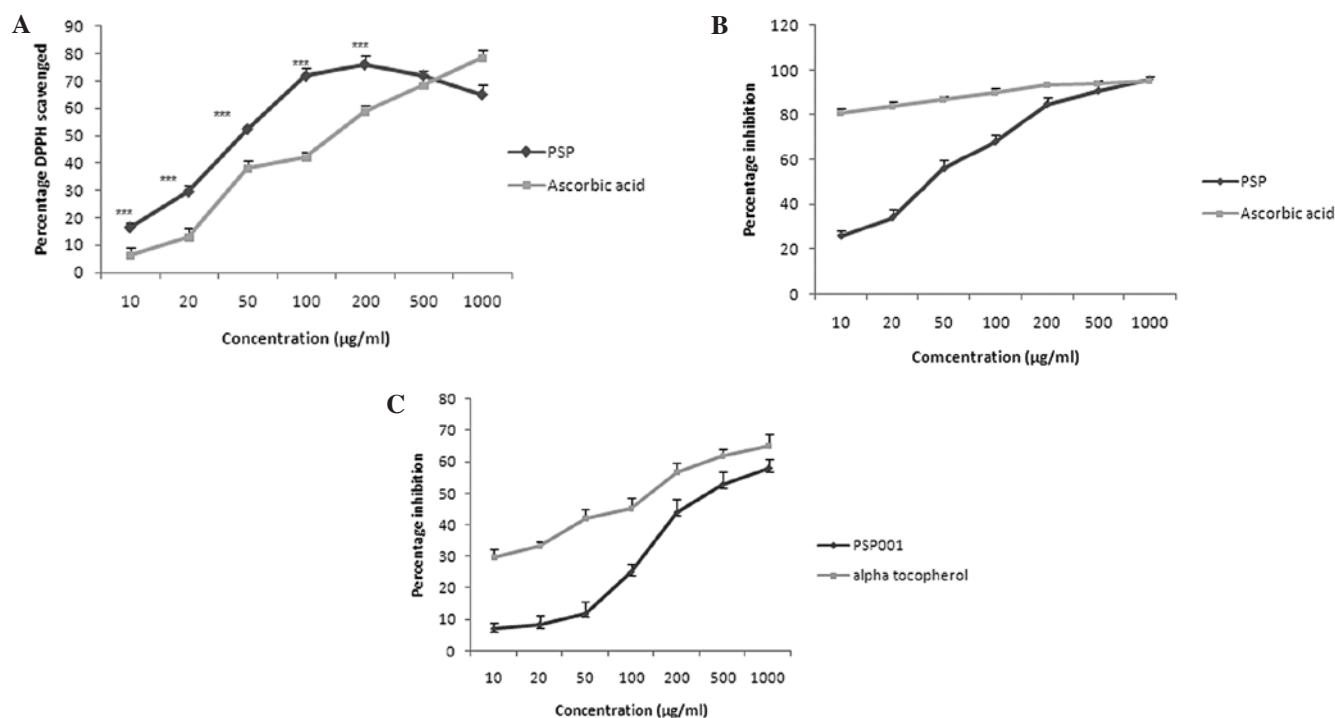
Figure 2. Antioxidant activity of PSP001. (A) DPPH radical scavenging activity of PSP001 and ascorbic acid at various concentrations. (B) FRAP assay using ascorbic acid as the standard. (C) Superoxide radical scavenging assay using α tocopherol as the standard. Results were expressed as the mean ± SD. Differences between the groups were compared using the Tukey test. p<0.001.

Antioxidant activity of PSP001. The total antioxidant capacity of a compound cannot be determined using a single method, due to oxidative processes. In this study, the radical scavenging ability of PSP001 was evaluated using various antioxidant assays.

DPPH radical scavenging assay. The radical scavenging activity of PSP001 against experimentally generated DPPH ions (Fig. 2A) showed marked scavenging activity even at low concentrations. The scavenging activity increased in a concentration-dependent manner up to 200 μg/ml, which exhibited the highest activity (76.09±3.4%); further increases in concentration exhibited no increase in the antioxidant capacity. PSP001 exhibited significantly (p<0.001) high DPPH scavenging activity compared to the standard ascorbic acid even at low concentrations. The IC50 value of PSP001 was found to be 44.2±0.8 μg/ml, whereas for ascorbic acid it was 149.7±1.2 μg/ml. PSP001 was capable of reducing the stable radical DPPH at an approximately 4-fold higher capacity than ascorbic acid.

