Elucidation of the anticancer property of *Abrus* agglutinin in oral cancer cell lines

Thesis submitted to **Department of life science for** ***the partial fulfillment Of the M.Sc. Degree in Life Science***

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CERTIFICATE

This is to certify that the thesis entitled "**Elucidation of the anticancer property of *Abrus* agglutinin in oral cancer cell lines**" which is being submitted by Miss Himadri Tanaya Panda, Roll No. 411LS2056, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him 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.

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DECLARATION

I, **Ms. Himadri Tanaya Panda (Roll No.411LS2056)** here by declare that the project report entitled '**Elucidation of the anticancer property of *Abrus* agglutinin in oral cancer cell lines'** submitted by me, is an original work done and submitted by me in partial fulfillment for the Degree of Master of Science in Life Sciences to the NIT ,Rourkela. This is a project work done by me under the guidance of **Dr. Sujit Kumar Bhutia**, Assistant Professor, NIT Rourkela. This thesis has not formed the basis for the award of any Degree /Diploma/ Associateship/ fellowship or other similar title to any candidate in any university.

Himadri Tanaya Panda
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Date:

Place:

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Himadri Tanaya Panda

411ls2056

DEDICATED TO MY
BELOVED PARENTS

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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

DAPI: (4,6) diamino-2-phenylindole

MTT dye: 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide

ABSTRACT

Abrus agglutinin, a ribosome inhibiting protein II (RIP II) family lectin, is a heterotetrameric glycoprotein of molecular weight 134 kDa which is composed of two A chains (N-glycosidase activity on 60S eukaryotic ribosome) and two B chains (galactose binding site) linked through a disulphide bridge. Here we studied the anti cancer activity of *Abrus* agglutinin in various oral cancer cell lines. The protein synthesis inhibitory activity of *Abrus* agglutinin (IC_{50}) in various oral cell lines is 7.5 µg, 6 µg , 1.25 µg, 56.66 µg in HEp2, RPMI-2650, FaDu, HaCaT respectively. The study confirmed *Abrus* agglutinin acts as a magic bullet for combating oral cancer .

Key words: Abrus, agglutinin, RIP, glycosidase activity, $IC_{50,}$ combating

1. INTRODUCTION

It is a widely accepted concept that lectins are carbohydrate binding proteins. The name of the lectin comes from the two Latin words i.e. *legere*, means, "To select". The protein lectin was first discovered by Stillmark in 1888. As lectins are carbohydrate binding proteins they have the structure that can simply bind to the any type of sugars & polysaccharides. Lectins are found in different organisms ranging from microorganisms & plants to humans (Van Damme et al., 2004). Lectins are very abundant in the plant kingdom & are mostly found in the stems, leaves, bark, tubers, seeds & bulbs (Adenik et al., 2009; Wong et al., 2008; Audrey and Sharon., 2002; Thakur et al., 2007). Lectins bind to the monosaccharaides more weakly, but it has a more affinity towards the complex carbohydrate molecules like polysaccharides & oligosaccharides. Each lectin differs in their subunit structure, composition, number of sugar binding sites per molecule & molecular weight. It is reported by some researchers after doing the biological analysis that lectins are abundantly found in legumes. It is found that lectins are universally present in plant species, but they have different structures & specific functions according to the plants that they originate from. So that scientists show a special interest towards the purification & characterization of lectins from a variety of plant species in the field of glycobiology. Plant lectin have a specific interaction with carbohydrates, there is a broad scientific study on the plant lectin. It is found that the high impacts of plant lectins on biological research are due to two main reasons. At first plant lectins are a readily accessible carbohydrate binding proteins. Lectins help in distinguishing between the malignant tumor cells & normal cells based on their agglutination behavior. This distinguishing property of the lectin is due to variable glycosylation on the cell surface associated with malignancy, invasion and metastasis (Hakomori., 2002; Kim et al., 1997). Lectns help in the detection of many other diseases which involve certain sialytion & increase in branching patteren of complex sugars. For this act of identification of various disease lectins are known as biomarkers (Strauchen., 1984; Comunale., 2006). Lectins have a good capacity to agglutinate (can help in clumping together) erythrocytes (R.B.C.). High levels of lectins may be found in legumes, dairy, grains amount of lectin bearing seeds to rabbits, increased their partial resistance to the toxicity. This shows that

lectins are immunogenic which means that it can be able to induce antigen antibody reaction. Among different lectin origins, lectins derived from leguminous plants are most extensively studied (Sharon et al., 1990). It is found that these glycoproteins are about 3% of the total weight of a mature seed.

A major priority has given to the legume lectins as they abundantly found in many crop plants. It is also found they involve in the symbiosis between the legumes & nitrogen fixing bacterium *Rhizobium* (Diaz et al., 1989). So it ensures that lectins are generally wide distributed among leguminous plants species (Toms., 1971). The lectins of legume plants shows many similar properties like metal ion binding, tetrameric glycoproteins possessing identical subunits (Liener., 1976; Lis.,1972). Lectins have a greater affinity to bind with the carbohydrate as it has many carbohydrate moieties. So that lectins vary in their molecular weight molecular weight subunit structure and number of sugar sites binding site per molecule.

It is found that *Abrus precatorius* is a very good source of lectin. Majorly it comes under the family Fabaceae & it is mostly famous as it is a major source of lectin. The *Abrus precatorius* seeds are famous in other names as e.g. Indian Liquorice, Giddee Giddee or Jumbie Bead in Trinidad & Tobago (Mendes 1986), also known as Gunja in Sanskrit and Ratti in Hindi (Nadkarni 1976). The *Abrus precatorius* is mostly found in different parts of the India & perhaps it may be found in some parts of tropical Asia. This *Abrus* plant is mainly a high climbing creeper like plant having trailing woody vine, slender, herbaceous branches. The leaves of the Abrus plants are alternate, petiolate , 5-13 cm long and pinnately compound with 5-15 pairs of leaflets & these are oval to oblong in shape,1.8 cm long, entire marginate. Flowers are mainly arranged in clusters & the flower may be in white to pink or reddish in colour. The fruits are mainly short & oblong pod, splitting before falling to reveal 3-8 shiny hard seeds, 6-7 mm (< 1 in) long. The seeds are may be black, red or white in colour. The seeds are with attractive scarlet or red colour with black bases. The seeds are highly poisionous. They have very toxic effect on hyper concentration of conjuption.

Fig: 1: Plant of *Abrus precatorius*

Fig: 2: Mature Abrus seeds

Fig: 3: Abrus seeds

Fig: 4: 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.**

1.1 Physiological role of plant lectin

According to many researchers plant lectins have a wide effect on the growth & development of the insect by their oral uptake. The effect of plant lectins on the insects & animals gives a very wide idea about the protective nature of the lectin against the predators. So that it is widely found that when the insects & higher individuals are attacked by the plant lectin; the lectins act as a specific defense protein.

1.2 In the field of biotechnology

Plant lectins have a wide use as a tool in the field of recombinant DNA technology & in enhancing the quality of transgenic plants. These tools are mainly targets specifically on the carbohydrate binding sites. Biological effects of lectins on cells, tissues and organs, used as inducers of specific process in animal or human cells (Kilpatrick et al, 1991). Plant lectins play a very important role for the purification of glycoconjugates. Plant lectins play a very powerful role in the purification of the monosaccharide & polysaccharides by the affinity chromatography method (Schumacher et al,1991; Gabius and Gabius,1991).

1.3 Antiviral activity

The plant lectins show a great protection against the viral infection by preventing the replication of the viral DNA.

1.4 Anti bacterial activity

The cell wall of the bacteria prevents the proteins from penetrating the cytoplasm. Plant lectins cannot alter the permeability of the bacterial membrane. Plant lectins play an important role in the defense of plant through indirect mechanism; which is based upon the interaction with cell wall carbohydrate or extra cellular glycans.

Several methods are employed for the isolation of the of lactose binding protein from *Abrus Precatorius* seeds. Protein is isolated from the *Abrus Precatorius* seeds by the help of sepharose 4B affinity chromatography, ion exchange & gel filtration steps (Olsnes *et al*.,1974; Wei *et al*.,1974; Roy *et a*l.,1976; Lin *et al*.,1978; Lin *et al*.,1981). Isolated proteins have to be identified by using various methods & also by knowing the properties of the protein. Affinity chromatography by sepharose 4B column is a

convenient method for the separation & purification. Gel filtration & SDS- PAGE is done for further confirmation of getting the targeted protein. Then the desired protein was targeted on some cell lines.

SCC4, SCC9, SCC15, SCC25, RPMI 2650, FaDu , HEp2 are the major oral cancer cell lines which are maintained by us. The oral mucosal presentations of these cells lines are squamous cell carcinoma. The main characteristics features of these cell lines are:

SCC cell lines

These squamous cell carcinoma cell lines are mainly originated from human tongue carcinoma. These cell lines have adherent properties. There are mainly various types of SCC cell lines i.e. SCC4, SCC9, SCC15, SCC25.

RPMI 2650 cell lines

These cell lines are mainly originated from the human nasal epithelial cell line. These cell lines have adherent properties.

FaDu cell lines

These cell lines are mainly originated from human hypopharyngeal tumor or this disease is mainly pharyngeal origin. It is mainly found in head & neck portion. These cell lines also show the adherent properties.

HEp2 cell lines

These cell lines are mainly originated from human larynx carcinoma. These cell lines also show the adherent properties.

The table represented below gives a brief idea about the origin & maintenance of the cell lines (table-1).

