Research article

Characterization of antioxidant, anti-cancer, and immunomodulatory functions of partially purified garlic (*Allium sativum* L.) lectin

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**ABSTRACT**

**Introduction and Aim:** The metabolic and oxidative stress induces highly reactive free radicals that are known to harm normal physiology and play a role in the development of cancer. Elevated levels of these free radicals contribute to excessive neovascularization leading to angiogenesis mediated cancer progression. Targeting these free radicals through dietary source is important strategy in regulation of cancer. *Allium sativum* L. (AsL) garlic has important multi pharmacological properties. On the other hand, dietary lectins are proven to be the best anti-cancer molecules. The study presents investigation that focus to assess the antioxidant, immunomodulatory and anticancer activities of partially purified garlic lectin (PPAsL).

**Materials and Methods:** Fresh garlic bulbs were processed and evaluated for lectin induced HA activity. Further the garlic lectins (AsL) were partially purified by ammonium sulphate precipitation and dialysis and analyzed through SDS-PAGE. Further lectins were characterized by producing Anti-AsL polyclonal antibodies and purification by affinity chromatography. Pharmacological evaluations of the lectins were assessed through antioxidant, anti-proliferative and antiangiogenic mediated anti-cancer activity.

**Results:** Lectin positive activity was confirmed by HA activity and partial purification lectin identified ~12kDa protein having Glc/Man glycan specificity. The polyclonal antibodies raised against PPAsL, confirmed that it has potent immunogen. Pharmacological evaluation confirmed that PPAsL has potent antioxidant, antiangiogenic and antiproliferative effect both in-vitro and in-vivo.

**Conclusion:** PPAsL is potent antioxidant, anti-proliferative and anti-cancer molecule. The dietary recommendation of the garlic lectin is an important therapeutic strategy against the cancer.

**Keywords:** Immunostimulatory; neovascularization; antioxidant; anti-angiogenic; anti-cancer.

**INTRODUCTION**

The molecules with antioxidants property are considered to have a vital role in physiological defense mechanism against free radicals induce oxidative damage. These oxidents are catastrophic by-products raised through aerobic metabolic activity during normal energy deriving reactions of the cells. Antioxidants can neutralize and prevent oxidative damage that causes various inflammatory stress and degenerative diseases. Uptake of various types of antioxidants through diet can maintain the normal physiological functions of living system. Several edible fruits and vegetables are abeyant to decrease the hazardous effect of several diseases, like inflammation and cancer (1). The lectins present in the dietary constituents were known to have antioxidant property providing with possible protective role in disease tolerance (2). Elevated expression of free radicals induced such as peroxides contribute to neovascularization and thereby increase progression of the malignancy. Comprehensive studies have emphasized the vital interrelationship among immunomodulatory and angiogenesis. Substantial evidence from many experiments demonstrates that T-cells are the key for anti-angiogenic immune response. This recommends the conjugative approach embracing immunostimulatory and anti-angiogenic aspects has convincing immunotherapeutic strategies for treating cancer (3, 4) with immunostimulatory, anti-angiogenic dietary constituents.

In the last decades, dietary lectins with specific glycan binding potential have gained an importance as indispensable tools in biology research. Lectins have
applications in cellular research as they represent high sensitivity and specificity during cellular interaction, that allows selective and signaling modulation which induce various cellular and biochemical manifestation. The most isolated and characterized dietary lectins with identified biological functionality are from the dicotyledonous seeds. In spite, the dietary lectins from non-seed plant parts like tubers, leaves are also emerging as promising research tools as they represent unique sugar-specificities with differential cellular binding capacity that explore valuable information on distinctive biological roles of dietary lectins that is still elusive (5).

Garlic (Allium sativum L.), belongs to Liliaceae family, is widely used spice across the globe as dietary flavoring agent to enhance physical and mental health (6,7). Consequential studies have shown that garlic exhibit wide range of pharmacological properties (8-13). Earlier reports have mentioned the presence of mannose-binding lectin in garlic bulbs. The protein biochemical studies have reported a lectin with relative M. wt of 25 kD as the predominant protein in bulbs with high specificity to mannose sugar (6). The heterodimer Allium sativum lectin (AsL) contains two subunits with M. wt. of 11.5 and 12.5 kD, interestingly, both subunits of AsL were derived from a single precursor containing two tandemly arrayed lectin domains (14).

