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Antiproliferative activity of cytotoxic tuber lectins from *Solanum tuberosum* against experimentally induced Ehrlich ascites carcinoma in mice

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Cytotoxicity of tuber lectins from two potato cultivars was assessed and their anti-tumor potential against experimentally induced Ehrlich ascites carcinoma in Swiss albino mice was evaluated. Twenty (20) kDa chitin-binding lectins from *Solanum tuberosum* tubers, STL-S and STL-D were purified through ion-exchange and affinity chromatographic methods, hemagglutinating activity and blood group specificity of the lectins were checked whereas the cytotoxicity was determined using brine shrimp (*Artemia salina* L.) nauplii lethality assay. The lectins showed no specificity to animal and human erythrocytes. LC_{50} values for STL-S and STL-D were found to be 75 and 90 µg/ml, respectively with a dose-dependent intermediary toxic effect. After inducing ascites by intraperitoneal propagation, the Swiss albino mice were treated by administering the lectins at a dose of 1.38 mg/kg/day for five consecutive days. STL-S and STL-D showed 79.84 and 83.04% of growth inhibition of EAC cells, respectively. Additionally, hemoglobin and RBC levels became considerably increased with a drop off in the WBC levels in the treated mice group indicating moderate anticancer activities exhibited by the potato lectins.

Key words: Chitin-binding lectins, antitumor activity, LC_{50}, cell growth inhibition.

INTRODUCTION

Plant lectins have been widely studied for pharmacological applications as well as their immuno-potentiating and anti-tumor activities (Karasaki et al., 2001; Suen et al., 2000; Chang et al., 2007; Li et al., 2011; Dhuna et al., 2005; Liu et al., 2009; Zhang et al., 2010; Faheina-Martins et al., 2011) due to their effects on animal cells.

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Abbreviations: STL-S and STL-D, Lectins from potato cultivars Sheelbilatee and Deshi, respectively; **GlcNAc,** *N*-acetyl D-glucosamine; **DEAE,** diethylaminoethyl; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **EAC,** Ehrlich's ascites carcinoma; **RBC,** red blood cells; **WBC,** white blood cells; **LC_{50},** lethal concentration 50 (concentration in water having 50% chance of causing death to aquatic life).

Lectins from different sources were found to have an effect on cell functions. There are well-established relationships in their biological effects on a number of tumor cell lines.

The degree of glycosylation of malignant tumors is related with metastasis and differs from benign cells. This difference can be identified by lectins through specific recognition to glycoconjugates.

Particularly, plant lectins have antitumor activities as those can bind to cancer cell membrane proteins and receptors. They can inhibit cell growth or kill the cells by creating cytotoxic environment and through the activation of apoptotic pathways (Kiss et al., 1997; De Mejia and Prisecaru, 2005; Ferriz-Martinez et al., 2010). Lectins from different sources inhibit cancer cells growth depending on their concentration. At lower concentrations, they exert an immunostimulatory effect whereas at high concentrations, a cytotoxic effect is shown to take place (Lyu et al., 2001, 2002; Pryme and Bardocz, 2001).

Plant lectins can be subdivided into four major families; the legume lectins, the chitin-binding lectins composed of hevein domains, the type 2 ribosome-inactivating proteins and the monocot mannose-binding lectins. Each family has its own characteristic carbohydrate recognition domain. Chitin-binding lectins can bind to chitin oligomers or *N*-acetyl-chito-oligosaccharides (GlcNAc)n, where, n = 2 to 5 (Van Damme et al., 1998) and have distinct biological activities including antineoplastic activities (Abdullaev and de Mejia, 1997; Yao et al., 2010; Wang et al., 2000).

A number of chitin-binding lectins are found in Gramineae and Solanaceae species (Van Damme et al., 1998). There are a variety of lectins recognizing oligomeric *N*-acetyl D-glucosamine (GlcNAc) as chitobiose (GlcNAc disaccharide), chitotriose (GlcNAc trisaccharide) and chitin (GlcNAc polymer) in fruit bodies and tubers of the plant family Solanaceae. In case of potatoes (*Solanum tuberosum* L.), there are multiple varieties of chitin-binding lectins with the molecular mass of 45 to 65 kDa in different cultivars (Allen et al., 1996). They were specifically reported to be glycoproteins with an unusual ratio of glycan portions of 50% by weight.