Table-2: showing the origin & maintenance of the cell lines

Cell line	**Tissue**	**Media**
FaDu	Pharynx	MEM (E) with NEAA +FC
HEp-2	Larynx	MEM (E) with NEAA +FCS
RPMI-2650	Human nasal septum	MEM (E) with NEAA +FCS
SCC-4	Tongue	DMEM:F12 with hydrocortisone + FCS
SCC-9	Tongue	DMEM:F12 with hydrocortisone + FCS
SCC-15	Tongue	DMEM:F12 with hydrocortisone + FCS
SCC-25	Tongue	DMEM:F12 with hydrocortisone + FCS

2. REVIEW OF LITERATURE

2.1 Definition of the lectin protein

From the early history & according to the characteristic lectins are known as carbohydrate binding proteins. Many plant enzymes are fusion proteins & there are composed by a carbohydrate-binding and a catalytic domain. There are many class of chitins are found out of which, 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. There are several carbohydrate binding proteins which have only one binding site, so that they are not capable of precipitating the glycoconjugates or agglutinating cells. It is widely found that some leguminous plant species have some proteins that are clearly related or similar to that of proteins but these are lack of carbohydrate-binding activity. There are many well-known examples which represent the proteins lack of carbohydrate binding protein; out of which *Phas eolus vulgaris* arcelins and the alpha-amylase inhibitor are the common examples of these category (Mirkov et al., 1994).

2.2: The plant lectin

It is found that all plant proteins having at least one non catalytic domain, which binds to a specific carbohydrate (may be mono or oligosaccharide), which are considered as lectin protein (Peumans et al., 1995). It is widely known that the plants are the most abundant source of lectin. Definitely the plant lectin is very useful because they are easily separable & they can form easily glycoconjugate bond in solution and on cell surface. Protein lectins have a great capacity to agglutinate with the erythrocytes of human or animal. From this simple theory we can measure the agglutination activity of the lectin protein using blood cells. As it is well known that lectins are found in the leguminous plants & these lectins are mainly located in the cotyledons. It is widely found that lectins have specificity for carbohydrates which can be checked by simple monosaccharide and oligosaccharides disaccharide, or glycopeptide. Also lectins are differing in their composition, molecular weight and number of sugar binding sites per molecule. There are several plant lectins which are found to having non-carbohydrate ligands & these are having

first & foremost water hating nature which including cytokinin, auxins, and indole acetic acid, as well as water-soluble porphyrins. Globally it has been recommended that these intractions may be physically applicable, since it is found that some of the molecules play a very important role as phytohormones in case of the plant species (Komath *et al.*, 2006). It is found that all the purified plant lectins have a very demand in science, medicine & technology.

It has been also proved that according to the structure, plant lectins can be differentiated into four different categories which are namely given as merolectins, hololectins, chimerolectins and superlectin (Van Damme *et al.*, 1997).

2.3: Uses of lectin

It has a widely use in the blood typing (N.Sharon). Commercially available lectins have been widely used in affinity chromatography for purifying glyco proteins (GE Healthcare Life Sciences, powerless lectin). Generally, proteins may be considered in respect to glycoforms & carbohydrate structure by the process of blotting, electrophoresis, affinity chromatography and affinity immunoelectrophoreis with plant lectins in addition to microarrays as in evanescent-field fluorescence-assisted lectin microarray (Glyco Station, Lec Chip, Glycan profiling technology). It has a wide use in the biochemical warfare. It has a wide use in studying carbohydrate recognition by proteins. It is widely used as a useful tool 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 the purification process, the protein can be separated by the help of affinity chromatography process. Then the protein is further followed by gel filtration. From many journals it is widely found that the molecular weight of abrin A is about 64.000, which are about of two agglutinins (Hegde et al., 1991; Lin et al., 1978). It was widely investigated by various scientists that the crysal structure of protein abrin A is under the monoclinic space group of P2 (Tahirov et al., 1995). It is found that 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.

2.5: *Abrus*-Agglutinin

It is found that 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 chains. (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.

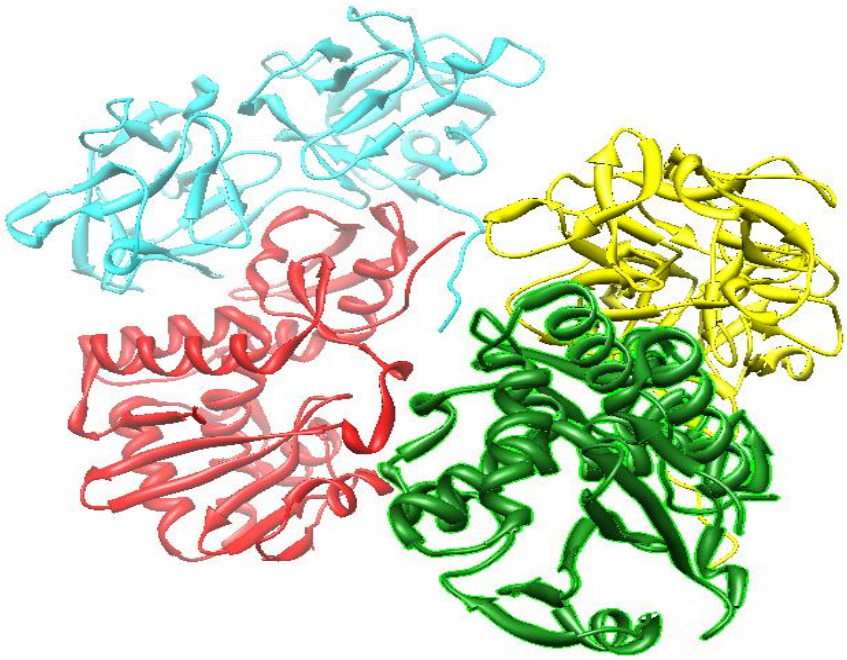

Fig. 5: PDB structure Agglutinin

2.6: Medicinal Uses of *Abrus precatorius*

According to many researchers Fevers, coughs and colds are cured by tea is made from the leaves (Mendes,1986). In the Indian System it has a wide use in traditional Medicine. The seeds are mainly used for the diseases like paralysis, headache, diarrhoea, leprosy, ulcer, dysentery, nervous disorders, sciatica, alopecia, in addition to antibacterial, anti-inflammatory, ant diabetic, antitumor, sexual stimulant and abortifacient. As the seeds are poisonous, therefore are used after alleviation. (Verma et al., 2011). Again it is found that

the extract which is produced from the seed methanolic dose-dependent bronchodilator action.(Mensah et al.,2011).

Various Properties of lectin

Again the effect of Abrus protein extract on growth inhibition of Yoshida sarcoma and Ehrlich ascites tumors in mice were investigated (Reddy et al, 1969 and Lin et al, 1982). Abrus agglutinin protein which are obtained from 10 kD molecular weight cut off membrane permeate (10 kMPP), & Abrus agglutinin was found to have anti proliferative activity (1–10 μg /ml). These proteins are again found to have cytotoxic effect on normal cell lines with dose of 100 μg/ml (Bhutia et al, 2008).

2.7: Antitumor properties

According to some doctors, surgery, radiation and chemotherapy are the three main treatment modalities of cancer. But again it is found that none of these treatment modalities alone is very much effective in controlling the disease. There are another treatment modality namely immunotherapy, though now in infancy stage, has got potential application in combination with other cancer treatments. Only surgical removal is successful mostly for benign tumors and radiation therapy kills the normal cells also. So that these therapies can't widely use as it has various side effects. Today, chemotherapy in cancer treatment is of well-established value and a highly specialized field. So that today natural peptides or plant derived proteins are widely used against these cancer treatments. Mainly lectins are found to be inhibiting the growth of tumours in experimental animals at sub lethal doses. They can also cause apoptosis and the antitumor activity, which is significantly related with apoptosis. Again according to some researchers Abrin is more toxic than to normal cells (Nicolson et al., 1975) & it is found that Abrin has greater cytoagglutination against human cultured cell lines but weak agglutination against normal lymphocytes (Kaufman and McPherson, 1975). Mainly these selective antiproliferative properties of lectins toward tumor cells attract to be a potential source for anticancer agent. Abrus lectins have a great capacity to reduce the tumor (Lin et al., 1969; Tung et al., 1981; Lin et al., 1882; Ramnath et al., 2002; Ghosh and Maiti, 2007). 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, 2007).

According to Bussing *et al*., (1996) there might be two different ways of cell killing, operative in Viscum album L (VAL)-mediated cytotoxicity: (a) effect on cell membrane causing loss of membrane integrity and subsequent influx of Ca^{+2} phosphatidylionositol, and/or (b) induction of apoptotic pathways. Though, colon and breast carcinoma cells was reported to be inhibited by *Agaricus bisporus* lectin (Yu et. al., 1993), peanut lectin can show either stimulatory or inhibitory effects on cancer cells, depending upon the dose of administration.

Effects of natural products on oral cancer cell lines:

Mainly berberine is a plant product which was originally obtained from the Chinese herb *Rhizoma coptidis* (Leng et al., 2004). According to various experiments berberine has a wide range of pharmacological action i.e antimicrobial, anticancer, anti-inflammatory, and antiarrhythmic properties which help in various ways (Lau et al., 2001). The berberine, which is a plant product, its effect was mainly examined on cell growth, apoptosis and cell cycle regulation in human oral squamous carcinoma cells. It is also found from previous studies that berberine can induce autophagy in various cancer cells i.e. in human gastric cancer & is in leukemia cells (Lin et al., 2006). Again it was also reported that berberine can inhibit growth, & it can also induces apoptosis by the disruption of mitochondrial membrane & by the cleavage of the PARP (Mantena et al., 2006).

On treating berberine on the oral squamous carcinoma cancer cell lines, the cell lines shows lower survival rate. Also it induces apoptosis in a dose dependent manner (Lin et al., 2007).

According to many researchers Curcumin, which is a major active component of turmeric Curcuma longa, it has also found to have inhibitory effects on cancers. Besides the anticancer activity it is used as an antirheumatic alleviating sinusitis, respiratory problems, hepatic disorders even anorexia & anti-inflammatory(Goel et al., 2008). Again from in vitro studies suggest that curcumin can inhibit cancer cell growth by activating apoptosis pathway, but the mechanism underlying the anticancer effects of curcumin is still in an unclear stage. Recently, it has been reported that autophagy may play an important role in cancer therapy. Many researchers have shown that curcumin has

anticancer activity against oral squamous cell carcinoma (OSCC). Further, by some researchers it has been proved that curcumin shows anticancer activity against OSCC via both autophagy and apoptosis. Again it has been reported that curcumin has been shown to induce cell death in malignant cancer cell lines including K562, MCF-7, and HeLa cells (Duvoix et al., 2003 & Choudhuri et al., 2002).

