"Isolation and characterization of lectins from white seeds of "*Abrus precatorius*"

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CERTIFICATE

This is to certify that the thesis entitled "**Isolation and Characterization of lectins from white seeds of *Abrus precatorius***" which is being submitted by Abhipsa Mishra, roll no. 410LS2059, for the award of the degree of master science from National Institute of Technology, Rourkela, is record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Sujit K. Bhutia

DECLARATION

I hereby declare that this project work entitled "Isolation and characterization of lectins from white seeds of "*Abrus precatorius*" is a record of original work done by me under the guidance of Dr S.k.Bhutia, Assistance Professor of Department of Life science, National Institute of Technology (NIT), Rourkela. This project work has not formed the basis for the award of any degree/diploma/Associate ship/Fellowship or similar to any candidate in any University.

Date: ABHIPSA MISHRA

CONTENTS

A.ACKNOWLEDGEMENT

Although theory is heard in many ways which seems to be very simple and effortless but when it comes to real ground then it matters and competes to retrospect the system in each and every way to realize the real retention process which leads to perfection. I feel myself speechless before them to enumerate their help and guidance who are real pedagogues.

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Finally I bow my head before God and my parents, who grew me up physically, mentally and spiritually with prayers and Devine love.

Date: **ABHIPSA MISHRA**

B. LIST OF FIGURES

C.LIST OF TABLES

D.ABBREVIATION

PBS: Phosphate Buffer Saline

et al: And others

Rpm: Rotation Per minute.

Conc: Concentration

Hrs: Hours

L: litre

Mg: Milli gram

pH: Hydrogen concentration

NaOH: Sodium hydroxide

Na_2CO_3 : Sodium carbonate

APS: Ammonium per sulphate

TEMED: N,N,N',N'-tetramethylenediamine

$KNaC_4H_4O_6$: Potassium sodium tartarate

SDS-PAGE: Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis.

BSA: Bovine serum albumin

KH_2PO_4: Potassium Dihydrogen Phosphate

K_2HPO_4: Potassium hydrogen phosphate

$(NH_4)_2SO_4$: Ammonium Sulphate

Pvt .Ltd: Private limited

kDa: kilo dalton

E. ABSTRACT

The present study indicates that *Abrus precatorius* was found to be the potential source for lectins. The lectin called *Abrus* agglutinin was isolated and characterized from white *Abrus* seed by the lactamyl sepharose 4B chromatography followed by SDS-PAGE. Lectin bind with the sepharose bead and it was separated out by the process of dialysis. The concentration of protein was measured. Agglutinins are sensitive to RBC of blood. Haemagglutination assay is another method for tittering protein based on their ability to attach to molecules present on the surface of red blood cells. The protein was identified by the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. The relative molecular weight of agglutinin was found 33kDa, 29kDa which corresponds to A chain and B chain of Agglutinin.

1. INTRODUCTION

Lectins are sugar-binding proteins.The name is derived from the Latin word i.e. *legere*, means, "To select". It was discovered by Stillmark in 1888. Lectins have the structure which can bind by any simple sugars and oligosaccharides. Because of the specificity each lectin has towards a particular carbohydrate structure even oligosaccharides with identical sugar composition can be distinguished or separated. The real structure is identified by the binding site of the lectin when it combines with its natural ligand, which is generally large and more complex than a single monosaccharide. Lectins, which have specificity towards the monosaccharide, may differ in their affinity for a particular disaccharide, oligosaccharide or glycopeptides. Different Lectin has different composition, molecular weight, subunit structure and number of sugar binding sites per molecule. Most of the reports and biological analysis confine that the abundance of lectin in legumes. Generally found in the seeds, roots and cotyledons of leguminous plants. Considering the huge number of lectin available in the nature, the ease with which they could be prepared in the purified form, their amenability to chemical manipulation and the fact that they can be inhibited by simple sugars makes them attractive as an important tool in biological research. Although lectins are found ubiquitously in plant species, they have different structures and specific actions according to the plants they originate from. Thus purification and characterization of lectins from a variety of plant species interests researchers in the field of glycobiology. These are very useful as they are easy to isolate and reagents for glyco conjugates in solution and cell surface. Lectins have the capacity to agglutinate erythrocytes. To determine the lectin activity, agglutination of a panel of human or animal red blood cells are the most convenient method.