In Bangladesh, potato is regarded as one of the main foods and about 27 cultivars with familiar local names are cultivated in different parts of the country according to cooking habits with different physiological and morphological properties (Salahuddin, 2000). The cultivar named 'Deshi' is a representative variety in this country with relatively small size (2 to 4 cm in diameter) and bright red in color whereas the Sheelbilatee cultivar is more elongated and irregular in shape. It is pale red and stickier than the Deshi cultivar.

As the detailed characterization of pharmacological activities of these varieties have not been reported in literature, the present work describes isolation of chitin-binding lectins from two local potato prototypes cultivated in Bangladesh and their significant *in vivo* antiproliferative activities against Ehrlich ascites carcinoma cells in mice.

MATERIALS AND METHODS

Materials

The tubers of indigenous potato cultivars 'Sheelbilatee' and 'Deshi' were collected from Rangpur and bought from the local market in Rajshahi, respectively. The specimens were identified by Prof. M. Manzoor Hussain, Department of Botany, University of Rajshahi, Bangladesh. Potato tubers were stored in a refrigerator at 4°C.

Chemicals and reagents

DEAE-cellulose and chitin were procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals and reagents used throughout this study were of analytical grade from BDH, UK; Merck, Germany and Sigma Aldrich, US.

Test animals and ethical clearance

Adult male Swiss albino mice, 6 to 8 weeks old with 25 (± 5) g body weight were bought from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B). The protocol was approved by the Institutional Animal, Medical Ethics, Bio-safety and Bio-security Committee (IAMEBBC) for experimentations on animals, humans, microbes and living natural sources (286/320 -IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of 10 animals in a cage. They were maintained under standard laboratory conditions (temperature 25±2°C, relative humidity 48% with dark and light cycle 12/12 h). The mice were allowed free access to standard dry mice food-pellets and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before commencement of the experiment.

Purification of potato lectins

300 g of potato tubers were peeled off, sliced and homogenized in 10 mM Tris-HCl (pH 8.2) containing 50 mM NaCl. The homogenates were centrifuged at 12,000 × *g* for 20 min; the supernatants were collected and dialyzed overnight against distilled water and 10 mM Tris-HCl buffer at 4°C. Crude supernatants were applied to diethylaminoethyl (DEAE)-cellulose column (2 × 25 cm) and eluted by a linear gradient of 0 to 400 mM NaCl containing 10 mM Tris-HCl buffer. The fractions (2.5 ml each) having hemagglutination activity were subjected on to a chitin column (2 × 25 cm) previously equilibrated with the same buffer and were eluted by 0.5 M acetic acid (Allen et al., 1996; Allen and Neuberger, 1973). The pH of the eluted fractions was neutralized by the addition of the aliquot of 1 M Tris-HCl buffer (pH 8.2) in each tube and then dialyzed overnight. The molecular mass and hemagglutination activity of the purified lectins from both potato cultivars were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% separating gel under reducing condition (Laemmli, 1970).

Hemagglutination assay and blood group specificity

The hemagglutination assay was performed in 96-well microtiter U-bottomed plates in a final volume of 100 µl containing 50 µl of protein solution serially diluted with equal amount of hemagglutination buffer (20 mM Tris-HCl buffer, pH 7.8 containing 0.9% NaCl and 10 mM $CaCl_2$) and 50 µl of 2% red blood cells (RBCs)

suspension previously washed with 0.15 M NaCl. After a gentle shaking, the plate was kept at room temperature for 30 min. The visual agglutination titer of the maximum dilution giving the positive agglutination was recorded.

RBCs of human (A, B, AB and O groups), rat, chicken and cow were collected, washed in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl. pH 8.2) and centrifuged at 3,000 rpm for 10 min. The erythrocyte pellet was washed thrice and re-suspended in the same saline to make a 2% RBC suspension.