On treating curcumin on the oral squamous carcinoma cancer cell lines, the cell lines shows lower survival rate. Also it induces apoptosis & autophagy in a dose dependent manner.

After ROS analysis of it was found that curcumin-induced ROS production is mainly responsible for the autophagy activation (Kim et al., 2012). So that on treating with curcumin on oral cancer cell lines it induces autophagy.

According to various researchers quercetin (Qu) is the natural flavonoid compound which is commonly extracted from the cranberries, blueberries, apples & onions. Again it is found that it possesses a wide spectrum of bio-pharmacological properties (Ishizawa et al., 2009). According to a report which reveals that A previous report also revealed that quercetin (Qu) plays a role as an inhibitor of Hsp synthesis and has protective effects in mouse liver injury for cellular homeostasis (Hsu et al., 2011).

On treating quercetin (Qu) the oral squamous carcinoma cancer cell lines (SCC 25), the cell lines shows lower survival rate. On treating the quercetin (Qu) it shows that the expression of MAPK decrease & (MAPK is a pathway whose expression increases the cell proliferation) increases the expression of caspase 3 which leads to apoptosis. So that on the treatment of quercetin increases the apoptosis of the cancer cells (Chen et al., 2012).

Effects of natural products on cervical cancer cell lines:

Again it is found that Abrus agglutinin (AAG), which is isolated from the seeds of *Abrus precatorius* is a hetero-tetrameric glycoprotein of 134-kDa molecular weight, composed of two A and two B chains linked through disulphide bridges (Bagaria et al., 2006; Hegde et al., 1991). *Abrus* agglutinin is a natural product which has both antitumor activity & anticancer activity.

According to researchers, a peptide mixture derived which is derived from AAG was able to inhibit the growth of tumor cell lines in a dose-dependent manner. Later, it is found that the

growth inhibitory activity of peptide was again associated with the induction of apoptosis in HeLa cells. It is again found that it also induces apoptosis in a dose depended manner.

Again the fluorescence microscopy study showed that the natural peptides penetrate cells by a yet unknown mechanism, and this peptide can induce apoptosis without disturbing membrane integrity showed by hemolysis assay and LDH release (Elmquist et al., 2006; Lehmann et al., 2006). According to the researchers, the incubation of the peptides with HeLa increased the ROS generation, which causes the decrease of mitochondrial membrane potential resulting in release of cytochrome C and activation of caspase-3 (Bhutia et al, 2007).

Anti-cancer activity of AGG is broadly studied in HeLa cell lines while no work has been done in oral cancer cell lines. In our experimental work, the effect of AGG is being explored on various oral cancer cell lines. The molecular pathway through which the AGG inhibits the cell proliferation is a route to its findings.

OBJECTIVES

- To isolate oral abrus agglutinin by white abrus seeds
- To characterize the isolated agglutinin protein
    - SDS PAGE
    - Native PAGE
- To validate cell cytotoxicity post agglutinin treatment on oral cancer cell lines.
    - MTT assay
    - Trypan blue exclusion assay
- To study anti-proliferative activity of *abrus* agglutinin by colony forming assay
- To determine nucler fragmentation and nuclear condensation by DAPI stain
- To monitor whether agglutinin induces autophagy by acridine orange stain

4. MATERIALS AND METHODS

Collection of the sample:

Mainly the sample that is here used, white Abrus precatorius seeds were collected from the deep forest of Angul, Odisha. India.

Chemicals:

Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3), Glycine, Cuppersulphate ($CuSO_4$) ,Potassium sodium tartarate ($KNaC_4H_4O_6$) were purchased from the company namely 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 company, USA. Potassium Dihydrogen Phosphate (KH_2PO_4), Potassium hydrogen phosphate (K_2HPO_4) were 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 was from Trimurty Chemicals, India. Pre stained molecular weight marker were purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from nice chemicals Pvt .Ltd. India. Cell culture media (i.e. MEM, DMEM) were purched from the Gibco. Trypsin & antibiotics were purchased from the Himedia. Acridine Orange & DAPI were purchased from the Sigma.

PREPARATION OF LACTAMYL SEPHAROSE AFFINITY MATRIX

AFFINITY CHROMATOGRAPHY:

Epoxy activation of sepharose 4B:

About 8g of sepharose 4B was washed by PBS in filtration unit and stored in 40ºC in alcohol **.** After that sepharose 4B was mixed in 12 ml of distilled water and mixed thoroughly through pipette. After that 5.2ml of 2M NaOH was added to it. Aproximately 1.3ml of epichlorohydrin was added so that the final concentrations of the various components were 30% (v/v) sepharose, 5% epichlorohydrin, 0.4M NaOH. Suspension was incubated at 40ºC for 2 hours with continuous shaking. Incubated suspension was transferred to a glass filter funnel and after that the gel was washed with 500ml of distilled water.

Preparation of amino sepharose 4B:

The epoxy activated sepharose 4B was mixed in 1.5 volume of concentrated ammonium solution (12ml).The suspension was then incubated at 40ºC for one and half hour. The incubated suspension was again transferred to glass filter funnel and the gel was washed with distilled water.

Coupling of lactose with amino sepharose 4B:

After that 8g of dried amino sepharose 4B was mixed in 6ml of 0.2M K_2HPO_4 buffer containing 208mg of lactose and 102mg of $NaCNBH_3$.The suspension was then incubated at room temperature for 10 days with occasional shaking. After that the free amino groups which were remaining in the gel were acetylated by adding 2ml of acetic anhydride. Then the suspension was again incubated at room temperature for 1 hour. The lactamyl sepharose 4B thus obtained was subsequently washed with distilled water, 0.1 M NaOH, distilled water and 10 mM PBS. The prepared column was stored in distilled water with traces of sodium azide at 4ºC.

Fig : 6 preparation of lactamyl sepharose affinity matrix

Purification of *Abrus* agglutinin:

Abrus agglutinin was purified from the seeds of red *Abrus precatorius* following the methods described by (Hegde *et al*. 1991). For the protein isolation process, 100 gms of white *Abrus* seeds were taken and decorticated. The uncoated seeds (72gms) were soaked in PBS overnight and grinded with PBS in a blender for 5 minutes. Then it was centrifuged at 10,000 rpm for 20 minutes at 4^{o} C. The supernatant was collected and then it was subjected to ammonium sulfate fractionation (first 0-30% and then 30-90%).

30% cut off

The total amount of ammonium sulphate required for achieving 30% cut off were taken. Then the crystalline ammonium sulfate was made to amourphous powder. Abrus supernatant was transferred to a 500ml beaker. Pinch wise ammonium sulfate salt was added to it. By the help of magnetic stirrer & bead the supernatant was thoroughly mixed.

90% cut off

The incubated 30% ammonium sulphate cut was centrifuged & again required amount of ammonium salt was added to the supernatant (of 30% cut off) in a pinch manner. By the same process the supernatant was thoroughly mixed by the help of the magnetic stirrer & bead.

After 90% cut off, the precipitate or the pellet was resuspended in PBS (p^{H} 7.4 Mm) and dialyzed for 72 hrs against 50mM PBS (pH 7.4) till all the salts to be released. The dialyzed sample was loaded onto Lactamyl Sepharose affinity column which was pre equilibrated with the buffer and eluted by adding 0.4M lactose solution. The affinity elusion profile showed a single peak.

Gel filtration chromatography:

The gel filtration chromatography was carried out by packed Sephadex G-100 which was preequilibrated with PBS to get further purified protein. We found two sharp peak while talking OD at 280 nm by using FPLC(GE,AKTA PRIME PLUS). The Peak I sample corresponds to Agglutinin which showed its highest peak at 800mAu and following brief stationary phase tha second peak found at 300mAu which is toxin . The two samples were collected at following floe rate 0.5 ml/min. The purity of the proteins were tested by both SDS and native Polyacrylamide Gel Electrophoresis (PAGE).

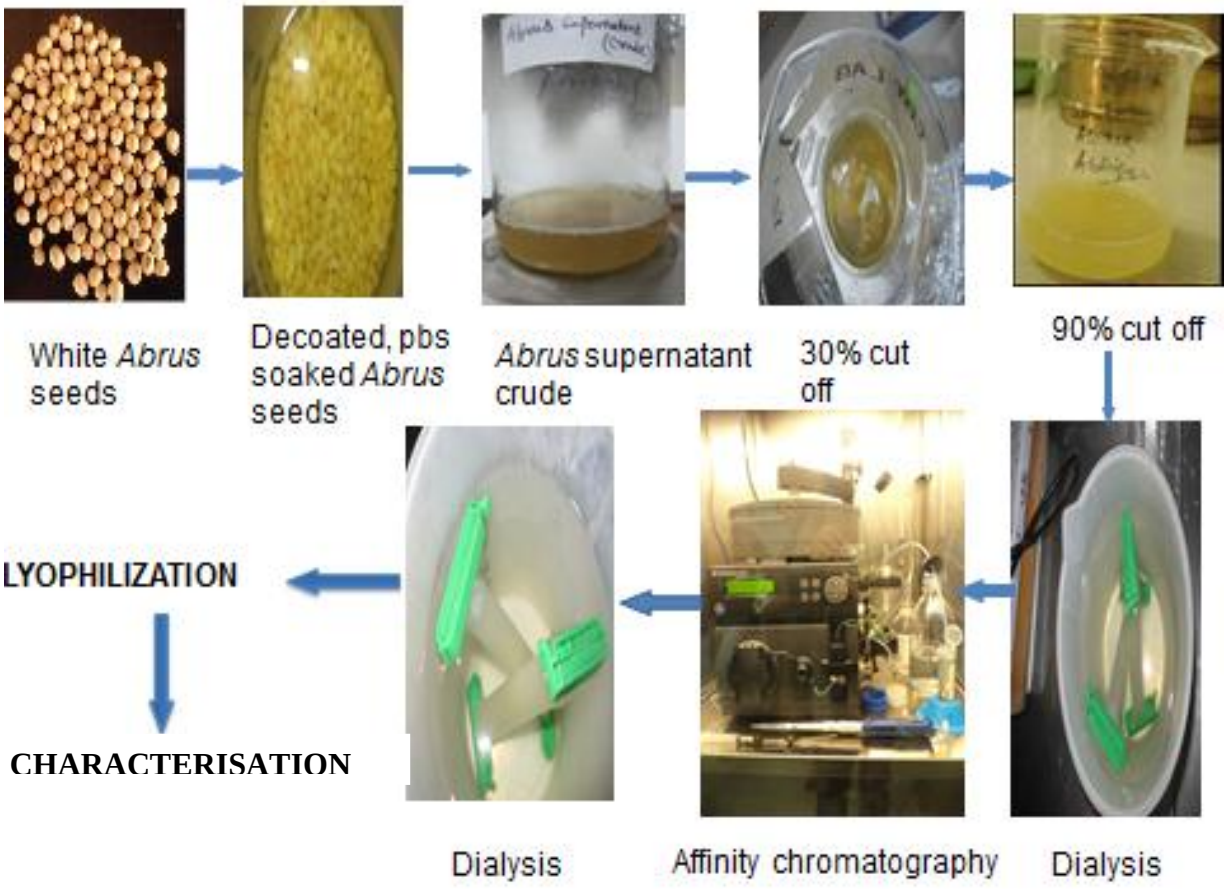

Fig:7 Purification of protein

Analysis of anti-cancer activity of Agglutinin in oral cancer cell lines

MTT assay:

Principle

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT dye enters the cells and then the dye passes into the mitochondria where it is reduced to form an insoluble, coloured (dark purple) formazan product. After that the cells are then mixed with an organic solvent (eg. isopropanol) and the released. Then the solubilized formazan reagent is measured

spectrophotometrically. As the reduction of MTT occurs, then it can only seen in the metabolically active cells, the level of activity is a measure of the viability of the cells.

Methodology

Cells were seeded in each well for Hacat, FaDu, RPMI 2650, HEp2. It was kept for incubation. The agglutinin was added in various concentrations. After treatment, cells were kept for 78 hours of incubation. MTT dye was added in each well and was kept for some hours of incubation. The violet crystal was dissolved in DMSO. Then the reading was taken at 562nm.

Trypan blue exclusion assay

Principle

This test is used to determine the number of viable cells present in a cell suspension. The main principle of this process is that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells cannot do it. According to this test, a cell suspension is simply mixed with dye and then the cell suspension is visually examined to determine whether cells take up or exclude dye. It is again found that, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Methodology

In aliquot of cell suspension is being tested for viability 5 min at 100 ×g & it is then centrifuged. Supernatant was discarded. Then size of the aliquot depends upon the no of the cells present. The cell suspension was mixed with 0.4% trypan blue and 1 part cell suspension (dilution of cells) were mixed. Then under the microscope the unstained & stained cells were counted.

Colony forming assay

Principle

The assays are based on the principle that certain proteins when expressed stably cause either cell cell cycle arrest or cell death, hence a reduction in colony number.

Methodology

The main method for the colony forming assay, cells were treated with agglutinin at various concentrations. After being rinsed with fresh medium, cells were allowed to grow for 14 days to form colonies. Following which post crystal violet staining, quantification of colonies was done.

DAPI staining:

Principle

The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA, specifically, with little or no cytoplasmic labeling; it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. The DAPI/dsDNA complex shows fluorescence at 460 nm.

Methodology

Preseeded cells were treated with AGG in various concentrations. Discard the old media. Paraformaldehyde treated for 30 minutes at 37^{o}c. washed with PBST. PBS wash was done for two times. DAPI was added. Dark incubated for 5 minutes at room temperature. PBS wash was done for three times. Pictures were obtained.

Acridine Orange staining:

Principle

Acridine orange is used in autophagy assays. It crosses into lysosomes (and other acidic compartments) and becomes protonated. Autophagy is characterized by increased formation of AVOs (lysosomes and autophagolysosomes). AVOs (acidic vesicular organelles) can be quantified by flow cytometry after staining the cells with AO (acridine orange). AO is a weak base that accumulates in acidic spaces and fluoresces bright red. The AVOs can be quantified based on the fact that the increase in intensity of the red fluorescence is proportional to the degree of acidity.

Methodology

Cells were seeded in 12-well plates and treated with Agglutinin in various concentrations for 24 hours. Acridine orange was then added at a final concentration of for a period of

15 minutes. Pictures were obtained with the help of a fluorescence microscope (olympus IX 71).

RESULTS

Purification of Agglutinin:

ELUTION PROFILE OF ABRUS LECTIN FROM LACTAMYL SEPHAROSE AFFINITY MATRIX

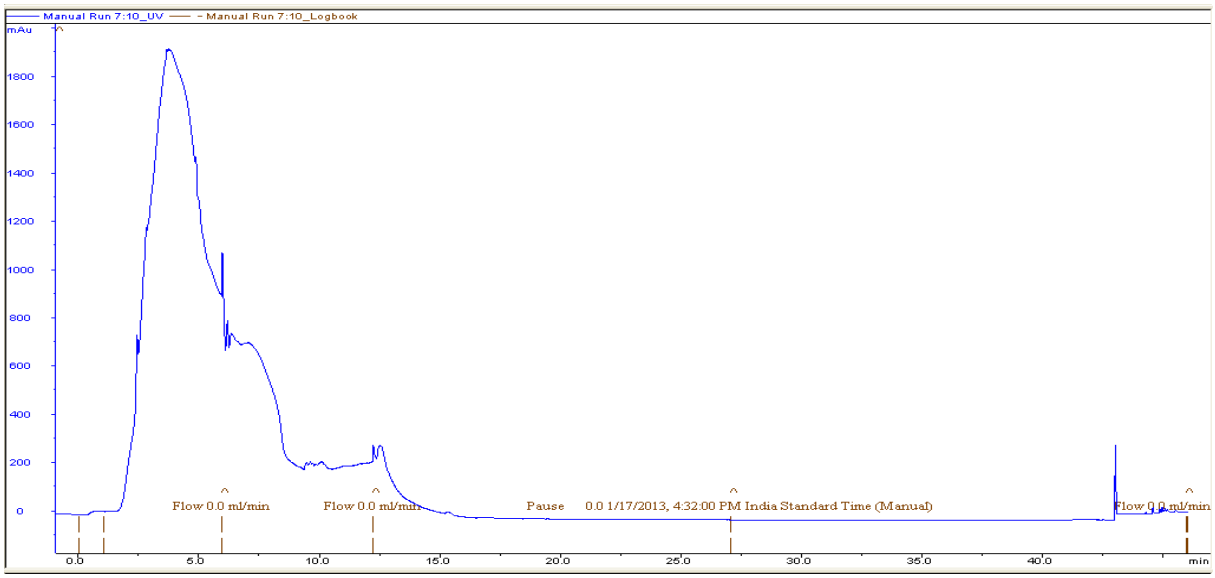

Fig: 8 peak showing elution of the *Abrus* lectin

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

ISOLATION OF AGGLUTININ AND TOXIN BY GELFILTRATION CHROMATOGRAPHY

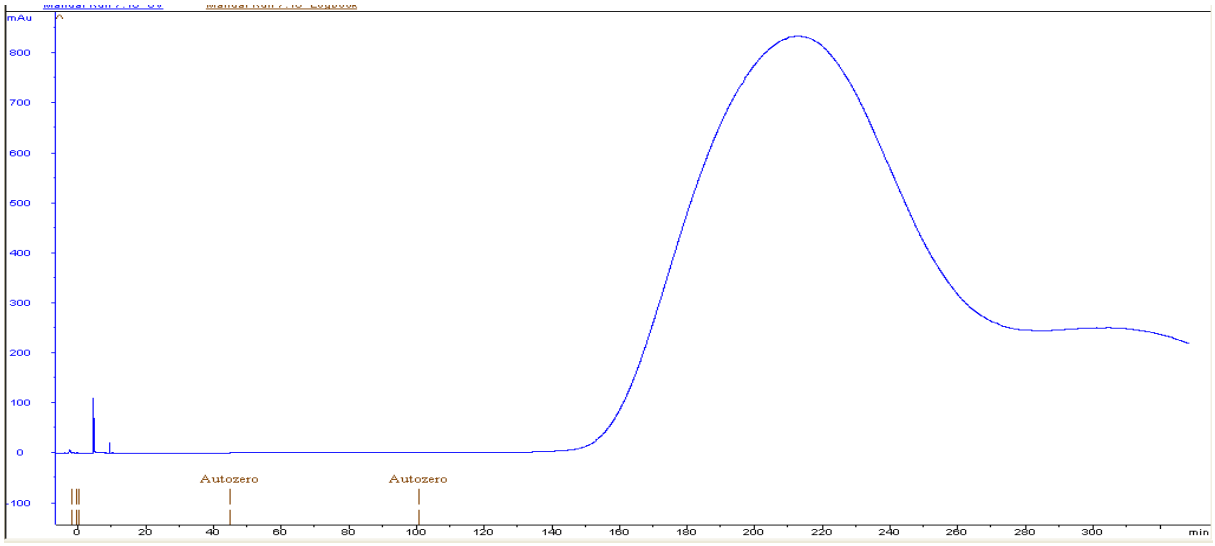

Fig: 9 peak showing isolation of the *Abrus* agglutinin & toxin

After gel filtration chromatography we got two peaks i.e. one peak for agglutinin the other peak is for the toxin.

SDS PAGE

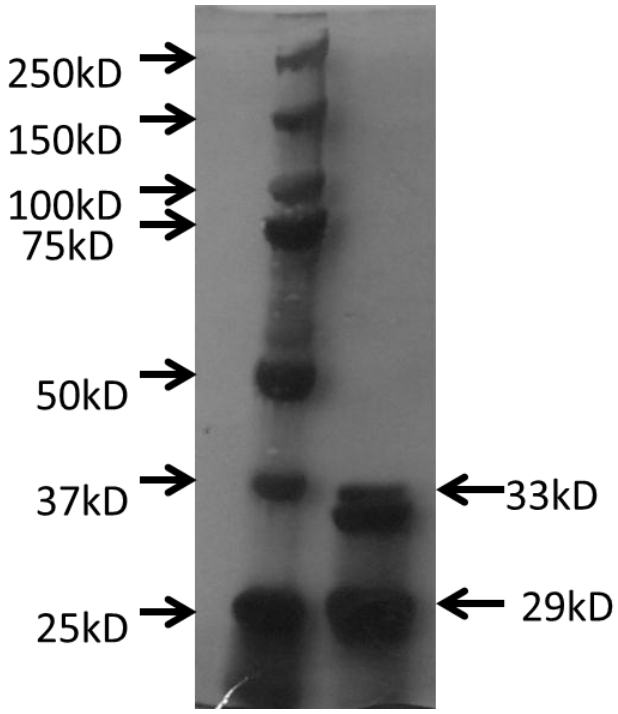

Fig: 10 Showing SDS PAGE

Fig: Photograph taken by Bio-Rad Gel documentation system. Lane 1 depicts the Bio-Rad prestained protein molecular weight marke rand Lane 2 depicts the two bands developed which are two chains of agglutinin having molecular weight 33 kD and 29 kD respectively.