Abrus Precatorius is the potential source for lectin. It is under the family Fabaceae (Leguminosae)/Pea Family and the major source for lectins. Abrus are well-known as Jequirity, Rosary Pea, John Crow Bead, Precatory bean, Crab's Eye, Indian Liquorice, Giddee Giddee or Jumbie Bead in Trinidad & Tobago (Mendes 1986), also known as Gunja in Sanskrit and Ratti in Hindi (Nadkarni 1976). These are found generally in India, and perhaps other parts of tropical Asia. *Abrus Precatorius* is a high-climbing tree having twining, or trailing woody vine, slender, herbaceous branches. Leaves are alternate, having petiole, 5-13 cm (2-5 in) long and pinnately compound with 5-15 pairs of leaflets, which are oval to oblong in shape,1.8 cm (< 1 in) long, entire margin. Flowers arranged in clusters,

white to pink or reddish in colour, small, in short stalked dense clusters at leaf axils. Fruit are short, oblong pod, splitting before falling to reveal 3-8 shiny hard seeds, 6-7 mm (< 1 in) long. The seed pod curls back when it opens and reveals the seeds with attractive scarlet or white colour with black bases. They are highly poisonous.

Fig: 1: Plant of *Abrus precatorius*

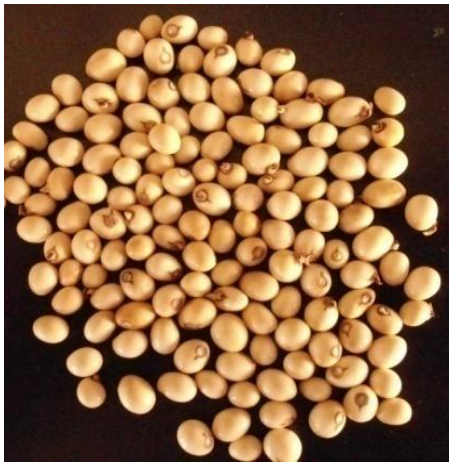

Fig: 2: Abrus seeds

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Fig: 3: Immature pods

Table.1: Scientific classification of *Abrus precatorius*

Kingdom:	**Plantae**
(unranked):	**Angiosperms**
(unranked):	**Eudicots**
(unranked):	**Rosids**
Order:	**Fabales**
Genus:	***Abrus***
Species:	***precatorius***
Binomial name	***Abrus precatorius* L.**

There are several methods for the isolation of lactose binding protein from *Abrus Precatorius*. Protein from these seeds are isolated by combination of sepharose 4B affinity chromatography, ion exchange and gel-filtration steps.(Olsnes *et al*.,1974; Wei *et al*.,1974; Roy *et a*l.,1976; Lin *et al*.,1978; Lin *et al*.,1981). These isolated protins have to identify by different methods by knowing the properties of the protein. Affinity matix are more suitable method for purification of Abrus lectins in fewer steps. The sepharose 4B matrix is the good method for the isolation of this lectin as it has better yield. Haemagglutination Assay is another convient method used in this isolation.

2. REVIEW OF LITERATURE

2.1. Terminology and Definition of Lectin

According to earlier definition Lectins are carbohydrate binding proteins of no immune origin that agglutinate cells or as carbohydrate-binding proteins other than antibodies or en- zymes require an update, so the molecular structure of lectins and lectin-related proteins has led to new way. There are some plant enzymes are fusion proteins which are composed by a carbohydrate-binding and a catalytic domain. Class I chitinases are built up of a chitin-binding domain and a catalytic domain, which are detached by a hinge region (Collinge et al., 1993). Similarly, another type is 2 RIPs, such as ricin and abrin, are fusion products of a toxic A chain (which has the N-glycosidase activity characteristic of a11 RIPs) and a carbohydrate-binding B chain (Barbieri et al., 1993). In some cases several carbohydrate-binding proteins have only one binding site, so they are not capable of precipitating glycoconjugates or agglutinating cells. For example the nonagglutinating Man-binding proteins from orchids are very similar to the dimeric Man-specific lectins from the same species except that they occur as monomers (Van Damme *et a*l., 1994). In some legume species there are some proteins that are clearly related to the lectins but are devoid of carbohydrate-binding activity. Well-known examples of this group of proteins are the *Phas eolus vulgaris* arcelins and the alpha-amylase inhibitor (Mirkov *et al*., 1994).

2.2: Plant lectin

According to the new definition all plant proteins possessing at least one non catalytic domain, that binds to a specific mono- or oligosaccharide are consider as lectin (Peumans *et al.*, 1995).The most abundant source of lectins are the plants. The plant lectins are very useful as they are easily separable and for their glycoconjugate bond in solution and on cell surface. Lectins have capacity to agglutinate erythrocytes of human or animal. By this simple method we can measure the agglutination activity of the lectin protein using red blood cell. Lectins are found in leguminous plants, where they are localized in the cotyledons of the seeds and roots.