Brine shrimp nauplii lethality assay

Lethality assay was studied using brine shrimp (*Artemia salina* L.) nauplii. The *Artemia* cysts were hatched in artificial seawater at 28°C under constant light and aeration. The artificial sea water was prepared by dissolving 38 g of NaCl in 1 L of DW and pH was adjusted to 7.0 by sodium tetraborate. The cysts were incubated in a glass tube with 1 g cysts per liter of artificial seawater. After a period of 48 h, the aeration was halted, and the light was directed to the bottom of the tube. The phototropic nature of nauplii caused them to migrate in the direction of light toward the bottom of the tube, facilitating their separation from unhatched cysts. 60, 125, 250, 500, and 1000 µl of STL-S and STL-D vials were taken containing artificial sea water and 10 *Artemia* nauplii were added to each vial. Finally the volume of each vial was adjusted to 4 ml by the addition of artificial sea water. The experiments were performed in triplicate, and negative control was the artificial water and the *Artemia* nauplii in the absence of the lectin. After 24 h, the number of dead nauplii was counted for each concentration and the LC_{50} values were determined by using Probit analysis as described by Finney (Finney, 1971).

Ehrlich's ascites carcinoma (EAC) cell growth inhibition assay

Ehrlich ascites carcinoma cells were maintained in Swiss albino mice by a bi-weekly intraperitoneal propagation. The cells were diluted with normal saline and adjusted to a number of 3×10^6 cells/ml by the help of a hemocytometer. Then, 0.1 ml of tumor cells having viability above 90% was injected intraperitoneally to each mouse. After 24 h of tumor inoculation, the mice were randomly distributed into three groups consisting of 6 mice per group. Two groups of the mice were treated with STL-S and STL-D at a concentration of 1.38 mg/kg/day for five consecutive days while the remaining group was used as a negative control (injected with 10 mM Tris-HCl buffer). Mice in each group were sacrificed on the 6^{th} day and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified with Trypan blue and then counted by a hemocytometer under an inverted microscope (XDS-1R, Optica, Italy). The percentage of inhibition was calculated by using the following formula:

Percentage of inhibition = 100 - {(cells from lectin treated mice/cells from control mice) × 100}

Hematological assay

To elucidate the cytotoxic effect of potato lectins on EAC-bearing and control mice, the study of hematological parameters were carried out. Treatment started after 24 h of tumor transplantation and continued for 10 consecutive days as the previous experiment. On the 12^{th} day of tumor transplantation, blood was collected from each mouse by tail puncture to determine the hematological parameters namely: WBC, RBC and hemoglobin content following standard methods using appropriate cell dilution fluids and hemocytometer (Rusia and Sook, 1988).

Statistical analysis

The experimental results are presented as mean ± SEM for three replicates for studied parameters. One way analysis of variance (ANOVA) was used to calculate the data followed by Dunnett 't' test using statistical package for social sciences (SPSS) software (Chicago, IL) version 10.

RESULTS AND DISCUSSION

Purification of potato lectins

The crude supernatants extracted from both potato cultivars had strong hemagglutinating activity against mice erythrocytes. In anion-exchange chromatography, the supernatants were applied to the DEAE-cellulose column and four peaks were eluted from the column by the linear gradient of NaCl concentration (0-400 mM). Peaks with significant hemagglutinating activity were collected together and applied separately on to the chitin column. After washing the chitin columns with 10 mM Tris-HCl buffer extensively, one single peak was eluted with the help of 500 mM acetic acid. Eluted fractions were collected, neutralized using 1 M Tris-HCl buffer and dialyzed against 10 mM Tris-HCl buffer. SDS-PAGE showed that, both STL-S and STL-D consisted of a major 20 kDa band with two other faint 22 and 17 kDa bands in reducing condition (Figure 1).

Twenty (20) kDa potato lectins isolated from both the cultivars were found to be non-specific to human and animal erythrocytes. Similar result was found in case of ADL which is a 15 kDa *N*-acetyl- D-glucosamine specific lectin with antiproliferative activity (Kaur et al., 2005). It also agglutinated all the tested erythrocyte samples from human and animals. Another GlcNAc-specific mitogenic lectin, BBL was reported to have this property (Banerjee et al., 2004). Besides different sugar-binding lectins like NNTL, PCSL, TRA and *Ficus cunia* lectin also possess these two common properties, non-specificity and antiproliferative activity (Kabir et al., 2011; Chen et al., 2009; Bhowal et al., 2004; Ray et al., 1992).