FRAP assay. The antioxidant capacity of PSP001 based on the ability to reduce ferric ions was calculated as the percentage of inhibition, as shown in Fig. 2B. There was a gradual increase in antioxidant activity with an increase in concentration, whereas the standard ascorbic acid showed a relatively stable activity even from lower concentrations. PSP001 exhibited an inhibitory activity of 96.05±1.1% at a concentration of 1000 μg/ml, which was higher than that of ascorbic acid (95±2.4%).

Superoxide radical scavenging assay. The scavenging ability of PSP001 against superoxide ions generated in the PMS-NADH-NBT system is shown in Fig. 2C. The superoxide

anion scavenging activity of PSP001 exhibited a concentration-dependent increase, and its highest activity of 58±3.9% was observed at 1000 μg/ml. However, the superoxide radical scavenging activity of PSP001 was observed to be less than the standard α tocopherol. The IC50 values were 399±2.3 and 143±1.4 μg/ml for PSP001 and α tocopherol, respectively.

Total antioxidant activity in the linoleic acid emulsion system. The inhibitory effect of PSP001 and ascorbic acid at various concentrations between 0 and 120 h is shown in Table I. PSP001 exhibited an increase in activity from 0 to 72 h, whereas ascorbic acid exhibited more activity with a 24-h incubation period, after which the activity decreased. PSP001 showed a significant (p<0.01) increase in the activity at 72 h compared with 24 h and the maximum inhibition was observed at a concentration of 1000 μg/ml (69.53±2.5%). Since PSP001 was capable of scavenging the radical even up to 72 h as compared to 24 h of the standard, its action is promising for use in biological systems.

Nitric oxide radical scavenging. The scavenging ability of PSP001 against experimentally generated nitric oxide ions is shown in Fig. 3A. PSP001 showed relatively favourable scavenging activity and is comparable to that of the potent antioxidant α tocopherol. The radical scavenging ability exhibited a concentration-dependent increase and showed 74.90±3.9% inhibition at a concentration of 1000 μg/ml. The IC50 values were 184±1.1 and 136.7±1.8 μg/ml for PSP001 and α tocopherol, respectively.

Hydroxyl radical scavenging assay. PSP001 exhibited concentration-dependent scavenging activity against hydroxyl

Table I. Antioxidant activity of PSP001 by linoleic acid emulsion system.[a]

Concentration (μg/ml)	0 h		24 h		48 h		72 h		96 h		120 h	
	PSP	ASC	PSP	ASC	PSP	ASC	PSP	ASC	PSP	ASC	PSP	ASC
10	10.8±2.5	13.0±1.9	11.4±3.0	42.1±1.5	9.0±4.2	42.0±1.6	15.3±3.2	21.3±2.8	11.8±4.5	24.3±3.8	24.3±4.6	16.0±3.2
20	11.6±3.2	17.0±2.1	13.6±1.9	37.5±2.0	13.3±2.1	47.0±1.7	19.3±3.5	29.3±3.2	22.7±4.0	28.8±4.0	28.8±3.1	20.6±2.0
100	12.7±1.5	25.5±1.0	10.9±2.3	74.19±2.0	29.8±1.9	42.8±2.7	29.0±.3.0	34.1±1.8	37.8±3.2	42.1±3.2	42.1±4.0	24.3±3.0
200	23.6±1.9	26.7±2.0	28.0±2.6	74.4±2.1	30.2±3.5	47.8±1.0	42.6±4.2	45.3±1.9	50.0±1.9	43.5±4.6	43.5±3.8	28.03±2.0
500	36.4±2.9	33.9±3.0	64.0±1.3	70.7±3.2	64.8±1.0	41.0±4.2	65.9±3.5	39.8±2.5	33.3±4.2	23.4±3.1	23.4±3.1	29.0±1.9
1000	39.6±1.5	56.0±4.0	62.5±2.9	71.5±4.2	74.1±3.8	32.5±3.9	69.5±2.5	22.0±2.9	32.7±3.2	26.1±4.1	26.1±3.9	43.5±3.4