Lectins have specificity for carbohydrates which can be checked by simple monosaccharide and oligosaccharides disaccharide, or glycopeptide. Lectins have binding sites for their ligands, which are more difficult like simple sugar to polysaccharides. Lectins vary in composition, molecular weight, subunit structure and number of sugar binding sites per molecule. Several plant lectins have been found to have non-carbohydrate ligands that are first and foremost water hating in nature, including adenine, cytokinin, auxins, and indole acetic acid, as well as water-soluble porphyrins. It has been recommended that these interactions may be physiologically applicable, since some of these molecules role as phytohormones. (Komath *et al.,* 2006).The purified Lectins have high demand in science, medicine and technology.

On the basis of structure, lectins can be grouped as four distinguished categories namely merolectins, hololectins, chimerolectins and superlectin (Van Damme *et al.*, 1997).

2.3: Uses of lectin

It is used in Blood typing(N.Sharon).Commercially available lectins have been widely used in affinity chromatography for purifying glyco proteins.(GE Healthcare Life Sciences, powerless lectin).In general, proteins may be considered with respect to glycoforms and carbohydrate structure by means of, blotting, electrophoresis, affinity chromatography and affinity immunoelectrophoreis with lectins in addition to in microarrays as in evanescent-field fluorescence-assisted lectin microarray(Glyco Station, Lec Chip, Glycan profiling technology).Use in biochemical warfare. Use in studying carbohydrate recognition by proteins. Useful tools in immunological studies (Moreira *et al.*, 1991).

2.4: Abrin

The *Abrus* seed is a mixture of at least five lectins, abrin A - D, and abrus-agglutinin. The toxicity of the seed is due to the abrin. The abrins have two peptide chains joined by a disulfide bridge. Abrin A-chain have N-glycosidase activity, which inert protein synthesis, and lectin-like B-chain binds with cell-surface receptors and responsible for penetrating of abrin-A molecule inside the cell (Ohba *et al*., 2004). After purification they can be separated by affinity chromatography followed by gel filtration. The relative molecular weights of abrin A. C are around 64.000, that of two agglutinins 128.000. (Hegde *et al*., 1991). (Lin *et al*., 1978).It was investigated by the crystal structure (Tahirov *et al*., 1995). The abrin A crystal is under the monoclinic space group P 2 (Tahirov *et al*., 1995). The sequence of amino acids of the B-chain in both abrin-A and abrin-B were clear up by the enzymatic digestion activity with trypsin. They have 268 amino acids and contribute to 256 identical residues (Komira *et al*., 1993).To clear up the activity of intoxication the active glycotopes for the attachment

were found out. This chemical structure is assumed to be responsible for the toxic effects. Abrins immobile the protein biosynthesis by inhibiting the 60S-ribosomes of animal cells, permanently. The toxicity of these abrins is conflicting, but they are the most fixed toxins. The abrus agglutinin is not so very toxic against cells, but it exhibits agglutination toward animal erythrocytes (Herrmann *et al*., 1981).

2.5: *Abrus*-Agglutinin

The lectin present in *Abrus* is *Abrus*-agglutinin, which is less toxic to eukaryotic cells (Olsnes, 1978). It is a heterotetrameric glycoprotein of size 134 kDa. This lectin has two chains A chain and B chain having size 30kDa, 31kDa respectively. (Lin *et al*., 1981). There is a disulfide between both the chain. (Bagaria *et al*., 2006).Both the proteins, agglutinin and abrin have carbohydrate specificity towards [Gal(β1-3) Gal/NAc]. *Abrus* agglutinin is weaker than Abrin the protein synthesis inhibitory concentration.

