

ISOLATION AND CHARACTERIZATION OF LECTIN FROM *PUNICA GRANATUM* (POMEGRANATE)

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ABSTRACT

Lectins are glycoproteins which recognize carbohydrates, agglutinate the cells and are detected in biological materials by hemagglutinating activity (HA) assay. This study aimed to isolate and characterize the *P. granatum* testa lectin. The lectin was isolated and extracted from the pomegranate seeds by extraction with phosphate buffered saline (PBS) at pH 7.2. The sugar and the protein content was estimated. The hemagglutination activity was performed on human erythrocytes of blood groups A, B, O, and AB +ve. It was found that pomegranate lectin was A and O +ve blood groups specific. The physicochemical characterization of the pomegranate seed lectin was studied with respect to pH and temperature stability and optimum pH and temperature was found to be 5 and 50°C. The hemagglutination inhibition assay was performed and pomegranate seed lectin was found to be lactose and arabinose specific. The interaction between the lectin and the sugars lead to the immuno modulation. The pomegranate lectin showed cytotoxic effect against the MCF-7 cell line and the IC50 value of 185.4 µg/ml dose-dependent inhibition of growth in MCF-7 cells. The anti mitogenic nature of this lectin needs to be experimented in further studies. Thus the present study may help to contribute in the future day's cancer therapy.

Keywords-Lectin, hemagglutination, physicochemical characterization, heat labile, immune-modulation, dose dependent inhibition

No: of Graphs: 5**No: of Tables: 1****No: of References: 12**

INTRODUCTION

Dietary lectins act as protein antigens which bind to surface glycoproteins (or glycolipids) on erythrocytes or lymphocytes (Etzler, 1985). They function as hemagglutinins and are present in small amounts in 30% of foods, more so in a whole grain diet (Nachbar and Oppenheim, 1980). In plants, lectins are particularly localized in seeds. Some of them reported that seed lectins are particularly seen in cotyledons where they appear during the later stage of maturation of the seeds. In addition to cotyledons, in some cases appreciable amounts of lectins have been reported in the embryos and small amounts in the seed coats. A few lectins are being investigated for their use in cancer research and therapy. Lectins are ubiquitous in nature. These are found in all kinds of organisms, from virus to humans (Sharon N 1972).

Lectins can be inhibited by specific carbohydrates like monosaccharides or oligosaccharides which can bind and prevent protein from attaching to the carbohydrate on cell membrane.

Lectins are difficult to degrade. They are resistant to stomach acids and enzymes. These are dynamic contributors to tumour cell recognition (surface markers), cell adhesion and localization, signal transduction across membranes, mitogenic stimulation and augmentation of host immune defence, cytotoxicity and apoptosis (Mody et al., 1995). Lectins are presently used in the clinical laboratory to

type blood cells and are used in a wide spectrum of applications, including, in part, as carriers of chemotherapeutic agents, as mitogens, for fractionation of animal cells and for investigations of cellular surfaces. Numerous studies have shown that lectins can be used to identify rapidly certain microorganisms isolated from a clinical specimen or directly in a clinical specimen. Lectins have been demonstrated to be important diagnostic reagents in the major realms of clinical microbiology. Thus, they have been applied in bacteriology, mycology, mycobacteriology and virology for the identification and/or differentiation of various microorganisms. Lectins have been used successfully as epidemiologic as well as taxonomic markers of specific microorganisms. Lectins provide the clinical microbiologists with cost effective and potential diagnostic reagents.

MATERIALS AND METHODOLOGY

Materials

Pomegranate (Bhagwa variety) was purchased from local market of Bangalore, India. Chemicals used include BSA, Sodium hydroxide, Dextrose, Fructose, Lactose, Maltose, Arabinose, were purchased from HiMedia, India Ltd, Ethanol [Jiangsu Huoxi international Trade co. Ltd], Sodium Dihydrogen phosphate [Merck specialties Pvt. Ltd], Disodium hydrogen phosphate from Thomas baker Pvt. Ltd, Sodium potassium tartarate, HCl, FC reagent from Merck, Sodium carbonate, copper sulphate from Rankem, MTT Powder (the solution is filtered through a 0.2 μ m filter

and stored at 2–8 °C for frequent use or frozen for extended periods) (Skanda Lifesciences) DMSO (Skanda Lifesciences). The MCF-7 cell line was obtained from Skanda Lifesciences Pvt. Ltd, Bangalore.