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 33kD and 29kD.

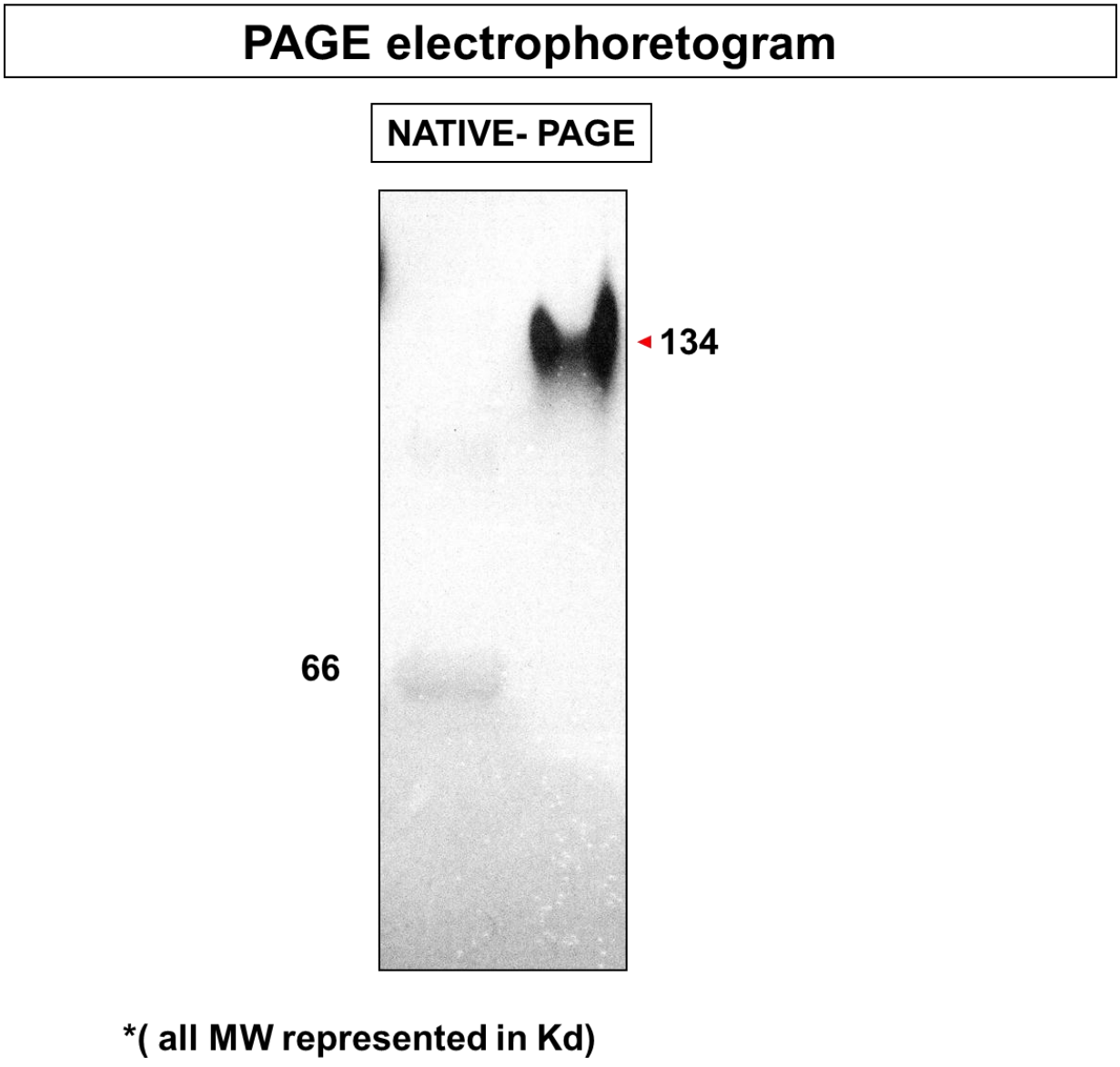

Fig: 11 showing Native PAGE

Native page showing the molecular weight of protein is about 134 KD (Fig: 10).

MTT Assay

Table: 3 showing MTT Assay for HaCaT

Concentration	Cell viability[%]
Control	100
10ng	98
100ng	98
1ug	75
10ug	57
100ug	44

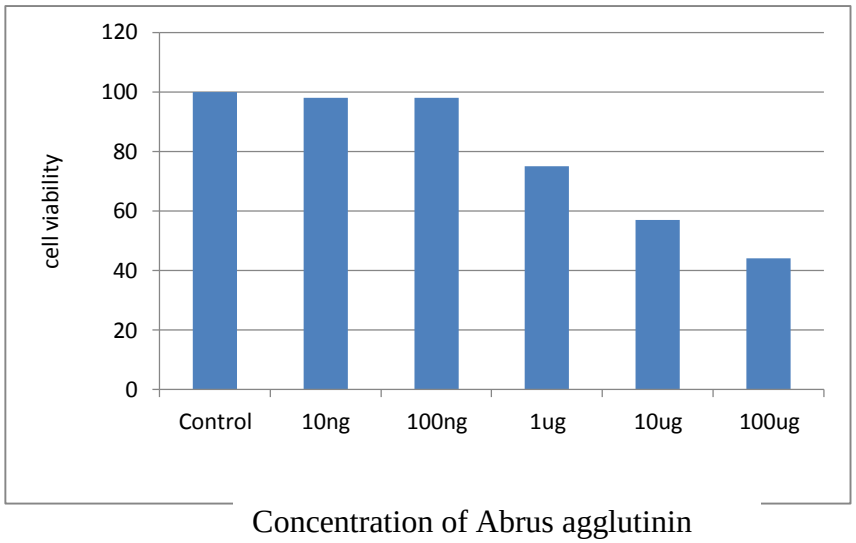

Fig: 12 Histogram showing MTT Assay of HaCaT

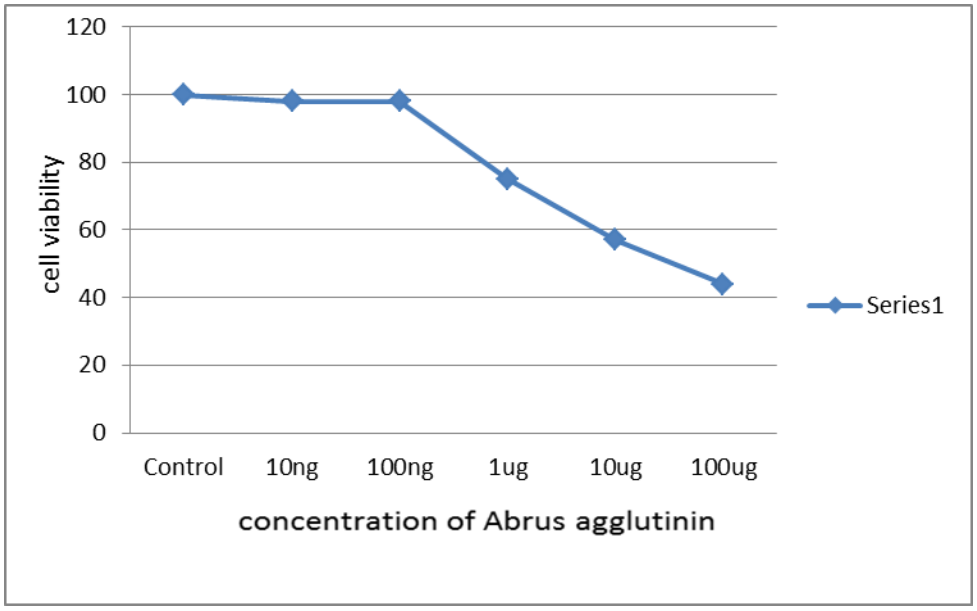

Fig: 12 graph showing MTT Assay of HaCaT

Table: 4 showing MTT Assay for FaDu

Concentration	Cell viability[%]
Control	100
10ng	82
100ng	53
1ug	52
10ug	40
100ug	37