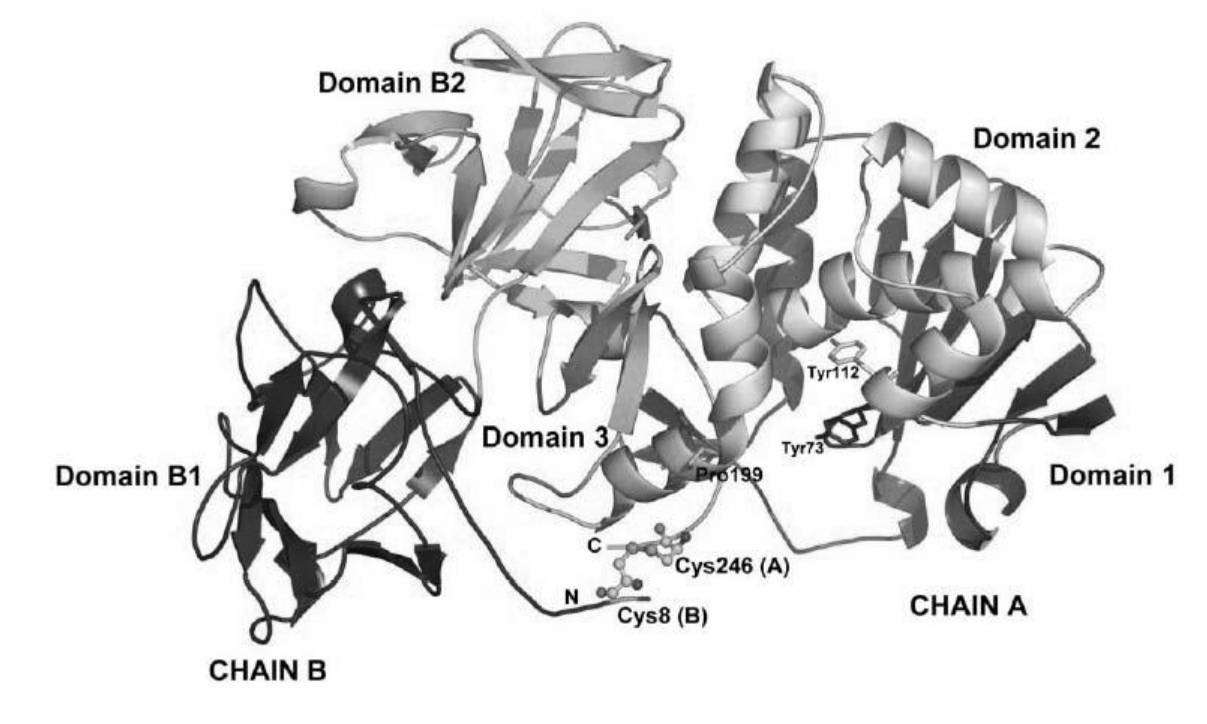

Fig.4: PDB structure Agglutinin

2.6: Uses of *Abrus precatorius*

Abrus precatorius jewellery also as medicinal proposes. Traditionally these seed are used as decorative, gold weighing purpose, herbal formulation. Disease like Leucoderma,

tetanus and rabies are treated by this seed (Chopra *et al*., 1956).These seed are also used to fight against the disease trachoma (Acharya, 2004).As this is a very attractive plant it is cultivated as ornamental garden plant.

2.7: Medicinal Uses

Fevers, coughs and colds are cured by tea is made from the leaves.(Mendes ,1986).The white variety seeds are used in Siddha medicine.(Raamachandran, J).In the Indian System of traditional Medicine, the seeds are used for the dieses like paralysis, headache, diarrhoea, leprosy, ulcer, dysentery, nervous disorders, sciatica, alopecia, in addition to antibacterial, anti-inflammatory, antidiabetic, antitumor, sexual stimulant and abortifacient. As the seeds are poisonous, therefore are used after alleviation. (Verma *et a*l., 2011).The extract produced from methanolic dose-dependent bronchodilator action.(Mensah *et al*.,2011).

2.8: Antitumor properties

Both lectins are found to be inhibiting the growth of tumours in experimental animals at sublethal doses. They cause apoptosis and the antitumor activity, which is significantly related with apoptosis. Abrin is more toxic than to normal cells (Nicolson *et al*., 1975). Abrin has greater cytoagglutination against human cultured cell lines but weak agglutination against normal lymphocytes (Kaufman and McPherson, 1975). These selective antiproliferative properties of lectins toward tumor cells attract to be a potential source for anticancer agent. *Abrus* lectins reduce the tumor (Lin et al., 1969; Tung et al., 1981; Lin et al., 1882; Ramnath et al., 2002; Ghosh and Maiti, 2007a, 2007b). Further, it is reported that *Abrus* agglutinin show a very significant antitumor properties with heat denatured condition in Dalton's lymphoma ascites model (Ghosh and Maiti, 2007a, 2007b).

