Mechanism of tumor inhibitory potential of *Abrus* agglutinin in oral squamous cell carcinoma

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Mechanism of tumor inhibitory potential of *Abrus* agglutinin in oral squamous cell carcinoma

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This is to certify that the work presented in the dissertation entitled, "Mechanism of tumor inhibitory potential of *Abrus* agglutinin in oral squamous cell carcinoma" submitted by Niharika Sinha, Roll Number 511LS105, is a record of original research carried out by her under our supervision and guidance in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Life Science. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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Declaration of originality

I, Niharika Sinha, Roll Number 511LS105 hereby declare that this dissertation entitled "Mechanism of tumor inhibitory potential of *Abrus* agglutinin in oral squamous cell carcinoma" presents my original work carried out as a doctoral student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections "Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation. I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

April 1, 2017 Niharika Sinha

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Dedicated to the budding scientists working on oral cancer prognosis and diagnosis for the complete eradication of the disease

April 1, 2017

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Appendix A: List of symbols

AGG	*Abrus* agglutinin
ATM	Ataxia telangiectasia mutated serine-protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CSC	Cancer stem cell
CO_2	Carbon dioxide
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetly sulphoxide
DNA	Deoxyribonucleic acid
DHE	Dihydroethedium
DHR123	Dihydrorhodamine 123
DSB	Double-strand break
ECL	Enhanced chemiluminiscence
EDTA	Ethylene diamine tetra acetate
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
h(s)	Hour(s)
HHV	Human herpes virus
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
kDa	Kilo Dalton
kg	Kilogram
L	Liter
MEM	Minimal essential medium
mg	Milligram
min	minute
ml	Milliliter
mM	Millimolar
MMP	Mitochondrial membrane potential
MnSOD	Manganese-dependent superoxide dismutase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium
NaCl	Sodium chloride
NAC	N-acetylcysteine
°C	Centigrade
OD	Optical density
OSCC	Oral squamous cell carcinoma
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PBST	Phosphate buffered saline with Tween-20
PI	Propidium iodide
PUMA	P53 upregulated modulator of apoptosis
Rh 123	Rhodamine123
RIP II	Ribosome inhibiting protein
ROS	Reactive oxygen species
Rpm	Rotation per minute

s.c.	Subcutaneous
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) amino methane
v/v	Volume/volume
w/v	Weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

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CONTENTS

Abstract

Abrus agglutinin (AGG), isolated from *Abrus precatorious*, a medicinal plant induces antitumor activity in oral squamous cell carcinoma *in vitro* and *in vivo*. P53 being mutated in oral cancer, the focus of our study represented to p73, the sibling of p53 and its regulation in DNA damage mediated programed cell death in AGG treated FaDu cells. AGG effectively inhibited the cell viability of different oral squamous cell carcinoma with IC_{50} value 1-10 µg/ml. AGG selectively inhibited growth and, caused cell cycle arrest and mitochondrial apoptosis through reactive oxygen species (ROS) mediated ATM-p73 dependent pathway in FaDu cells. AGG-induced ROS accumulation was identified as chief mechanism of its effect on apoptosis, DNA damage and DNA-damage response which significantly reversed by ROS scavenger N-acetylcysteine (NAC). Moreover, AGG found to interact with mitochondrial manganese-dependent superoxide dismutase which might inhibit its activity and upshot ROS in FaDu cells. Further, AGG was found to inhibit epithelial-mesenchymal transition (EMT) in p73 dependent manner in epithelial growth factor (EGF) stimulated FaDu cells. Importantly, AGG induced Snail degradation through ubiqutination and Snail overexpression rescued suppression of EMT phenotypes. Confocal imaging and immunoprecipitation data elaborated about the Snail interaction with p73 in EGF stimulated FaDu cells and AGG found to inhibit the interaction of Snail and p73 through Snail degradation. In addition, our work demonstrated the efficiency of AGG on cancer stem-like cells which has high tumorigenic capacity in tumor population. We showed that AGG has a potential role as an integrative therapeutic approach for combating oral cancer by eliminating self-renewal capacity accompanied with apoptosis in orospheres of FaDu cells. Importantly, AGG induced ROS accumulation in orospheres and pretreatment of NAC inhibited AGG mediated caspase-3 activity and β-catenin expression. The present study provided deep insight into the mechanism of AGG-mediated tumor inhibition and elucidated the further scope for the development of cancer therapeutics against oral squamous cell carcinoma.

Keywords: Oral cancer, apoptosis, p73, ROS, DNA damage, Snail, orospheres

Chapter 1

Introduction

1.1. Introduction

India has one of the highest incidences of oral cancer in the world. The Indian subcontinent is the global epicenter of oral cancer where it is the most common cancer of males and the fifth most common in females (Coelho et. al., 2012). One of the most common types of head and neck cancer is oral squamous cell carcinoma (OSCC) and is diagnosed worldwide with relatively low 5-year survival rates. 90 % of presentations among all oral mucosal origin are squamous cell carcinoma (SCC). OSCC most commonly occurs at the lips, tongue, salivary glands, gums, oropharynx gingival, soft palate, roof or floor of the mouth, buccal surfaces and other intra-oral locations (Mehrotra et. al., 2006; Aruna et. al., 2011). The conventional practices of betel quid chewing especially tobacco and other risk factors have showed high prevalence of oral cancer due to the influence of carcinogens and region-specific epidemiological factors. These include genetic predisposition, immunodeficiency, diet and viral infections, e.g. HPV (human papillomavirus) and HHV (human herpes virus) (Goon et. al., 2009). Tobacco carcinogens get accumulated in mitochondria upshooting the increased reactive oxygen species (ROS) productions and DNA damage. In the multidisciplinary armamentarium against OSCC, the most suitable treatment approaches; chemotherapy plays a vital role alongside surgery, radiotherapy, biological therapy are the predictable treatment options available for oral cancer patients at both primary and late stage of the cancer. Despite of efficacy in broad range of malignant tumorigenesis, chemotherapeutic agents' usefulness is limited due to acquired resistance and severe side-effects. Consumption of vegetables and fruit-based diet has remarkably reduced the overall risk of cancer proven from various epidemiological studies and animal experiments (Lanou and Svenson, 2011). Therefore, numerous preclinical and clinical revisions have also focused on the documentation of non-toxic phytochemical and authenticating their worth against numerous tumors. The lectins derived from plants are universal in diverse plant classes, are extremely different and non-toxic. They belong to proteins that are of non-immunogenic origin and have specificity towards carbohydrate moieties. These phyto-derived proteins are of great attention to biomedical research in cancer associated glitches. Plant lectins modulate plethora of signaling pathways involved in p53, caspase family, Bcl-2 family, PI3K/Akt, Ras-Raf, ERK, BNIP3 and ATG families in cancer through apoptosis and autophagy.

Stillmark first described the lectins in 1888 by working with castor bean extracts (Gorakshakar et al., 2016). Plant lectins are found in diverse organisms, with potent biological activity, and belong to a unique group of proteins and glycoproteins. The diversification in terms of structures, functions and dynamic properties of carbohydrates allow lectins to play a crucial role against cancer cell proliferation. Phytohemagglutinins are a non-immune group of proteins with carbohydrate-binding glycoproteins and potent biological activity. They possess the ability to agglutinate erythrocytes preferentially agglutinate and aggregate malignant cells with known carbohydrate specificity since they have at least one non-catalytic domain that binds reversibly to specific monosaccharides or oligosaccharides (Liener et. al., 1976). They are ubiquitous as they mark their presence in foods like banana, pea, lentil, soybean, mushroom, rice, wheat, corn, tomato, potato, peanut, kidney bean. Thus, dietary intakes by humans play a significant role. Some lectins are tenacious to heat and proteolytic enzymes; henceforth, resist digestion, overcomes gut passage and easily move to bind gastrointestinal cells and/or can enter the circulatory system intact. Lectins thwart cancer cell growth as upon ingestion sequesters the available body pool of polyamines. Lectins might find noticeable practical applications in a diversity of activities including inhibition of viral enzymes, immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), and α- and β-glucosidases, antitumor, immunomodulatory, antifungal and anti-insect activities. Moreover, antibacterial and anti-nematode activities are also exhibited by a small number of lectins (Lam et. al., 2011). A number of lectins are used as therapeutic agents since they favorably bind to cancer cell membranes or their receptors, causing cytotoxicity, inhibition of tumor growth via programmed cell death-apoptosis, autophagic cell death possessing anticancer properties *in vitro, in vivo,* and in human case studies. Lectins inhibit protein synthesis by binding to ribosome. They amend the non apoptotic G1 phase accumulation mechanisms of cell cycle by inducing, G2/M phase cell cycle arrest and apoptosis, and can trigger the caspase cascade. They distress the immune system by oscillating the production of various interleukins, or by activating certain protein kinases. Lectins bind to the T-cell receptor and presumably appear mitogenic complex and activate lymphocytes. Amongst the innumerable identified lectins, major lectins that have attracted the research repertoire are; leguminous lectins (Con A, S. flavescens, Phaseolus coccineus L), plant enzymes [like the type 2 ribosome inactivating proteins, *Abrus* agglutinin, RIP Mistletoe lectins and the class I chitinases] and GNA-related

lectins [Garlic associated lectins; Ophiopogon japonicus lectin, Liparis noversa lectin and Polygonatum cyrtonema lectin] (Chan et. al., 2012). Plant RIPs are classified into three main categories according to the physical composition and characteristics. Most commonly RIPs are type I RIPs, single polypeptide chain proteins of molecular mass around 30 kDa composed of the toxophoric A subunit with N-glycosidase activity; such as cucurmosin (*Cucurbita moschata*), curcin (*Jatropha curcas*), trichosanthin (TCS) (*Trichosanthes kirilowii*), and trichomislin. Ricin (*Ricinus communis*), agglutinin, and abrin (both from *Abrus precatorious*) belong to II RIPs comprising of polypeptide subunits, homologous A chain that is functionally similar to type I RIPs while B chain marginally larger than A with a galactose-specific lectin that binds to cell surfaces. After post-translation modification, single gene corresponds to A chain and B chain and are linked through disulfide bond. Maize, and barley, atypical type I RIPs are derived from inactive proprotein and activated after proteolysis are Type III RIPs. Two polypeptide subunits are present in the mature type III RIPs with their extra domains of unknown function acting as an N glycosidase jointly. Therefore, the division of RIPs into types I and II RIP is taken into consideration of biological activities (Walsh et. al., 2013).

Abrus agglutinin (AGG), protein isolated from the seeds of *Abrus precatorius* shows low-toxicity with medicinal effectiveness in India. The heterodimer protein is a glycoprotein having molecular weight of 134-kDa. Two A chain 30 kDa toxic and two B chains 31 kDa are connected through disulfide bridges; carbohydrate moieties are the sites were the B chain binds to the two galactose binding sites per molecule of the cell surface receptors of glycoproteins and glycolipids, enter the cells by receptor mediated endocytosis, and are transported by the retrograde pathway to the endoplasmic reticulum (ER), hence the entire toxin is internalized into the cell. In the ER, the reduction of the A-B intersubunit disulphide bond takes place which is an essential parameter for the cytotoxicity that takes place followed by the translocation of A chain to the cytosol. rRNA *N*-glycosidase activity is exhibited by A chain and irrevocably inhibits protein synthesis by catalytical inactivation of ribosomes by depurinating the universally conserved α-sarcin loop through selectively cleaving the *N*-glycosidic bond of the adenine-4324 residue in the conserved GAGA hairpin loop of 28S rRNA of eukaryotes in the eukaryotic ribosome. The elongation factors are being inhibited from binding of ribosomes because of the depurination, thereby arresting protein synthesis causing cytotoxicity. In addition to inhibition of protein

synthesis, RIPs are also capable of inducing apoptosis. It has specificity towards [gal (β 1→3) gal NAc] and belongs to type II ribosome inactivating protein family (RIP II) with a protein synthesis inhibitory concentration (IC_{50}) of 0.469 μg/ml and a lethal dose (LD_{50}) of 5 mg/kg body weight in mice (Bhutia et. al., 2008). A chain of AGG bearing the 13 amino acid with catalytic function was completely conserved among RIPs. The A chains from *Abrus precatorius* were expressed in *Escherichia coli*, and their isolated cDNAs have been cloned. The low toxicity of AGG comparative to abrin is difference in the amino acid residues at Pro199 that correspond to Asn200 of abrin-a at proposed active sites were evaluated with the introduction of site-directed mutagenesis for studying the structure and function of these RIPs. The impairment in the activity of protein synthesis is due to the Pro199 that plays a crucial role in A- (or C-) chain of AGG because of a kink created due to the hydrophobic residue results in steric hindrance since it is bound with the cleft in a more compact complementary relationship. Bagaria et al reported the less toxic X-ray crystal structure of 3.5 Å, because of the fewer interactions involved with the substrate adenine. On reference to the description of the abrin-a, and to the CATH database the structure of the AGG A (or C)-chain was divided into three folding domains γ1, γ2, and γ3. Six β-sheets are present in domain γ1, one β-hairpin and two α-helices, composed of residues 2 to 108. The N terminus of AGG is one residue shorter than that of abrin, first two residues are severely disordered. Domain γ2 consists of 109 to 196 residues, and is the most conserved part of A chain. It has total of five helices. Domain γ3 has 197-261 residues. It has two helices and two antiparallel strands and a random coil at the C terminus. The C terminus of agglutinin is 10 residues longer than that of abrin. The structure of B chain, the lectin chain, is highly conserved across the type II RIP family. It is divided into two homologous domains, δ1 and δ2, mainly formed by β-sheets and loops; each of which consists of four subdomains namely λ, α, β, and γ. The three subdomains, β, and γ possess pseudo 3 fold symmetry around a hydrophobic core. Agglutinin B chain comprehends three N-glycosylation sites, namely Asn-100, Asn-140, and Asn-214. The crystal structure reveals the presence of two glycosylation sites Asn-100 and Asn-140 holding sugar chains in 1β and 2β subdomains. Moreover, agglutinin B chain contains two sugar-binding sites at Asn-51 and Asn-260 present in 1α and 2γ subdomains, respectively. The architecture of sugar binding pockets in agglutinin is similar to that of abrin. Structure of active site is exactly the cleft formed by the intersection of all 3 domains in AAG A (or C)-chain. The location of the active

site region of the AGG A (or C)-chain is five invariant residues (Tyr73, Tyr112, Arg166 and Glu163) and five conserved residues (Gln159, Glu194, Asn71, Arg123, and Asn195) are located in the active site cleft (Bagaria et. al., 2006; Cheng et. al., 2010).

