Ricinus agglutinin induced Autophagy in Glioblastoma cells.

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ROURKELA, ODISHA.

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CERTIFICATE

This is to certify that the thesis entitled "***Ricinus agglutinin induced autophagy in glioblastoma cell***" which is being submitted by Ms Puja Sahoo, Roll No. 413LS2049, 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 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.

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DECLARATION

I do hereby declare that the Project Work entitled **"*Ricinus* agglutinin induced autophagy in Glioblastoma cells"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr.Sujit Kumar Bhutia, Head of Department, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

Date: 11 /05/2015

Place: Rourkela PUJA SAHOO

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At the end, I bow down my head to the almighty God for blessing me with my beloved parents and whatever I have.

Date: 11 /05/2015 PUJA SAHOO

Place: Rourkela

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LIST OF ABBREVIATIONS

et al	And others
PBS	Phosphate Buffered Saline
Rpm	Rotation Per minute
Conc.	Concentration
mg	Milligram
pH	Hydrogen ion 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 tartrate
BSA	Bovine serum albumin
KH_2PO_4	Potassium Dihydrogen Phosphate
K_2HPO_4	Potassium hydrogen phosphate
$(NH_4)_2SO_4$	Ammonium Sulphate
KD	Kilo Dalton
RA	Ricinus agglutinin
°C	Degree Celsius
mM	Milimolar
ml	Millilitre
Kg	Kilogram
gm.	Gram

ABSTRACT

Ricinus agglutinin (RA) is a heterodimer consisting of two chains known as chain A and chain B respectively. The molecular weight of RA is 120 kD. RA is highly toxic to normal cells due to its ability to bind to the carbohydrate moieties present on the cell surface and causes the agglutination of the erythrocytes. Toxicity of ricin is due to its catalytic activity on eukaryotic ribosomes. RA is also known as type II Ribosome Inhibiting Protein (Type II RIP).The purpose of our study was to see whether RA induces autophagic cell death or not .There were amazing results that proved that RA showed both antiproliferative activity and autophagic cell death when treated with Glioblastoma cells. Hence, RA could be effective in the treatment of Glioblastoma.

Keywords: *Ricinus* agglutinin, type II RIP, toxic,autophagy

INTRODUCTION

The term “Lectin” has been derived from the Latin word “legere”, which means “to select”, by William Boyd in 1954. Lectins possess the ability to bind to carbohydrates. These proteins have the capability to agglutinate red blood cells.

Lectins are mainly proteins/glycoproteins, found in a diversity of animals. It has one non-catalytic domain that binds reversibly to specific monosaccharides or oligosaccharides. They can bind to the carbohydrate moieties present on the surface of erythrocytes and agglutinate them, without altering the properties of the carbohydrate moieties.

Chromatographic technique is used to isolate lectins from their natural sources. The yield of animal lectin is low in comparison to that of plant lectins. The various roles exhibited by lectins include antitumor, immunomodulatory, antifungal, HIV-1 reverse transcriptase inhibitory, and anti-insect activities.

Lectins specific to carbohydrate specificity have been purified from various plant tissues and other organisms. The lectins accordingly can be classified on the basis of their carbohydrate specificity or can also be classified according to the overall structures into merolectins, holoectins, chimerolectins and superlectins, or can be grouped into different families such as monocot mannose-binding lectins, legume lectins, type II ribosome-inactivating proteins, and other lectins. The content of lectin varies from organism to organism. Depending upon the properties of lectins, they have a wide variety of applications such as anti-fungal, anti-insect, anti-viral, and anti-tumor and many more.Lectins are also present in seeds (Sze and Tzi, 2010).

Lectins are extremely useful in the investigation of carbohydrates moieties present on cell surfaces. Recent studies have demonstrated the function of lectins as recognition molecules in cell–molecule and cell–cell interactions in a variety of biological systems (Nathon and Halina, 2004).

James B. Sumner at Cornell University is well known for being the first to crystallize an enzyme, urease in 1926 (for which he was awarded the Nobel Prize 21 years later) also isolated a crystalline protein in its pure form and from jack bean (Canavalia ensiformis) named it as concanavalin A (Nathon and Halina., 2004).

REVIEW OF LITERATURE

Ricinus communis

A flowering plant that belongs to the family Euphorbiaceae.It is mostly found in the south eastern Mediterranean Basin, Eastern Africa and India but is widespread in the tropical regions and wastelands. It is also grown as an ornamental plant.

The systemic classification of *Ricinus communis* is as follows:

Kingdom: Plantae

Class : Magnoliopsida

Order : Malpighiales

Genus : *Ricinus*

Species : *Ricinus communis*

Figure 1: Images of the fruit and the seeds of *Ricinus communis* or castor plant.

The constituents of the castor beans are: Fatty oil, tocopherols (Vitamin E), lectins, alkaloids, proteins, ricinoleic acid, and ricin. *Ricinus communis* is commonly called as "Rendi" in Hindi. The parts of the plant that are commonly used are leaves, seeds, roots, oil, and fruit. Not only does *Ricinus communis* has medicinal values but also helps in the production of biodiesel as it is cheap and environment friendly.

Structure of ricin agglutinin

Ricin, a potently toxic substance is found in the seeds of the castor oil (Ricinus communis) plant. The term ricin was coined by Stillmark while working with the isolated toxic protein from the castor seeds (Stillmark et all, 1888).Toxicity of the ricin from castor seeds was believed due to its ability to agglutinate the red blood cells. Toxicity of ricin is due to its catalytic activity on eukaryotic ribosomes.

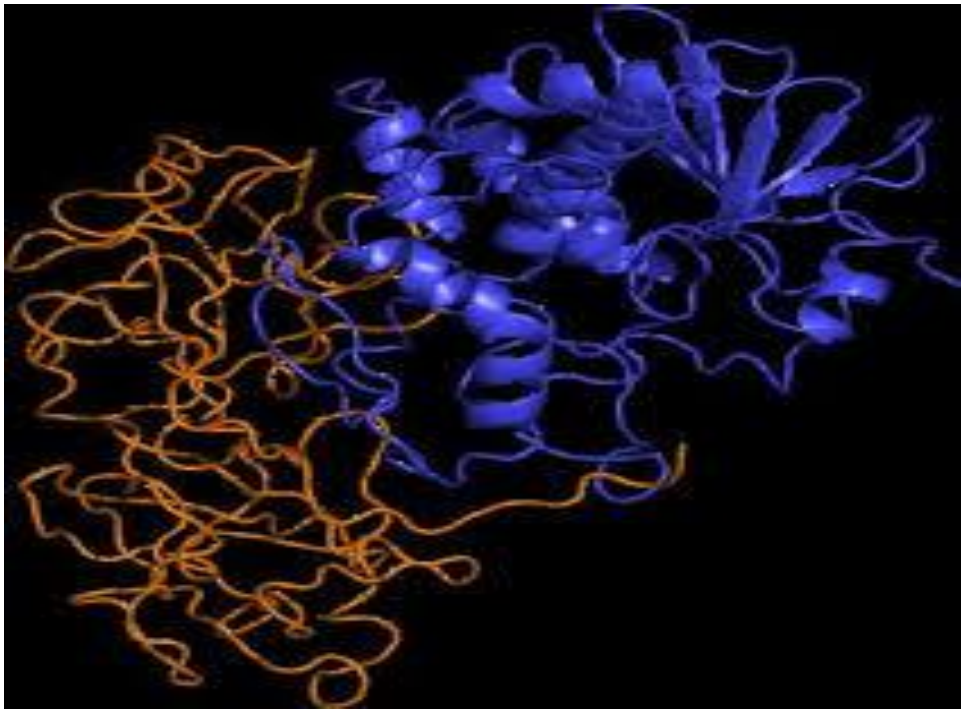

Figure 2: Structure of *Ricinus* agglutinin.