2.9: Immunostimulatory properties

Agglutinin and abrin have a practical responsibility in tumour defences by immunomodulation. They begin immune cells upon binding to the carbohydrate moieties, which is localized in the cell surface. After long-lasting contact with lectins, lymphocytes get proliferate and become mature effector cells, exude lymphokines, and show characteristic functions of meticulous cells such as cellular cytotoxicity, immunoglobulin construction, and suppressor characteristics. The mitogenic properties of *Abrus* abrin and agglutinin are reported in human also in mice.

3. OBJECTIVES

- Isolation and purification of lectin from red seed of *Abrus precatorius.*
- Measurement of concentration of protein
- Characterization of protein:

  a) Haemagglutinin assay

  b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

4. MATERIALS AND METHODS

4.1: Sample Collection

The white Abrus precatorius seeds were collected from deep jungle of Angul ,Odisha. India.

4.2: Chemicals

Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3), glycine,Cuppersulphate($CuSO_4$) ,Potassium sodium tartarate ($KNaC_4H_4O_6$) were purchased from SRL, Sisco Research laboratories Pvt. Ltd., Mumbai. Acrylamide, bisacrylamide, Ammonium per sulphate (APS), Sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), Bovine serum albumin(BSA), Tris were purchased from Sigma Aldrich, USA. Folin-Ciocalteau phenol reagent, Potassium Dihydrogen Phosphate (KH_2PO_4), Potassium hydrogen phosphate (K_2HPO_4) was purchased from S.D. fine chem. Ltd., Mumbai. Acetic acid, Bromophenol blue, and agarose were purchased from Himedia, Mumbai. Glycerol was purchased from RANKEM Pvt Ltd. Ethanol from Trimurty Chemicals, India. Pre stained molecular weight marker was purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from nice chemicals Pvt .Ltd. India.

4.3: Purification of Abrus agglutinin:

Abrus agglutinin was purified from the seeds of red *Abrus precatorius* following the methods described by (Hegde *et al*. 1991). In brief, 100 gms of red *Abrus* seeds were taken and decorticated. The uncoated seeds (50gms) were soaked in PBS overnight and grinded in minimum volume of PBS in a blender for 5 minutes. Then it was centrifuged at 7000 rpm for 20 minutes at 4^0c. The supernatant was collected and subjected to ammonium sulfate fractionation (first 0-30% and then 30-90%). The precipitate formed by 90% ammonium sulfate cut was dissolved in minimum volume of water and dialyzed against PBS. The dialyzed sample was loaded onto Lactamyl Sepharose affinity column and eluted by adding 0.4M lactose solution. The activity of the lectin was determined by haemagglutinin assay and the purity of the protein was tested by native Polyacrylamide Gel Electrophoresis (PAGE) .