[a]Radical scavenging ability of PSP001 and ascorbic acid at various concentrations from 0 to 120 h incubation period. Results are expressed as mean ± SD.
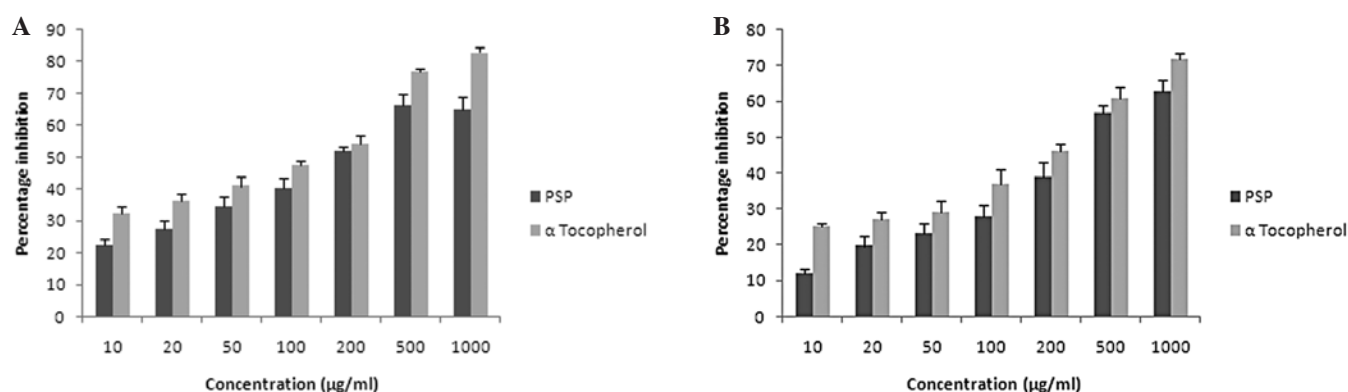

Figure 3. Antioxidant activity of PSP001. (A) Nitric oxide radical scavenging assay using α tocopherol as the standard. (B) Hydroxyl radical scavenging assay using α tocopherol as the standard. Results are expressed as the mean ± SD. Differences between the groups were compared using the Tukey test.

radical generated in a Fenton reaction system (Fig. 3B). At a concentration of 1000 μg/ml, PSP001 demonstrated a radical scavenging ability of 63.90±3.1%, and IC50 values of 385.4±2.3 and 283.8±1.3 μg/ml for PSP001 and α tocopherol, respectively. The radical scavenging activity of PSP001 is promising and may aid in the prevention or reduction of the progress of various oxidative stress-induced diseases, thereby benefiting human health.

Antitumor and lymphocyte proliferative activity in vitro. The anticancer activity of PSP001 was evaluated by *in vitro* techniques on MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562 (leukemia) cancer cell lines using the MTT assay. Cytotoxicity on cancer cell lines was evaluated at 24, 48 and 72 h incubation with various concentrations of PSP001. The effect of PSP001 on the viability of cells is shown in Fig. 4. Doxorubicin, a well-known anticancer agent, was used as a positive control at equal concentrations (Table II). A substantial increase in cell growth inhibition was observed after the treatment of PSP001 on MCF-7 cells and an IC50 value of 97.21±1.06 μg/ml was obtained after 72 h of incubation (Fig. 4A). The cytostatic effect increased in a concentration-dependent manner up to 200 μg/ml during the incubation periods, after which the activity decreased. Similarly, growth inhibition on oral cancer cell line KB

showed the highest value at a concentration of 200 μg/ml (36.4±2.4%) at 72 h of incubation, but no IC50 value was obtained (Fig. 4B). PSP001 showed its maximum cytotoxicity to be approximately 200 μg/ml for MCF-7 and KB cell lines, whereas in leukemia cell line K562 it exhibited a dose- and time-dependent increase in activity up to a concentration of 1000 μg/ml (63±2.9%). An IC50 value of 52.8±0.9 μg/ml was obtained during 72 h of incubation, which was even less than that of MCF-7.

Since the majority of anticancer compounds are toxic to normal cells their application in medicine is highly restricted. The effect of PSP001 at various concentrations on normal lymphocytes was also evaluated using the MTT assay. The compound was found to be both non-toxic to lymphocytes as well as to enhance the *in vitro* growth of lymphocytes when administered with a mitogen PHA. Fig. 5 shows a significant growth enhancement (p<0.001 in all concentrations compared with the control) on normal lymphocytes. A proliferative index (PI) of 1.21±0.01 at a concentration of 1000 μg/ml was obtained. The results of this experiment show that PSP001 exhibited higher growth stimulation even at low concentrations, indicating its potential as an immunomodulatory compound. T lymphocytes play a crucial role in enhancing the immune functions of organisms. Therefore, examination of alterations in lymphocyte proliferation is the

Table II. Inhibition rate of doxorubicin on *in vitro* cell lines.[a]