Ricinus agglutinin consists of two chain i.e., chain A and chain B.The combined molecular weight is 120 kDa. It is highly toxic due to its ability to agglutinate the red blood cells**.**These are specific in action and irreversibly inactivate the eukaryotic chromosomes inhibiting protein synthesis. Such proteins are known as ribosome inhibiting proteins (RIPs).RIPs is of two types:

(a) Type I RIPs, and

(b) Type II RIPs.

Type I RIPs are usually monomers of about 30 KDa and are frequently N-glycosylated (Lord et all, 1991). These are non-cytotoxic as they do not have any means of reaching the ribosomes in a eukaryotic cell. Plant tissues rich in Type I RIPs such as barley grain or wheat germ are consumed worldwide.

Type II RIPs are toxins that are heterodimeric as they are covalently joined to a second polypeptide through a disulphide bond. These enter the cytosol of eukaryotic cells by interacting with the galactosides present on the cell surface, inhibiting protein synthesis leading to cell death. Some of the examples of Type II RIPs are abrin, modeccin and ricin.

Ricin, a heterodimeric type II RIP is composed of a ribosome-inactivating enzyme, designated as the A chain or RTA having a molecular weight of 32 KDa and a galactose/N-acetylgalactosamine-binding lectin ,designated as the B chain or RTB and has a molecular weight of 34 KDa. Both the chains are linked by a single disulphide bond but differ in their biological properties. There exists several isoforms of ricin such as ricin D, ricin E, and *Ricinus communis* agglutinin i.e., RCA. The molecular weight of *Ricinus* agglutinin is 120 kD.

Type-I RIPs exhibit low toxicity to a whole cell system as comparison to Type-II RIP due to lack of internalization facility.

Glioblastoma is also known as grade IV astrocytoma. It is the most common and deadly form of cancer involving glial cells**.**

Autophagy

Autophagy is derived from Greek where "auto" – self and "phagia" – eating. The term autophagy originated when the Nobel laureate Christian de Duve used it while attending the *Ciba Foundation Symposium on Lysosomes*, which took place in London on February 12–14, 1963. It is an evolutionarily conserved catabolic process where a cell self digests its cytoplasmic contents (Bhutia et al., 2013; Panda et al., 2014).

The cytoplasmic constituents are delivered to the lysosome for bulk degradation (Mizushima & Klionsky, 2007; Mizushima et al., 2002). Autophagy helps recycle energy and nutrients during starvation or stress for cell survival (Bhutia et al., 2013; Panda et al., 2014).

Autophagy is classified into three main types: chaperone-mediated autophagy, micro autophagy, and macro autophagy (Yorimitsu et al., 2005).

The molecular basis of autophagy has been studied extensively in yeasts that revealed that autophagy is regulated by a number of highly conserved genes called ATGs (for AuTophaGy genes). Thirty-six ATGs have been identified in yeasts that are essential for the process of autophagy to occur. Various stimuli such as damaged organelles, cytokines, misfolded proteins, stress pathogens, and even inhibition of protein synthesis induce autophagy (Bhutia et al., 2013; Panda et al., 2014).

Autophagy plays a dual role. It can be both protective and lethal. It is essential for the removal of damaged or long-lived organelles and proteins to prevent susceptibility to defects such as tumorigenesis, genome damage, and metabolic stress. Autophagy, besides being a tumor suppressor can also lead to stress tolerance, which allows tumor cells to survive under unfavourable conditions. The role of autophagy in stress tolerance hinders successful cancer therapy (Bhutia et al., 2013).

Autophagy involves the formation of autophagosomes and autolysosomes.Autophagosome is a double-membrane cytoplasmic vesicle that engulfs cytoplasmic organelles along with other cellular components. Fusion of the autophagosomes with lysosomes form autolysosomes. All the cellular materials are digested within the autolysosomes (Mizushima et al., 2002).

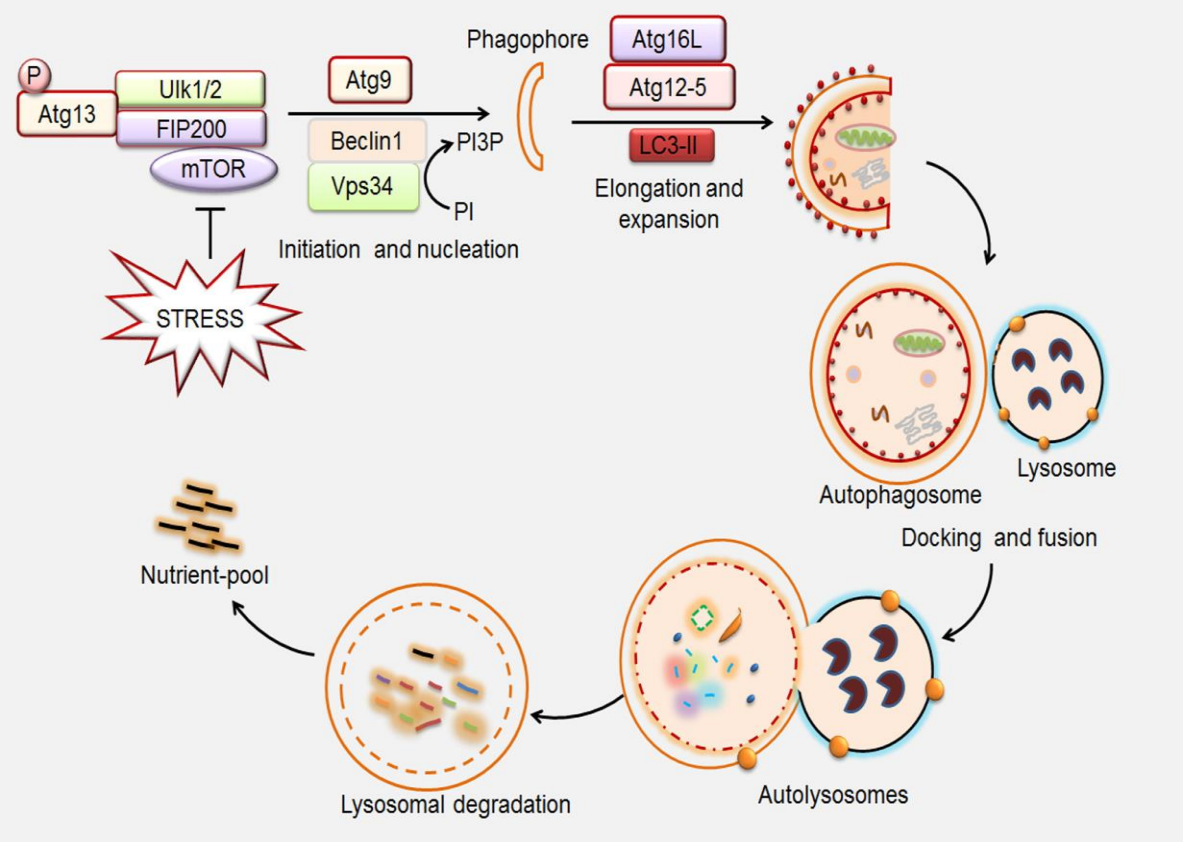

Figure 3: Schematic representation of the autophagy process.