The sublethal doses of *Abrus* agglutinin AGG initiated direct killing of tumor cells through extrinsic and intrinsic apoptotic pathways have prompted antineoplastic property in various tumor models (Bhutia et. al., 2008b). It has been stated that AGG has shown pro-apoptotic effects in many cancers. The use of AGG as an anti-cancer molecule regulated tumor growth inhibition for liver cancer treatment. It inhibited cell proliferation *in vitro* as well as *in vivo* (Mukhopadhyay et. al., 2014a). AGG repressed cell proliferation and angiogenesis in the growth of distant tumors of cancer cell xenografted mice, with decreased CD31 and Ki-67 staining as well as enhanced TUNEL, showcasing its competence in apoptosis induction (Mukhopadhyay et. al., 2014a). AGG promoted ROS mediated AKT-dependent extrinsic apoptosis in breast cancer cells and decreased angiogenic phenotypes both *in vitro* and *in vivo* (Bhutia et. al., 2016). Peptide fractions obtained from AGG induced apoptosis through mitochondrial pathway in the mice model of Dalton's lymphoma (DL) (Bhutia et. al., 2008).

AGG displays immunostimulatory properties and upon binding to the carbohydrate moieties showcases its strong mitogenic activity to lymphocytes localized in the cell surface reported in human as well as mice. Lymphocytes become mature effector cells by proliferating on prolonged exposure to lectins, secrete lymphokines, and functions of a specialized cells are exhibited in terms of cellular immunoglobulin production, cellular cytotoxicity, and helper/suppressor characteristics. Moreover, AGG bearing heat denatured and tryptic digested peptides show strong antitumor and immunomodulatory activities activate splenocytes and induce production of cytokines like TNF-α, IL-2, IFN-γ and in normal as well as DL bearing mice. It exhibits both humoral and cellular immunity with propensity to stimulate the innate effector arms like macrophage and natural killer cells by activating splenocytes, leading to Th1 response (Bhutia et. al., 2011b).

Chapter 2

Review of Literature

2.1. Introduction

2.1.1. Molecular mechanism of plant lectin induced apoptosis in cancer

Apoptosis is known as tumor suppressor mechanism in cancer and apoptosis induction can be used as important targeted cancer therapy (Wong et. al., 2011). Apoptosis the program cell death type-I is associated with membrane blebbing, nuclear break down and chromatin condensation and is regulated by different cellular signaling (Elmore et. al., 2007). Apoptosis is a programmed cell death which follows two pathways; intrinsic (mitochondrial) and extrinsic (death receptor mediated). It is found to be mutated in different cancer cells. Recent discoveries have explored the lectin science to target the obnoxious cancerization (Lichtenstein and Rabinovich et. al., 2013).

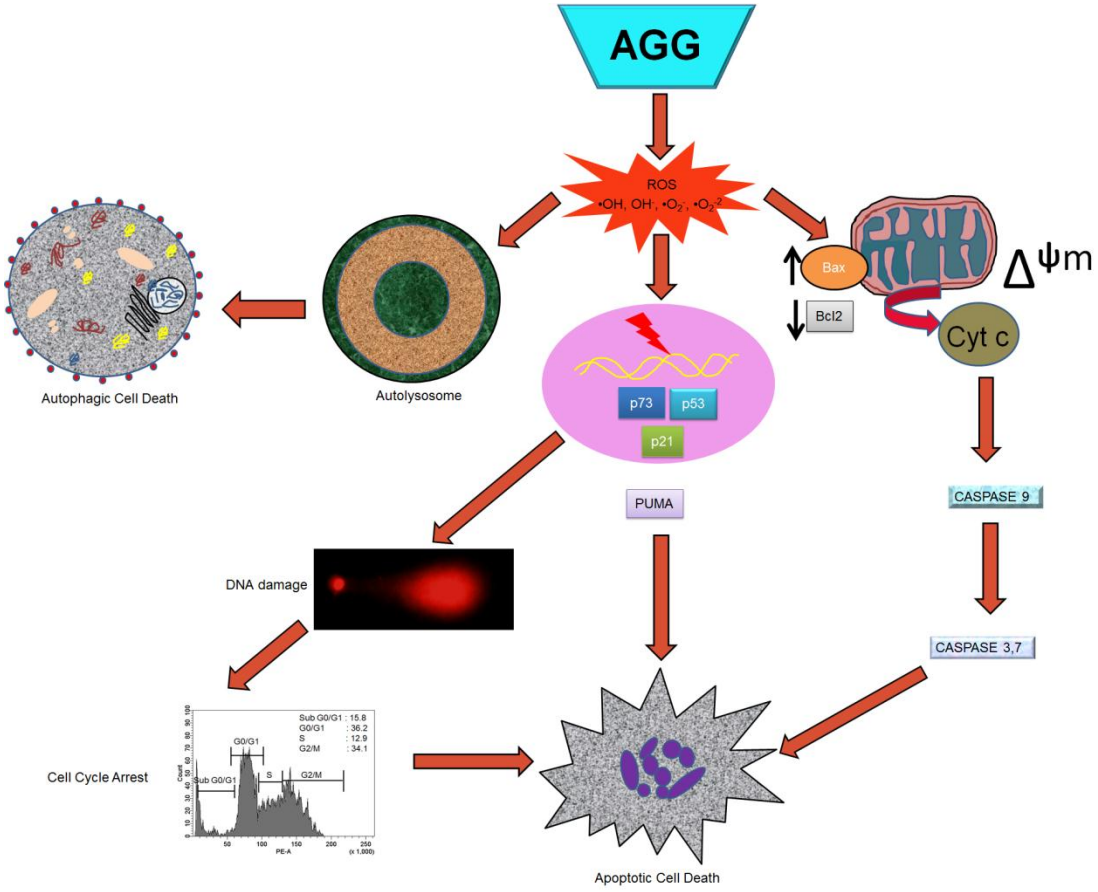

Figure-2.1. *Abrus* agglutinin (AGG) induces programmed cell death in a cancer cell: AGG treatment in a cancer cell upshots ROS production causing DNA damage. Double strand break activates the stress adaptive molecules ATM-p73/p53 resulting in cell cycle arrest followed to depolarize membrane potential triggering release of cytochrome c followed to caspase mediated apoptosis. ROS also results in autophagic cell death that occurs independently and or simultaneously to apoptosis.

2.1.2. Ricin B family lectins

Mistletoe species are semi-parasitic plants which grow on deciduous trees all over the world. Mistletoe lectins (MLs) are type II ribosome inactivating proteins (RIPs II), primarily of three types; ML-I, ML-II, ML-III amongst which ML-I and ML-II show anti-proliferative activity. European mistletoe lectins mostly belong to ML-I group (Gal-specific) whilst Korean mistletoe lectin is categorized as ML-II type specific to both Gal and GalNAc (Lee et. al., 1999; Yoon et. al., 1999). The accumulating data showed ML-I showed apoptosis inducing activities and sensitize the effects of chemotherapeutic drugs towards different cancer cells. Similarly, ML-II found to show the anticancer effect through apoptosis by activating ERK/p38MARK pathway. European mistletoe lectin, ML-I was identified as the active component of the *Viscum album* extract VAA-I, was shown to induce apoptosis by shutting down the synthesis of proteins, including the anti-apoptotic protein Mcl-1. The aqueous extract of European mistletoe (*Viscum album,L*.) has been applied in cancer therapy (Lyu et. al., 2004). However, for the efficient practical use of lectin in the clinical stage, a delivery system is required to lower toxicity, extend exposition, and improve efficacy.

Korean mistletoe lectin (*Viscum album coloratum* agglutinin, VCA), belonging to ML-I, has been reported to trigger apoptosis by a mitochondrial pathway independently of p53 in hepatocarcinoma Hep3B cells. It enhanced its anti-proliferation activity in COLO cells by activating caspase- 2, -3, -8, and -9 in a time- and dose-dependent manner, downregulating the expression of anti-apoptotic nuclear factor- κB (NF- κB), X-linked inhibitor of apoptosis protein (XIAP), and Akt/protein kinase B (Khil et. al., 2007). When U937 cells were treated with Korean mistletoe lectin II, it elaborated the apoptosis induction through activation of ERK1/2, p38 MAPK and stress-activated protein kinase (SAPK) / c-Jun N-terminal kinase (JNK) in the cells. It further showed its anti-apoptotic agent in several other cancer cell lines including RAW 264.7 cells, HL-60 cells, DLD-1 cells and Jurkat T cells, primary acute myelocytic leukemic cells (Pryme et. al., 2006). The lectin has furnished its anti-tumor activity in B16-BL6 melanoma treated cells. Henceforth, VCA, a potential candidate for the chemotherapeutic agent for cancer cells triggers molecular changes resulting in the inhibition of cell proliferation and the induction of apoptotic cell death. In an experiment using C57BL6 mice inoculated with B16-BL6 melanoma cells, Korean VAA inhibited tumor growth and metastasis by increasing apoptosis or type I programmed cell death and inhibiting angiogenesis. It has progressed in inducing

apoptotic cell death resulted from dephosphorylation of Akt in correlation with the inhibition of telomerase activity and the activation of caspase-3 in A253 cells (Choi et. al., 2004; Pryme et. al., 2006). Similarly, Chinese mistletoe lectin-I (CM-I), an ML-I type *Viscum articulatum* has showed antitumor activity toward colorectal cancer (CLY and HT-29 cells) through suppression of wnt signaling pathway by downregulating miR-135a and b expression and upregulating the expression of their target gene adenomatous polyposis coli (APC) along with the phosphorylation of related effector β-catenin (Li et al., 2011). Interestingly, Japanese ML-I inherits the similar N-terminal sequences of A-chain and B-chain of Korean isoform C (KML-U/A and KML-U/B chains) but strongly discriminates from European mistletoe. It induced chromatin condensation and nucleosomal fragmentation associated apoptosis which was blocked by a caspase inhibitor in U937 cells, similar to the findings with European ML-I (Pervin et. al., 2015).

The seeds of *Abrus precatorius* contains two galactose specifc lectins; namely 63 KD toxic lectin, abrin and 134 KD less toxic lectin, agglutinin which are heterodimer and heterotetramer respectively. Abrin initiated apoptotic cell death via a mitochondrial pathway toward various types of cancer cells such as Jurkat, CCRF-HSB-2, MOLT-4, RPMI8402, and BALL-1 cells (Bagaria et. al., 2006). It induced apoptosis by stimulating caspase-3 expression and blocking Bcl-2 expression in murine Dalton's Lymphoma Ascites (DLA) cells (Ramnath et. al., 2009). More recently, abrin has frustrated Jurkat cells by inducing apoptosis in a caspase dependent manner, and induced programmed caspase independent necrosis in U266B1 cells in a manner even when there was ROS production and loss of MMP. The data revealed that abrin-mediated necrosis is associated with the permeabilization of lysosomal membrane that causes release of cathepsins. Importantly, it has been suggested that abrin-mediated death pathway by apoptosis or necrosis appears to depend on which of the two events occurs firstly lysosomal membrane permeabilization or loss of MMP. It induces apoptosis in HeLa cells by targeting mitochondria in caspase dependent pathway. After its infiltration, oxidative stress results in production of reactive oxygen species, a drop in mitochondrial transmembrane potential downregulation of Bcl-2 followed by upregulation of Bax. Abrin induced apoptosis was found to be dependent on p38 MAPK but not JNK. It induced Bid cleavage with the activation of caspase-2 and caspase-8 leading to mitochondrial membrane potential loss, and thus connecting

the signaling events from ER stress to mitochondrial death machinery (Fu et. al., 2011).

RIPs II lectins having specificity towards D-galactose like; *Momordica charantia* lectin (MCL), Ricin A have shown their anti-proliferation attribute in different cancer cell lines (Kabir et. al., 2015). Fang et al in 2012 unveiled the potent cytotoxicity of MCL, for the first time towards NPC CNE-1 and CNE-2 cells with minimal action to normal NP 69 cells. Ricin A, which binds to D galactose containing glycans, including many glycoproteins expressed on the surface of enterocytes is obtained from castor bean plant. One of the subunits of lectin ricin, was found to affect tumor growth by binding to ribosomes, thus inhibiting protein synthesis, and was described to persuade apoptotic cell death through upregulating caspase-8 and its downstream caspase 3/7 in L540 Hodgkin's lymphoma cells (Polito et. al., 2009). Additionally, it could inhibit the proliferation of human promyelocytic leukemia cell line (HL-60 cells) via cytotoxic mechanisms involving caspase activation and apoptosis. Unlike Ricin A**,** other Ricin B family members, like; Rice bran agglutinin (RBA) has been gradually brought into *in vitro* experiments**,** RBA executed apoptotic mechanism resulted in chromatin condensation, nuclear disassembly, degradation of nuclear DNA into nucleosomal units in human monoblastic leukemia U937 cells. RBA along with wheat germ agglutinin WGA caused G2/M phase cell cycle arrest with increased expression of Waf1/p21, while cell cycle arrest was not observed for VAA (Miyoshi et. al., 2001). Moreover, aralin from *Aralia elata*, is a lectin specific for galactose (Gal) identified as type II ribosome-inactivating protein induced apoptosis in various cancer cells. The study focussed on the aralin receptor which functions as a 110-kDa high-density lipoprotein-binding protein (HDLBP) and mediates RIP cell entry through endocytosis. The specific target of aralin was the 110-kDa HDLBP, the tumors formed by cells without aralin were significantly smaller than those formed by the cells expressing the 110-kDa HDLBP, symbolising the HDLP plays an essential for tumor growth. It was further demonstrated that forced expression of the 110-kDa HDLBP (Asp475-Arg1328) in aralin-resistant Huh7 cells, depicted aralin sensitivity influencing tumorigenesis (Otsuka et al., 2011).