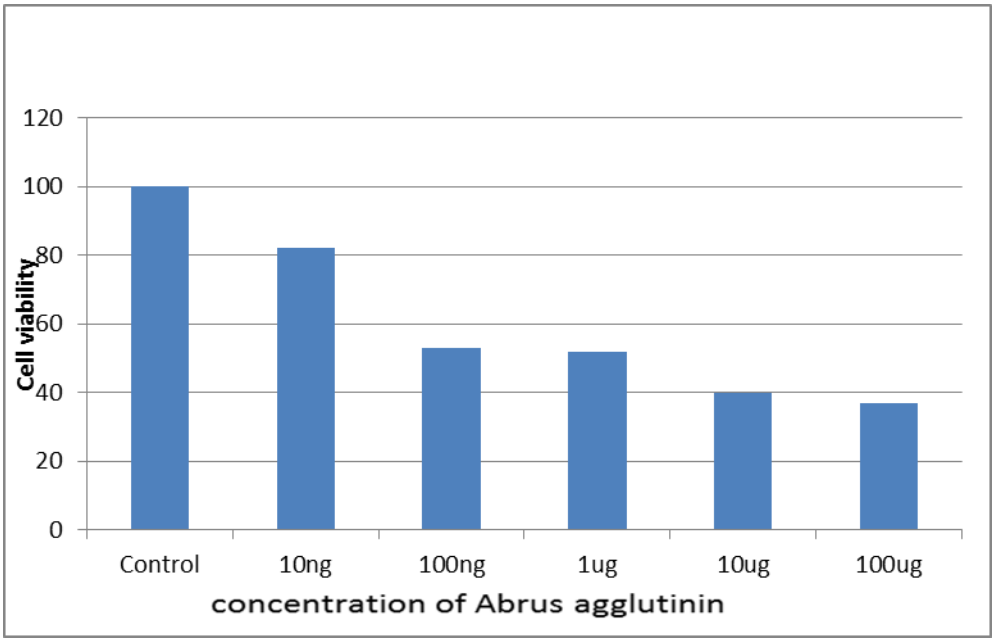

Fig: 13 Histogram showing MTT Assay of FaDu

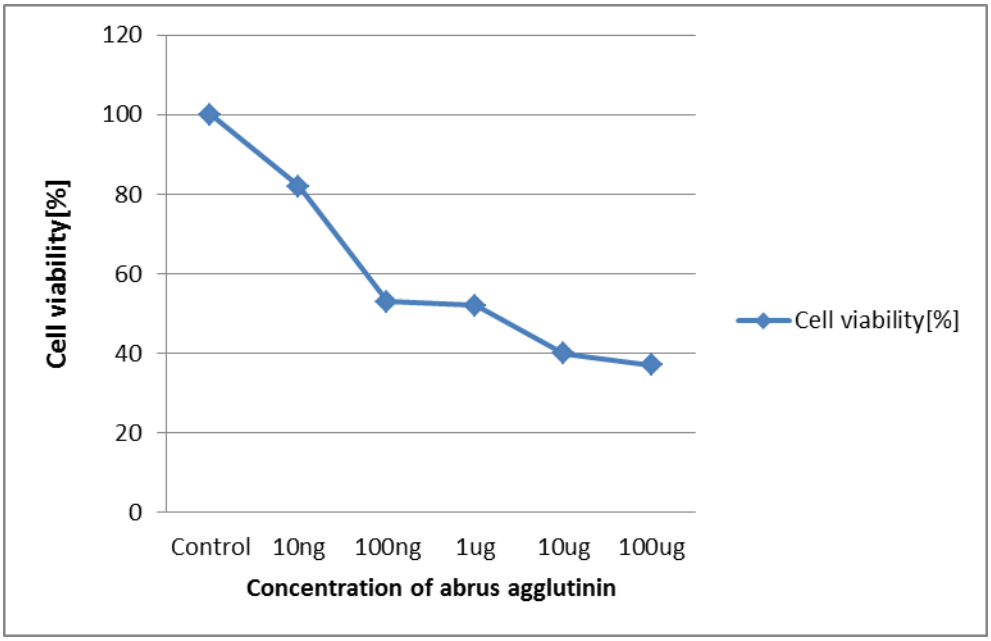

Fig: 13 graph showing MTT Assay for FaDu

Table: 5 showing MTT Assay of RPMI 2650

Concentration	Cell viability[%]
Control	100
10ng	77
100ng	72
1ug	69
10ug	43
100ug	38

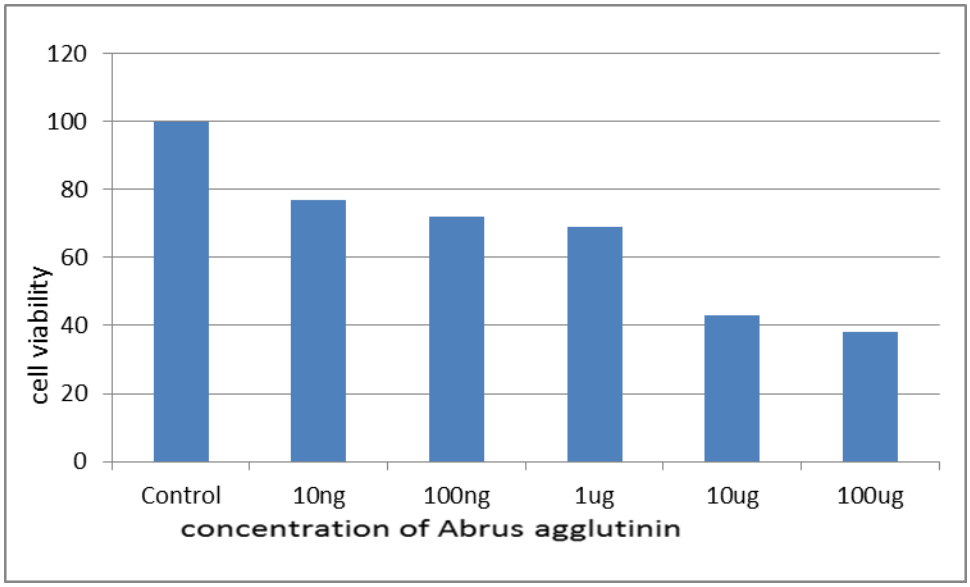

Fig: 14 Histogram showing MTT Assay of RPMI 2650

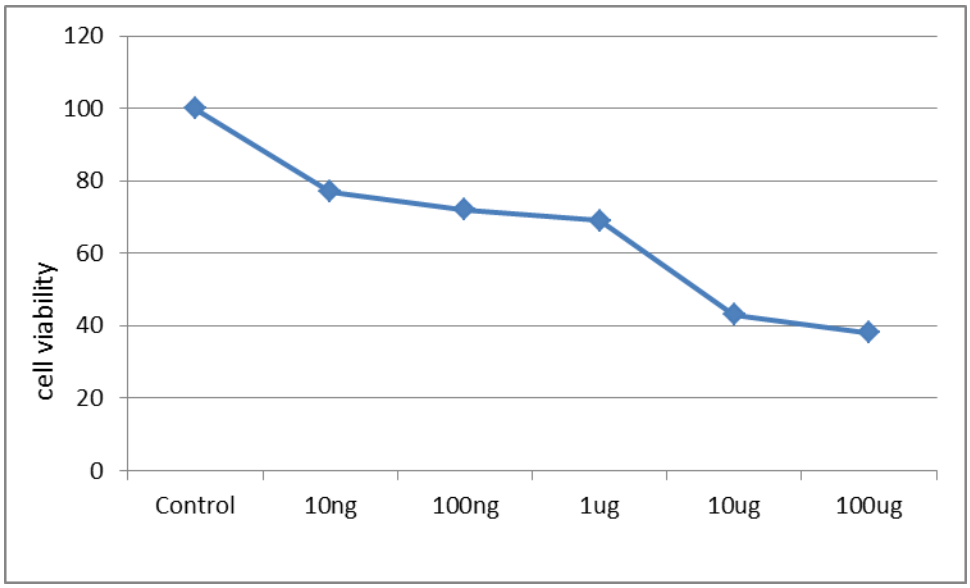

Fig: 14 graph MTT Assay for RPMI 2650

Table: 6 showing MTT assay for HEp2

Concentration	Cell viability[%]
Control	100
10ng	89
100ng	84
1ug	70
10ug	49
100ug	40

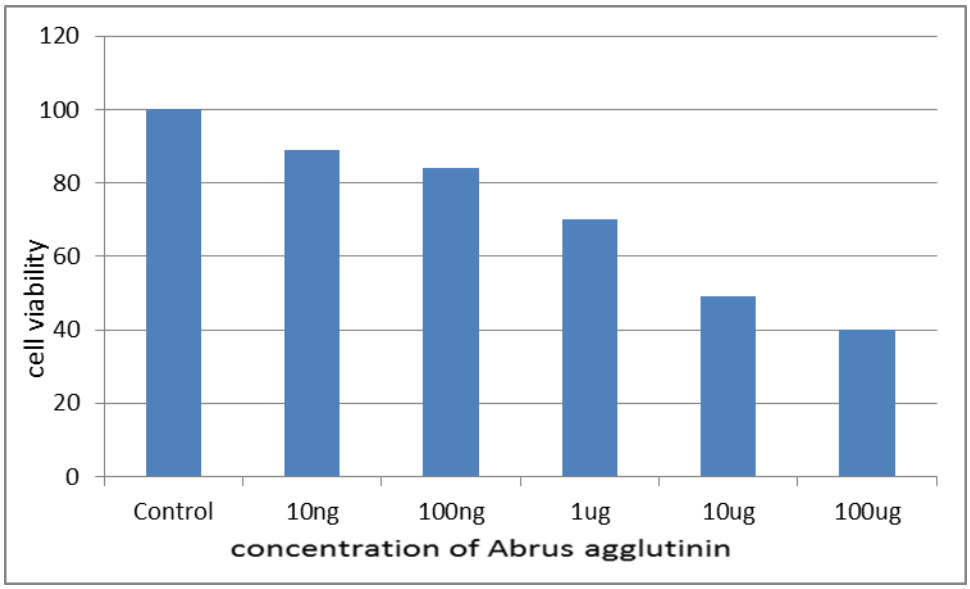

Fig: 15 Histogram showing MTT Assay of HEp2

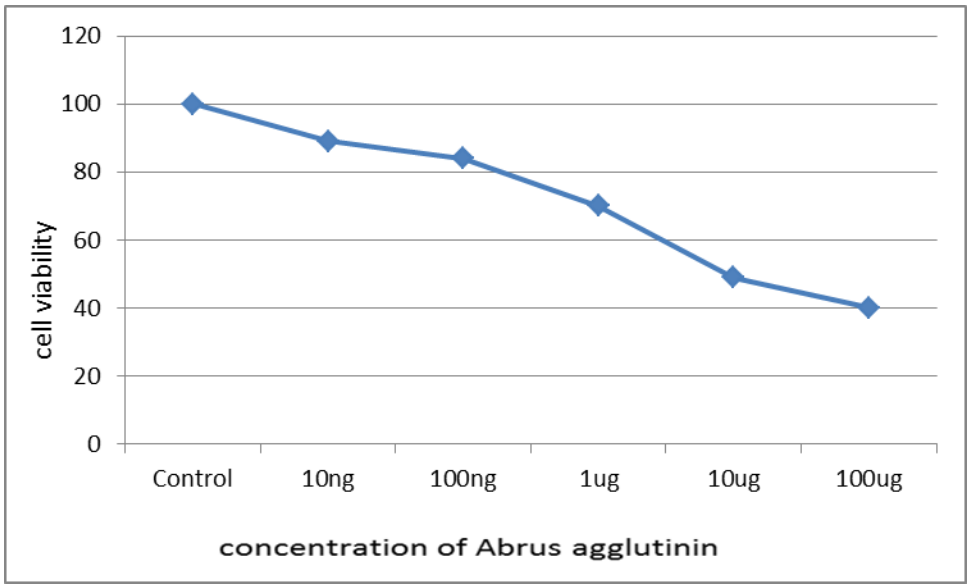

Fig: 15 MTT Assay of HEp2

Comparison

Table: 7 showing comparison between different oral cancer cell lines

Concentration	HaCaT	FaDu	RPMI2650	HEp2
Control	100	100	100	100
10ng	98	82	77	89
10ng	98	82	77	89
1ug	75	52	69	70
10ug	57	40	43	49
100ug	44	37	38	40