Phagophore nucleation is the first step of autophagy. During stress the cellular stress sensor i.e. mammalian target of rapamycin (mTOR) gets inactivated and results in the in hypophosphorylation of Atg13 .Atg13 binds to Atg1 with the help of Atg7.The newly formed phagophore gets elongated and matured in the presence of class III phosphoinositide (PI) 3-kinases, such as vesicular protein sorting 34 (Vps34) that interacts with Beclin-1 and harvests phosphatidylinositol-3-phosphate (PI3P) (Simonsen and Tooze., 2009).

Mature autophagosomes fuse to lysosomes and form autolysosomes, where the cytoplasmic contents and the cell organelles are degraded into the acidic lysosomal compartment supplying energy and essential nutrient to the cells to face adverse stress conditions (Bhutia et al., 2013 and Kimura et al., 2008). Autophagy plays a protective role during stress conditions but can also be lethal during extreme stress conditions leading to autophagic cell death (Ouyang et al., 2012 and Bhutia et al., 201

OBJECTIVES:

1. Isolation and purification of *Ricinus* agglutinin (RA) from the seeds of *Ricinus communis*.

2. Characterisation of the protein-

    - Haemagglutination (HA) assay.
    - NATIVE-Polyacrylamide Gel Electrophoresis (Native-PAGE).

3. Study of anti-proliferative activity of RA on Glioblastoma cells via MTT assay.

4. Study of autophagic cell death induction by RA through-

    - Acridine orange staining.
    - GFP-LC3 transfection.
    - Inhibitory effect of Chloroquine and 3MA on Glioblastoma cells treated with RA.

MATERIALS AND METHODS:

REAGENTS:

Sodium Phosphate monobasic dehydrate ($NaH_2PO_4.2H_2O$), Sodium Phosphate dibasic dehydrate ($Na_2HPO_4.2H_2O$) were purchased from SRL, Sysco Research Laboratories Pvt. Ltd. Mumbai. Sodium chloride (NaCl) was purchased from Fischer scientific. Bovine serum albumin (BSA) was purchased from Himedia, Mumbai, India. Sodium hydroxides (NaOH), Sodium carbonate (Na2CO3), Sodium potassium tartarate (KNaC4H4O6), Copper sulphate (CuSO4) were bought from Himedia, Mumbai, India. Folin-Ciocalteau phenol reagent purchased from Himedia, Mumbai. Acrylamide, Bisacrylamide, Ammonium per sulphate (APS), N, N, N', N'-tetra methylene diamine (TEMED), Ammonium sulphate $(NH_4)_2SO_4$, and Tris were purchased from Sigma Aldrich, USA. Bromo phenol blue, acetic acid, Glycerol was purchased from RANKEM Pvt Ltd.β-mercaptoetanol blue was purchased from Pre- stained molecular weight marker was purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from Nice chemicals Pvt. Ltd. India. Ethanol purchased from Trimurty Chemicals of India.

CELL LINE:

T98G (glioblastoma) was cultured in Modified Eagle Medium (MEM), and HaCaT (human keratinocyte cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with antibiotic-antimycotic and 10% fetal bovine serum. All the cell lines were purchased from the National Centre for Cell Science, Pune, India.

1. Isolation of lectin from *Ricinus* seeds

REMOVAL OF SEED COAT:

About 300 gm. of *Ricinus communis* seeds were taken and grinded in a mixer so as to remove their seed coat. The decoated seeds were soaked in PBS for 12hrs at 4°C.The next day the seeds were grinded into a fine paste. The grounded paste was collected in 500 ml centrifuge bottles and centrifuged at 7500 rpm, 4°Cfor 20 minutes and the supernatant was collected. The supernatant was centrifuged again at 5000 rpm, 4°C for 25 minutes to eliminate the undesired

pellet .About 2 ml from the supernatant was taken and stored at 4°C for estimation of proteins and haemagglutination assay and the remaining supernatant was used for the process of salting out (Figure 4).

AMMONIUM SULPHATE FRACTIONATION OR SALTING OUT:

The solubility of proteins is affected by ions. The solubility of the proteins decrease with an increase in the ionic strength and this process is known as "salting-out". When the concentration of the salt is increased, the water molecules are attracted by the salt ions, which leads to a decrease in the number of water molecules available to interact with the charged part of the protein resulting in an increased demand for solvent molecules, the protein molecules coagulate by forming hydrophobic interactions with each other. It is the basis of protein purification procedures.

Ammonium sulphate $(NH_4)_2SO_4$ is mostly used for the proteins to salt out. It is highly soluble in water, is not affected by temperature, and has no harmful effect on the proteins. The supernatant collected from the crude extract of *Ricinus* seeds was taken for 30% cut-off. The required amount of ammonium sulphate was weighed by following the salt chart and was grinded into a fine soft powder with the help of mortar and pestle. The fine amorphous powdered salt was added to the supernatant pinch wise and the solution was stirred continuously to ensure that the salt dissolves well. After the completion of the addition of the salt, the sample was incubated overnight in 4°C .The sample was centrifuged again the next day and the supernatant was taken for the next cut-off i.e., 90% cut off. The procedure for 90% cut-off was similar to that of the 30% cut-off. The incubated 90% cut-off was centrifuged and the pellet was collected. The pellet was dissolved using minimal amount of PBS and was prepared for dialysis (Figure 4).

DIALYSIS:

Dialysis helps in the separation of the dissolved molecules via semi-permeable membrane. The semi-permeable membrane contains pores that allows smaller molecules, such as salts and solvents to diffuse across the membrane but the diffusion of the larger molecules is restricted. Cellophane, also known as cellulose acetate is commonly used semi-permeable membrane for dialysis. Other substances such as collodion or nitrocellulose can also be used.

It is a method in which an aqueous solution containing both small and large molecules are placed in a dialysis bag made out of the cellulose acetate membrane. The dialysis bag in turn is placed in a large vial containing either distilled water or buffer. The smaller molecules will easily diffuse out of the membrane. After sometime the concentration of the smaller molecules present inside the bag will be same to that present outside the bag whereas the macromolecules still remain inside the dialysis bag. During dialysis the fluid containing the dialysis bag must be changed at regular intervals of time.

The pellet obtained from 90% cut-off was dissolved in PBS and taken for dialysis. It was kept in dialysis for 3 days. First two days the sample was dialyzed against water and the third day against PBS.One important thing to be taken care of while dialysis is that the dialysis bag containing the sample should immerse completely in water or PBS being used. Upon completion of dialysis the sample was centrifuged at 7500 rpm, 4°C for 20 minutes. The supernatant was collected and filtered using Whatman filter paper followed by filtration using a 0.22µ syringe filter. The filtered supernatant was stored in -20°C to be used for affinity chromatography (Figure 4).