The garlic lectin exhibits leuco and hemagglutinating properties indicating its interaction with immune cells. Previous studies displays that the garlic lectin shows high affinity in binding to cells which contain oligomannosides and high mannose N-glycans on the cell surface (15). The garlic bulb in traditional medicine is connected to the health-promoting properties of this allium vegetable that may corresponds to some unique cellular modulation by the presence of garlic lectins. These have enchanted considerable interest from scientific medical investigators, asserting that garlic lectin plays a certain role in immunomodulation and even in cancer prevention. There are no studies reported on the anticancer and immunogenic properties of garlic lectin. The present study focused to investigate the antioxidant, immunomodulatory and anticancer property of partially purified garlic bulb lectin (ppAsL) and to enumerate the effect of garlic lectin on immune response in rabbits to assess its immunogenicity. This study attempts to identify a natural dietary immunomodulator with anticancer potential, that promises garlic lectin as a future pharmaco-potential biomolecule as immune-stimulator and dietary preventive agent for cancer therapeutics.

MATERIALS AND METHODS

Freud’s complete and incomplete adjuvants, goat anti-mouse IgG-ALP conjugate and BCIP/NBT liquid substrate were from the product of Sigma-Aldrich (St. Louis, MO, USA). Cell culture wares purchased from Eppendorf. Nitrocellulose membrane was obtained from Amersham Protran. Protein A agarose, p-nitrophenyl phosphate were obtained from Bangalore Genei, India. All other experimental reagents and chemicals used in the present study were of analytical grade and were procured from HiMedia, Sigma-Aldrich and SRL, India.

Purification and characterization of garlic lectin

Collection and preparation of garlic extract

The hybrid garlic (Allium sativum) was collected from the local market of Shivamogga. The samples were authenticated by the botanist from Sahyadri Science College, Kuvempu University, Shivamogga, India. The collected samples were processed for isolation of lectins. Exactly 100 grams of garlic cloves were separated and were peeled to remove the bulbs coat, washed thoroughly with sterile distilled water, and homogenized in 200 ml of sterile 10 mM PBS (phosphate buffered saline) with pH-7.4. After two hours of slow stirring at 4°C, the crude extract was initially filtered with muslin cloth and further centrifuged (8000 rpm x 20 min) to remove the particulate debris. The obtained pale-yellow supernatant was stored at 4°C and used for further purification.

Ammonium sulphate precipitation and dialysis

The clear supernatant containing garlic lectin in the extract was subjected to sequential 20% to 90% ammonium sulphate fractionation as described in previously with some modification (14). Briefly, addition of NH42SO4 was done accordingly to the protein supernatant solution to achieve 20% saturation at 4°C with gentle agitation using magnetic stirrer for 1h and allowed the mixture to stand at 4°C for overnight. Further, centrifuged at 3000 rpm for 20 mins, precipitate was collected and resuspended with Phosphate buffered saline (10mM PBS; pH 7.4); that supernatant was followed by the addition of ammonium sulphate accordingly to produce 50% and 90% saturation at 4°C respectively (16). The precipitates with fractioned protein and salt mixtures were subjected to Amicon stirred ultra-filtration with 3K cut-off membrane for concentration and removal of salt molecules. The purified fraction was labeled as partially purified garlic lectin (PPAsL).

Hemagglutination (HA) assay

Preparation of trypsinized RBC suspension

Fresh 5 ml of chicken blood samples was collected from local slaughter house. 5ml of human blood sample (all blood groups) was drawn by clinically trained technician from the respective volunteer after obtaining the consent form by them. About 2ml of rabbit blood samples were drawn as per CPCSEA
guidelines and with ethical clearance from IEC. All blood samples were added with an equal volume of Alsever’s solution in sterile tubes and centrifuged at 1000g for 10 mins at 4°C. The supernatant containing plasma was discarded and the pelletted erythrocytes were resuspended and washed 3-4 times using saline solution (0.9% NaCl), and finally RBCs were resuspended in 10 mM PBS with pH 7.4. The RBC suspension was incubated with 1% trypsin at 37°C for 10mins. The trypsinized cells were then centrifuged at 1000g for 10 min at 4°C, washed 2 times with saline and readjusted to 2% based on OD values with PBS for use in the Hemagglutination (HA) and HA inhibition assay (17).