2.1.3. Legume family lectins

Amongst the important lectin families; the type II ribosome inactivating proteins and GNA-related lectins, legume lectins are extensively studied *in vitro* and *in vivo*

dissecting the different molecular pathways being triggered en route to substantiate its forte in anti-cancer activity. Legume lectins have the capabilities to bind to the glycoconjugates of cancer cell surface; with mannose/glucose binding specificity; *Canavalia ensiformis* (ConA), *Canavalia brasiliensis* (ConBr), *Sophora flavescens* (SFL), *Vicia faba* agglutinin (VFA), *Canavalia ensiformis* (ConA).

Concanavalin A (ConA), the first reported legume lectin, induced apoptosis by breakdown of mitochondrial potential (MMP) and releasing cytochrome c in human hepatocellular liver carcinoma HepG2 cells, PU5-1.8 murine macrophage and in human melanoma A375 cells (Liu et. al., 2009a). It was demonstrated that SHPS-1 was identified as the receptor of Con A which reduced and regulated ConA dependent modulation of different signaling molecules, like; NF-κB, ERK, and JNK, and increased p53 and p21 levels. Moreover, Con A unveils the role of p73 in p53 null cells, both ovarian cancer SKOV3 and Li-Fraumeni syndrome MDAH041cells accelerating apoptosis by increasing the Bax/Bcl-2 ratio followed to inhibiting the survival Akt pathway and activating Foxo1a-Bim signaling. Similarly, in MOLT-4 and HL-60 cell lines, the antiproliferative activity of lectins *Canavalia brasiliensis* (ConBr) was studied. Both lectins appeared cytotoxic to the leukemic cell lines compared to the normal human peripheral blood lymphocytes, the lectins were not cytotoxic even at 10 folds higher concentration. The mechanism behind the anti-proliferative activity of ConA and ConBr was apoptosis which encompassed DNA damage, DNA fragmentation, increased sub G1 phase and altered mitochondrial membrane potential (Martins et. al., 2012). *Sophora flavescens* (SFL) with molecular weight 32KD is a traditional Chinese medicine isolated from *Sophora flavescens* Ait roots. Apart from its hemagglutinating and anti-fungal activities, it induced apoptosis by death receptor mediated caspase regulated apoptotic pathway in treated HeLa cells. SFL inhibited the growth of MCF-7 cells with no effect on MCF-10A cells. Treatment with SFL propelled the changes in NF-κB, ERK, p53, and p21 levels (Shi et. al., 2014). Another lectin, *Vicia faba* agglutinin (VFA) with affinity for D mannose and D glucose, increased the morphological differentiation and diminished the malignant phenotype of colorectal cancer cells. Dietary or therapeutic VFA may prompt slow progression of colon cancer and henceforth, can reverse the malignant phenotype of these cells possibly through an interaction with an adhesion molecule, epCAM.

Legume lectins with galactose binding specificity have demonstrated their anti-proliferating activity by inducing apoptosis in various cancer, some of them are; *Bauhinia forficate* (BfL), *Phaseolus vulgaris* cv. extra-long autumn purple bean lectin (EAPL), *Astragalus membranaceus* lectin (AML), *Astragalus mongholius* (AMML), *Sophora alopecuroides* lectin (SAL), *Lotus corniculatus* lectin (LCL). *Bauhinia forficata* lectin (BfL) was reported to induce DNA damage and caspase-9 inhibition resulting inhibition of G2/M phase arrest through modulation pRb and p21 which leads to necrosis in MCF7 cells. Moreover, BfL also found to inhibit adhesion on different types of matrix including laminin, fibronectin and collagen type I with reduced α1, α6 and β1 integrin subunit expression in MCF-7 cells (Silva et. al., 2014). Similarly, a 60 KDa lectin from seeds of *Phaseolus vulgaris* cv. extra-long autumn purple bean lectin (EAPL), galactose specific can inhibit proliferation of HepG2 cells by inducing assembly of apoptotic bodies (Lam and Ng et. al., 2011). Similarly, a 60 kDa dimeric hemagglutinin with a blocked N-terminus was isolated in large yield (190 mg/60 g) from the common edible bean *Phaseolus vulgaris* cv. Hokkaido large pinto bean inhibited the proliferation of CNE2, MCF7 and HepG2 cells (Yin et. al., 2015). Likely, *Astragalus membranaceus* lectin (AML), its anti-proliferation properties have been excavated explaining the mechanism of apoptosis initiation in K562 cells. It prompted caspase dependent apoptosis in the chronic myeloid leukemia cell line, K562. Furthermore, cytotoxicity and apoptosis of K562 cells induced by AML were completely abolished in presence of lactose or galactose. Subsequently, AML could act as a potential anti-cancer drug (Huang et. al., 2012). Another glycoprotein *Astragalus mongholius* (AMML) isolated from the roots *Astragalus mongholicus* has been reported to induce apoptosis toward human carcinoma cells (HeLa), human osteoblast-like cells (MG63) and human leukemia cells (K562) (Yan et. al., 2009). A novel lectin *Sophora alopecuroides* lectin (SAL) resembling other legume lectins has been isolated from the seeds of *Sophora alopecuroides* suppressed the proliferation of human cervical cancer cells (HeLa and Eca109). Moreover, recombinant lectin was also showed strong anticancer effect against cancer cell without affecting the normal cells (Li et. al., 2012). A tetrameric 70KD lectin isolated from *Lotus corniculatus* seeds, *Lotus corniculatus* lectin possessed strong anti-proliferative activity towards human leukemic (THP-1) cancer cells followed by lung cancer (HOP62) cells and

HCT116. Induction of apoptosis by LCL was examined by flow cytometry analysis with an increase in sub G0/G1 percentage of cells. Treatment with *Lotus corniculatus* lectin (LCL) displayed in treated THP-1 cells showed morphological annotations that displayed appearance of membrane enclosed apoptotic bodies, nuclear fragmentation, and DNA fragmentation elaborating induction of apoptosis. The wound healing assay reflected that LCL effectively restricted the cell migration in a dose dependent manner (Rafiq et. al., 2013).

Legume lectins with sialic acid binding specificity have canvassed their restricting attribute towards tumor cell proliferation, like; Phytohaemagglutinin from Phaselous cultivars (*Phaseolus vulgaris* lectins: (PHA-E), (PHA-L), *Phaseolus coccineus L., Phaseolus acutifolius,), Arachis hypogea* agglutinin (PNA), *Glycin max* lectin (SBL), *Griffonia simplicifolia* 1-B4 (GS1B4), *Datura stramonium*. Phytohemagglutinin (PHA-L, PHA-E) from *Phaseolus vulgaris* (red kidney bean) are classified as a "complex-type" specific group, since these lectins show preference for complex-type N-glycan. A 64-kDa hemagglutinin from a *Phaseolus vulgaris* cultivar, the northeast red bean exhibited anti-proliferative activity on different cancer cell lines; cell proliferation was strongly inhibited in MCF7 (breast cancer), CNE1 and CNE2 (nasopharyngeal cancer) cells compared to HepG2 (liver cancer). The hemagglutinin showed growth arrest in the G0/G1 and G2/M phases and initiation of early apoptosis, mitochondrial depolarization and DNA condensation in MCF7 cells. At high hemagglutinin concentrations, the MCF7 cells condition ameliorated and witnessed severe DNA damage and entered into late apoptosis stage (Chan et. al., 2013). *Phaseolus coccineus* L. (P. *multiflorous* willd) specificity towards sialic acid induces anti neoplastic activity via caspase dependent pathway in L929 murine fibrosarcoma cells (Chen et. al., 2009). *Phaseolus acutifolius*, the tepary bean seeds exhibits apoptotic induction on colon HT-29 cancer cells caused a significant increase in caspase 3 activity (Arteaga et. al., 2016). Recently our lab has published research articles documenting the anti-neoplastic activities of common culinary item derived molecules with sialic acid specificity, like; peanut and soybean. Peanut agglutinin (PNA) isolated from *Arachis hypogea* binds to terminal galactose-B1,3-N-acetylgalactosamine induced apoptosis and autophagic cell death in HeLa cells (Mukhopadhyay et. al., 2014). Likely, Soybean lectin (SBL) isolated from *Glycin max* specifically binds to N- acetyl galactosamine, inhibited tumor cell proliferation in

various cancer cell lines (Panda et. al., 2014) Moreover, it impeded proliferation of MCF7 cells and HepG2 cells (Lin et. al. 2008). *Griffonia simplicifolia* 1-B4 (GS1B4) is reactive with the-galactose (Gal) and N-acetylgalactosamine (GalNAc) moieties. Legume GS1B4 induced apoptosis with DNA fragmentation of sensitive CL8-1 melanoma cells, but not resistant tumour cell lines. The lectin GSI-A4 specifically recognizes terminal alpha-linked N-acetylgalactosaminyl groups and was toxic to human colon cancer cells-LS174t and SW1116 composing its potential candidature as a diagnostic agent against colorectal cancer. *Datura stramonium* agglutinin (DSA) isolated from the seeds of *Datura stramonium* (Jimson weed) is the most prominent and well characterized protein among the three different carbohydrate-binding proteins found in the seeds of *Datura stramonium*, which all exhibit specificity towards oligomers of N-acetyl-D-glucosamine. DSA inhibited proliferation of C6 glioma cells and induced irreversible differentiation, which resulted in differentiated human glial tumour cells (Sasaki et. al., 2002).

2.1.4. GNA family binding lectins

The GNA binding lectins with a β-prism structure have high reactivity with mannose and mannose-containing N-glycans. Vegetables in our daily life have several bioactive compounds including different forms of mannose binding lectins have been identified for their anticancer potential. For example, the first being, *Allium sativum*-L garlic dimeric lectin with moleclur weight 11.5 kDa found to inhibit DNA synthesis and induced apoptosis in human U937 and HL-60 cells (Yedjou and Tchounwou et. al., 2012.). Similarly, another garlic lectin named ASl50 showed strong apoptotic activity through caspse-3 activation in oral carcinoma KB cells and it had no cytotoxic effect towards normal HEK 293 cells as well as human erythrocytes (Kumar et. al., 2015). Initially *Ophiopogon japonicas* lectin (OJL) and subsequently, *Liparis noversa* lectin (LNL) *Polygonatum cyrtonema* lectin (PCL) and *Polygonatum odoratum* lectin (POL) were reported to possess remarkable inhibitory effect on growth of different human cancer. All of these lectins have conserved motif of ‘QXDXNXVXY required for mannose recognition. The LNL and POL have three such motifs, OJL has two and PCL has only one motif which might regulate the differential growth inhibition effects on MCF-7 cells, indicating role of sugar binging capacity on cell death signaling pathways. At molecular level, PCL-induced mitochondrial apoptosis was mediated by upregulating Bax and downregulating Bcl-2, Bcl-xL proteins through ROS generation in human melanoma A375 cells. Further, it showed that PCL-accumulated ROS that

activated series of signaling molelcues including p38 and p53, indicating PCL induces intrinsic apoptosis through ROS-p38-p53 dependent pathway. In addition, PCL also induced apoptosis by inhibiting the key negative regulator Ras-Raf and PI3K- Akt signaling pathway in L929 cells. GNA family lectin POL was reported to promote caspase dependent apoptosis in L929 cells (Liu et. al. 2009b). It signaled involvement of death receptors with increasing identities; FasL and FADD followed to caspase 8 dependent extrinsic pathways. It also disrupted MMP stimulating the release of cytochrome c successively leading to the stimulation of initiator and effector caspases; caspase 9 and caspase 3. A homotetramer with mannose-binding agglutinin of GNA family, *Lycoris aurea* agglutinin (LAA) from bulbs of *Lycoris aurea* up-regulated p21 expression and triggered G_2/M phase cell cycle arrest via inhibiting interaction between cdk1-cyclinA, and induced apoptotic cell death in human lung adenocarcinoma A549 cells through inhibiting PI3K-Akt survival pathway (Li et al., 2013). LAA amplified the antineoplastic effects of cisplatin toward A549 cells with no comparable cytotoxic effect toward normal human embryonic lung fibroblast HELF cells. In Bangladesh, an eatable tuberous rhizome with medicinal property, *Kaempferia rotunda* Linn., has been used to purify a lectin from the extracts christened as KRL with specificity towards methyl-α-D-mannopyronoside. It is the thermo-stable rhizome lectin that showed similarities with Concanavalin A lectin, some mannose/glucose at its N-terminal sequence, but the main differences is with their molecular masses and sugar content. KRL encumbered the cancer cell proliferation by cell cycle arrest at G0/G1. Apoptosis was monitored with the morphological changes in the treated Ehrlich ascites carcinoma cells (EAC), confirmed by fluorescence and optical microscope. RT-PCR revealed the downregulation of Bcl-2, Bcl-X, and upregulation of Bax (Kabir et. al., 2011).