AFFINITY CHROMATOGRAPHY:

Affinity chromatography technique is used for the purification of a molecule of interest from complex mixtures. It is based on highly specific reversible biological reactions between two molecules such as enzyme and substrate, receptor and ligand, or antibody and antigen. In this technique the affinity ligand or the interacting molecule is covalently bound onto a solid matrix, referred as the stationary phase while the target molecule in the mobile phase (Urh et al., 2009). The matrix must be chemically inert, porous, and have a variety of functional groups suitable for coupling with diverse ligands. Ligands used in affinity chromatography, depends on the protein to be purified (Voet & Voet., 1995). The major advantage of this process is that it significantly saves time and provides higher purification (Urh et al., 2009).

Affinity chromatography technique using carbohydrate ligands or matrices for the purification of lectins (or glycan-binding proteins) is known as carbohydrate affinity chromatography. These lectins bind non-covalently and reversibly to the carbohydrate moieties so that the lectin is released from the column by competitive elution using specific free carbohydrates (Varki et al., 2008).

The filtered and stored dialyzed sample was used for affinity chromatography by Akta prime. The column used was Sephadex G100 that had been prepared previously. The Sephadex G100 column was thoroughly washed with PBS and 20% ethanol solution. After washing with PBS and 20% ethanol the dialyzed sample was loaded onto the column. After finishing the sample the column was washed again with PBS.The protein was eluted with 10 ml of 0.2M of lactose solution. The eluted protein was stored at 4°C for lyophilisation (Figure 4).

DIALYSIS:

The eluted protein sample was stored at 4°C and was dialyzed against PBS for 24 hours (Figure 4).

Estimation of Protein concentration by Lowry's method

Principle:

Under alkaline conditions the copper [II] ions reacts with the peptide nitrogen[s] and reduces to a monovalent ion.The monovalent copper ions and the aromatic amino acids (tyrosine, tryptophan, and cysteine) reduce the Folin-Ciocalteau phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue. The blue colour complex formed gives a maximum absorption in the region of 660 nm wavelength.

Protocol:

The concentration of crude, 30% cut-off, 90% cut-off and 90% cut-off affinity was estimated by Lowry's method. Bovine serum albumin (BSA) was used as the standard protein.
The reagents required:
1. BSA stock solution (1 mg/ml)
2. Analytical Reagents
Reagent A: Sodium hydroxide (0.5%)
Sodium carbonate (2%)
Reagent B_1: 1% copper sulphate
Reagent B_2: 2% sodium potassium tartarate
The analytical reagents were prepared by mixing 1ml of both reagent B_1 and B_2 and 100 ml of reagent A.

3. Folinciocalteau reagent: 1N of this reagent was prepared by mixing equal volume of the reagent and water i.e., 5 ml of the reagent was mixed with 5 ml of distilled water.

Different dilutions of BSA stock solutions were prepared. ml of protein sample was taken to which 2ml of Lowry's reagent was added, mixed and then kept for 15 minutes incubation. Then 200 µl of Folin's reagent was added to it and incubated for 30 minutes. O.D was taken at 595 nm. The concentration of the unknown protein was plotted in a graph taking absorbance in the Y-axis and concentration in the X-axis (Figure 5).

Haemagglutination assay:

Principle:

Lectins are well known for their ability to agglutinate the erythrocytes i.e., Human RBC's. The lectin isolated from *Ricinus* forms a mesh like structure in between the blood erythrocytes which does not allow the blood to agglutinate. Higher is the concentration of lectin more will be the agglutination.

Protocol:

1. About 2 ml of human blood from different blood groups was collected from Common Welfare Society Hospital, Rourkela in a 15 ml falcon tube and EDTA was added to it.
2. About 1 ml of blood was taken and centrifuged at 1000 rpm for 5mins at room temperature. The serum was discarded and pellet was collected. About 10 ml of PBS was added to the pellet and centrifuged again for 5 mins at 1000 rpm in room temperature.
3. The supernatant was discarded and the pellet was collected. About 200 µl of the pellet was taken and mixed with 10 ml of PBS. This was the final blood sample for the assay.

The haemagglutination assay was carried out in 96 well round bottom microtitre plate. The first well of each row served as positive control and the last well as negative control .The positive control contains sample and RBC's whereas the negative control contained PBS and RBC's. Individual wells were provided with 100µl of PBS and then the protein sample was added and serially diluted till the negative control. About 100µl of prepared blood was added to each of

the well. Care should be taken so that the final volume of each well should be 200µl (Figure 6).

NATIVE–polyacrylamide gel electrophoresis (Native-PAGE)

Principle:

Electrophoresis is one of the most common methods for the analysis of biomolecules such as proteins and nucleic acids (RNA OR DNA). The term electrophoresis describes the migration of a charged particle under the influence of an electric field.

Native-PAGE involves the electrophoresis of proteins in the absence of SDS.The mobility of the proteins not only depends upon the charge-to-mass ratio but also on the primary amino acid sequence and the pH of the running buffer used during electrophoresis. Each protein has an isoelectric point and molecular weight particular to its primary structure.

Protocol:

Stacking gel (5%) of pH 8.8 and resolving gel (6%) of pH 6.8 were prepared respectively. The native gel loading dye (5x) contained 1M Tris, 100% glycerol, β-mercaptoetanol, and bromophenol blue. Electrophoresis was carried out until the marker i.e., bromophenol blue reached the bottom of the resolving gel. The stacking gel was run at 90v and resolving gel at 140v.The electrophoretically separated proteins on polyacrylamide gels were detected by silver staining (Figure 7).

Cell survibility Assay MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide)

Principle:

MTT assay is a colorimetric assay which measures the reduction of yellow coloured 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple coloured formazan product by the enzyme succinate dehydrogenase present in mitochondria. MTT upon entering the cells passes into the mitochondria and gets reduced to an insoluble, dark purple coloured formazan product only in a live cell. It is insoluble in water so needs to be solubilised by DMSO (an organic solvent), it imparts purple coloured with the solubilisation of formazan. Then the absorbance of the solution is measured spectrophotometrically at 500- 600 nm.

Protocol:

The cells (T98G and HaCaT) were sub cultured and when cells were80-90% confluent, they were trypsinized. The cells were seeded in 96 well plates. After 24 hours, the cells were treated with different concentrations of ricin agglutinin (RA) and incubated for 72 hours. . The efficacy of RA on the viability of various cancer and normal cell lines was determined using MTT dye reduction assay by measuring the optical density at 595 nm using a micro-plate reader spectrophotometer (Perkin-Elmer) (Bhutia et al., 2008). The control group was treated with PBS that was used to dissolve RA (Figure 8).

Measure of RA induced autophagy via Acridine orange staining

Principle:

Acridine orange enters acidic compartments such as lysosomes and become protonated and sequestered. At low pH, the dye will emit orange light when excited by blue light. Thus, acridine orange can be used to identify engulfed apoptotic cells, because it will fluoresce upon engulfment.

Protocol:

The development of acidic organelles were quantified by seeding T98G cells in a 6-well plate and incubated with RA for 24 h. The cells were treatment with various doses of RA for 24 h and then stained with 10 mM acridine orange at 37 °C in the dark for 15 min followed by washing twice with PBS. Images of acridine orange staining were taken immediately using a fluorescence microscope (Olympus IX71) (Bhutia et al., 2010) (Figure 9).