**Hemagglutination (HA) activity**

HA activity of PPAsL was investigated using trypsinized 2% chicken, human and rabbit blood erythrocytes suspension. In a concavity agglutination plate, the 50 µL of protein solution was serially diluted with PBS and were added with 50µl of 2% erythrocytes suspension, gently mixed and incubated at 37°C for 1h to visualize the appearance of agglutination. Erythrocytes without the protein solution incubated only with PBS were used as control. The minimum amount of protein required to produce visualize agglutination at the highest dilution represents the hemagglutination titer value.

**Leucoagglutination assay**

Lectin mediated leucoagglutination assay was performed with isolated human peripheral blood lymphocytes through density gradient centrifugation method using Ficoll-Hypaque. The separated lymphocytes (100µl) in 10mM PBS was taken and then added with equal volume of PPAsL in a concavity agglutination plate, the samples were mixed and incubated at 37°C for 1h. Further, standard leucocyte staining was performed for the cells and photographed microscopically.

**Protein estimation and SDS-PAGE**

Protein quantification was done following Lowry’s assay method using BSA as standard. PPAsL were subjected to reducing 15% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), using Bio-Rad mini electrophoresis unit. The gel was stained with Coomassie brilliant blue R-250 (18).

**Estimation of free radical scavenging efficacy of PPAsL by anti-oxidant assays**

**DPPH radical scavenging assay**

The PPAsL was evaluated for free radical scavenging property by DPPH assay. At various concentration (12.5 to 200µg), the PPAsL samples were added to marked test tubes and final volume was made to 2ml with distilled water. The tubes were added with 3ml of alcoholic DPPH (0.004% DPPH prepared in 95% ethanol) and the reaction mixtures were incubated under dark condition at room temperature for 30min. The DPPH radical scavenging activity of garlic lectin was determined by measurement of absorbance at 517 nm. The natural antioxidant ascorbic acid was used as standard and the DPPH radical scavenging activity of garlic lectin was compared with Ascorbic acid.

**Lipid peroxidation inhibition assay**

In-vitro lipid peroxidation assay was employed to estimate the lipid peroxidation inhibitory capacity of PPAsL. About 10% of liver homogenate with 0.15M potassium chloride (KCl) was prepared, to 0.5ml of homogenate solution 2ml of PPAsL at varied concentrations (0-50µM) were added into the tubes. To the mixture, exactly 100µl of ferric chloride (0.2mM) was added to induce lipid peroxidation and further the reaction mixtures were subjected for incubation for 30 min at room temperature. Following incubation, the reaction was seized by addition of 2ml of ice-cold 0.25N HCl containing TCA (15%), TBA (0.38%), and BHA (0.5%). The contents of samples were vortexed and heated for 60 min on boiling water bath. Further samples were cooled and centrifuged for 10 min at 3000 rpm to collect the clear supernatant. The absorbance of the supernatants were measured at 532 nm to calculate the percentage inhibition in lipid peroxidation.

**Animal experiments**

All the animal (New Zealand white rabbit and Swiss albino mouse) experimentations were approved by the Institutional Animal Ethical Committee (IAEC), in accordance with the committee for the purpose of CPCSEA guidelines for laboratory animal facility (Ref No. KSHEMA/IAEC/01/2020 Date: 04.06.2020).

**Generation of polyclonal antibody against PPAsL in rabbit**

Polyclonal antibodies against PPAsL were produced by subcutaneous injection of 1 mL garlic lectin protein solution (0.1 mg/mL) with complete Freund's adjuvant to rabbits. Booster doses of antigen with the same concentration of 0.1 mg/mL with incomplete Freund's adjuvant were administered subcutaneously at 15-day intervals for four weeks after primary dose injection (19). The rabbit was bled to collect blood by marginal ear vein puncture before a week of antigen administration to obtain pre immune sera. A week after administration of each antigen booster dose, the animal was bled to obtain immune sera. The antibody titer is monitored and immunoglobulin (IgG) from antiserum was isolated by (NH₄)₂SO₄ precipitation method. Briefly, 2ml of rabbit antisera was gradually added with (NH₄)₂SO₄ was gradually added with

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stirring at ice-cold temperature. The solution was left overnight at 4°C with occasional stirring. The sample was subjected for centrifugation at 3000rpm for 30min at 4°C and the supernatant was discarded. Pellet was resuspended with 10mM Phosphate buffer saline. Sample was dialyzed against the 10mM Phosphate buffer Saline for extensively with frequent change of buffer. Dialyzed sample was collected and stored at -20°C.