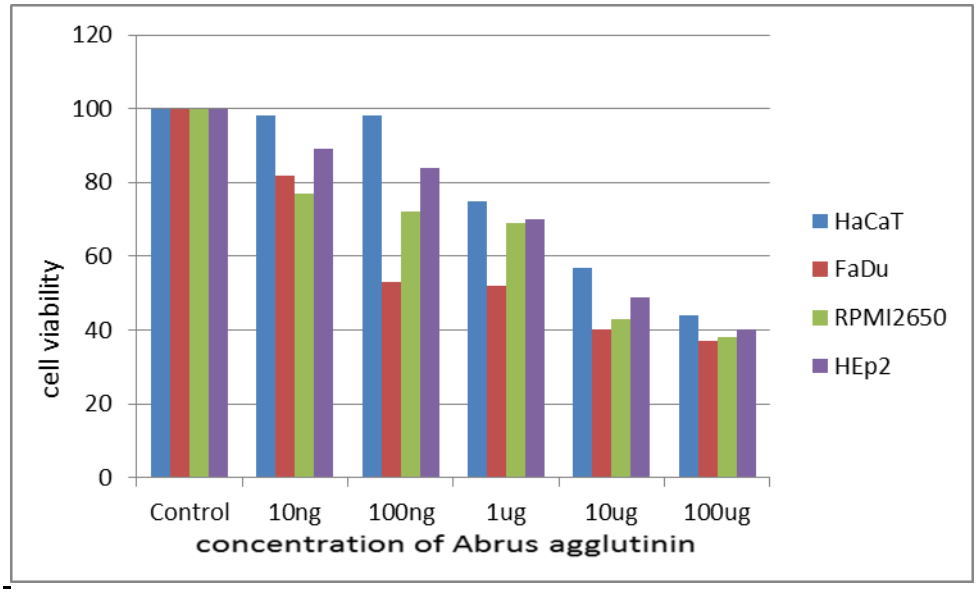

Fig: 16 histogram showing comparision between different oral cancer cell lines

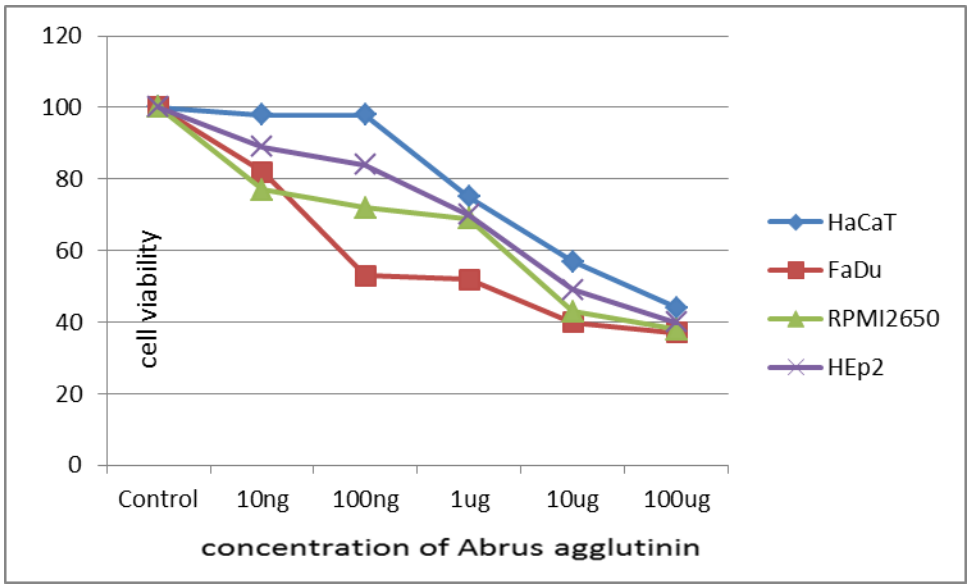

Fig: 16 graph showing comparison between different oral cancer cell lines

IC_{50} of oral cancer cell lines

Table: 8 showing IC_{50} of oral cancer cell lines

Cell lines	**IC_{50}**
HaCaT	56.66 µg
FaDu	1.25 µg
RPMI-2650	6 µg
HEp2	7.5 µg

IC_{50} of HaCaT cell lines is much more higher than the oral cancer cell lines. Therefore the concentration at which drug can be administered to the patient will have no effect on normal cells (Table-8).

Trypan blue exclusion assay

Table: 9 showing trypan blue exclusion assay

concentration	**vability**	**Non-viability**	**% of viability**
control	48	0	100
100 ng	12	3	80
1 µg	4	6	40
10 µg	1	7	13

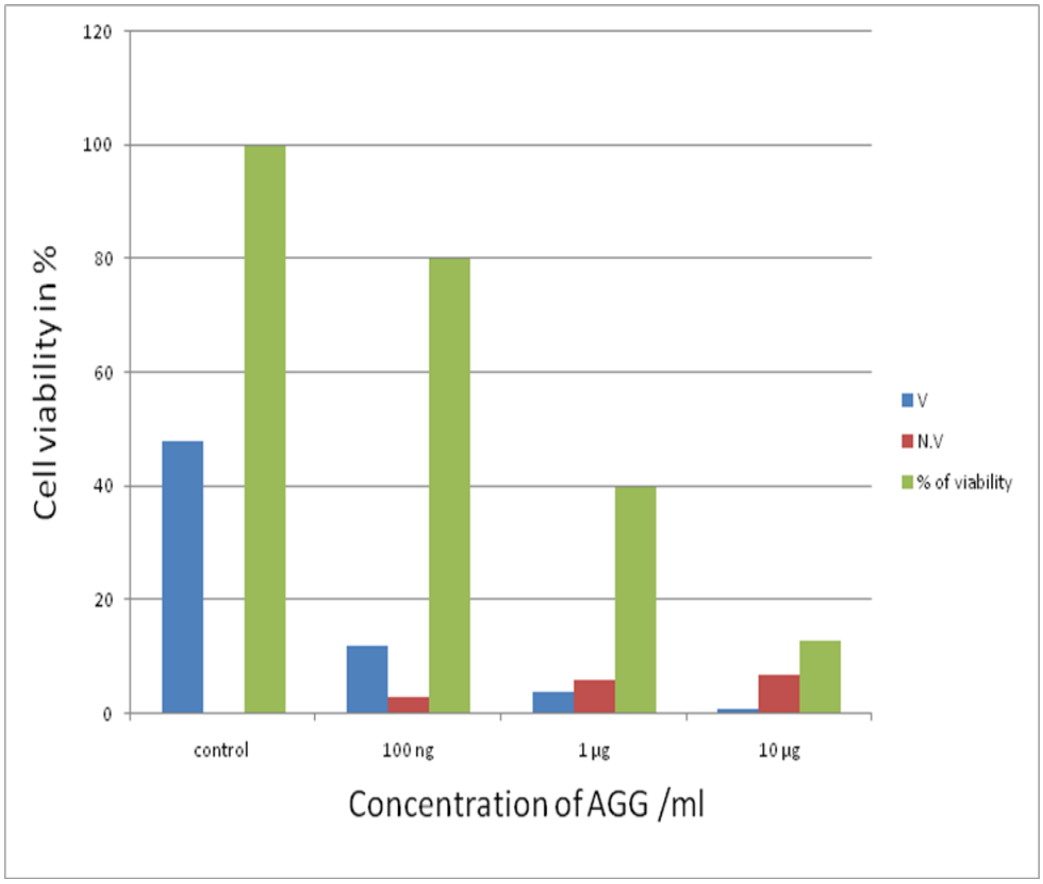

Fig: 17 Histogram showing trypan blue exclusion

Trypan blue dye is being taken up by non-viable cells while the viable cells don't take up the dye depending upon the cytoxicity is generated by the agglutinin; percentage cell viability is calculated by counting the number of viable and non-viable cells. Thus dose dependent decrease in percentage of viability is seen with agglutinin treatment (Table: 9) (fig: 17).