Measurement of RA induced autophagy by GFP-LC3 transfection

Principle:

The study of autophagy has led to the discovery of certain proteins that can be used as molecular markers that are useful in the study of the autophagic structures more precisely under a fluorescent microscope.LC3 is a commonly used marker for the study of autophagy.LC3 is present in both the inner and the outer membrane of the autophagosome.There are two forms of LC3 i.e., LC3I and LC3II.During the process of autophagy the concentration of LC3II increases within the cell.

Protocol:

T98G cells were transfected with pEGFP-LC3 (Addgene plasmid 11546, Jackson et al., 2005) using Lipofectamine 2000 reagent® (Gibco) according to the instruction of the manufacturer. After 48 h of transfection, T98G cells were treated with different doses of RA for 24 h, and the level of autophagy was estimated by counting the mean number of puncta that displayed intense staining (Bhutia et al., 2010) (Figure 10).

Effect of autophagic inhibitors on RA treated cells.

Autophagy inhibitors could be a new approach in the treatment of cancer. These can be classified into two broad categories:

- Early stage inhibitors ,and
- Late stage inhibitors.

3-methyadenine (3-MA) inhibits the early stage of autophagy by inhibiting the recruitment of class III PI3K (Vps 34) to the isolation membrane (Figure 11).

Chloroquine (CQ) is a late stage autophagic inhibitor preventing autolysosomal formation. (Boya et al., 2005) (Figure 12).

RESULTS AND DISCUSSION

1. Isolation of RA

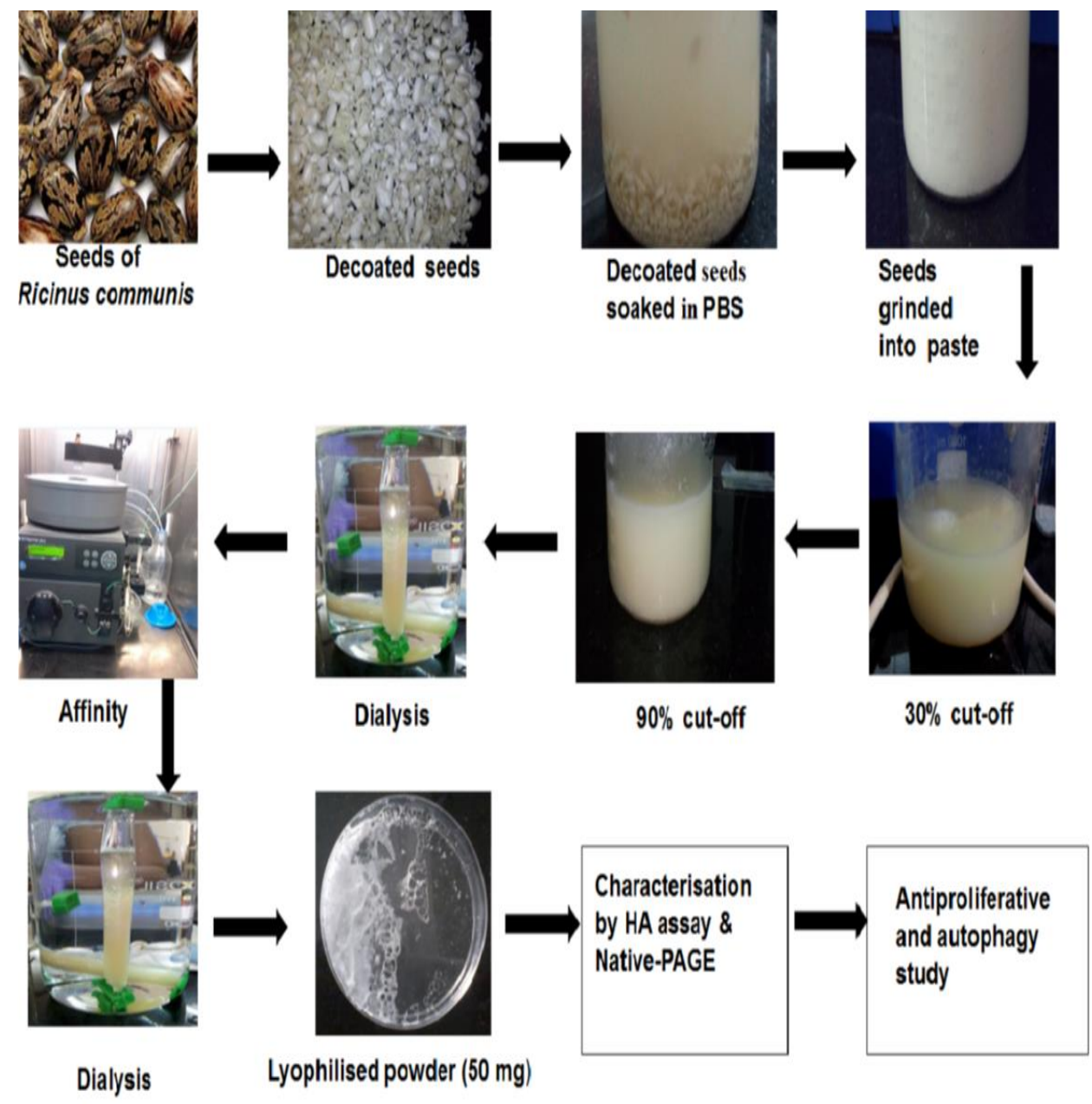

Figure 4: Schematic representation of isolation, purification, characterisation as well as anticancerous activity of *Ricinus agglutinin.*