Methodology

Extraction of agglutinin

Punica granatum (Pomegranate) was obtained from the local market sold as food and cleaned with water to remove dirt. 10% homogenate was prepared by crushing the arils of pomegranate along with seeds with 1 M PBS in Mortar- pestle.

Preparation of erythrocyte suspension

The human blood was obtained in sodium citrate. 2ml blood was taken in a centrifuge tube and 8ml of 0.9% NaCl was added to make the volume 10ml. The blood was properly resuspended with the help of Pasteur pipette. It was centrifuged in cooling centrifuge at 10,000rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was again resuspended in 0.9% NaCl to make up the volume upto 10ml. 5 ml of blood was transferred into another tube and 5 ml of 0.9% NaCl was added to each tube to make it 10 ml.

Hemagglutination Assay

The hemagglutination assay was performed in 96 well polystyrene U bottomed microtitre plate (Chatterjee et al, 1979). The series of decreasing dilutions of agglutinin was made with 100µl of PBS (0.1M, pH-7.2). A control was prepared by using only PBS. 100µl of 2.5% erythrocyte

suspension was added to every well and mixed by resuspending the mixer. The plate was kept for incubation at 37°C for 1 hour. Hemagglutination was examined visually and reciprocal of maximum dilution of the agglutinin solution showing hemagglutination was recorded as titre.

Hemagglutination Inhibition Assay

Hemagglutination inhibition assay was performed by carrying out hemagglutination assay of agglutinin, along with equal volume of inhibitors (Dextrose, Fructose, Maltose, Arabinose and Lactose) and keeping PBS provided with respective concentration of inhibitor as control. The degree of agglutination was examined and maximum dilution of agglutinin showing inhibition was recorded.

Physicochemical properties

pH Stability

The pH stability of lectin was found out by using 0.1M PBS of differ pH ranging from pH 3-9, in which the agglutinin is serially diluted. The hemagglutination assay was performed as described above.

Temperature stability

The agglutinin in 0.1 M PBS (pH- 7.2) was incubated in water bath at temperature ranging from 20°C- 60°C for 15 minutes. The aliquots (100µl) was withdrawn, cooled and hemagglutination assay was performed as described above.

Protein Estimation

The protein concentration in all the agglutinin fractions was determined by the

Lowry et al method. To 500µl of the sample solution 500µl of distilled water was added to make up the volume up to 1ml. To this, 5ml of Alkaline copper reagent and followed by 5 minutes incubation at room temperature. 0.5ml of FC reagent was added to this mixture. Tube was kept in dark for 30 minutes and the absorbance was measured at 660nm. BSA (100µg/ml) was used as the standard and a blank was prepared with water. The amount of protein was determined from the standard curve obtained from the absorbance of BSA. (Lowry et al method)

Sugar Estimation

The sugar concentration in the pomegranate extract was determined by Anthrone method. The absorbance was read at 720nm. D- Glucose was taken as standard (1mg/ml). (Morse et al method)

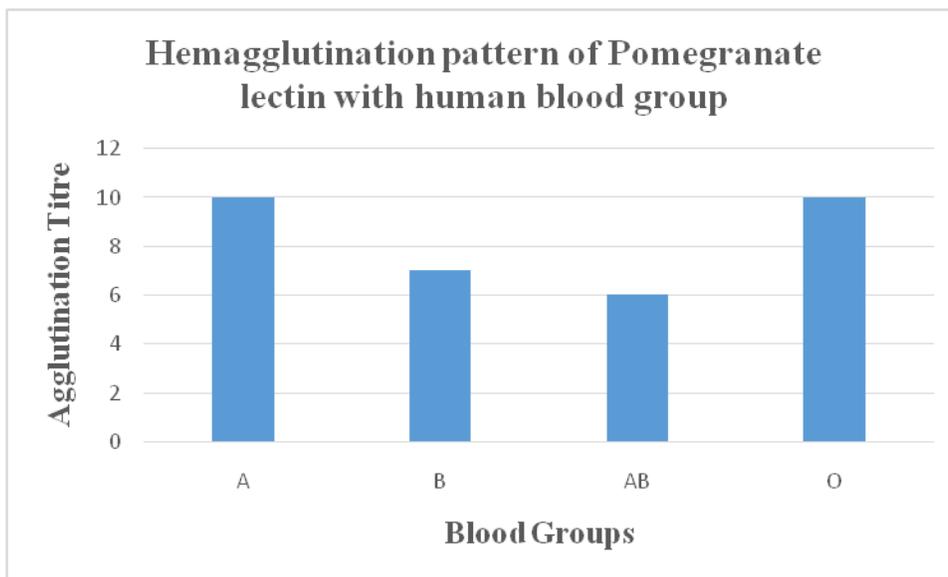
Cytotoxic assay on MCF-7 cell line by MTT method