2.1.5. Chitin family binding lectins

Triticum vulgaris, wheat germ agglutinin (WGA) is a chitin binding lectin with hevein domains, reactive with N-acetylglucosamine (GlcNAc) and sialic acid/ neuraminic acid moieties, mediate apoptosis of various murine and human tumor cell lines (Charland et. al., 2015). WGA treatment constrained the cell proliferation in AR42J which was revealed by estimation of the S-phase fraction by DNA flowcytometry and also induced differentiation of cells. It instigated growth inhibitory mechanism *in vitro* in lymphoma, leukaemia and breast cancer cells showcasing chromatin condensation, nuclear distortion and DNA fragmentation (Ebert et. al., 2009). Another potent lectin

of this group, *Setcreasea purpurea* lectin (SPL), exhibited a high cytotoxicity to CNE-1 cells by MTT method, cell morphological analysis and LDH activity-based cytotoxicity assays and induced apoptosis in a dose-dependent manner. Moreover, due to the caspase inhibitors analyses, caspase was also found to play an important role in the potential apoptotic mechanism of SPL (Yao et. al., 2010).

2.1.6. Jacalins family

The first member of Jacalins family to be identified is *Artocarpus integrifolia* (Jackfruit). The family of Jacalin-related lectins (JRLs) are basically subdivided into two groups; Gal- and Man- specific jacalins. The gal-specific JRLs are confined to small taxonomic group while the man-specific JRLs cover wide range of taxonomic group. Van Damme et al showed that both group of sub classes of JRLs are present as a major vegetative storage proteins in the bark of the black mulberry tree. *Artocarpus heterophyllus lectin* (ArtinM), D-mannose-binding lectin from *Artocarpus heterophyllus* treated human myelocytic leukemia, NB4 cells showed anti-proliferation activity, disruption of mitochondrial membrane potential associated with caspase activation and DNA fragmentation. In the treated cells, stress results in release of ROS orchestrating to autophagy, as indicated by the detection of acidic vesicular organelles (Carvalho et. al., 2011). *Del Monte* banana lectin (*Musa acuminate*) (BanLec) from banana binds to fructose or mannose and mannose-containing oligosaccharides. It retarded proliferation of L1210 cells and HepG2 cells (Cheung et. al., 2009). Another JRL, lectin from Mulberry leaf (MLL) is specific to galactose, galactosamine and N-acetyl galactosamine (GalNAc) inhibited cell proliferation in human breast cancer (MCF-7) and colon cancer (HCT-15) cells. The mechanism of inhibition was caspase dependent apoptosis. Apoptosis was scrutinized by the nuclear changes, positive annexin V staining, acridine orange/ethidium bromide staining and flow cytometry; that confirmed the apoptosis by delivering increased population of sub G0-G1 phase in MLL treated cells (Deepa et. al., 2012).

2.1.7. Other family lectins

A novel homodimeric rhizome lectin isolated from traditional Chinese herb *Pinellia ternata* (PTL) might be a novel araceae lectin since its has not occupied a space in any reported lectin family. Zhou et al, showed PTL induced apoptosis in the human tumor Bel-7404 cells. Further, *in vitro* antineoplastic activity of PTL was modulated through induction of G0/G1 cell cycle arrest in sarcoma 180, HeLa and K562 cells. A new monocot plant lectin from the tubers of family Araceae, *Sauromatum venosum*

(Voodoo lily), impeded proliferation of nine human and four murine cancer cells; OVCAR-5(ovary), SK-N-MC (CNS), SiHa (cervix), HT-29 (colon), SK-N-SH (CNS), SW-620 (colon), HEP-2 (liver), T-47D (breast), and PC-3 (prostate) whilst of murine being; WEHI, J774, P388D1 and A-20 (Martinez et. al., 2011). Another novel lectin from family Araceae, *Arisaema flavum* exhibiting specificity towards asialofetui, a complex glycoprotein, showed significant *in vitro* antiproliferative activity in WEHI-279 (B-cell lymphoma) and A20 (T-cell lymphoma), and P388DI and J774 (macrophage cancer cell line) murine cancer cell lines. A 70-kDa galactose-specific lectin was purified from the tubers of *Dioscorea opposita* cv. Nagaimo which inhibited the growth of breast cancer MCF7 cells, hepatoma HepG2 cells and nasopharyngeal carcinoma CNE2 cells. Nagaimo lectin evoked apoptosis in MCF7 cells was deciphered by the externalization phosphatidylserine and mitochondrial depolarization. However, the anti-proliferative activity of nagaimo lectin was not blocked by application of galactose, signifying that the activity was not related to the carbohydrate binding specificity of the lectin classified to the monocot mannose-binding lectin family (Chan et. al., 2013).

2.2. Lectins in preclinical stage

Enormous studies proclaim the anti-proliferation activity of plant lectins *in vitro*. But, only few lectins have been explored in the animal model to predict their efficacy as potential candidature for cancer therapy. RIP II lectins from ricin family illustrate the potential role in prevention and therapy of various tumors. Recently, our group successfully studied the nude mice bearing HepG2 xenografts with reduced tumor growth by inducing apoptosis, thus manifesting the agglutinin family as an alternative natural remedy for the treatment of human hepatocellular carcinomas. Hitherto, we have revealed the effectiveness of peptide derived fraction (10kMPP) from *Abrus* agglutinin in Dalton's lymphoma (DL) mice model by showing apoptotic bodies, condensed nuclei, membrane blebbing in treated DL cells compared to untreated one. The survival rate in AGG treated mice was significantly higher because of decreased ascites contents in comparison to control tumor bearing mice. Supporting the apoptotic activity of tryptic digested, abrin derived peptide fractions; our group corroborated the tumorcidal activity in Dalton's Lymphoma bearing mice with concomitant low toxicity in normal mice. *Momordica charantia* lectin (MCL), type II ribosome inactivating protein of ricin family, at a physiologically safe dose abated the size and volume of nasopharyngeal carcinoma (NPC) in xenograft nude mice (Fang et. al.,

2012). Application of another ricin family member, mistletoe lectin, exerts both local and systemic effects in tumor bearing mice resulting immunomodulation along with tumor nodule inhibition as well as inhibits cancer cell proliferation. In an experiment, C57BL6 mice were inoculated with B16- BL6 melanoma cells, Korean VAA inhibited tumor growth and metastasis by increasing apoptosis or type I programmed cell death and inhibited angiogenesis. Oral administration of 4 % (430 mg/kg/day) enteric-coate Korean mistletoe (*Viscum album* L. var. coloratum) drastically reduced tumor volume showing higher survival rate compared to the negative control group in B16F10 melanoma-containing BDF1 mice (Park et. al., 2001). Recombinant mistletoe lectin (rML) shows potential anti-tumor activity in human ovarian cancer cells transplanted into severe combined immunodeficient (SCID) mice. Different commercial VAE (*Viscum album* L extracts, European mistletoe) preparations and a recombinant ML (rML) drug are currently being developed and tested in animals and are in clinical trials. Hara and Seon found that treatment with immunotoxins containing ricin A-chain completely or partially suppressed solid tumor growth in nude mice inoculated with MOLT-4 human T-cell leukemia cells. Study shows that on treatment with *Ricinus communis* agglutinin I (RCA I) that interacts very firmly and preferentially gets internalized to endothelial cells of RIP-Tag2 transgenic tumor mice. As a result of this selective binding of RCA1 to endothelial cells, it leads to reduction in expression level of VEGFR-2. Reduced expression of VEGFR-2 leads to apoptosis in endothelial cells catapulted to tumor regression (You et. al., 2010). Future studies are required to determine the applicability of ricin and its derivatives as anticancer drugs. Likely, administration of leguminous proteins; ConA and SFL significantly decreased the subcutaneous tumor mass volume and weight in MCF-7 bearing nude mice. Treatment of ConA in severe combined immune deficiency (SCID) mice model reduced the liver tumor formation in comparison to control one. More number of tumor nodules was found in $CD8^+$ T depleted mice in comparison to non-depleted one, suggesting that $CD8^+$ cells played an important role in the ConA mediated cytotoxicity. *Phaseolus vulgaris* lectin (PHA), legume lectin, reduced the growth of a murine non-Hodgkin lymphoma tumour in both intra-peritoneal ascites tumour and a solid subcutaneous tumour developed in mouse model. The encapsulation of legume lectin *Cratylia mollis* with liposomes lowered toxicity in the liver and kidney, and improved its antitumor activity in Swiss mice inoculated with sarcoma. We genuinely evaluated the anti-neoplasticity of dietary legume lectins; soybean lectin (SBL) and peanut agglutinin

(PNA) in mice model, both enhanced cell death by apoptosis and autophagic cell death. Further, legume lectin, *Pisum sativum* lectin (PSA) binds to N-glycans containing α-linked mannose with α-fucose residue linked to N-acetylchitobiose; at 2.8 mg/kg body weight showed reduced growth of Ehrlich ascites carcinoma, accompanied by increased red blood cell numbers and normal white blood cell numbers in mice, suggesting the usefulness of this lectin for cancer therapy. The mechanism of inhibition of tumor growth in mice was shown to involve apoptosis by cell cycle arrest at G*2*/M phase via increased expression of pro-apoptotic *Bax* and reduced expression of anti-apoptotic *Bcl-2* and *Bcl-XL (Kabir et. al., 2013).* A legume lectin with sialic acid specificity, lectin fraction (LF) from Tepary bean seeds (Phaseolus acutifolius) inhibited 53 % of early stages tumors in male Sprague Dawley rats afflicted with colon cancer while no prominent effect was seen in late stage of carcinogenesis (Ferriz-Martínez et. al., 2015). *Solanum tuberosum*, 20 KD chitin binding lectin, STL-S and STL-D recognizes oligomeric N acetyl D glucosamine, it demonstrated 79.84 and 83.04% of growth inhibition in swiss albino mice afflicted with Ehrlich ascites carcinoma at 1.38 mg/kg/day (Hasan et. al., 2014).

2.3. Lectins in clinical significance:

The poor diagnosis and prognosis constrain the current conventional treatment to seek for options to defer the accompanied side effects. Anticancer effects of mistletoe ML-1 (*Viscum album* L.) galactoside-specific lectin extracts (VAE) and V. *album* agglutinins (VAA) have shown pronounced effect in the long run of the cancer therapeutics. Interestingly, intratumoural injections with Viscum album L extract (Quercus; Iscador®Qu) were administered to a 78-year-old Caucasian male twice in an attempt to restrict tumour growth (Schoen-Angerer et. al., 2014). Eight months after the second intratumoural injection was given, the adenoma had disappeared and biopsy revealed no intraepithelial dysplasia or adenocarcinoma. Adjuvant therapy, in combination of V. *album* extract therapy and conventional therapy in the treatment of breast cancer patients after surgery and radiotherapy appeared satisfactory. The venture saw improvement in HRQoL, alleviated side effects with enhanced longevity. Another clinical significance of mistletoe lection could be noticed by patient suffering from stageIII/IV glioma prolonged the relapse of cancer occurrence by increasing survivability after V. *album* agglutinins (VAA) treatment.

Objectives of the PhD work

With the background, *Abrus* agglutinin (AGG) has been taken to study the mechanism of tumor inhibitory potential of AGG in oral squamous cell carcinoma; the objectives of the thesis are as follows:

1. *In vitro* and *in vivo* anti-tumor activity of *Abrus* agglutinin in oral squamous cell carcinoma
2. *Abrus* agglutinin promotes irreparable DNA damage by triggering ROS generation followed by ATM-p73 mediated apoptosis in oral squamous cell carcinoma
3. p73 induction by *Abrus* agglutinin facilitates Snail ubiquitination to inhibit epithelial to mesenchymal transition in oral squamous cell carcinoma
4. *Abrus* agglutinin targets cancer stem-like cells by eliminating self-renewal capacity accompanied with apoptosis in oral squamous cell carcinoma

Chapter 3

In vitro and *in vivo* anti-tumor activity of *Abrus* agglutinin in oral squamous cell carcinoma

Abstract

Abrus agglutinin (AGG), a type II ribosome-inactivating protein isolated from the seeds of *Abrus precatorius* established its anti-tumor activity with decreased cell viability in a dose dependent manner in different oral squamous cell carcinoma cell lines. We found that AGG exhibited its long term effect by inhibiting colony forming potential in FaDu, a human pharyngeal cancer cells. At molecular level, AGG was shown to cause apoptosis as analysed by annexin V staining, DAPI, and DNA laddering. AGG induced apoptosis by upregulating the expression of PUMA, Bax, and cytochrome c followed with downregulation of Bcl-2, cleaved PARP and elevated initiator and executioner caspase activities. Interestingly, cell cycle arrest lead to apoptotic cell death was validated by alternation in cell cycle regulated proteins and the decreased number of cells in the G0/G1 and G2/M phase. Moreover, administration of AGG (50 µg/kg body weight) significantly inhibited the growth of FaDu xenografts in athymic nude mice. In immunohistochemical analysis, xenografts from AGG-treated mice showed a decrease in the expressions of CD-31, PCNA, Epithelial-to-Mesenchymal-Transition (EMT) modulators, and CD44-β-catenin as compared to controls. In conclusion, we confirmed that AGG effectively exhibited its anti-tumor potential against oral squamous cell carcinoma both *in vitro and in vivo,* and can be regarded as a potent candidate for the development of novel cancer therapeutics to treat oral cancer.