Colony forming assay:

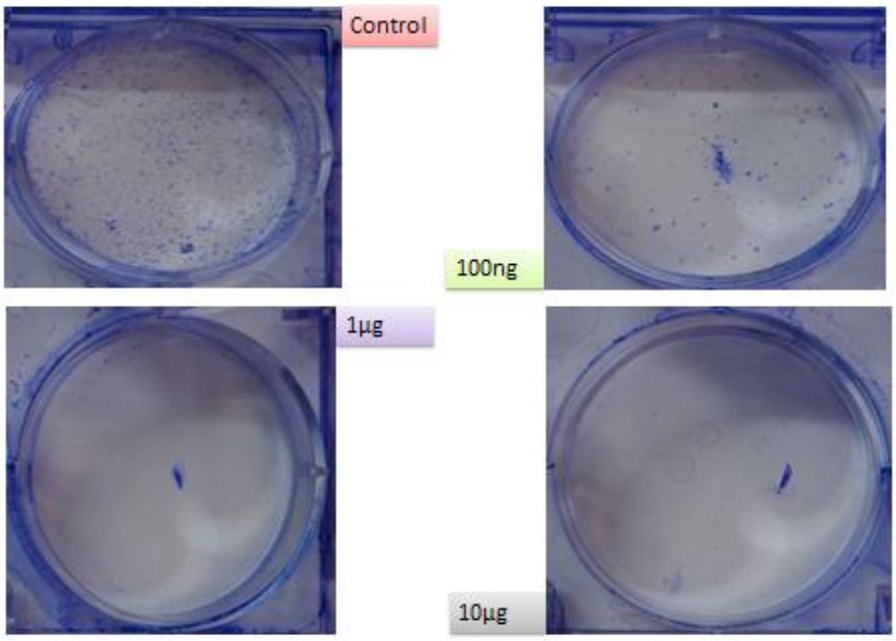

Fig: 18 showing colony forming assay

Table: 10 showing colony formation assay

concentration	**No of colonies**
control	200
100ng	120
1µl	12
10µl	3

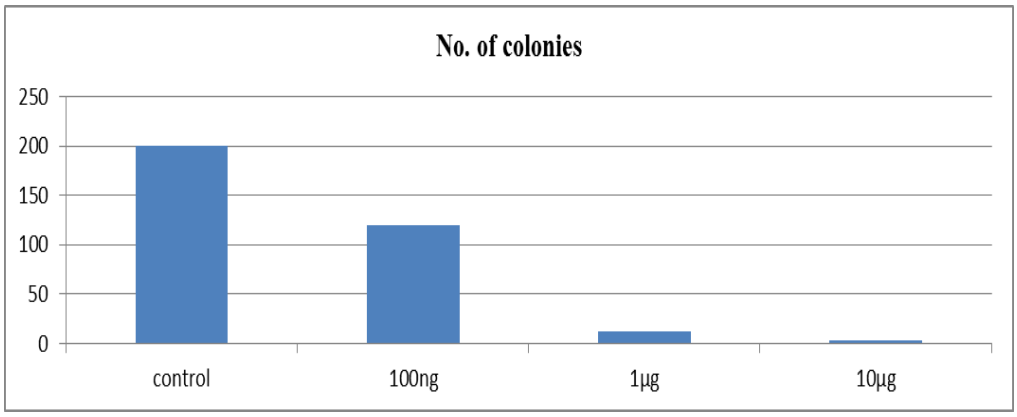

Fig: 18 Histogram showing antiproliferative activity of *abrus* agglutinin on HEp2 cell lines

Cancer cells have high proliferative proliferation rate. The inhibitory effect of agglutinin on proliferation of HEp2 is monitored by dose dependent reduction of colonies (Table: 10) (Fig:18).

DAPI Staining for nuclear fragmentation

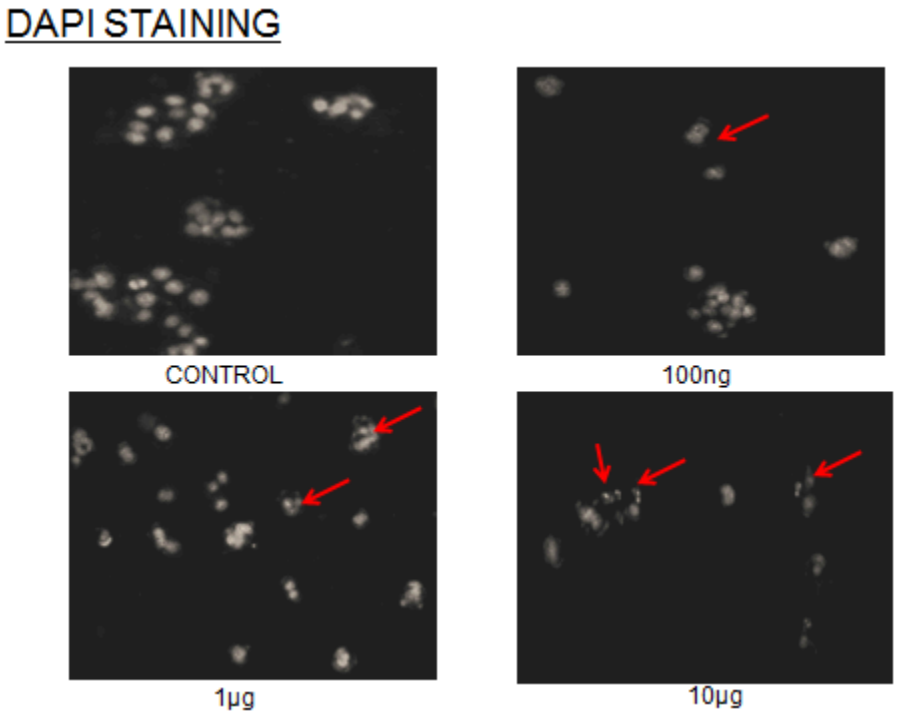

Fig: 19 showing DAPI staining

Nuclear condensation is a DNA fragmentation membrane blebbing is assign of pre apoptosis. Thus, on agglutinin treatment, at higher concentration maximum fragmentation is seen (Fig: 19).

Acridine Orange Staining

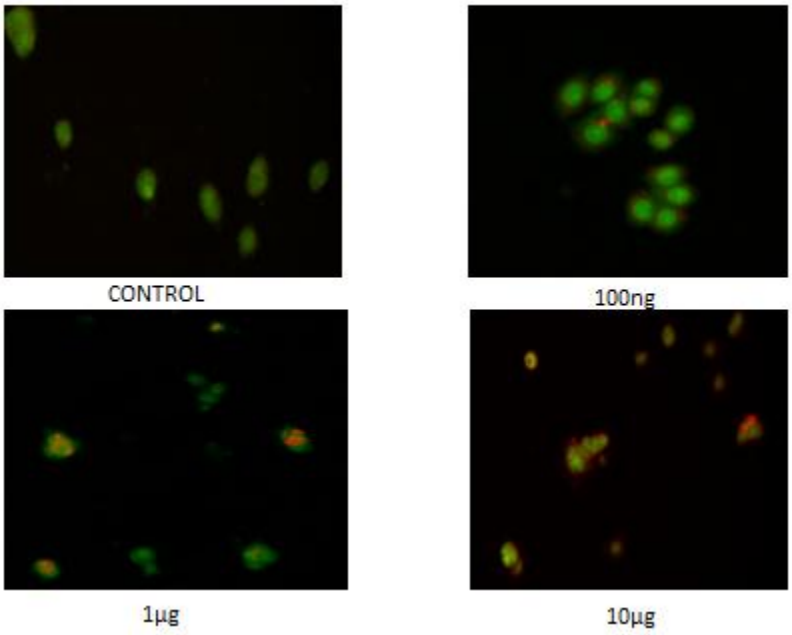

Fig: 20 showing acridine orange staining

Acridine orange is a marker of an autophagy. As the dose of agglutinin increases, formation of acidic vacuoles is increased. The shift in the redness with the increase in the agglutinin treatment indicates the auto lysosome formation, thus depicting autophagy (fig: 19).

DISCUSSION

Abrus agglutinin, a ribosome inhibiting protein II (RIP II) family lectin, is a heterotetrameric glycoprotein of molecular weight 134 kDa which is composed of two A chains (N-glycosidase activity on 60S eukaryotic ribosome) and two B chains (galactosebinding site) linked through a disulphide bridge (Liu et al., 2000). The protein synthesis inhibitory activity of agglutinin is (IC50 = 3.5 nM) and the LD50 is 5 mg/kg of body weight (Hegde et al., 1991). Tryptic digest of *Abrus* agglutinin (TDA) stimulates macrophage to increase the phagocytic and bactericidal activity as well as hydrogen peroxide production. Tryptic digest of *Abrus* agglutinin also proliferates splenocytes leading to Th1 response and NK cell activation *Abrus* agglutinin (TDA) (Tripathi et al. 2004).In human cervical cancer cells *Abrus* agglutinin induces mitochondrial apoptosis (Bhutia et al. 2008). *In vivo* therapeutic effectiveness *Abrus* agglutinin peptide fractions were deciphered in Dalton's lymphoma (DL) mice model which mediate through apoptosis (Bhutia et al.,2008). In mouse model *Abrus* agglutinin showed non-specific immunostimulatory activity both native (NA) and heat-denatured (HDA) condition (Ghosh et al.,2009).

Although many work has been done on cervical cancer cell lines and also lymphoma of *Abrus* agglutinin but no work has been found in literature on oral cancer cell lines till date. Here we showed the anticancer activity of *Abrus* agglutinin after isolating and characterizing by both SDS and Native PAGE. For cell viability property of Abrus agglutinin we performed MTT assy in various oral cancer cell lines. We also performed anticancer activity of *Abrus* agglutinin in normal cell line. The protein synthesis inhibitory activity of *Abrus* agglutinin (IC_{50}) in various oral cell lines is 7.5 µg, 6 µg , 1.25 µg, 56.66 µg in HEp2, RPMI-2650, FaDu, HaCaT respectively.Intrestingly we also noticed *Abrus* agglutinin show different anticancer activity among the various oral cancer cell lines. Among the three oral cancer (HEp2,RPMI-2650,FaDu) cell lines the IC_{50} of *Abrus* agglutinin in FaDu is very less simultaneously the IC_{50} *Abrus* agglutinin in HEp2 is very high. To explore our interest and to know anticancer activity of *Abrus* agglutinin in normal cell and we found the IC_{50} of *Abrus* agglutinin in HaCaT is highest in comparison to any oral cancer cell lines. We also performed dose dependent cell viability by trypan blue exclusion assay of *Abrus* agglutinin in HEp2 and

we noticed dose dependent decrease of cell viability. Long term effect of agglutinin has on anti-proliferation of HEp2 has been studied by colony forming assay. DAPI showcased the DNA fragmentation ensuring pre-apoptosis which will be validated by western blotting. Acridine orange staining is not a confirmatory result for autophagy so we will investigate expression of autophagic proteins.

CONCLUSION

Chemotherapy, radiation and operation are three treatment modalities in practice for defending cancer now a days but the above three practice not fruitful and unable to give successful result. Therefore plant lectins plant lectins are one of the most promising candidates as immunomodulators and anti-cancer agents. In a semideveloped country like India which is the hub of oral cancer. Therefore the study is the novel one and *Abrus* agglutinin acts as a magic bullet for combating oral cancer and acts as a new potential weapon in 21st century.

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