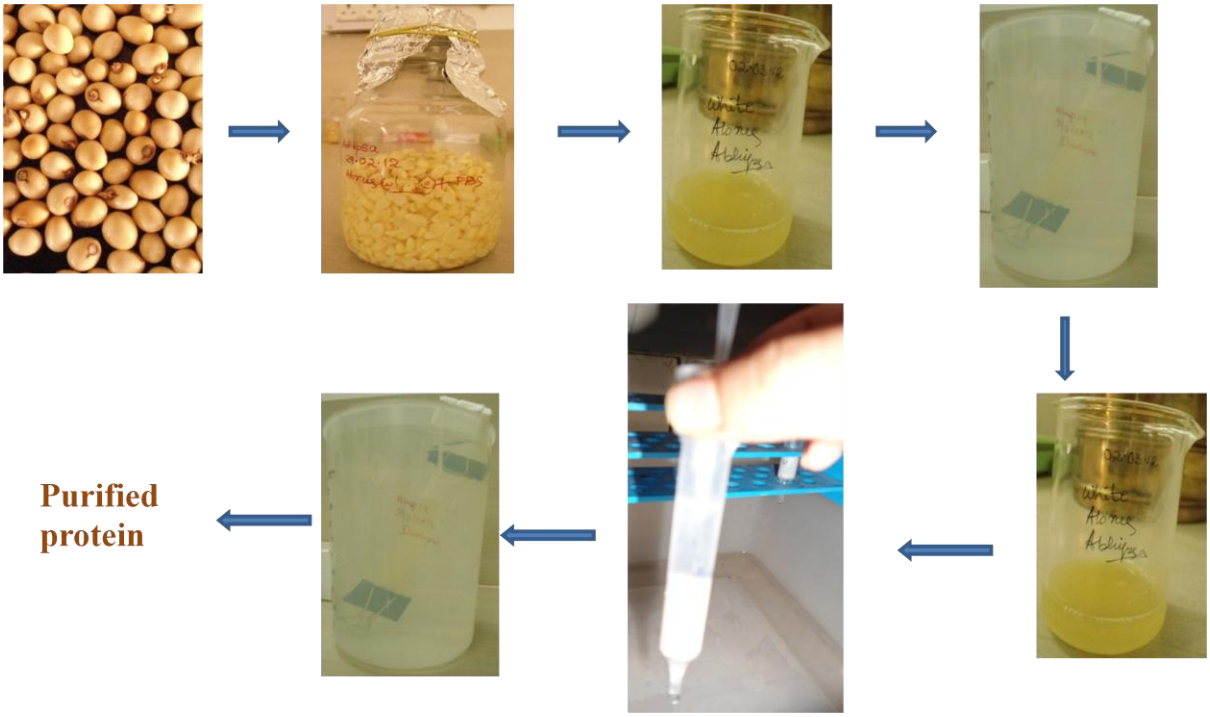

Fig.5: Purification of protein

4.4: Haemagglutination assay:

Human erythrocyte suspension (10^6cells/ml) of blood was used for the haemagglutination assay. The assay was carried out in a 96 well round bottom known as microtitre plate. Agglutinin solutions (both native and heat denatured solution at concentration of 1mg/ml) were serially diluted (double dilution) and 100µl volume of agglutinin solutions at different concentration were added to 100µl of cell suspension. The plates were incubated at room temperature for 4 h.

4.5: Sodium dodecyl sulphate polyacrylamide gel electrophoresis: (SDS-PAGE)

The molecular weights of lectin were determined by SDS-PAGE (Laemmli, 1970). Samples (Crude, 30%, 90%, affinity) were prepared for gel loading by boiling with sample buffer for three minutes at 100^0c. Samples were loaded into wells of a stacking gel of 5% above the separating gel of 12% acrylamide. Loaded samples were electrophoresed at 140 V for approximately 60 minutes. The gels were stained by performing silver staining method. Estimation of molecular weight of the lectin subunit band in comparison to molecular weight standards was made using Quantity one Imaging Software (BIORAD).

5. RESULT

5.1: Determination of protein concentration

The concentration of the proteins (crude, 30% cut, 90% affnity) was determined by measuring OD at 280 nm. as depicted in Table- 2.

Table-2: Concentration of proteins by measuring OD at 280 nm.

Sample	volume	OD at 280 nm	Conc (mg/ml)	Total conc(mg/ml)
Crude	**30 ml**	**123.375**	**98.7**	**2961**
30%	**25 ml**	**105**	**84**	**2100**
90%	**23 ml**	**5.736**	**4.59**	**105.57**
Affinity	**15 ml**	**1.156**	**0.92**	**13.8**

The total conc .of affinity sample is 13.8 mg/ml. which is the lowest from the sample of crude, 30%, 90%. This conc.of protein may contain the our desired protein.

5.2: Elution graph

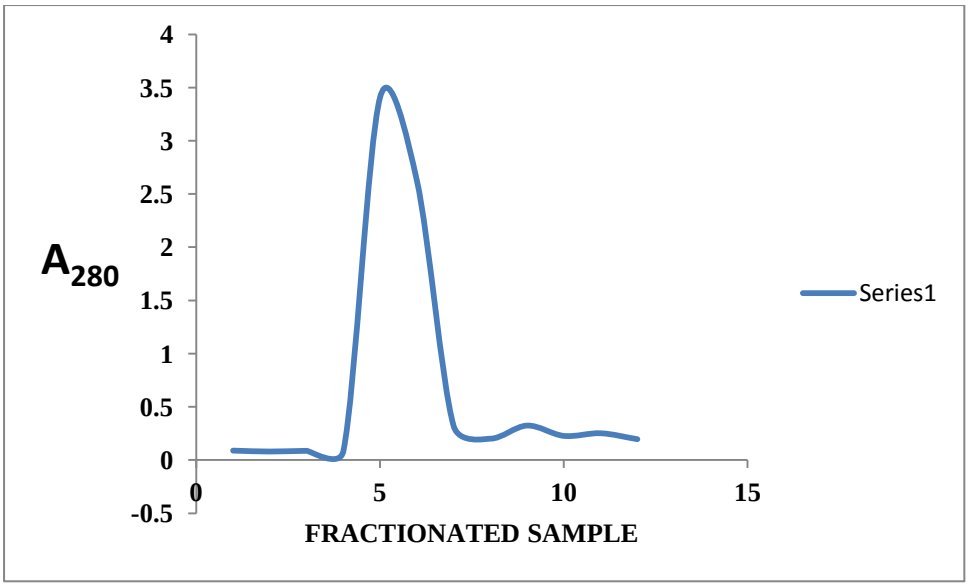

Fig.6: Lectin is desorbed with 0.4M lactose giving high peak.