Hemagglutination and blood group specificity assay

STL-S and STL-D showed no specificity to any type of blood and agglutinated RBC cells from every blood type significantly. This result supports the previous findings that this lectin agglutinates erythrocytes of different animals including mice, chickens, cows, sheep, horses, rabbits as well as the human (A, B, AB and O groups) erythrocytes indicating its non-specificity to any blood group (Sharon et al., 2000). The minimum protein concentration to agglutinate Swiss albino mice erythrocytes was 4 µg/ml.

Brine shrimp nauplii lethality assay

Higher mortality rates were found for STL-S comparing to

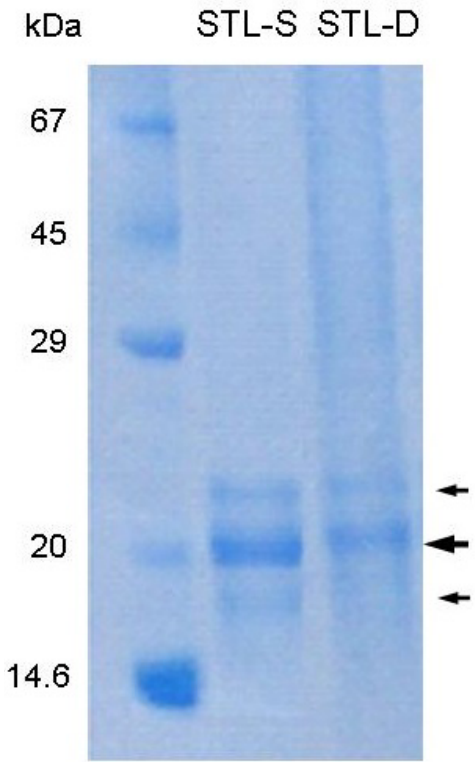

Figure 1. The molecular mass of potato lectins by SDS-PAGE. Standard protein markers: Bovine serum albumin (67 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Trypsin inhibitor (20 kDa) and Lysozyme (14 kDa). Lectins from Sheelbilatee potato cultivar (STL-S) and Deshi potato cultivar (STL-D): Arrows indicate major (20 kDa) and minor (22 kDa and 17 kDa) proteins.

STL-D. The mortality rate was found to be increased with the increase of concentration of the lectin as shown in Figure 2. The LC_{50} values were determined as 75 µg/ml for STL-S and 90 µg/ml for STL-D. Both these lectins have a molecular weight of around 20 kDa and showed similar inhibitory effects on EAC cell growth; but STL-S was found to be slightly more toxic than STL-D. May be this difference took place because of their genetic diversity. Plant lectins are reported with a wide range of cytotoxicity values. A lectin from *Curcuma amarissima* rhizomes showed *in vitro* antiproliferative activity against a breast cancer cell line (BT 474) with an LC_{50} value of approximately 21.1 µg (Kheeree et al., 2010). Highly toxic lectins were isolated from *Abrus pulchellus* and Mulberry seeds having LC_{50} values of 3.5 mg/ml and 21.87 µg/ml whereas other lectins from the seeds of *Dioclea lasiophylla* and tubers of *Nymphaea nouchali* had much lower values like 45.85 and 120 µg/ml, respectively (Ramos et al., 1998; Absar et al., 2005; Pinto-Junior et al., 2013; Kabir et al., 2012). Presence of a non-toxic lectin from *Sebastiania jacobinensis* bark to *Artemia nauplii* is also reported (Vaz et al., 2010). Even two lectins (Halilectin 1 and Halilectin 2) from the same source, a marine sponge, had been purified with quite different toxicities (6.4 and 142.1 µg/ml) (Carneiro et al., 2013). Comparing different LC_{50} values of these lectins, it became evident that STL-S and STL-D exhibited an intermediary toxic effect against *Artemia* nauplii.

Ehrlich's ascites carcinoma (EAC) cell growth inhibition assay

At the dose of 1.38 mg/kg/day, significant growth inhibition of tumor cells was found by STL-S and STL-D with a reduction percentage of 79.84 and 83.04% respectively. Figure 3 shows the reduced numbers of viable EAC cells in the lectin-treated mice comparing the control mice group.

Plant lectins are recently being used to evaluate novel cancer therapeutics as well as to identify predictive biomarkers in early stages of drug development (Chang and Lei, 2008). A lectin from *Ficus cunia* agglutinated EAC cells and agglutination was inhibited by chitin oligosaccharides [(l → 4) linked β-GlcNAc] and glycopeptides containing GlcNAc residues indicate the presence of these oligosaccharides as receptors (Ray et al., 1992).