Concentration (µg/ml)	24-h inhibition rate			48-h inhibition rate			72-h inhibition rate		
	MCF7	KB	K562	MCF7	KB	K562	MCF7	KB	K562
0.001	6.2±1.2	6.07±1.8	6.0±1.8	8.0±2.1	7.27±2.2	11.7±0.8	12.8±1.5	19.3±2.1	28.5±1.2
0.01	8.2±1.4	8.24±0.9	8.8.±1.1	21.2±2.4	29.3±1.8	18.3±1.5	14.5±2.1	22.0±1.4	40.0±3.0
0.1	13.0±2.3	12.6±2.8	13.5±2.3	47.3±1.2	32.5±1.1	24.8±1.9	41.5±1.9	50.0±2.1	45.2±2.1
1	29.1±1.1	28.16±2.1	19.2±1.3	58.0±1.9	61.25±2.1	26.9±2.4	60.2±1.1	77.0±1.8	48.8±1.8
10	33.5±1.9	41.8±0.7	21.0±0.9	63.4±0.9	64.88±2.7	30.46±2.9	63.5±1.8	76.5±2.4	53.5±1.1
50	37.9±2.8	47.8±1.2	23.6±2.1	64.4±2.4	67.27±3.4	33.6±1.8	65.0±2.1	77.7±3.1	56.0±2.4
100	45.8±3.0	51.4±1.8	26.9±1.7	67.7±3.4	69.4±1.7	39.8±1.3	66.0±1.7	78.0±1.7	62.3±2.5
200	51.2±1.4	53.1±2.1	31.0±1.3	69.1±2.4	70.8±0.9	49.9±2.9	67.8±2.3	77.0±2.4	66.0±2.7
500	49.2±2.7	53.0±3.2	34.1±2.9	68.0±1.5	68.9±1.4	52.0±2.0	66.5±1.9	76.6±1.5	69.7±1.8
1000	45.8±2.3	50.0±2.4	36.7±2.4	66.7±2.0	68.6±2.3	54.9±2.1	66.1±3.2	76.1±3.1	72.0±2.6

[a]MCF7 (breast cancer), KB (nasopharyngeal carcinoma), K562 (leukemia) with various concentrations for 24-, 48- and 72-h incubation periods. Results are expressed as the mean ± SD.
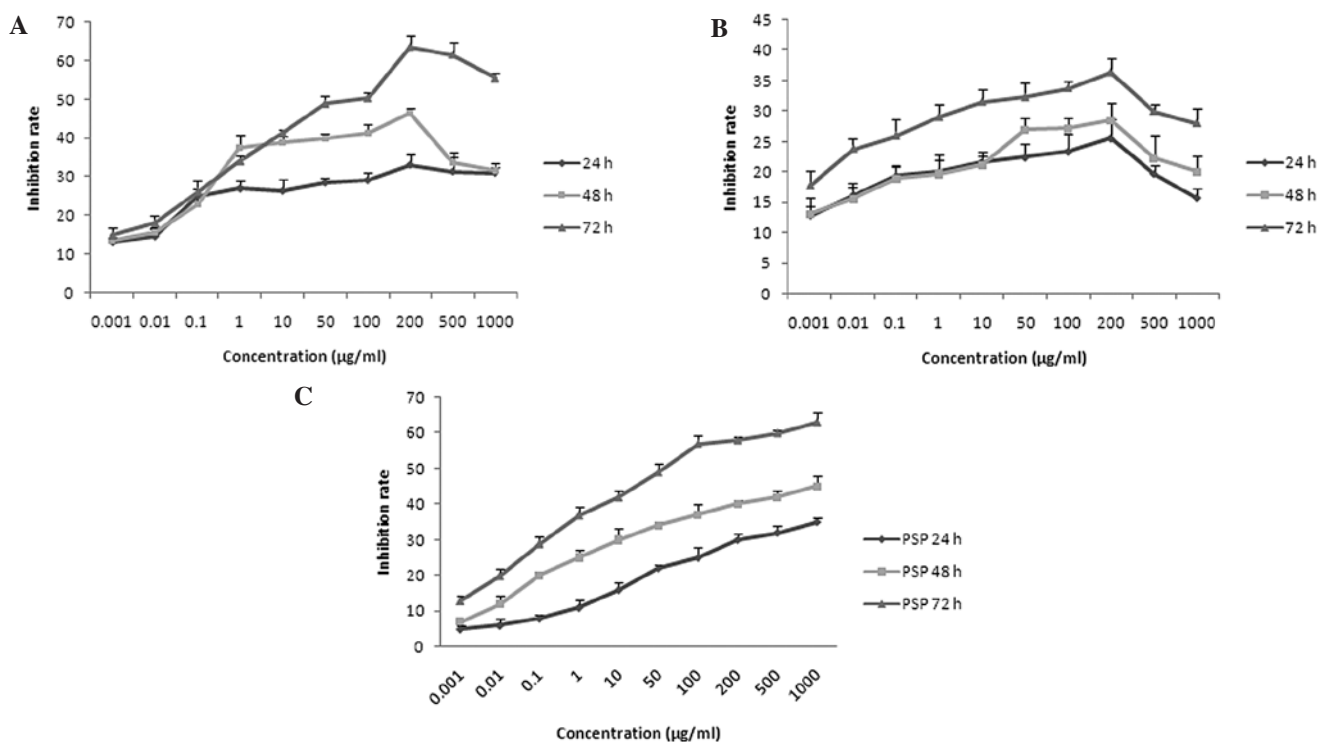