**Immunoadfinity chromatography**

The dialyzed IgG sample was further purified by subjecting to affinity protein A agarose column chromatography. In brief, dialyzed IgG fraction was loaded and unbound sera proteins were collected at flow rate of 1ml/ 3min until the OD value at 280nm reaches zero. Bound fraction was eluted by changing the elution buffer with 0.05M citrate buffer, pH 3 at flow rate of 1ml/3min and OD was read at 280nm. The eluted bound IgG fractions were pooled and used as IgG antibody sample.

**Analysis of purified anti-AsL antibody by SDS-PAGE**

Purified anti-AsL antibody IgG quantification was done by Lowry’s method of protein assay using BSA as comparative standard. IgG were subjected to reducing 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE), using Bio-Rad mini electrophoresis unit (14).

**Ouchterlony double Immunodiffusion**

Double immunodiffusion assay was performed in accordance with standard established methods (20). Briefly, 0.8% agarose was prepared using 10mM PBS. The solution was poured in 30mm petri dish and kept at room temperature. After solidification, wells were made on gel. Wells were loaded with samples respectively, the plate was incubated at 37°C in humidified environment for 24 h, the gel was observed for opaque precipitation line between the antigen and antisera wells.

**Immunoblot analysis**

The immunoreactivity and specificity of rabbit polyclonal antiserum was assessed by Dot blot assay. The PPAsL (5-10 µg) sample was loaded on to the nitrocellulose membrane and the membrane was blocked and analyzed like the western blot development as explained. For knowing antibody specificity and affinity for garlic extract and purified AsL, 10 µg of protein samples were separated by SDS-PAGE and were transferred onto nitrocellulose membrane. Membrane was blocked immediately using blocking buffer (5% skimmed milk in TBST) washed with TBST and the membrane was incubated with primary antibody (rabbit anti-AsL serum 1:500 dilution) for overnight at 4°C. The membrane was washed 3-4 times and subjected incubation at 37 °C for 1 h with 1:30000 diluted goat anti-rabbit IgG-ALP conjugated as source of secondary antibody. After washing, the immunoblots were developed with BCIP/NBT substrate solution.

**Effect of PPAsL on cell proliferation**

Lymphocyte proliferation for garlic lectin was done following previously published protocol (21). Briefly, peripheral lymphocytes were isolated from healthy blood based on lymphotrap density gradient separation method. The cells were adjusted to 2x10⁶ cell/mL and were seeded as 100µl of cell suspension in 96-well culture plates and challenged with crude and PPAsL. Following incubation, 1/10⁸ volume of MTT were added and further plate was incubated at 37 °C, 5% CO₂ for 4 h. Following incubation, 200µl of DMSO was added to the wells to dissolve the insoluble blue formazan crystals. The absorbance of the plate was read at 570nm using a microplate reader. The experiment was simultaneously carried out with Human Melanoma (A375) cell lines and the readings were recorded.

**In ova CAM assay**

The preliminary Angio modulatory effectiveness of PPAsL was investigated by in-Ova CAM assay as described previously (22). CAMs were separated into three systems and treated with extracts as Control, Dose-1(2µg-PPAsL) and Dose-2 (3µg-PPAsL) were added on 5th day to the growing fertilized eggs. The changes in the appearance of new blood vessels or neovascularization patterns were evaluated and photographed in comparison with the control.

**Ehrlich ascites carcinoma (EAC) tumour model development and treatment**

Ehrlich Ascites carcinoma (EAC) tumour model was developed as described before using Swiss albino mice. The animals with tumour were grouped experimentally and administrated with doses of 0, 25 and 50 µg/kg body weight (b.w.) PPAsL (through intraperitoneal (i.p.), n = 6 on every alternative day. At the end of experiment, tumour growth parameters such as volume, ascites, cell number and survival rate were documented (23).