Several typical GlcNAc-binding lectins such as mistletoe lectin and wheat germ agglutinin were found to possess significant antitumor activities that can stimulate apoptosis to kill the cancer cells (Liu et al., 2010). Each lectin displays variation in their antitumor activities due to the differences in sugar specificity (Yan et al., 2010). Like mistletoe lectin, STL-S and STL-D are also capable of binding with GlcNAc polymers and exerting antitumor effects.

The aggregated cancer cells may become trapped in microvascular vessels, which facilitate their extravasation. These lectins showed a resemblance with another lectin, TRA that agglutinated EAC cells with a minimum concentration of 3.12 µg/ml. The glycan part of TRA contained mannose (16.8%), GlcNAc (1.07%) and arabinose (0.67%). This may be a consequence to the change in surface structure of the tumor cells, occurring with the progress in tumor growth or because of the clustering of the lectin receptors present on cell surfaces (Alderson and Green, 1978; Mastromarino et al., 1980).

It became evident that these two potato lectins (STL-S and STL-D) had inhibitory effects on EAC cell growth (79.84 and 83.04%) at a dose of 1.38 mg/kg/day (equivalent to 50 µg/day). Some other lectins like Jackfruit lectin inhibited EAC cell growth by 21.8, 40.2 and 57.5% at 50, 100 and 150 µg/day respectively (Ahmed et al., 1988). Pea lectin showed 44 and 63% decrease in EAC cell growth at 1.4 mg/kg/day and 2.8 mg/kg/day (Kabir et al., 2013). *Nymphaea nouchali* tuber lectin (NNTL) was found to inhibit EAC cells by 56 and

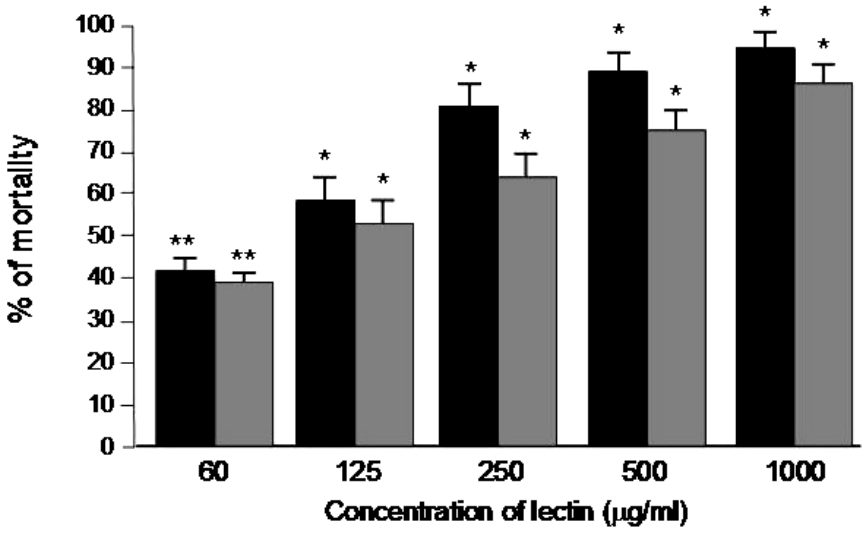

Figure 2. Toxic effect of STL-S and STL-D at different concentrations (60, 125, 250, 500 and 1000 µg/ml) on *Artemia* nauplii. Black and gray bars indicate the mortality rate for STL-S and STL-D, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