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24hrs in 5% CO₂ atmosphere. After incubation the test solutions in the wells were discarded and

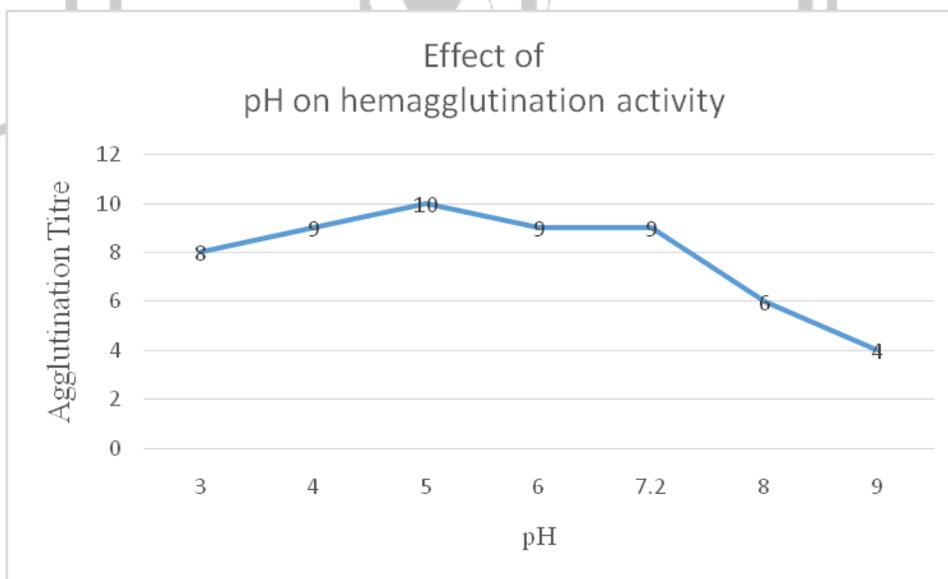
100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37° C in 5% CO₂ atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line.

Results and Discussion

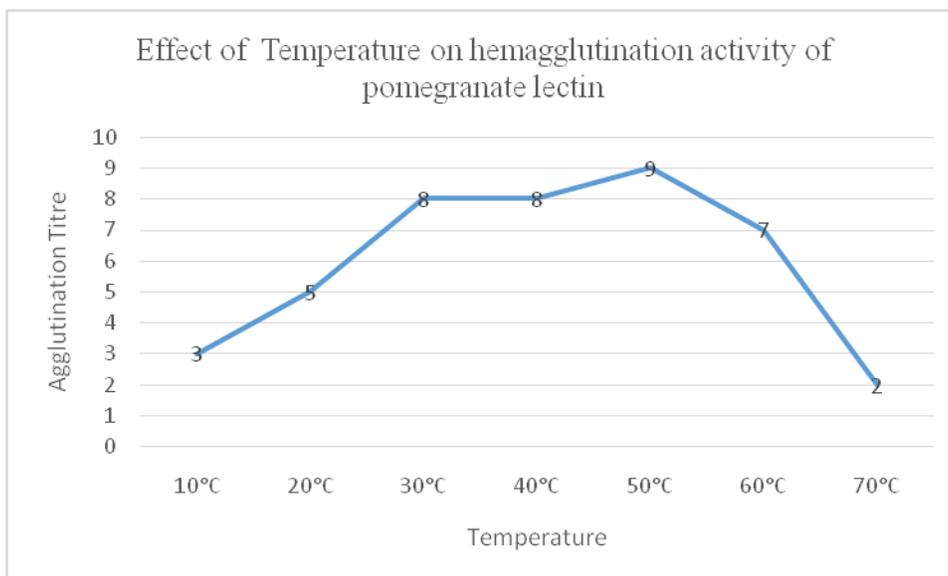
The present study was based on *Punica granatum* lectin which was isolated, purified and partially characterized. Lectin isolated from pomegranate has a maximum activity at pH range 3-7 (Graph 2) and temperature range 20-50°C (Graph 3). Lactose and Arabinose showed high binding activity in hemagglutination inhibition assay (Graph 4). The protein concentration estimated by Lowry's method has given the protein concentration of about 1mg/ml. The total sugar estimation was done by Anthrone method and the sugar concentration was found to be 2.2 mg/ml. The test sample Pomegranate seed extract has shown IC₅₀ value of 185.4 µg/ml dose-dependent inhibition of growth in MCF-7 cells. (Graph 5)