3.1. Introduction

Cancer that forms in tissues of the oral cavity or the oropharynx is referred as the oral cancer and among all oral mucosal origin; about 90 % are squamous cell carcinoma (SCC) (Mehrotra et. al., 2006). Oral squamous cell carcinoma (OSCC) most commonly occurs on the tongue, roof or floor of the mouth, soft palate gums, and other areas of the oral cavity (Posner et. al., 2007; Wein et. al., 2010; Aruna et. al., 2011). Symptoms are followed by a white (leukoplakia) or red patch (erythroplakia), unhealable tender lesions, sores, wounds characterized by painful chewing or swallowing. The major risk factor for these neoplasms is chronic exposure of oral mucosa to betel quid (paan) along with areca nuts chewing, a practice that is highly prevalent in different parts of India. Although tobacco and alcohol are independent risk factors, they have a synergistic effect in carcinogenic development. Human papilloma virus (HPV) infection (Goon et. al., 2009) with serotypes HPV 16, HPV 18

are found in a variable but small proportion of oral, and up to 50 % of tonsillar and oropharyngeal SCC (Syrjanen et. al., 2004). In addition, Epstein-Barr virus, gastro-esophageal reflux disease (GERD), exposure to various agents like - asbestos, paint fumes, gasoline fumes, wood dust, and plastic by-products are considered highly common possible risk factors (Mirzamani et. al., 2006). Under current therapeutics, the prognosis of OSCC remains dismal since more than 50 % of patients die with this disease or complications within a short period ranging from few months to three or five years. To worsen the condition, the malignancy in India shows only a small perspective of patients coming to cancer hospitals during early stages of carcinogenesis. Further, complicating the matter it is clearly reported that even surgical, chemo or radio treatment measures often do not give the desirable results compelling chances of multiple surgeries are common these days in cases of cancer relapse (Coelho et. al., 2012). Recent findings have explicated the natural molecules for the novel non-toxic natural approaches for treatment of cancer. China and India have published immense research work on lectin science for eradicating cancer; primarily mistletoe lectins have been brought to the clinical trials while con A, peanut lectin, soybean lectin and *Abrus* agglutinin have proved their candidature in pre-clinical trials. The apoptosis induction by natural therapeutics is mostly mediated through tumor-suppressor p53 pathway. But the p53 gene is the mostly mutated in human cancers including oral cancer. The hypopharyngeal (FaDu) cell line expresses only 50 % of the normal level of p53 mRNA, either because only one of the two alleles was transcribed, a mutation of CGG to CTG occurred at codon 248 (Kim et. al., 1993; Kropveld et. al., 1999; Min et. al., 1994; Partridge et. al., 2007; Reiss et. al., 1992). The laryngeal (HEp-2) cancer cell line translated very low levels of p53 protein compared to the normal counterpart. Sequencing of p53 cDNA for HEp-2 line showed no mutations (Kim et. al., 1993; Kropveld et. al., 1999; Min et. al., 1994). Southern and Northern analyses revealed that these cell lines harbored HPV-18 DNA and expressed the viral E6/E7 protein. The tongue carcinoma cell line (SCC-4) contained significantly higher p53 protein levels than did the normal counterpart, a mutation of CCC to TCC occurred at codon 151. SCC-9 had a deletion of 32 base pairs between codons 274 and 285; the cell line SCC-15 had an insertion of five base pairs between codons 224 and 225; the line SCC-25 had a deletion of two base pairs in codon 209; expressed negligible amounts of p53 transcripts compared to the normal counterpart

and undetectable levels of p53 protein (Kim et. al., 1993; Kropveld et. al., 1999; Min et. al., 1994).

The anticancer effects of AGG have been evaluated in several tumor models where this lectin inhibits the growth of tumors in experimental animals at sublethal doses by direct killing of tumor cells through extrinsic and intrinsic apoptosis (Costea et. al., 2006). AGG has a strong mitogenic activity to lymphocytes. It exhibits both humoral and cellular immunity in normal as well as tumor-bearing mice by stimulating tumor-associated macrophages and also activates splenocytes, leading to Th1 response and NK cell activation-directed immune response (Reya et. al., 2005; Zhang et. al., 2012; Hirschmann-Jax et. al., 2004; Ding et. al., 2010; Kong et. al., 2011). Interestingly, heat-denaturing and tryptic digested AGG showed potent antitumor and immunomodulatory activity in the murine model. The present work underpins the antineoplastic potential of AGG, a RIP II family member that inhibits proliferation and induces apoptotic programmed type-I cell death. Here, we report for the first time the novel *in vitro and in vivo* effects of AGG on oral squamous cell carcinoma.

3.2 Material and Methods

3.2.1. Chemical and reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), 6-Diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO), annexin V, N-Acetyl-L-cysteine (NAC) and trypsin were purchased from Sigma Aldrich, India. The fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), Dulbecco's modified Eagle's medium with high glucose (DMEM/high glucose), Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (*DMEM*/F-12), antibiotic–antimycotic solution were purchased from Invitrogen, India. Caspase-Glo assay kit for quantification of caspases was purchased from Promega, USA. Antibodies against Bcl-2, Bax, cytochrome c, PUMA, PARP, and actin were purchased from Cell Signaling Technology, USA; γ-H2AX from Millipore, USA; pRb, cyclin E, and p21 from BD Biosciences, USA. Other reagents used in the experiments were of analytical grade or the highest quality available.

3.2.2. *Abrus* agglutinin purification

AGG was isolated and purified from *Abrus precatorius* seeds by ammonium sulfate fractionation followed by lactamyl sepharose affinity chromatography and Sephadex G-100 gel permeation chromatography. The activity of isolated AGG was measured

by a hemagglutination assay, and the purity of the protein was subsequently analyzed by SDS-PAGE, native-PAGE and gel permeation by HPLC (Hegde et. al., 1991).

3.2.3. Cell culture

FaDu (pharynx), HEp-2 (larynx), RPMI2650 (nasal) were cultured in minimal essential medium (MEM), supplemented with antibiotic–antimycotic (1X) and 10 % fetal bovine serum. SCC4 and SCC9 were cultured in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (*DMEM*/F-12) supplemented with 400 ng/ml hydrocortisone. FaDu and SCC cell lines were procured from ATCC, USA; while the rest of the cell lines were purchased from National Centre for Cell Science, Pune, India. All the cells were maintained in their respective growth media supplemented with 10 % heat-inactivated fetal bovine serum (FBS) containing 1 % penicillin-streptomycin at 37°C in a humidified atmosphere, 5 % CO_2. All media, supplements and antibiotics were purchased from Invitrogen.

3.2.4. Cell viability by MTT assay

Cells were harvested from maintenance cultures in logarithmic phase and were counted by hemocytometer using trypan blue solution. About 1×10^4 cells/well were cultured in a 96-well plate at 37 °C, and exposed to varying concentrations of AGG for 72 h. After 72 h, MTT solution (5 mg/ ml) was added, post 4 h incubation, the resultant formazan crystals were dissolved in dimethyl sulfoxide and the absorbance was measured by a microplate reader (Perkin Elmer) at 595 nm. All experiments were performed in triplicate, and the relative cell viability was expressed as percentage relative to the untreated control cells.

3.2.5. Clonogenic survival determination in FaDu cells

FaDu cells were treated with different concentrations of AGG for 12 h and collected by trypsinization. The cells were counted and replated in triplicate in a 6-well tissue culture plate with 3000 cells/well. The cells were cultured for 14 days, with the growth medium being replaced every 3 days. The cells were then stained with 0.5 % crystal violet (in methanol/water, 1:1), and the colonies were counted.

3.2.6. Annexin V staining

It was performed using a FACScan (Becton Dickinson Immunocytometry Systems). After 24 h AGG treatment FaDu cells were washed with annexin V binding buffer and then centrifuged at 1200 rpm for 5 min at room temperature. Cell pellets were incubated with annexin V in binding buffer and analyzed by flowcytometer.

3.2.7. 4′, 6-Diamidino-2-phenylindoledihydrochloride (DAPI) staining

FaDu cells treated with varying doses of AGG were fixed in 4 % paraformaldehyde for 10 mins and permeabilised for 3 mins in 0.2 % of Triton-X 100. Intermittently cells were washed with PBS and incubated for 1-5 min at room temperature in DAPI labeling solution. The cells were imaged using blue filter of fluorescence microscope.

3.2.8. DNA fragmentation assay in AGG treated FaDu cells

The new DMSO (dimethyl sulphoxide)–SDS(sodium dodecyl sulphate)–TE (Tris-EDTA) method was used to study the DNA fragmentation assay since it retains smaller DNA fragments, reduced the overall time of processing. FaDu cells with cell count 1×10^6 cells were seeded in 60 mm petri plates and treated with varying doses of AGG. After 24 h incubation, cells were scrapped and washed in PBS. The pellet was subjected to 100 µl DMSO and mixed well followed by immediately addition of 100 µl of TE buffer (pH 7.4) with 2 % SDS followed by mixing and vortexing. The resulting solution was centrifuged at 12,000g at 4^0C and 40 µl of the supernatant was loaded on 1.5 % agarose gel.

3.2.9. Flow cytometry analysis of cell cycle distribution

After treatment with AGG, the FaDu cells were harvested and fixed in 70 % ethanol (stored at -20 °C). Then, the cells were washed with ice-cold PBS (10 mM, pH 7.4) and resuspended in 200 µl of PBS followed by incubation with 20 µl DNase-free RNase (10 mg/ml) and 20 µl of DNA intercalating dye propidium iodide (PI) (1 mg/ml) at 37 °C for 1 h in dark. The distribution of cells in the different cell cycle phases was analyzed from the DNA histogram using BD ACCURI C6 flow cytometer and FCS EXPRESS software.

3.2.10. Immunofluorescence

After 24 h incubation, FaDu cells treated with varying doses of AGG were fixed with absolute methanol for 2-3 mins. Cells were permeabilised using 0.1 % of Triton X-100 for 15 mins. Cells were blocked in 5 % BSA in PBST (PBS 1X, 0.1 % Tweens20) for 4 h. After overnight incubation with primary antibodies (Bcl-2, Bax, PUMA from Cell Signaling and cytochrome c from BD Biosciences), cells were incubated with secondary antibodies. Imaging was performed at 40X fluorescence microscope (Olympus IX71).

3.2.11. Caspase assays

FaDu cells were seeded in 6 well plates and were treated with AGG for 24 h. After treatment, caspase activity was measured using Caspase- Glo assay following the manufacturer's protocol (Promega Corp., Madison, WI).

3.2.12. Western blotting

Experimental sets of treated FaDu cells were scrapped and protein was estimated by Bradford's Reagent. Proteins (80 µg) were subjected to electrophoresis through the 10 % SDS polyacrylamide gel, followed by the transfer of proteins on to the poly-vinylidenedifluoride membranes. The membrane was blocked in a buffer containing 5 % non-fat milk in at room temperature for 1 h, and consequently incubated overnight in the primary antibody prepared in the blocking buffer. Membrane was then incubated with secondary antibody conjugated with a horse radish peroxidase at room temperature for 1 h. The blot was developed using GE X-ray films.

3.2.13. HNSCC cancer xenograft mouse model

Xenograft studies were performed as previously described (Bhutia et. al., 2016; Wang et. al., 2008). All mice were weighed before the start of experiment. Animal experiments were conducted in accordance with Singapore NACLAR guidelines (Law as of November 2004) for laboratory animal use and care. Good quality food and fresh, clean water were available at all times. Diets with oil rich foods and high in fat were avoided, milled pellets or blocks were preferred containing 10 % moisture and 3.8 to 4.1 kcal ME/g (16–17 kJ ME/g). Water is most easily made available and kept free from contamination by providing it in water bottles equipped with "slipper" tubes. Briefly, 4-wk-old athymic balb/c nude male mice (Biolasco, Taiwan) weighing 16-18 g were randomized into the following treatment and control group (n=5). FaDu cells were subcutaneously injected (5×10^6 cells/ mouse) to each mice. The control group was treated with normal saline (0.9 %) and treatment group mice received intraperitoneal plant lectin agglutinin at doses of 50 µg/kg five times a week. The treatment was continued for 2 weeks from the date of randomization. The mice body weight and tumor sizes were recorded every day and the tumor size was determined by Vernier caliper and calculated using the formula $[\text{length} \times (\text{width})^2]/2$. At the end of 2 week mice were sacrificed under isoflurane anaesthesia followed by cervical dislocation, tumor volume was measured and weighed. A part of the tumor tissue was fixed in phosphate buffered formalin for histology and the remaining tissue was stored in liquid nitrogen for future analysis.

3.2.14. Immunohistochemical staining and scoring

For the immunohistochemical analysis, formalin-fixed and paraffin-embedded specimens of 3–4 mm thickness were sectioned and staining was performed with anti-CD31 (Dako Corporation, Carpenteria, CA) and Ki-67 (BD Pharmingen, San Diego, CA) as described previously. IHC analysis was performed by finding out the percentage of positive cells by counting the number of positive stained cells (weak, moderate and strong) and the total number of cells from control and treated at 40X magnification. IHC score was analyzed for control and treated as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining).

Statistical analysis

All data were given as the mean ± SD. Experimental results were analyzed by Student's t-test. $P < 0.05$ was considered as the level of significance for values obtained for treated compound to control.

3.3. Results

3.3.1. Effect of AGG on growth inhibition in oral squamous cell carcinoma

To investigate the antineoplastic activity of AGG, we performed cell viability assay. Different doses of AGG were treated on different oral cancer cell lines for 72 h and the data showed that viability of cells declined in a dose-dependent manner (Fig. 3.1). The concentrations required for inhibiting cell growth by 50 % (IC_{50}) for FaDu, HEp2, SCC4, SCC9, SCC15, SCC25, and RPMI 2650 were 3.2 ±0.6, 8.9 ±0.6, 2.1 ±1.4, 1.0 ±0.2, 3.2 ±0.8, 4.4 ±0.4, 7.8 ±0.8 µg/ml, respectively (Table 1). Further, AGG exhibited its long term effect by inhibiting colony forming potential in FaDu, a human pharyngeal cancer cells in a dose-dependent manner (Fig. 3.2).