**Statistical analysis**

The data were generated with multiple experiments and were analysed using statistic tools and relevant graphs were prepared using MS Excel version 10. Statistical significance for the data sets represented were evaluated by one-way analysis of variance (ANOVA) following Student’s t-test. All the values are expressed as mean of triplicate values± standard deviation (S.D.). Values of significance of *P< 0.05 and **P < 0.01 were expressed in the represented data as statistically significant.
RESULTS

Purification and characterization of partially purified garlic lectins (PPAsL)

The homogenized crude garlic extract was obtained and hemagglutination activity was tested for the presence of lectin using chicken, human and rabbit erythrocytes. Agglutination was visualized only in the rabbit erythrocytes and was absent with chicken and all types of human erythrocytes (Fig 1A). Further, the crude garlic extract was subjected to the ammonium sulphate precipitation at different saturation fractions like 0-20%, 20-50% and 50-90%. The isolation of garlic lectins was confirmatory by hemagglutination activity which was high at 50-90% fraction (Fig 1B) Smaller molecular weight proteins were expected to separate at the last fraction (50-90%) confirms the presence of lectin. Followed by dialysis to obtain concentrated garlic fractions by using a Amicon ultracentrifugation unit. The analysis of PPAsL by SDS-PAGE showed more than 80% homogeneity with minor contamination protein at high molecular weight. Molecular mass of PPAsL was identified by reducing SDS-PAGE, it revealed a band with apparent molecular weight of 12kDa (Fig 1C). PPAsL examined agglutinating property with RBC and lymphocytes, which in turn exhibits both hemagglutination and leucoagglutinating properties. The PPAsL had exhibited interaction with lymphocytes and cause visible clumping under microscope (Fig.1D). The lectin recovery during purification steps was around 28% with high hemagglutination activity was summarized in Table 1.

The specific hemagglutination activity of PPAsL was observed to increase from 20 HA-units/mg (crude garlic extract) to 160 HA-units/mg (PPAsL) at the partially purification level. The yield of PPAsL was approximately 114.02mg from 100g of garlic bulbs.

![Fig. 1: (A & B) Confirmatory hemagglutination assay for lectin at different stages of PPAsL from garlic bulb. (C) Coomassie brilliant blue (15% gel) Lane profile: 1: crude Allium Sativum extracts, 2: Ammonium sulphate precipitation (0-20%), 3: Ammonium sulphate precipitation (20-50%), 4: Ammonium sulphate precipitation (50-90%), 5: Dialyzed. (D) PPAsL display leucocyte agglutination. The leucocytes were isolated with Ficol-hypaque density gradient centrifugation and microscopic view of PPAsL induced agglutinated leucocytes visualized at 40X optical magnification.](image-url)

### Table 1: Isolation and purification of garlic lectin (AsL) from garlic bulb (100g)

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Total HA activity (Units)</th>
<th>Concentration (mg)</th>
<th>HA-Specific Activity (Units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Garlic Extract (50% w/v)</td>
<td>64,780.0</td>
<td>3239.0</td>
<td>20</td>
<td>100.0</td>
</tr>
<tr>
<td>Precipitation by Ammonium Sulphate at 50%-90% saturation rate</td>
<td>28,034.4</td>
<td>350.43</td>
<td>80</td>
<td>43.27</td>
</tr>
<tr>
<td>Post-Dialyzed by Amicon Ultracentrifuge</td>
<td>18,243.2</td>
<td>114.02</td>
<td>160</td>
<td>28.16</td>
</tr>
</tbody>
</table>
Antioxidant activity of garlic lectin

The PPAsL was examined for free radical scavenging activity using DPPH assay. Different concentration of PPAsL (0.2 – 1.0 mg/mL), was taken in a total volume of 1.0 mL and were added with 3.0 mL of the DPPH solution. The samples were vortexed thoroughly to mix them. The reaction mixture was kept in the dark for 30 min. The OD values were read at 520 nm and compared against standard Ascorbic acid (Fig 2A). Free radical scavenging potential of PPAsLis almost 50% lower efficient than the standard control, Ascorbic acid; indicating weaker capacity in free radical scavenging properties. Result represents the percentage of scavenging activity of PPAsL, where PPAsL shows the significant radical scavenging property which was proportional with the increased concentration of PPAsL.