2.Purification of RA by affinity chromatography

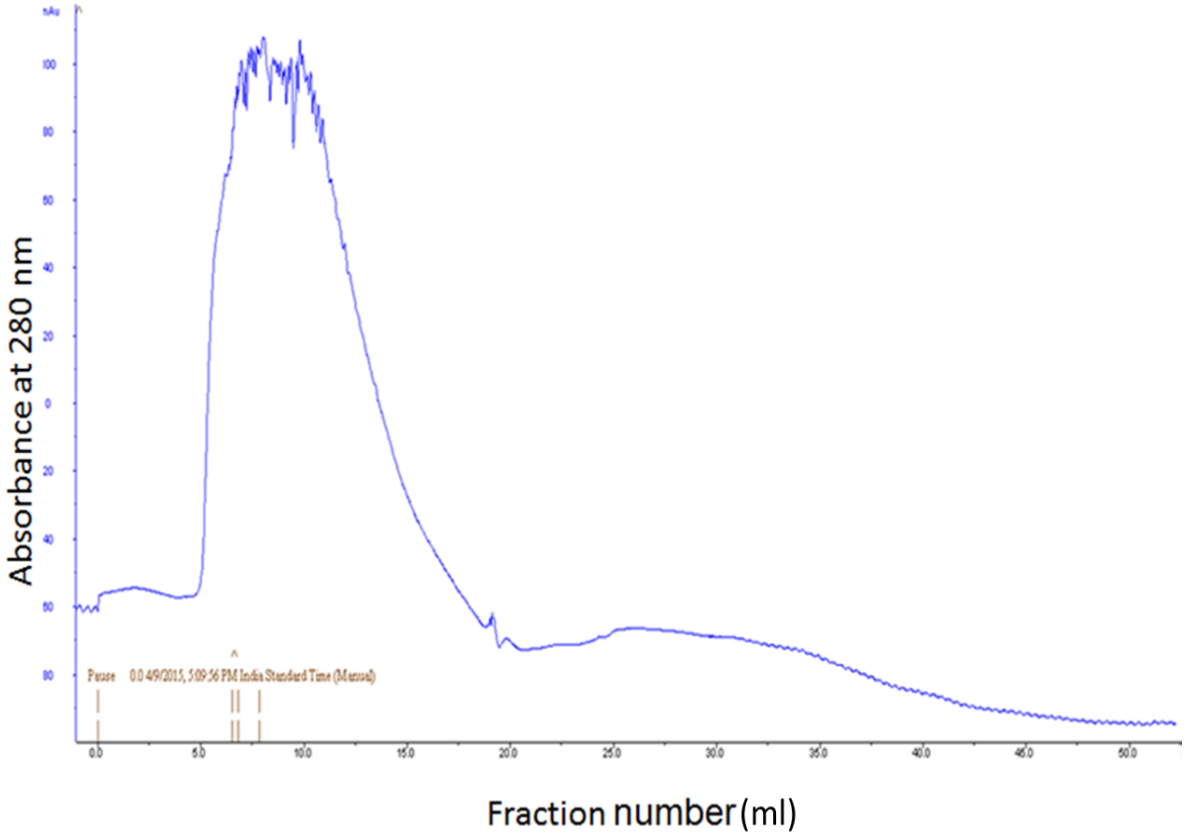

Figure 5: Affinity chromatography peak using lactamyl Sephadex G-100 column.

INFERENCE

The isolated protein is purified by affinity chromatographyusing lactamyl Sephadex G100 column and the graph of the eluted protein is prepared. This peak shows that the protein elution.

.

3. Estimation of protein concentration by Lowry's method

Table No-1

SAMPLE	OD at 595 nm	Concentration (mg/ml)
Crude	199.1	100.25
30%	160.7	80.28
90%	2.19	1.45
Affinity	1.09	0.786

INFERENCE

The eluted protein concentration check was done by Lowry's method and was found to 50mg when calculated with total volume and this was collected by lyophilisation and is further used.

4. Haemagglutination (HA) assay

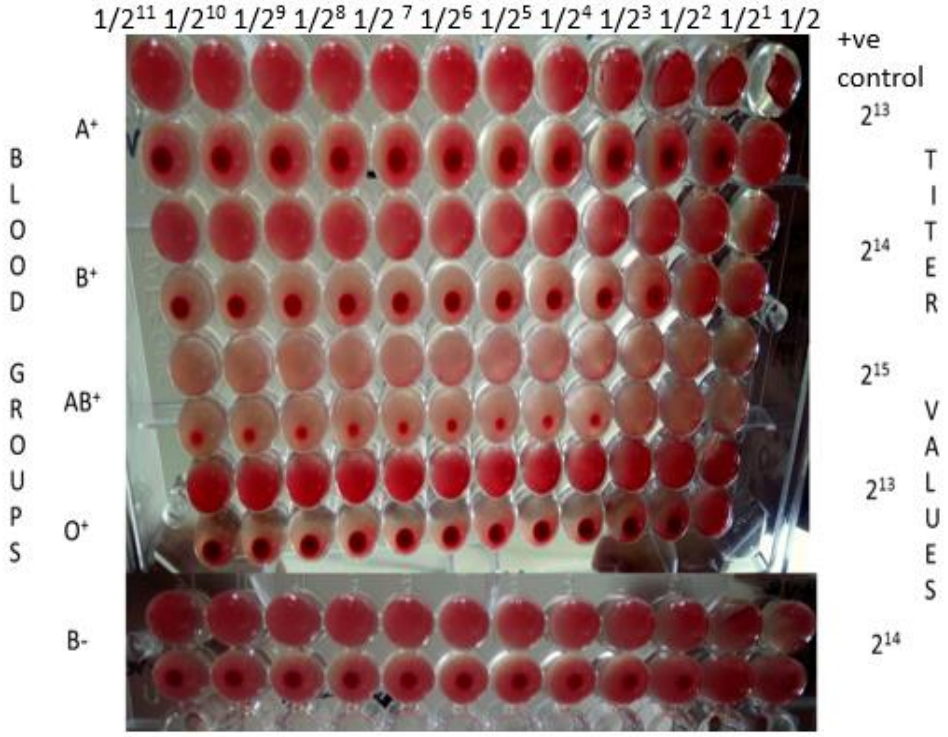

Figure 6: Haemagglutination assay of *Ricinus* agglutinin in different blood groups.

Titre value of blood groups:

Table No-2

Blood Group	Titre value
A^+	**2^{13}**
B^+	**2^{14}**
AB^+	**2^{15}**
O^+	**2^{13}**
B^-	**2^{14}**

Inference:

The isolated protein has the capability to agglutinate different blood groups which proves that the isolated protein is a lectin.The AB^{+} blood group has the highest titre value.

5. Native-PAGE analysis of RA

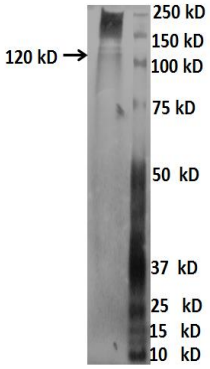

Figure 7: Native-PAGE analysis of RA.

Inference:

The molecular weight of our interested protein i.e., RA was found to be 120 kD.

6. Estimation of antiproliferative activity of RA using MTT assay

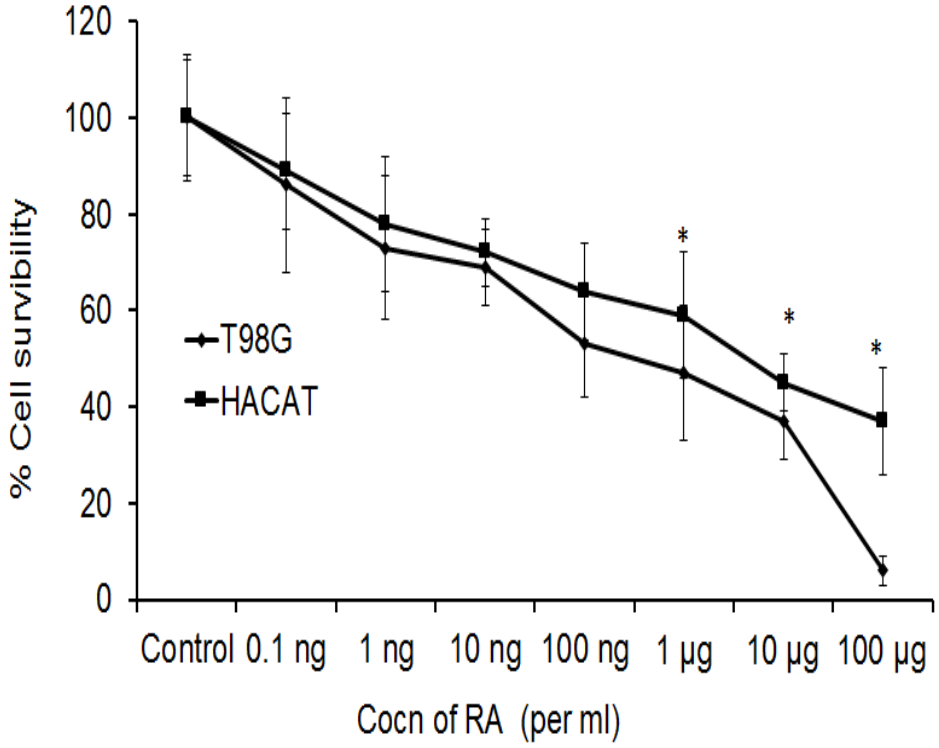

Figure 8: Antiproliferative activity of RA on both Glioblastoma cell line and the normal cell line.