Table-3.1. IC_{50} of *Abrus* agglutin in different oral squamous cell carcinoma

Cell lines	IC_{50} (µg/ml)
FaDu	3.2 ±0.6
HEp2	8.9 ±0.6
SCC4	2.1 ±1.4
SCC9	1 ±0.2
SCC15	3.2 ±0.8
SCC25	4.4 ±0.4
RPMI2650	7.8 ±0.8

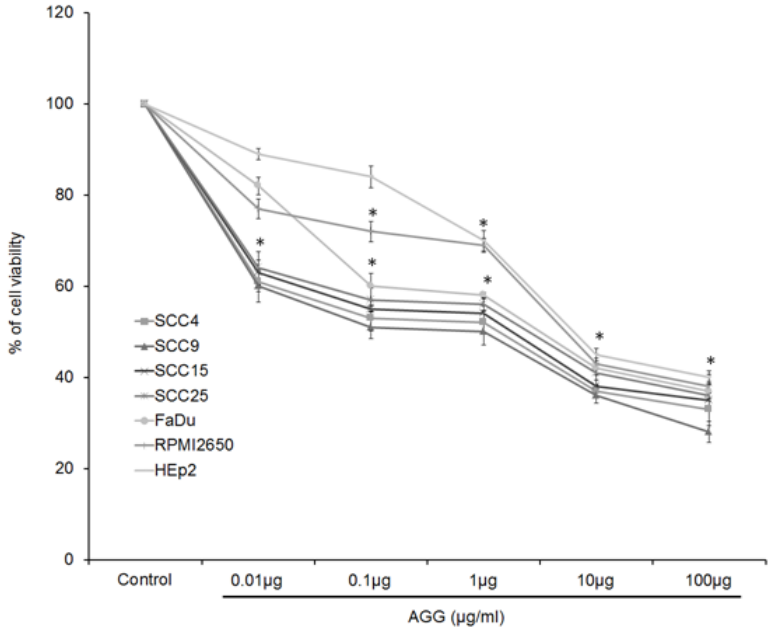

Figure-3.1. AGG inhibited cell proliferation of oral squamous cell carcinoma. The oral squamous cell carcinoma cell lines (FaDu, HEp2, RPMI2650, SCC4, SCC9, SSC15, SCC25) were treated with different concentration of AGG; 0.01 µg, 0.1 µg, 1 µg, 10 µg and 100 µg for 72 h and cell viability was performed by MTT assay. All data are represented as the mean ± SD of five different observations.

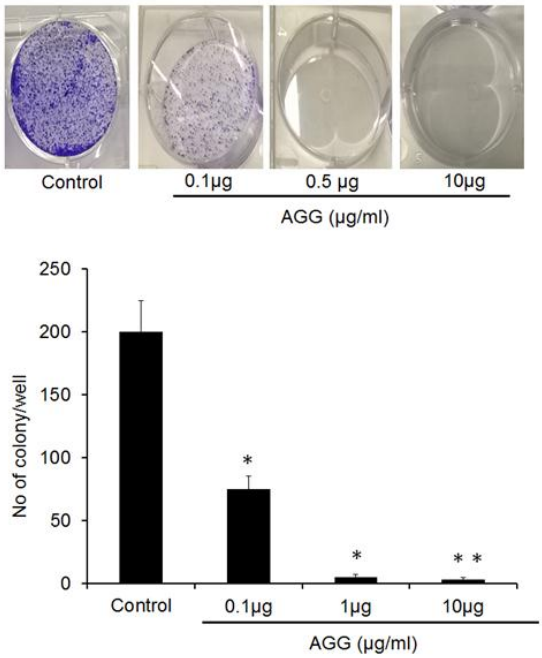

Figure-3.2. Growth inhibition induced by AGG on FaDu cells. The colony forming potential of FaDu cells was monitored. FaDu cells were treated with different concentrations of AGG for 12 h and collected by trypsinization. The cells were counted and replated in triplicate in a 6-well tissue culture plate with 3000 cells/well. The cells were cultured for 14 days, with the growth medium being replaced every 3 days. The cells were then stained with 0.5 % crystal violet (in methanol/water, 1:1), and the colonies were counted.

Next, we probed whether AGG induces programed cell death in FaDu cells. The data revealed that AGG increased apoptotic population in a dose-dependent manner after 24 h of its treatment. The annexin V population increased from 5.6 ±1.5 % in control to 11.3 ±1.8 %, 31.0 ±4.5 % and 40.0 ±2.1 % following 0.1, 1.0 and 10 µg/ml of AGG treatments, respectively (Fig. 3.3A). Furthermore, DAPI stained AGG treated FaDu cells showed apoptotic nuclei with condensed chromatin at the periphery of the

nuclear membrane, membrane blebbing or total fragmented morphology of nuclear bodies, in a dose-dependent manner (Fig. 3.3B). Lastly, DNA fragmentation assay was performed to further validate apoptosis, which showed that the treatment of FaDu cells with AGG results in DNA fragmentation in a dose-dependent manner (Fig. 3.3C).

3.3.2. Induction of mitochondrial apoptosis by AGG

Analysis of apoptotic proteins by Western blot and immunofluorescence elaborated the effect of AGG on FaDu cells. Mitochondria play an essential role in the propagation of apoptosis, and the disruption of the mitochondrial membrane initiates the release of cytochrome c in cells undergoing apoptosis. Our data showed there were an increase in the levels of pro-apoptotic protein Bax and a concomitant decrease in the levels of anti-apoptotic protein Bcl-2, thereby increasing the Bax/Bcl-2 ratio which was the driving force to apoptosis. Likewise, the expression of PUMA and cytochrome c was increased in a dose-dependent manner in the presence of AGG; indicating AGG induced alteration in apoptotic protein and regulated mitochondrial apoptosis. The upregulated and downregulated apoptotic proteins were also studied by fluorescence microscopy (Fig. 3.4B) observed the AGG induced cleavage of PARP as end-point for caspase-dependent apoptosis (Fig. 3.4A). Furthermore, our data showed that the caspase activity of initiator (caspases 8, 9) as well as executioner caspases (caspases 3/7) increased dose-dependently by AGG treatments, suggesting its efficacy in inducing both the extrinsic and more profoundly intrinsic pathways of apoptosis in FaDu cells (Fig. 3.5). Poly (ADP-ribose) polymerase (PARP) is the signature molecule of apoptosis. It is a nuclear enzyme involved in DNA repair, is a well-known substrate for caspase-3 cleavage during apoptosis. We analyzed PARP expression by Western blot and observed the AGG induced cleavage of PARP as end-point for caspase-dependent apoptosis (Fig. 3.4A). Furthermore, our data showed that the caspase activity of initiator (caspases 8, 9) as well as executioner caspases (caspases 3/7) increased dose-dependently by AGG treatments, suggesting its efficacy in inducing both the extrinsic and more profoundly intrinsic pathways of apoptosis in FaDu cells (Fig. 3.5).

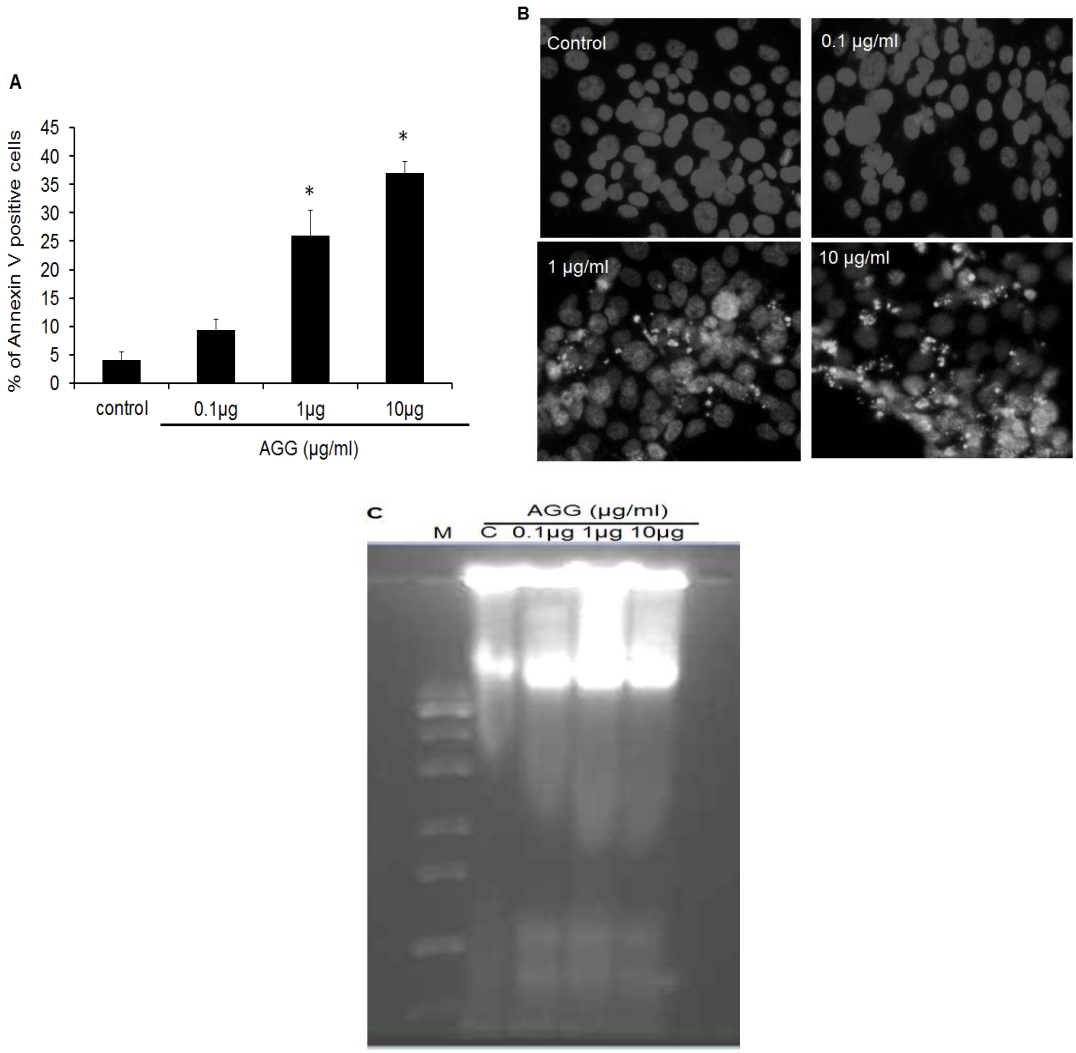

Figure-3.3. Apoptosis induction induced by AGG on FaDu cells: FaDu cells were treated with different concentration for 24 h and apoptosis was evaluated by annexin V/PI dual staining through flow cytometry (A). Morphological changes of nuclei by DAPI staining with arrow marks indicated condensed and fragmented nuclei and DNA fragmentation assay in a non-enzymatic manner was subjected to agarose gel electrophoresis (B and C). Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01 were considered significant as compared with control.

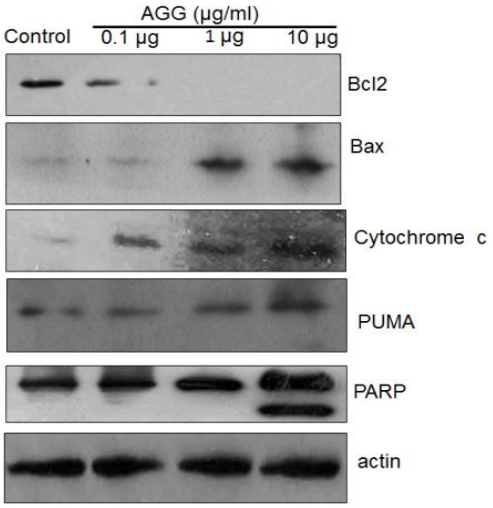

Figure-3.4. Mitochondrial apoptosis induction induced by AGG on FaDu cells: FaDu cells were treated with AGG for 24 h and analyzed for expression of apoptotic protein (Bcl-2, Bax, cytochrome c and PUMA) analyzed by Western blot.

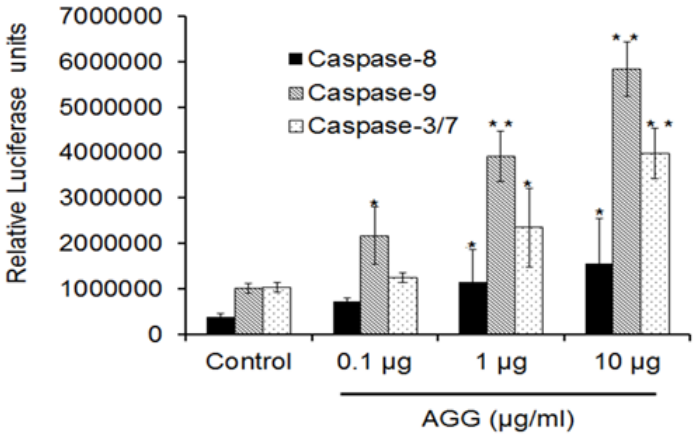

Figure-3.5. Caspase activity induced by AGG on FaDu cells: After treatment of FaDu cells with AGG for 24 h, caspase activity of initiator (caspases 8 and 9) as well as executioner caspases (3/7) were measured through Glo assay. Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01 were considered significant.

3.3.3. AGG treatment resulted in cell cycle arrest

Further, we analyzed the role of AGG in cell cycle progression in FaDu cells at different dose and time interval by PI staining through flow cytometry. The number of cells in the G0/G1 and G2/M phase decreased in a dose and time-dependent manner (Fig. 3.6A and 3.6B) with AGG treatment in FaDu cells. On the contrary, a dose and a time-dependent increase in the sub-G1 phase (the apoptotic cells) cell population were observed following AGG treatment of FaDu cells (Fig. 3.6C). In addition, our data showed that AGG effectively downregulated the expression of cyclin E and upregulated pRb and p21 protein levels (Fig. 3.6D), suggesting that AGG stimulated

the growth inhibition of FaDu cells by arresting cell cycle progression and apoptosis induction.