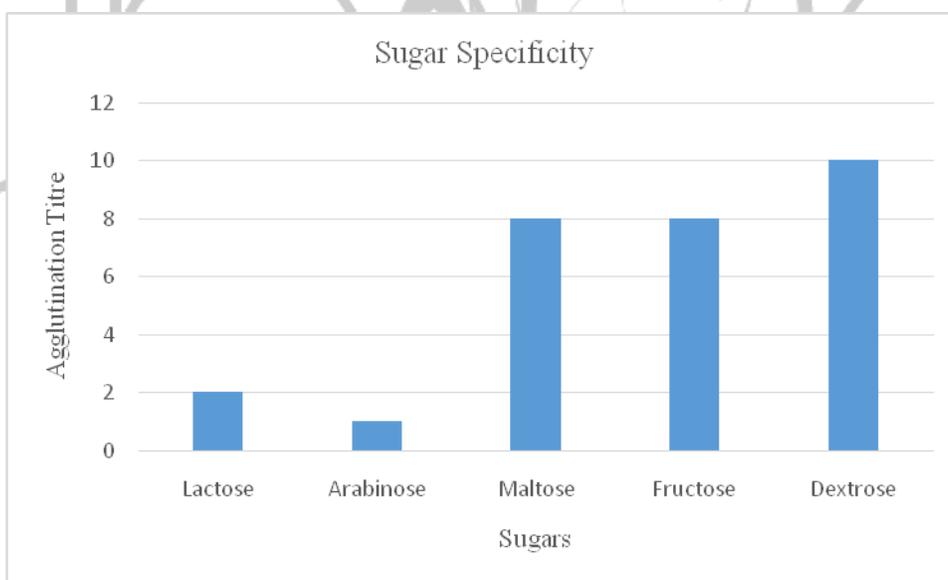
GRAPH 1: Showing hemagglutinating activity of Pomegranate lectin with human blood groups. A and O +ve blood groups showed optimum hemagglutination activity.



GRAPH 2: Results showing effect of pH on hemagglutination assay of pomegranate lectin. The pomegranate seed lectin was active in the pH range of 3-7. Optimum pH is 5



GRAPH 3: Results showing effect of temperature on hemagglutination activity of pomegranate lectin. The pomegranate seed lectin was stable in the temperature range of 20-50°C. The optimum temperature is 50°C.