The lipid peroxidation level in liver was measured based the reaction between malondialdehyde (MDA) and 2-thiobarbituric acid (TBA). MDA are the biomarkers for lipid peroxidation. PPAsL exhibits inhibitory lipid peroxidation were shown in Fig. 2B the lipid peroxidation inhibitory percentage increased from 56%, 72%, 76% and 80% with the increased concentration of PPAsL (25, 50, 100 and 150µg). This indicates the PPAsL can provide significant protection for membrane lipid peroxidation.

Production and purification of anti-rabbit polyclonal antibody against garlic lectins to assess immunogenicity

The antisera collected from immunized rabbit were purified to homogeneity by ammonium sulphate precipitation method and protein A agarose affinity chromatography, to obtain a pure anti-AsL IgG antibody (Fig 3A). Purified fraction of antibody was analyzed by SDS-PAGE under non-reducing condition (Fig.3B) obtained molecular weight of 150kDa. The lectin immunized anti-sera was checked for the presence of antigen specific antibody and its affinity to PPAsL by ODD (Fig 3C) and was further confirmed by Dot blot (Fig 3D) and immunoblot (Fig 3E) for the lectin specificity. Rabbit anti-AsL antibodies showed and demonstrated strong immunoreactivity to PPAsL as analyzed by dot blot and immunoblot technique. This confirms the above purified serum sample contains IgG antibody against PPAsL displays the immunogenic property.

PPAsL induced Mitogenic proliferation of peripheral blood lymphocytes (PBLs)

The PPAsL was identified to be mitogenic for human peripheral blood lymphocytes (PBLs) and was also found co-mitogenic with Con A. The results are shown in Fig 3A. PPAsL showed contemporary effect and had similar response as that of Con A (a well known mitogen). The garlic extract (RGE), PPAsL lectin and Con A were checked in the range of 0.01 to 0.01 to 10 µg/mL concentration. The results were showed in Fig. 4B. Both garlic lectin (PPAsL) and RGE exhibited lymphocyte stimulation effects and was observed to increase with concentrations (0.01 to 0.1 µg/mL). PPAsL behaves comparable in mitogenic response with Con A to induce proliferation of human PBLs.

Fig.2: Antioxidant activity of PPAsL: (A) Percentage of DPPH scavenging activity of PPAsL, where PPAsL exhibited slight significant in radical scavenging property which is proportional with increasing amount of AsL. (B) The result of the PPAsL inhibitory lipid peroxidation were shown in figure the lipid peroxidation inhibitory percentage increased with the increased concentration of PPAsL.
Fig. 3: Purification of IgG by Protein-A agarose chromatography: (A) Protein A affinity chromatography profile of for purification fractions of rabbit anti-AsL antibodies, the second peak was bound peak (IgG), which was eluted by changing the pH of the buffer. (B) Coomassie brilliant blue. (8% gel) under non-reducing condition Lane Profile: 1 - Crude serum sample, 2- Ammonium sulphate precipitated serum sample, 3 – Dialyzed serum sample, 4 - Peak-2 (purified anti-AsL-IgG fraction). (C) Ouchterlony double diffusion to analyse the immunoprecipitation of anti-AsL antibodies against various stages of antibody purification. a. pre-immune serum sample, b-crude serum sample, c- ammonium sulphate precipitation serum sample, d-Dialyzed serum sample. The precipitation line indicates the identical proteins from the crude, ammonium sulphate precipitation and dialyzed sample against antigen (centre well). (D) Dot blot analysis for the rabbit anti-AsL polyclonal antibodies 1.BSA and 2. PPAsL. (E) Immunoblot analysis of rabbit anti-AsL polyclonal antibodies. Lane 1-crude garlic lectin, 2- PPAsL.

Fig. 4: The immunomodulatory effects of partially purified garlic lectin (PPAsL) on human peripheral blood lymphocytes (PBLs) A: At protein amount at 10 μg/mL and B: The protein amount used in the range of 0.01 to 10 μg/mL. Con A was used as a reference positive control for proliferation of PBLs and control is not added with any protein.