The lactamyl–Sepharose 4B elution profile shows a peak value, which means lectins perfectly bind with the sugar. These peaked valued samples were taken. From this lactose was separated by dialysis. After it we get our desire lectin.

5.3: Haemagglutination assay

This assay helps to decide the needed protein, Haemagglutination Assay was performed by using human erythrocyte suspension. The assay was carried out in 96 well "U" bottom micro titre plates by serially diluting the lectin sample and allowing it to incubate for 2 hours. Then it was set up that there was an increasing order agglutination reaction from crude to affinity sample.

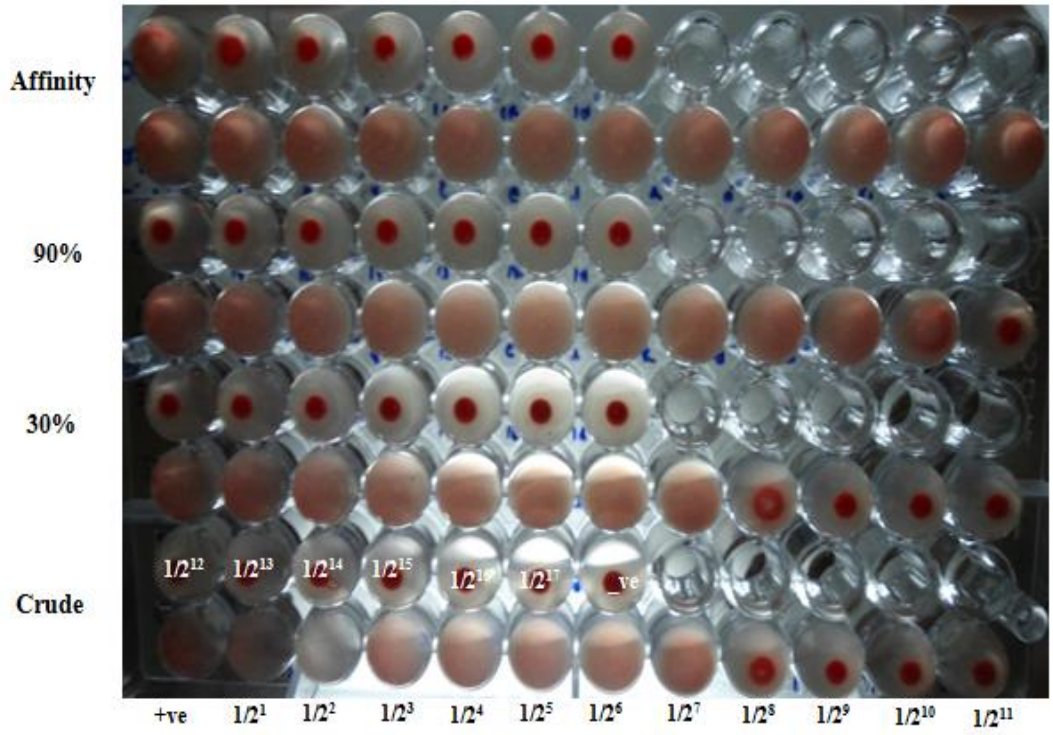

Fig.7: Haemagglutination assay

- **Crude sample:2^8**
- **30% sample:2^8**
- **90% sample:2^{11}**
- **Affinity sample:2^{13}**

5.4: SDS- PAGE :

To determine the size of the protein, SDS-PAGE was performed using 12% polyacrylamide as the resolving gel and 5% polyacrylamide as the stacking gel and the bands were stained by silver staining method. Then the bands were visualized by gel documentation system and the molecular wt of my desired protein was found 33kDa and 29kDa.