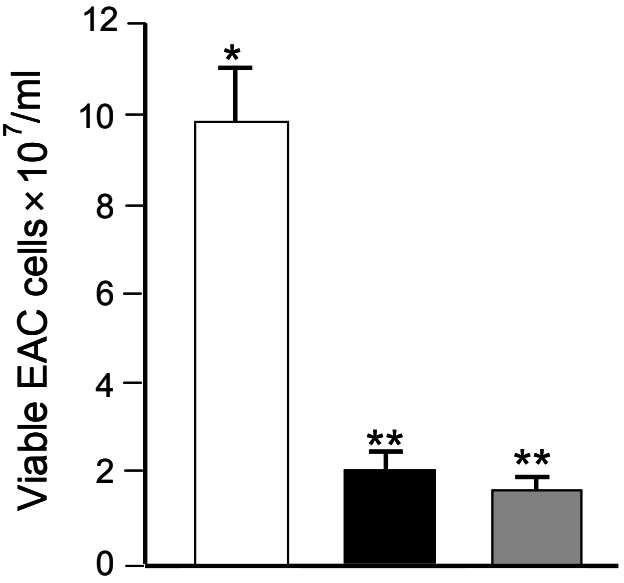

Figure 3. Number of EAC cells in non-treated (control) mice and lectin-treated mice counted by a light microscope on day 6 of tumor inoculation. White, black and gray bars indicate the values for non-treated (control), STL-S treated and STL-D treated mice, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

76% at a dose of 1.5 mg/kg/day and 3.0 mg/kg/day while *Trichosanthes cucumerina* seed lectin (TCSL) showed 28 and 72% growth inhibition against the same cell line (Kabir et al., 2011; Kabir et al., 2012). All these lectins were found weaker than STL-S and STL-D in terms of activity.

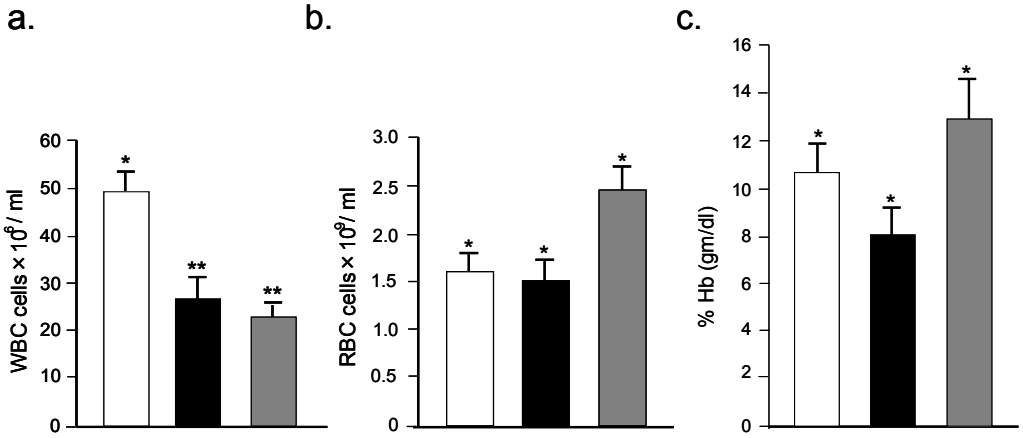

Figure 4. Hematological parameters of ECA-bearing non-treated (control) mice and lectin-treated mice on day 12 of tumor inoculation. White, black and gray bars show the values for non-treated (control), STL-S treated and STL-D treated mice, respectively. Error bars: SE calculated from three independent experiments (n = 6). *, P <0.05 and **, P <0.01; P <0.05 were considered statistically significant when compared with control.

Hematological assay

WBCs count was increased in the control mice as compared to the treated mice (Figure 4a). The RBCs count for the mice treated with STL-D was higher than the control mice but unexpectedly the mice treated with STL-S were found to have even lesser number of RBC cells than the control mice (Figure 4b). This result was corresponded by the amount of hemoglobin found in the treated mice group comparing the control group (Figure 4c).

In tumor-bearing mice, WBC count generally increases while anemia takes place due to the reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Fenninger and Mider, 1954). In the present study, both STL-S and STL-D decreased WBC count in the treated mice. STL-D increased the RBC count and hemoglobin level though this phenomenon was particularly absent in case of STL-S. The additional cytotoxicity of STL-S compared to STL-D can be responsible for this. It might also have happened due to the specificity of lectin-induced cellular cytotoxicity. A lectin triggers different clones of effector cells selectively, each with its own unique cytotoxic capability. Therefore, the specificity involves more than the presence of receptors on a RBC target for any particular lectin (MacDermott et al., 1976).

Conclusion

The present study indicates that potato lectins exhibited moderate anticancer activities. To our knowledge, there is no report available discussing growth inhibition of EAC cells mediated by potato lectins. However, further investigations are required to understand these phenomena considering all the relevant hypotheses and to declare potato lectins as potent anticancer agents despite their cytotoxicity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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