Figure 4. Cytotoxicity profile of PSP001 on *in vitro* cell lines after 24, 48 and 72 h incubation as measured by MTT assay. (A) MCF-7 (breast cancer). (B) KB (nasopharyngeal carcinoma). (C) K562 (leukemia). Results were expressed as the mean ± SD.

most effective method for the study of the activity and action mechanism of the compounds. This assay is relatively easy to use and is suitable for screening of the immune-enhancing potential of novel compounds.

Discussion

The present study investigated the antioxidant, antitumor and immunomodulatory activities of the polysaccharide isolated from the fruit rind of *Punica granatum*. Carbohydrates are a relatively untapped source of new drugs and therefore offer promising new therapeutic opportunities. The polysaccharide PSP001 was successfully isolated and characterized from the fruit rind of pomegranate and was found to be easily soluble in water. The factors that determine whether a polysaccharide is likely to exhibit antitumor activity are unclear. However, there appears to be a direct association between the antitumor activity of polysaccharides and their ability to interact with serum albumin (19,30). Since water-soluble polysaccharides were found to be therapeutically applicable (31), PSP001 is ideal for various pharmacological uses due to its immuno-modulatory, anticancer and antioxidant properties.
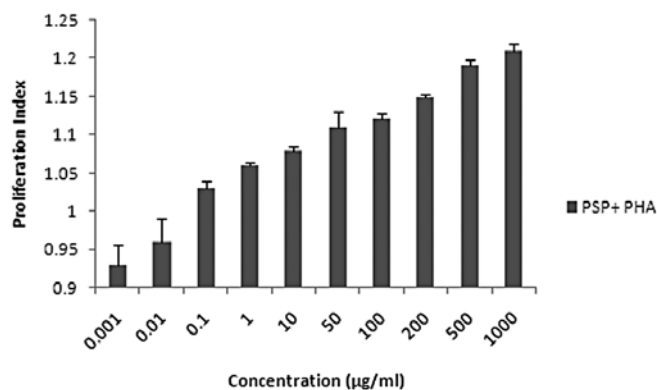
Figure 5. Lymphocyte proliferative activity assay of PSP001 *in vitro* after 72 h of incubation. Results are expressed as the mean ± SD.

Oxidative stress, predominantly caused by an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defence system (32) is the pathology behind the majority of human diseases. Radical scavenging activity is a significant functional property for bioactive compounds (33), and numerous polysaccharides have been reported to have marked antioxidant activity. The antioxidant properties of PSP001 were investigated using various *in vitro* radical scavenging assays, of which DPPH, FRAP, nitric oxide radical scavenging and linoleic acid emulsion thiocynate assay produced promising results. The DPPH radical scavenging activity is widely used to evaluate the antioxidant capacity of compounds (34). The DPPH radical was considered to be a model of the lipophilic radical and a chain reaction in lipophilic radicals was initiated by lipid autoxidation. PSP001 showed significantly (p<0.001) high DPPH-scavenging activity compared to the standard ascorbic acid, even at low concentrations and the IC50 values were found to be 44.2±0.8 and 149.7±1.2 μg/ml for PSP001 and ascorbic acid, respectively. PSP001 may be considered to be a potential scavenger against the stable DPPH ions. It appears that PSP001 exhibits hydrogen-donating activities as an antioxidant, as reported for other compounds such as cystine, glutathione, ascorbic acid, and tocopherol (35).