IC_{50} value of T98G cells and HaCaT cells:

Table No-3

Cell line	IC_{50} value (µg/µl)
T98G	0.5
HaCaT	10

Inference:

Ricinus agglutinin showed antiproliferative activity in dose wise in T98G Glioblastoma cell line. However it showed less cytotoxicity in normal human keratinocyte cell line (HaCaT). The IC_{50} of T98G and HaCaT is 0.5 and 10 (µg/µl) respectively.

7. Study of RA induced autophagic cell death

(a)

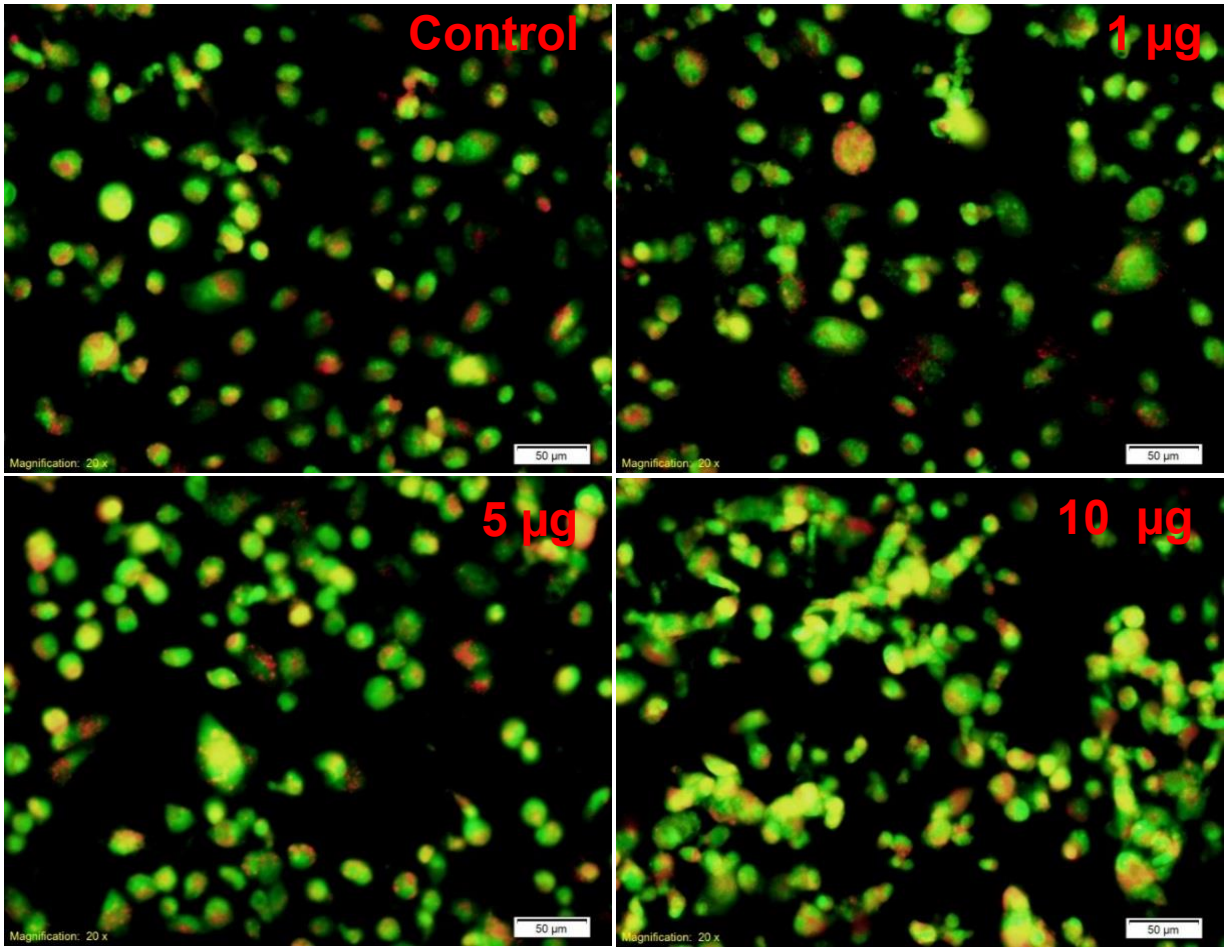

Figure 9: Acridine orange staining:

Inference:

With an increasing concentration of RA (dose-dependent) in T98G cells ,autophagic cell death also increased.T98G cells treated with 10 µg of RA showed occurrence of highest autophagy.

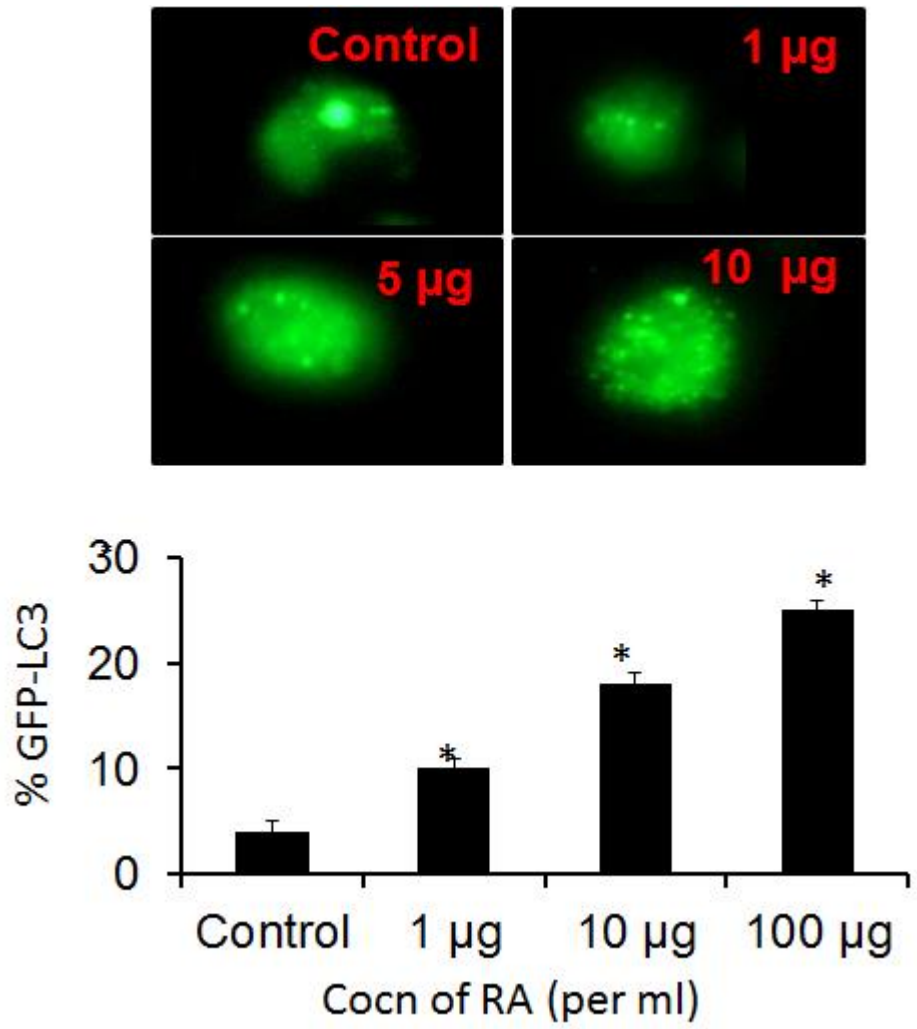

Figure 10: GFP-LC3 transfection study

Inference: No of GFP-LC3 puncta increases in increasing doses of RA. The above result clearly depicts RA induces autophagy in dose dependent manner in Glioblastoma cells.