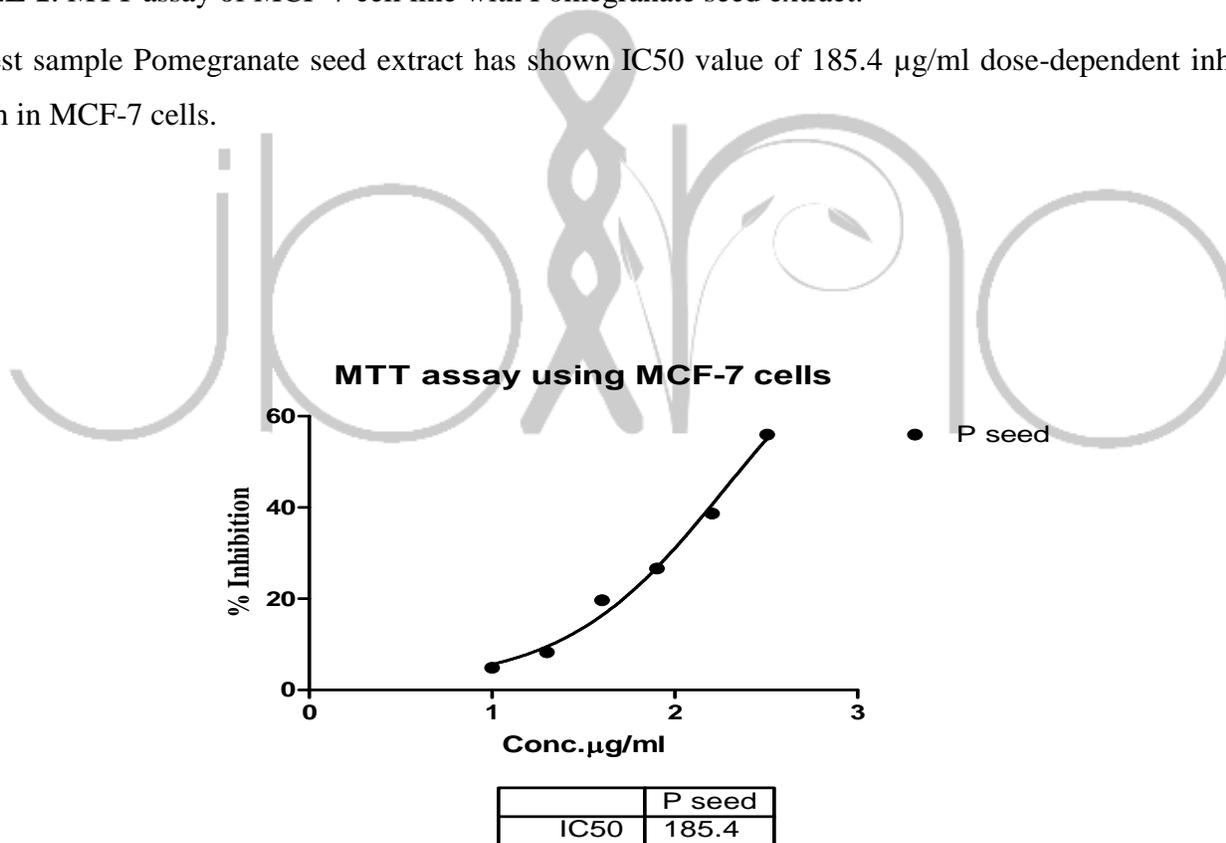


GRAPH 4: Results showing effect of hemagglutination inhibition activity of pomegranate lectin against various sugars. The pomegranate seed lectin was found to be arabinose and lactose specific.

Sample	Conc. (µg/ml)	O.D at 590nm	% Inhibition	IC50 (µg/ml)
Pomegranate seed extract	10	0.84	4.89	185.4
	20	0.81	8.27	
	40	0.71	19.66	
	80	0.64	26.62	
	160	0.54	38.66	
	320	0.39	55.97	

TABLE 1: MTT assay of MCF-7 cell line with Pomegranate seed extract.

The test sample Pomegranate seed extract has shown IC50 value of 185.4 µg/ml dose-dependent inhibition of growth in MCF-7 cells.



GRAPH 5 : Graph showing growth inhibition of MCF-7 cells

DISCUSSION

Lectins are unique proteins of varying biological importance. They are characterized by specific binding to carbohydrate residues, whether monosaccharides, disaccharides or polysaccharides. The sugar heads on the surface of the erythrocyte specify the different blood groups. Lectins, as an antigenic determinant of blood group, have come to be an important tool in the identification of different blood groups. . Lectin from *Vicia cracca* has been proved to be a good anti-A, lectin from *Dolichus biflorus* can be used as anti-A1, and lectin from *Griffonia simplicifolia* as anti-B. Similarly, pomegranate lectin was found to show high level of agglutination with human erythrocytes of 'A' +ve and 'O' +ve blood groups as compared to 'B' and 'AB' blood groups. (Khan F, 2002)