Anti-proliferative and anti-angiogenic properties of PPAsL

In the present study, the screening of cytotoxicity effect of PPAsL was analyzed by conducting MTT cell viability assay against human melanoma cell lines A375. The in-vitro anticancer activity of PPAsL was verified by cytotoxic assay. The cultured A375 cell lines were treated with different amounts of PPAsL and the effect on the survivability of cells was observed. Results showed the decreased cell viability against PPAsL treatment with approximate IC₅₀ value of ~12.5μg/ml. Detailed results is shown in Table 2.

Tumour neo-vascularization is one of the integral mechanisms in malignant tumour expansion and marked precocious capillary sprouting with convoluted, distorted, and excessive vascular branching.

Table 2: Effect of partially purified garlic lectin (PPAsL) in A375 cell proliferation

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Average Absorbance ±SD</th>
<th>Normalized viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.517±0.102</td>
<td>100±4.059</td>
</tr>
<tr>
<td>3.12</td>
<td>2.352±0.283</td>
<td>93.470±11.237</td>
</tr>
<tr>
<td>6.25</td>
<td>2.347±0.043</td>
<td>93.272±1.691</td>
</tr>
<tr>
<td>12.5</td>
<td>1.256±0.048</td>
<td>49.907±1.888</td>
</tr>
<tr>
<td>25</td>
<td>1.213±0.141</td>
<td>48.212±5.584</td>
</tr>
<tr>
<td>50</td>
<td>1.094±0.063</td>
<td>43.470±2.493</td>
</tr>
<tr>
<td>100</td>
<td>1.083±0.150</td>
<td>43.020±5.965</td>
</tr>
</tbody>
</table>

The anti-angiogenic effect of PPAsL in Ova CAM assay, PPAsL was found anti-angiogenesis, which was observed inhibit the vascularization and results inferred that blood vessel counts in Ova CAM was
remarkably regressed. Treatment with PPAsL displayed 86% and 75% inhibition of CAM vessel density. PPAsL treated displays the inhibition of vessel density in CAM (Fig. 5).

Cytotoxicity was measured by MTT assay against cell lines. Each value represents the three independent (n=3) measurement. DMSO was used as a vehicle control which exhibits very negligible cytotoxicity. All values were expressed as mean ± SD.

**Fig.5:** Antiangiogenic activity of PPAsL in Ova angiogenic assay models. Ova CAM, and Graphical images with anti-angiogenic potential. Treatment with PPAsL displayed 86% and 75% inhibition of CAM vessel density. PPAsL treated displays the inhibition of vessel density in CAM where the concentration of PPAsL was increased the density of the blood vessel drops.

**PPAsL exhibit potent anti-tumour effects on ascites tumour model**

The in-vivo tumour models are the critical for the evaluation of anti-proliferative efficacy. The in-vivo anti-proliferative potential of PPAsL was investigated using a reliable murine EAC tumour model. 0.5ml of EAC tumour cells were transplanted to mice via intraperitoneally (i.p.) route of administration. After the 4th day of tumour growth, PPAsL was administrated at three different doses at 0, 25 and 50 µg/kg body weight on every alternative day. Results revealed that PPAsL exhibited a dose dependent decrease in the tumour development as assessed by the physical morphology and body weight index as compared to that of control bearing animal. (Fig.5A). Ascites secretion in the PPAsL treated was very negligible whereas the control animal depicted plethoric secretion (Fig.5B). The reduced tumourigenic index was reflected in the decreased cell count (Fig.5C). The survival of untreated PPAsL bearing mice is maximum for 15-20days, but PPAsL regimen has prolonged the survival duration >32days (Fig.5D).