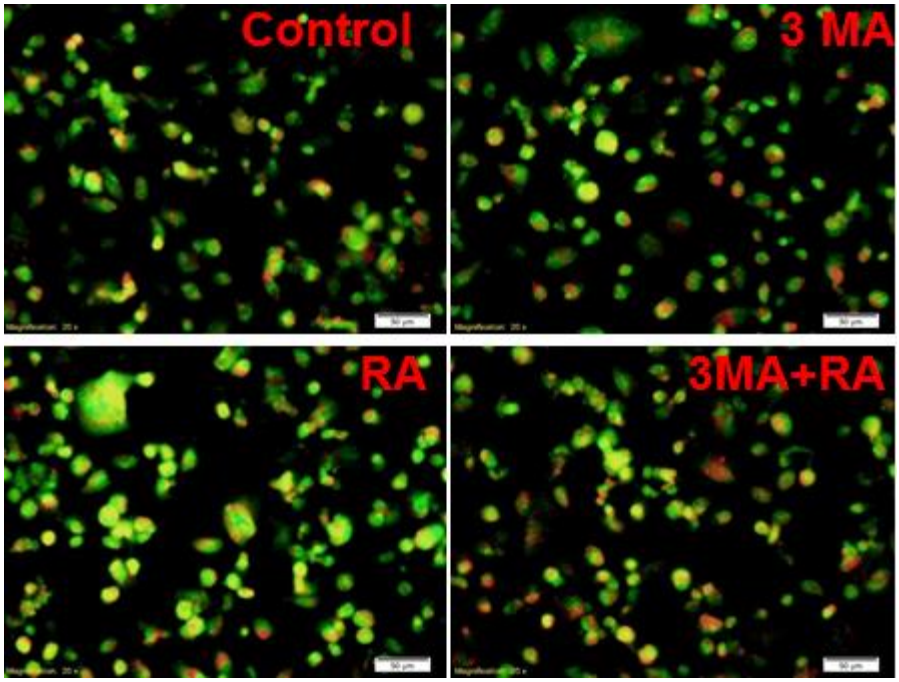

Figure 11: Inhibition of autophagy using early stage inhibitor.

Inference: 3-methyadenine (3-MA) is an early stage inhibitors of the autophagic pathway that target class III PI3K(Vps34) recruitment to the isolation membrane which confirms occurrence of autophagy induced by RA.

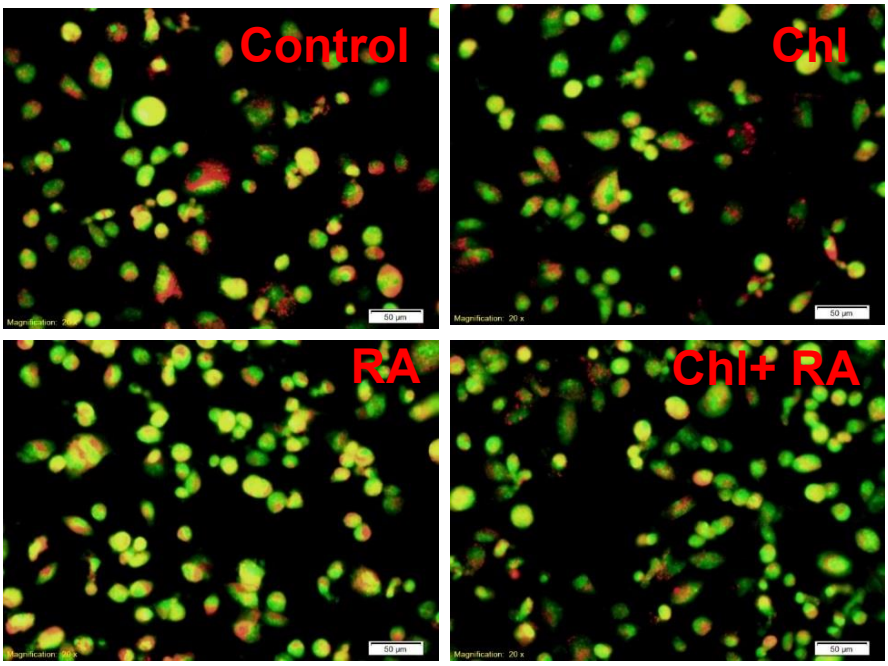

Figure 12: Autophagy inhibition by late stage inhibitor chloroquine(CQ)

Inference: Chloroquine (CQ) is a late stage inhibitor which prevents autolysosomal formation and interestingly above result confirms RA induced autophagy in Glioblastoma cell line.

DISCUSSION:

The important part of the project was to isolate the *Ricinus* agglutinin from the seeds of *Ricinus communis* and to see its antiproliferative activity against the cancerous cells. *Ricinus* agglutinin was isolated and purified by affinity chromatography using lactamyl Sephadex G-100 column.Hemagglutination assay further confirmed that the isolated protein was of our interest as it could agglutinate the erythrocytes. Native-PAGE was performed to determine the molecular weight of our protein and the molecular weight was found to be 120 kD. Reports are available other lectin like ConA and SBL induced autophagy (Chang et al; 2007 and Panda et al; 2014) .

The antiproliferative activity of RA was studied via MTT assay however it showed less toxicity toward human normal keratinocyte cell line (HaCaT).Induction of autophagic cell death by RA was studied by Acridine orange staining process, GFP-LC3 transfection and also in the presence of the autophagic inhibitors such as 3MA and CQ.This is the first report *Ricinus* agglutinin induced autophagy and also the first report induction of autophagy in Glioblastoma cells.

Conclusion:

From the above experiments performed it can be concluded that RA is successfully isolated and purified from seeds of *Ricinus communis* by affinity chromatography using Sephadex G 100 column. Haemagglutination assay showed that these proteins could agglutinate the human erythrocytes due to the presence of carbohydrate binding site. Native page gave the molecular weight of the protein and confirmatory result of presence of protein by silver staining. Treatment of *Ricinus* agglutinin on T98G cells reveals the dose dependent cell death of cancerous cells. The number of viable cancer cells is decreasing with increase in the concentration of the drug. *Ricinus* Agglutinin from *Ricinus communis* belongs to ribosome inactivating proteins-II (type II RIP) that needs many more applications to prove the anticancerous activity of *Ricinus* agglutinin. Autophagy inducing potential of plant lectin *Ricinus* agglutinin could be used as a substitute to fight against apoptosis resistant deadly crabs' disease. It could be used as a golden bullet in the treatment of Glioblastoma in current future.

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