Silva V Gomes in 2013 studied the pH range for pomegranate lectin and found that the Pomegranate lectin was stable at pH 5. (Silva V Gomes, 2013 In the present study the pomegranate lectin was found to be stable at a pH range of 3-7) and also the lectin is stable at a temperature range of 20-50°C (Ajay Pratap Singh, 2013).and was found to be inactivated below 20°C as well as above 50°C. (Irving V et al, 2007)The pomegranate lectin is stable at acidic pH (pH 3-7), hence lectin may remain active in the stomach and intestinal juices. This lectin is a thermo labile lectin. Hence, lectin may not require special preservation methods since it is stable at room temperatures 30-50°C. So the preservation costs may also get reduced

In the present study, the sugar specificity of the pomegranate lectin is studied by using different sugars viz. dextrose, fructose, maltose, arabinose, lactose and maltose. Lactose and Arabinose have showed high binding affinity followed by maltose, fructose and dextrose. The minimum concentration of lactose and maltose for the inhibition of hemagglutination activity of pomegranate lectin was found to be 0.25mg/ml. The hemagglutination inhibition activity was found up to the concentration of 1mg/ml of lactose and arabinose. (Martin S Nachbar, 1980) Dextrose on the other hand has shown less affinity and gave the positive hemagglutination activity.

Arishya Sharma et al in 2008, studied the cytotoxic effect of *Phaseolus vulgaris* cv. (Anasazi beans) lectin on MCF-7 cancer cell line and found the inhibition of MCF-7 cells with IC 50 value of 1.3µM and the lectin showed the anti -proliferative property against MCF-7 cell line. (Arishya Sharma et al, 2008)

The pomegranate lectin showed cytotoxic effect against the MCF- 7 cell lines. The test sample, Pomegranate seed extract has shown IC50 value of 185.4 µg/ml dose-dependent inhibition of growth in MCF-7 cells. Hence, the pomegranate seed lectin has shown the anti-proliferative activity against MCF-7 cell line. From the present study, it may be concluded that the lectin present in the pomegranate seed extract is having the inhibitory mitogenic activity when tested on MCF-7 cancer cell line.

The protein concentration estimated by Lowry's method has given the protein

concentration of about 1mg/ml. and sugar concentration is 2.2mg/ml.

The total sugar concentration was estimated by Anthrone method and the sugar concentration was found to be 2.2mg/ml.

From the present study it has been revealed that the pomegranate lectin which is arabinose and lactose specific is a potent cytotoxic agent inhibiting the growth of the cancer cells (MCF-7) at concentration of 185.4ug/ml. The anti mitogenic nature of this lectin may be due to its binding capacity with suitable membrane receptor thus influencing the signal pathway to inhibit mitosis in those cancer cells. The result also may suggest that the lectin may have initiated the apoptotic pathway to resist the growth of the cancer cells in vitro condition which needs to be experimented in further studies. Thus the present study may help to contribute in the future day's cancer therapy.

CONCLUSION

In the present study, it has been found that the pomegranate lectin has the ability to agglutinate the erythrocytes, especially of blood groups 'A' positive and 'O' positive. It has been seen that the hemagglutination activity of pomegranate lectin vary with respect to variation in physicochemical parameters such as temperature, pH of the buffer. The pomegranate lectin is stable at acidic pH(pH 3-7), hence lectin may remain active in the stomach and intestinal juices. This lectin is a thermo labile lectin. Hence, lectin may not require special preservation

methods since it is stable at room temperatures 30-50°C. So the preservation costs may also get reduced. The sugar inhibition tests shows that lectin from pomegranate has greater affinity towards monosachharides such as Arabinose and Lactose which explains the interactions of lectins with cell surface receptors. This interaction may be responsible for the immuno modulatory activity of this lectin. The pomegranate lectin showed cytotoxic effect against the MCF- 7 cell lines. The test sample, Pomegranate seed extract has shown IC50 value of 185.4 µg/ml dose-dependent inhibition of growth in MCF-7 cells. Hence, the pomegranate seed lectin has shown the anti-proliferative activity against MCF-7 cell line. From the present study, it may be concluded that the lectin present in the pomegranate seed extract is having the inhibitory activity when tested on MCF-7 cancer cell line.

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