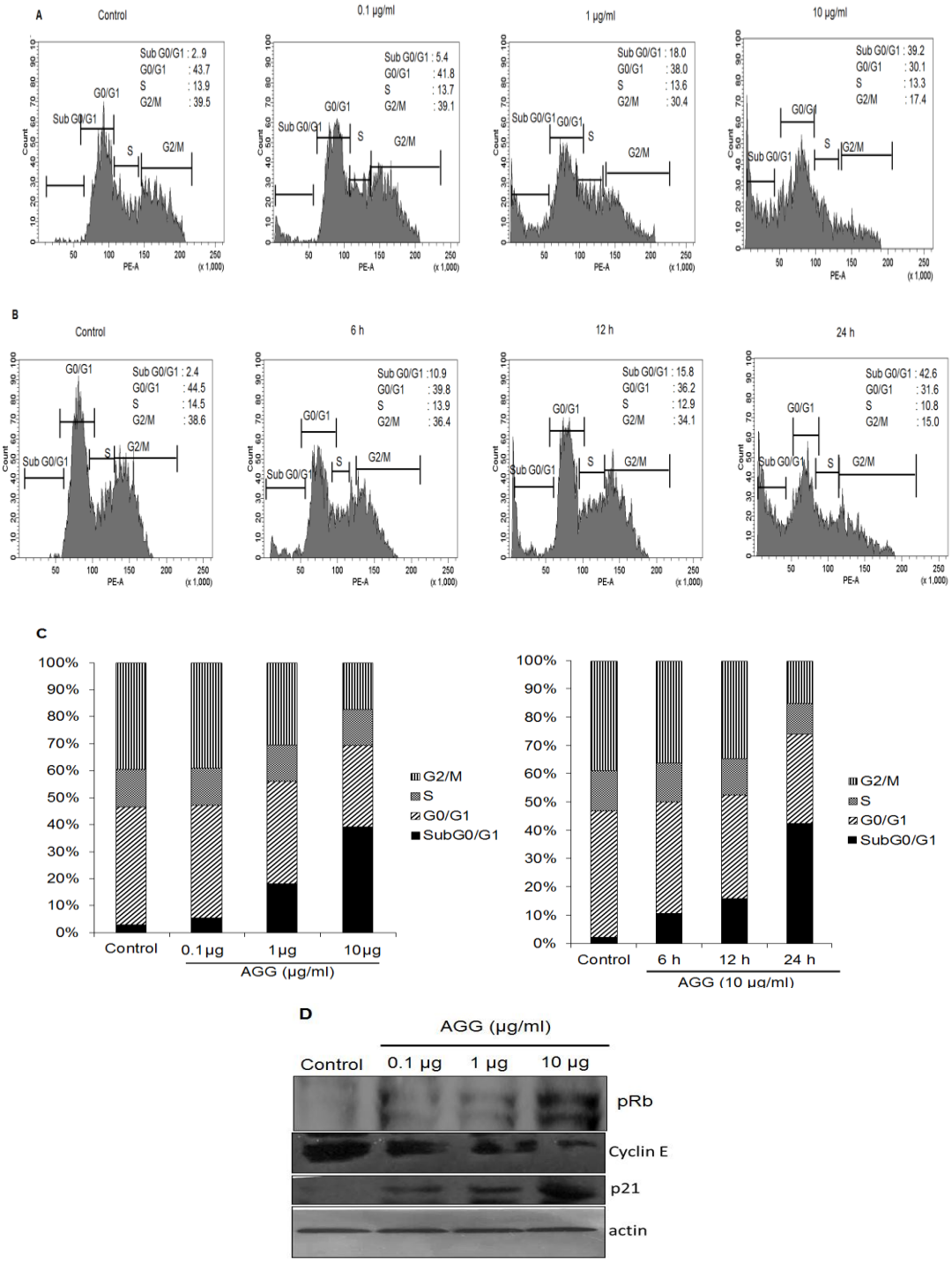

Figure-3.6. Cell cycle arrest induced by AGG on FaDu cells: FaDu cells were treated with different dose of AGG (0.1 µg, 1 µg, 10 µg/ml) for 24 h and different time (6 h, 12 h, 24 h) with 10 µg/ml of AGG, and were analyzed for cell cycle distribution by PI staining through Flow cytometry (A, B and C). After 24 h treatment with AGG, FaDu cells were analyzed for expression of pRb, Cyclin E and p21 by Western Blot (D). Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01 were considered significant.

3.3.4. AGG inhibited the growth of HNSCC tumor *in vivo*

Based on all completed cell culture studies showing strong efficacy of AGG and deciphering associated mechanisms, we also assessed AGG efficacy in inhibiting the growth of FaDu cell xenograft in nude mice. Male nude mice were injected subcutaneously with FaDu cells and treated with AGG (50 µg/kg body weight) administered intraperitoneally for 2 week (five times per week). We found that AGG significantly suppressed the tumor growth *in vivo* following 2 week of its treatment (Fig. 3.7A and 3.7B). The tumor tissues were subjected to immunohistochemistry for the angiogenic marker CD31 and cell proliferation marker PCNA. In the xenograft, the AGG-treated group showed a significant reduction in CD31 and PCNA staining compared to the control (Fig. 3.7C). The IHC score significantly distinguished the expression of CD31 in control and treated as high and low whiles the expression of PCNA in control and treated as high and moderate (Fig. 3.7D and 3.7E). To further support our *in vivo* finding, next we examined the effect of AGG on expression of E-cadherin, Snail, p73, Vimentin, SOX2, and Nanog, in FaDu xenograft tissues. The tumor tissues were subjected to immunohistochemistry analysis to check the status of molecules involved in the EMT phenomena. FaDu xenograft tissue sections revealed that there was a significant increase in the expression of E-cadherin, and p73 with decrease in the expression of Snail, Vimentin, and SOX2 in AGG treated group as compared to control group (Fig. 3.8A). The immunoreactivity score significantly distinguished the expression of E-cadherin as low and moderate; p73 as low and high in control and treated whiles the expression of Snail in control, and treated as moderate and low; followed by Vimentin and SOX2 with high and low immunoreactivity score in control and treated (Fig. 3.8B and 3.8C). Further we proceeded with immunohistochemical analysis to check the status of molecules involved in Wnt canonical pathway. FaDu xenograft tissue analyses revealed that there was a significant decrease in the expression of β-catenin and CD44 in AGG treated group as compared to control group (Figure. 3.9A). The IHC score significantly distinguished the expression of β-catenin in control and treated as high and low whiles the expression of CD44 in control and treated as moderate and low (Figure. 3.9B and 3.9C). Together, these findings clearly suggest that AGG decreased cell proliferation and angiogenesis, supressed the expression of the mesenchymal phenotypes and inhibited the expression of the CSC phenotypes *in vivo* along with

upregulation of E-cadherin and p73, and as a part of its anti-cancer efficacy against HNSCC.

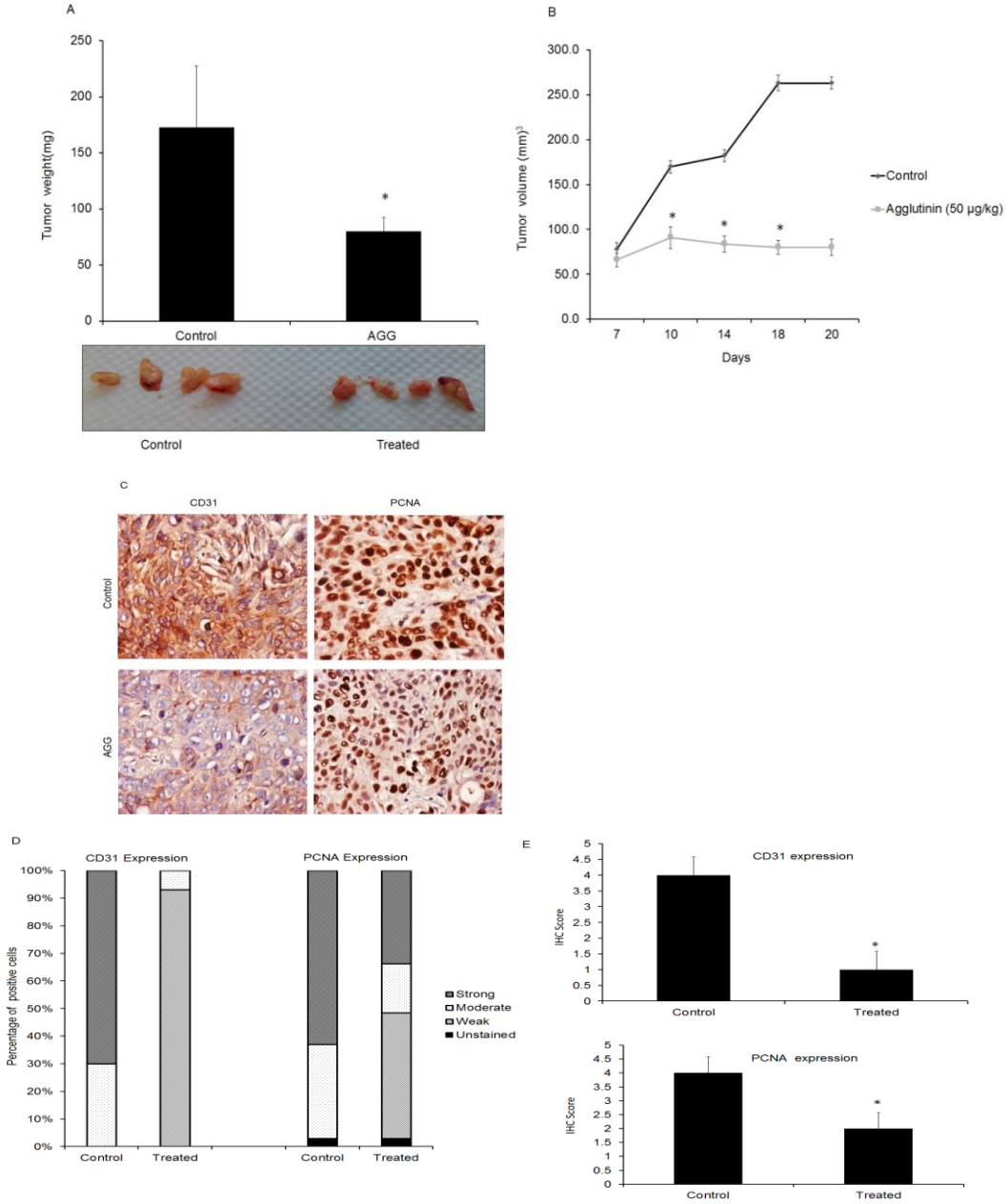

Figure-3.7. AGG inhibited the growth of HNSCC tumor *in vivo*. FaDu cells were subcutaneously injected (5×10^6 cells/mouse) to each mouse. The mice in control group were treated with normal saline (0.9 %) and in treatment group with intraperitoneal AGG at a dose of 50 µg/kg body weight. Tumor weight and tumor volume were plotted in the form of histogram and graph (A, B). Tumor tissues were harvested followed by fixation with formalin and paraffin-embedded sections were immunostained for CD31 and PCNA in control and treated groups (C). IHC analysis was performed by finding out the percentage of positive cells by counting the number of positive stained cells (weak, moderate and strong) and the total number of cells from control and treated at 40X magnification. IHC score was analyzed for control and treated as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining) (D, E).

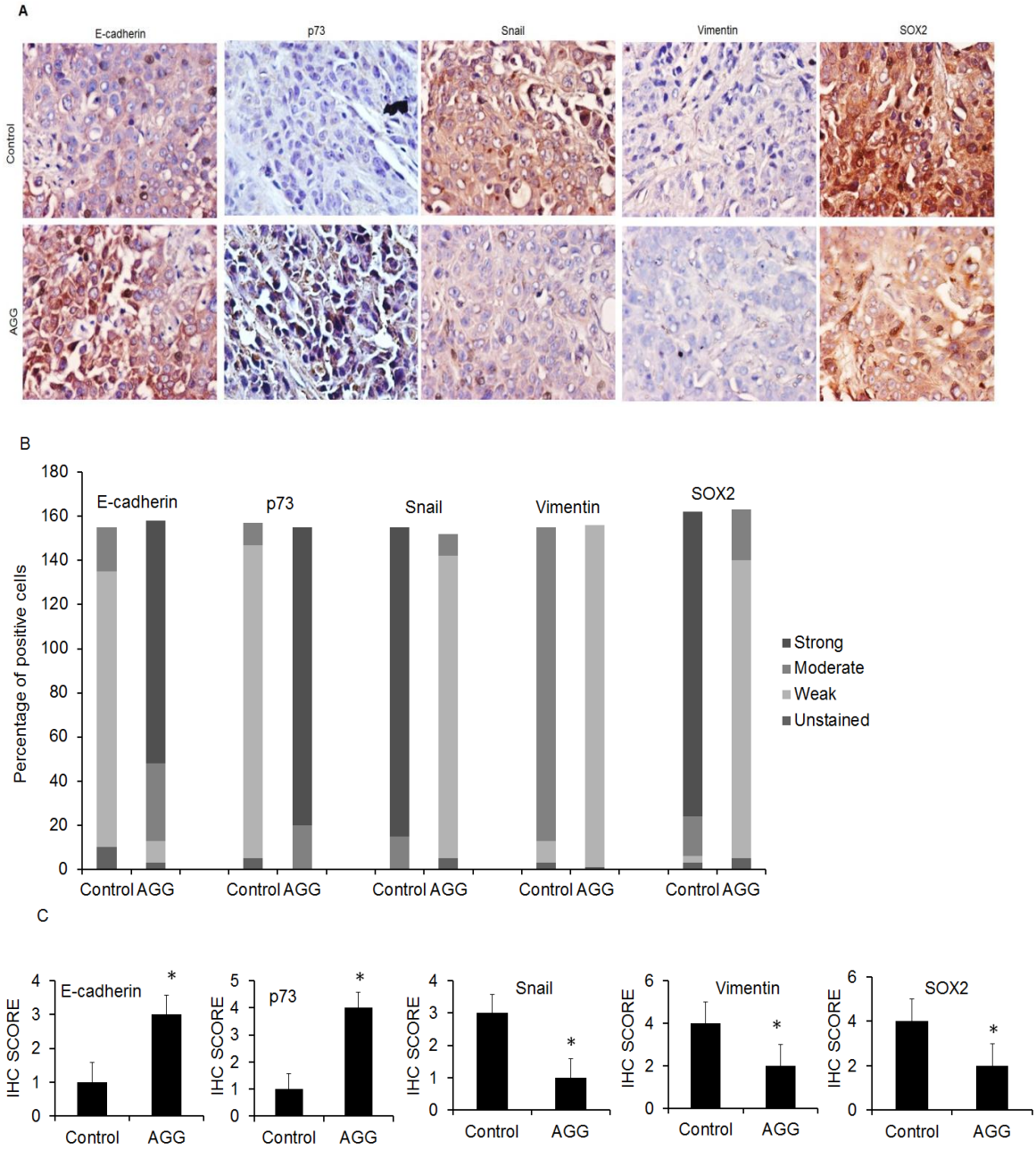

Figure-3.8. Immunohistochemistry analysis to check the status of molecules involved in the EMT phenomena. The tumor tissues were subjected to immunohistochemistry analysis to check the status of molecules involved in the EMT phenomena. FaDu xenograft tissue sections revealed that there was a significant increase in the expression of E-cadherin, and p73 with decrease in the expression of Snail, Vimentin, and SOX2 in AGG treated group as compared to control group (A). The immunoreactivity score significantly distinguished the expression of E-cadherin as low and moderate; p73 as low and high in control and treated whiles the expression of Snail in control, and treated as moderate and low; followed by Vimentin and SOX2 with high and low immunoreactivity score in control and treated (B and C).