The FRAP assay directly measures antioxidants or reductants in a sample (36). The FRAP assay measures the reducing ability of and antioxidant that reacts with ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produces a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ). PSP001 exhibited an increase in antioxidant activity with an increase in concentration as per the FRAP assay. Additionally, inhibition of up to 96.05±1.1% at a concentration of 1000 μg/ml was obtained, which was comparable to that of any known antioxidant. Superoxide anions indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide, serving as precursors of singlet oxygen and hydroxyl radicals (37) and have also been observed to directly initiate lipid peroxidation (38). Similar to the trend observed with the FRAP assay, PSP001 showed an increase in its radical scavenging ability against superoxide anions with an increase in concentration. PSP001 was not considered to be an effective superoxide anion scavenging compound as its highest activity was only 58%. Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves the formation

of lipid radicals, leading to membrane damage. Free radicals induce lipid peroxidation of polyunsaturated lipids. During the linoleic acid oxidation, peroxides formed and these compounds oxidize Fe^{2+} to Fe^{3+}. The latter Fe^{3+} ions form a complex, which results in an intense red colour and has maximum absorption at 500 nm. PSP001 was capable of scavenging the radicals in the linoleic acid emulsion system even up to 72 h as compared with 24 h of the standard; its long-lasting action may be promising for use in biological systems where the radicals are generated in a cascade manner. Nitric oxide is a potent pleiotropic mediator of a variety of physiological process and is a diffusible free radical that plays numerous roles as an effector molecule in diverse biological systems, including neuronal messenger, vasodilation and antimicrobial activities (39). Although nitric oxide ions are involved in the host defense, overproduction of this radical contributes to the pathogenesis of certain inflammatory diseases (40). Moreover, these ions form potentially cytotoxic molecules, peroxynitrite. PSP001 was also found to be a good scavenger of nitricoxide ions in a concentration-dependent manner, and an inhibition rate of up to 74.9 % was obtained. Hydroxyl radicals are known to be the most reactive of the reduced forms of dioxygen and are thought to initiate cell damage *in vivo* (41). These radicals attack proteins, DNA, polyunsaturated fatty acid in membranes and the majority of biological molecules in contact with them (42), are known to be capable of abstracting hydrogen atoms from membrane lipids, and cause peroxidic reaction of lipids. A concentration-dependent increase in activity exhibited by PSP001 against hydroxyl radicals (63.90±3.1%) may be the basis for promising applications in biological systems.

The anticancer properties of PSP001 evaluated on MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562 (leukemia) cells by MTT assay disclosed its therapeutic potential against cancer. The antitumor effects were observed even at low concentrations (0.001 μg/ml) and IC50 values of 97.21±1.06 and 52.8±0.9 μg/ml were obtained on 72 h incubation for MCF-7 and K562 cells, respectively. PSP001 was not capable of producing an IC50 value in KB cells. PSP001 showed its maximum cytotoxicity at approximately 200 μg/ml for MCF-7 and KB cell lines, whereas in the leukemia cell line K562 it exhibited a dose- and time-dependent increase in activity up to a concentration of 1000 μg/ml.

The transformation of a normal to a cancerous cell is likely not a critical event in the genesis of cancer, rather, it is the body's inability to destroy these newly formed cancer cells that lead to tumorigenesis. The majority of newly formed cancer cells do not grow beyond the microscopic stage, as immune cells of the body identify and destroy these cancer cells immediately when they are few in number. The risk of developing cancer is multiplied in cases where the immune system is weak. Thus, any compound that enhances the immune system of the body may be a potential facilitator cancer therapy. The antioxidant and anticancer properties of PSP001 can only be utilized therapeutically if it produces no toxicity to normal cells. The effect of PSP001 on isolated normal lymphocytes at various concentrations revealed that the compound was non-toxic to lymphocytes and also enhanced their proliferation when administered *in vitro* with a mitogen PHA; a PI of 1.21±0.01 at a concentration of 1000 μg/ml was obtained, indicating immunomodulatory activity. Early studies from our

laboratory revealed that PSP001 is a non-toxic compound to BALB/c mice. Conventional anticancer compounds suppress the immune system of the body and a rapid decrease in the lymphocyte count was observed following their administration. For the three biological properties tested with PSP001, the highest activity was found between 200-1000 μg/ml. A non-toxic antitumor compound with immunomodulatory and considerable antioxidant activity would be of marked significance in developing an anticancer agent. Therefore, further investigations are required to investigate the antitumor properties of PSP001 and to determine its potential in cancer treatment and management.

Acknowledgements

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