**Fig.5:** EAT (Ehrlich Ascites tumour) was induced by injecting EAC cells (5 x 10⁶ cells/mice) intraperitoneally in Swiss albino mice. (A) Regressed tumourigenic index indicative of the reduction in the tumour growth in the dose dependent manner. (B) Decrease in ascites secretion. (C) Declined tumour cell population. All the parameters assessed were evaluated and compared to the relevant control tumour bearing mice. (D) Kalper-Meier graph showed the prolonged life span of PPAsL treated animals. Results were expressed as the mean value of three determinations; each experiment is conducted in triplicates. Statistically significant values are *p< 0.05; **p< 0.01
DISCUSSION

Lectins are proteins having high degree of stereo specificity to recognize glycans structures bearing sugar complexes to form reversible linkages upon interaction with glycoconjugate complexes. Further, lectins exhibit unique carbohydrate binding specificity, and have been extensively utilized to regulate and modulate many biological and physiological functions. The ability of protein-carbohydrate interactions through lectins allow to explore their applications as immunomodulatory or anti-tumour molecules through various cellular interactions (24). In line with previous reports (16), in the present study we isolated and partially purified a mannose specific Allium sativum lectin (AsL) from garlic bulb. The PPAsL has molecular weight of ~12kDa with specific hemagglutination and leucoagglutinating property.

Oxidative stress was observed to be a causative result of impaired excessive production and accumulation of free radicals and reactive oxygen species (ROS) in inflamed cells and tissues. Over appearance of ROS induce oxidative damage to the cells or tissues through lipid peroxidation, denaturation of cellular proteins, enzymes and nucleic acids. PPAsL shows the significant antioxidant properties with increased concentration by DPPH assay, and it was 50% efficient compared to standard ascorbic acid. Lipid peroxidation also involved in the damage of cells and tissues. The thiobarbituric acid reacting substances test (TBARS) is used for the quantification of MDA. PPAsL inhibits increased lipid peroxidation percentage with the increased concentration of PPAsL. Garlic and Back seeds’ consumption had significantly reduced the Plasma MDA levels of normal postmenopausal women (3). This observed effect may be due to antioxidant potential of garlic constituents and also increasing activities of cellular antioxidant enzymes. The reports are in comparison with our data as PPAsL showed significant increase in the antioxidant activities and was increased with increased concentration of PPAsL.

Tumour neo-vascularization, one of the integral mechanisms in tumour malignant expansion and was mainly marked with appearance of precocious capillary sprouting with formation of new blood vessels which are convoluted, distorted and excessive vascular branching. Tumour cells are compelled to pass through a hypoxia phase that refer to low oxygen supply which is key trigger of cascade of events that leads to increased angiogenesis owing rapid growth of the cancer cells. The study tried to understand the anti-angiogenic effect of PPAsL in Ova CAM assay. The observed effect of PPAsL on angiogenesis, results in repressed or decreased blood vessel counts in Ova CAM. PPAsL treated displays the inhibition of vessel density in CAM where the concentration of PPAsL was increased the density of the blood vessel drops. The anti-tumour property was observed from the anti-angiogenic and anti-proliferative effect of PPAsL in in-vitro angiogenesis and cytotoxicity assay system, respectively. These results explain that PPAsL has abrogated the tumour progression possibly through inhibiting angiogenic factors and inducing immunological stimulation to suppress and control tumour growth (4).

PPAsL was then subjected to in-vivo anti-tumour studies Ehrlich ascites carcinoma (EAC) tumour model. Our studies indicated that PPAsL significantly regress the tumour progression which is indisputable from the tumourigenic index and cell count. Understudy, PPAsL treated mice showed significant reduction in the ascites secretion which was obviously linked to the expressive tumour inhibition in mice with extended survival. Consequently, PPAsL potently inhibited the tumour cell proliferation most evidently through the neovasculature blockade (22). Although the pathological effect of plant lectins is well-documented. This is the first report to our knowledge, that the study illustrates and demonstrates that the partially purified garlic lectin (PPAsL) acts as immunogenic, antioxidant and has anti-tumour properties. Future design of experiments with purified lectin with in-depth mechanistic study would open a new perspective field for development of lectin based novel agent and drug targeted for various diseases, specifically with dietary lectins from plant sources.

CONCLUSION

Allium sativum L., “garlic”, has well known medicinal and therapeutic plant with adequate compounds that are beneficial to boost immune system and treat numerous pathological conditions including cancer. However, presently PPAsL was identified as a promising anti-cancer with multiple modes of action such as anti-proliferative and anti-angiogenic, its consumption through healthy diet may have beneficial effects on cancer. Nonetheless, to explore better mechanism on the anticancer activities of garlic lectin, further studies on purified garlic lectin are necessary in future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.
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