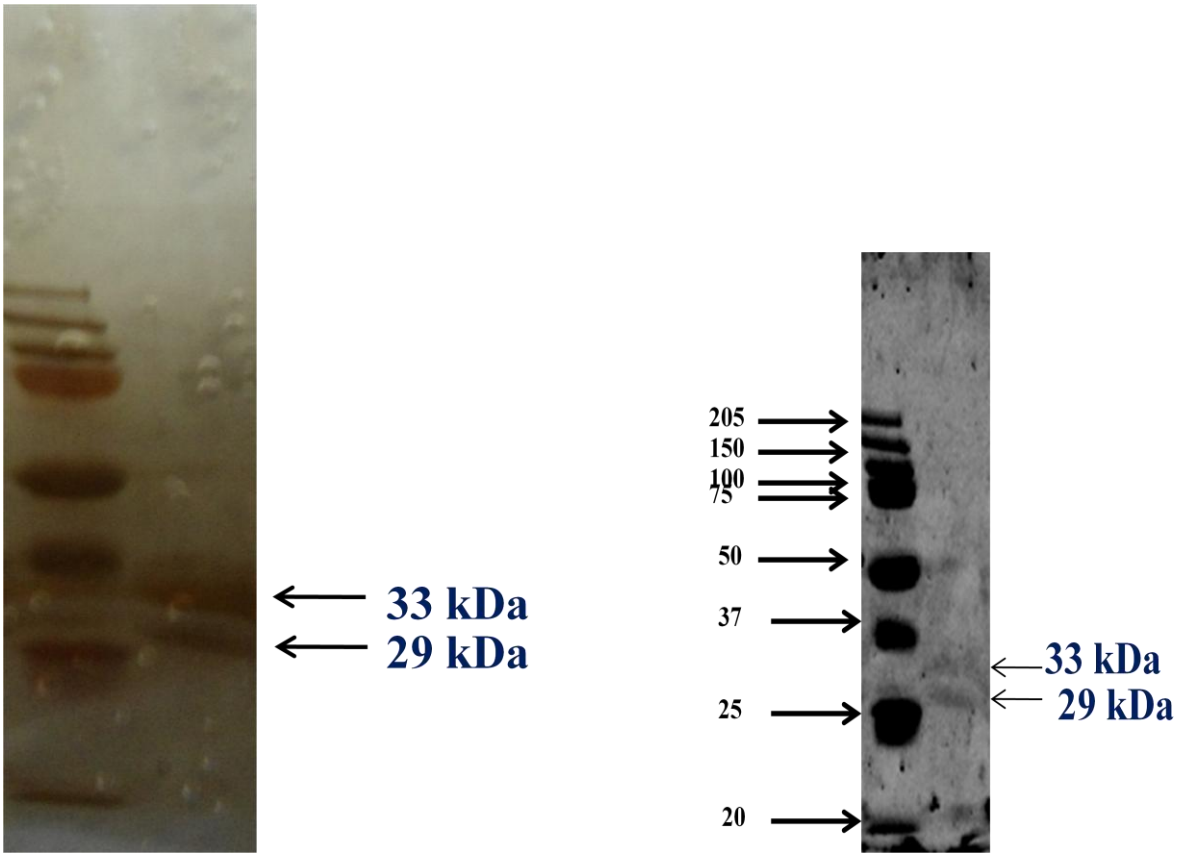

Fig: 8 Silver staining of prepared gel. Lane 1 depicts the marker and Lane 2 depicts the bands developed while running the affinity sample.

Fig: 9 Photo graph taken by Bio-Rad Gel documentation system.Lane 1 depicts the Bio-Rad prestained protein molecular weight markerand Lane 2 depicts the two bands developed which are two chains of agglutinin having mol wt 33 kDa and 29 kDa respectively.

6. DISCUSSION

Agglutinin and Abrin are two lectins present in the white *Abrus precatorius* seed. In the process of salting out, the desired protein was found by increasing the conc. of ammonium sulphate i.e 30% fractionated and 90% cut off. Lactamyl sepharose 4B affinity chromatography was done to make the lectin affinity with lactose for easy isolation. Dialysis was done to remove lactose from our sample. From elution graph we can noticed the graph gives a pick value and then it declines. We collect the eluted sample till it gives higher OD. In Haemagglutination Assay we noticed the eluted sample gave the highest tighter value. From SDS PAGE we quantify our protein of interest. The molecular weight of Agglutinin was 134 kDa yielded peptides of approximately 33 kDa and 29 kDa respectively from SDS-PAGE. From this we can assume that Agglutinin is probably a heterotetrameric glycoprotein.

7. CONCLUSION

The seeds of the Jequiriti bean (*Abrus precatorius* L.) have long been known for their medicinal use in Unani and Ayurvedic medicine. The selective tumor-targeting nature of Abrus lectins ensures its place as a potential anticancer agent, and both lectins (agglutinin and abrin) inhibit the growth of tumors in experimental animals through apoptosis induction. The immune adjuvant phenomena of Abrus agglutinin and abrin provide a challenge for potentiating the systemic immune response.

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