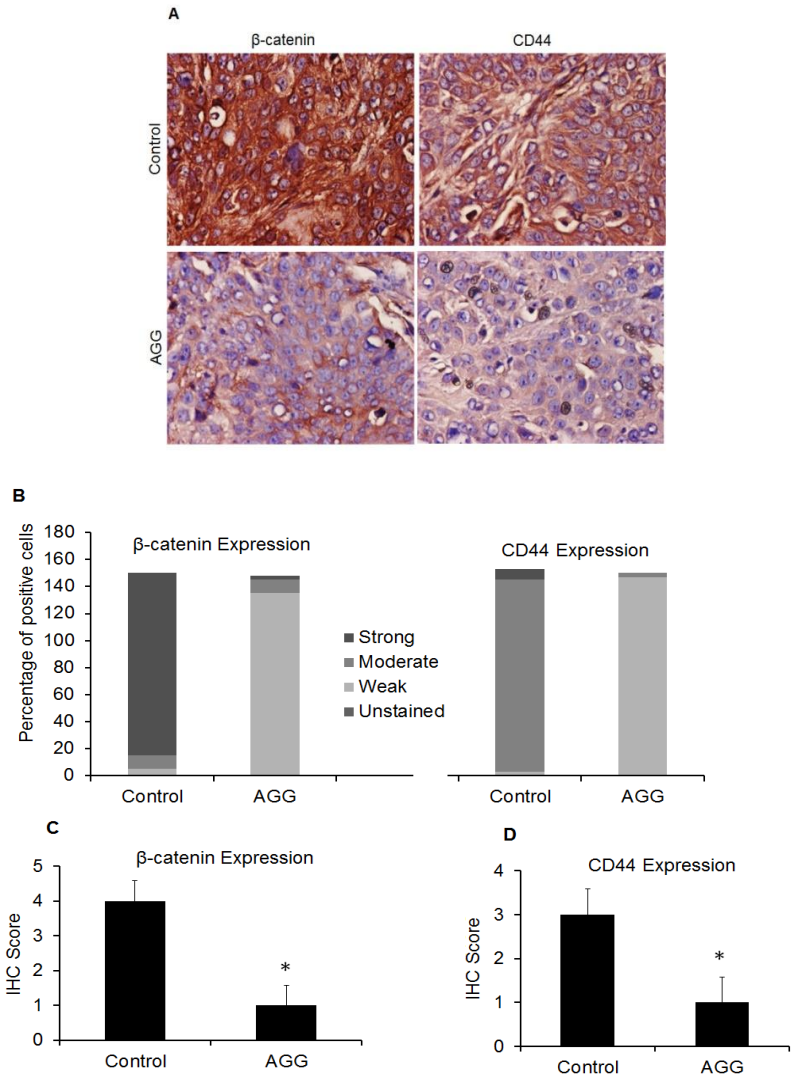

Figure-3.9. AGG inhibited the expression of CD44 and β-catenin in FaDu xenograft tissue. Tumor tissues were harvested followed by fixation with formalin and paraffin-embedded sections were immunostained for β-catenin and CD44 in control and treated groups (A). The IHC score significantly distinguished the expression of β-catenin in control and treated as high and low whiles the expression of CD44 in control and treated as moderate and low (B-D).

3.4. Discussion

New targeted therapies need to be developed with non-toxic agents for the prevention/intervention of both primary HNSCC and the recurrence of secondary primary tumors post-HNSCC therapy, since the five-year survival rate of OSCC patients remains abysmal. Lectins from traditional medicinal plant and plant-based diet are promising anticancer agents that have been adapted for its potent anticancer responses, and are widely used in the different region of the world.

The apoptosis induction is the prime target for cancer therapy. Korean mistletoe lectin (*Viscum album coloratum* agglutinin, VCA) has been reported to trigger apoptosis by a mitochondrial pathway independently of p53 in

hepatocarcinoma Hep3B cells. It enhanced its anti-proliferation activity in COLO cells by activating caspase- 2, -3, -8, and -9 in a time- and dose-dependent manner, downregulating the expression of anti-apoptotic nuclear factor- κB (NF- κB), X-linked inhibitor of apoptosis protein (XIAP), and Akt/protein kinase B (Khil et. al., 2007). Ginseng saponin extract (GSE) treatment strongly assessed apoptotic cell death by involvement of caspases of both intrinsic and extrinsic pathways. In the present study, AGG inhibited the proliferation and induced mitochondrial apoptosis in oral cancer cells convincingly. Additionally, inspecting the role of AGG on the survival of normal, non-tumor cell line, HaCaT (Human keratinocyte cell line) in comparison with tumor cell lines, we observed AGG to exhibit specific selective antitumor effect and did not induce any damages even at 10 µg/ml concentration (Mukhopadhyay et al., 2014a). AGG insult resulted in upregulation of pro-apoptotic proteins; PUMA and Bax, and release of cytochrome c; and downregulation of Bcl-2 along with cleaved PARP. Mistletoe lectins have showed apoptosis inducing activities and sensitize the effects of chemotherapeutic drugs towards different cancer cells by showcasing anticancer effect through apoptosis by activating ERK/p38MARK pathway. The aqueous extract of European mistletoe (*Viscum album*,L.) has been applied in cancer therapy (Lyu et. al., 2004). It induced apoptosis by shutting down the synthesis of proteins. VCA triggers molecular changes that resulting in the inhibition of cell growth and the induction of apoptotic cell death of cancer cells, it as a potential candidate for the chemotherapeutic agent for cancer cells. Rice bran agglutinin (RBA) along with wheat germ agglutinin (WGA) caused G2/M phase cell cycle arrest with increased expression of Waf1/p21 (Miyoshi et. al., 2001). *Bauhinia forficata* lectin (BfL) was reported to induce DNA damage and caspase-9 inhibition resulting inhibition of G2/M phase arrest through modulation pRb and p21 which leads to necrosis in MCF7 cells (Silva et. al., 2014). The 64-kDa hemagglutinin from a *Phaseolus vulgaris* cultivar hemagglutinin showed growth arrest in the G0/G1 and G2/M phases and initiation of early apoptosis with externalization of phophatidylserine, mitochondrial depolarization and DNA condensation in MCF7 cells (Lam and Ng et. al., 2011). *Lycoris aurea* agglutinin (LAA) from bulbs of *Lycoris aurea* triggered G_2/M phase cell cycle arrest via up-regulating p21 expression as well as down-regulating cdk-1cyclinA signaling pathway, and induced apoptotic cell death through inhibiting PI3K-Akt survival pathway in human lung adenocarcinoma A549 cells (Li et al., 2013). *Kaempferia rotunda* Linn., (KRL)

encumbered the cancer cell proliferation by cell cycle arrest at G0/G1 (Kabir et. al., 2011). Zou et al, displayed antineoplastic activity of *Pinellia ternata* (PTL) *in vitro* in sarcoma 180, HeLa and K562 cell lines. Flow cytometric analysis demonstrated that the inhibition mechanism involved induction of G0/G1 cell cycle arrest. Previously our group has reported that AGG induced G0/G1 cell cycle growth arrest in Dalton's lymphoma tumor model and in cervical cancer cells. Congruent to these findings, we found that AGG treatment caused cell cycle arrest with increased expression of p21, pRb and reduced expression of cyclin E in FaDu cells.

The clinical and preclinical studies demonstrated lectin as a potential alternative therapy for cancer treatment. We and other showed that lectins including *Abrus* agglutinin (Bhutia et. al., 2009b), *Abrus* abrin (Bhutia et. al., 2009a), Concanavalin A (Liu et. al., 2009c), Soybean lectin (SBL) (Panda et. al., 2014) and Peanut lectins (Mukhopadhyay et al., 2014b) induced apoptosis in different types of cancer. Specifically, our previous studies have established that AGG induced antitumour activity in lymphoma, melanoma, cervical, breast and hepatocellular carcinoma through generation of ROS-dependent intrinsic and extrinsic apoptosis. In an experiment using C57BL6 mice inoculated with B16- BL6 melanoma cells, Korean VAA inhibited tumor growth and metastasis by increasing apoptosis or type I programmed cell death and inhibiting angiogenesis (Park et. al., 2001). BfL inhibited adhesion on different types of matrix including laminin, fibronectin and collagen type I with reduced α1, α6 and β1 integrin subunit expression in MCF-7 cells. Chinese mistletoe lectin-I (CM-I), an ML-I type *Viscum articulatum* has showed antitumor activity toward colorectal cancer (CLY and HT-29 cells) through suppression of wnt signaling pathway by downregulating miR-135a&b expression and upregulating the expression of their target gene adenomatous polyposis coli (APC) along with the phosphorylation of related effector β-catenin (Li et al., 2011). In support to our previous findings, AGG significantly suppressed the growth of FaDu tumor xenograft in nude mice. Immunohistochemical analysis showed that AGG treatment on FaDu cell xenograft in nude mice downregulated the expression of CD31, PCNA, and mesenchymal phenotype with reintroduction of mesenchymal-epithelial transition by upregulation E-cadherin expression. AGG further inhibited the wnt canonical pathway molecule β-catenin and its target CD44, extending its role in inhibition of cancer stem cells. AGG efficacy in inhibiting human HNSCC tumor growth makes it a golden bullet that can be triggered for oral cancer treatment.

Chapter 4

Abrus agglutinin promotes irreparable DNA damage by triggering ROS generation followed by ATM-p73 mediated apoptosis in oral squamous cell carcinoma

Abstract

Oral cancer, a type of head and neck cancer has been ranked the top most malignancy in India. AGG selectively caused mitochondrial apoptosis through reactive oxygen species (ROS)-mediated ATM-p73 dependent pathway in FaDu cells. To examine any direct DNA damage caused by AGG, comet assay and γH2X staining were performed after AGG treatment in a dose as well as time dependent manner. AGG-induced ROS accumulation was a major mechanism of its effect on apoptosis, DNA damage and DNA-damage response, which was significantly reversed by ROS scavenger N-acetylcysteine (NAC). Moreover, *in silico* and co-localization studies showed interaction between mitochondrial manganese-dependent superoxide dismutase (MnSOD) and AGG which might inhibit its activity and upshot ROS accelerated apoptosis in FaDu cells. P53 being mutated in oral cancer, the focus was on p73. We observed that AGG treatment temporally upregulated the phosphorylation of DNA damage sensor kinases ATM (at Ser1981) and enhanced the expression of p73. Knock down of p73 caused a decrease in AGG-induced apoptosis. Interestingly, AGG-dependent p73 expression was regulated by ROS which was decreased by NAC. In order to delineate ATM expression with ROS generation and p73 induction, we found reduced p73 levels in AGG-treated shATM cells which also resulted in decreased apoptosis. Hereafter, a novel connection has been established among ROS, ATM and p73 mediated apoptosis by AGG.

4.1. Introduction

Oral cancer, a type of head and neck cancer, is the most common cancer in India and other South Asian countries, and may contribute up to 25 % of all new cases of cancer worldwide. Its high prevalence being attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco, betel quid chewing and other risk factors. These include genetic predisposition, immunodeficiency, diet and viral infections, e.g. human papillomavirus (HPV) and human herpes virus (HHV) (Datta et. al., 2015; Mirghani et. al., 2015). Treatments available for oral cancer chemotherapeutic drugs including cisplatin, 5-flourouracil, and paclitaxel in combination with radiotherapy; surgery is the conventional option for patients at both early and late stage of the malignancy. Besides, as an alternative approach, there is

significant interest in natural phytochemicals that have clinical potential in the prevention and treatment of cancer.

Lectins are ubiquitous, found in animals, plants, and microorganisms. Malignant cells are easily agglutinated than normal cells and this selective agglutination can be used in cancer therapy (Jiang et. al., 2014; Ouyang et. al., 2014). *Abrus* agglutinin (AGG), isolated from the seeds of *Abrus precatorius*, a herbal medicinal plant in India, is a heterodimeric glycoprotein of 134-kDa molecular weight. It is composed of two A and two B chains linked through disulfide bridges; out of which only B chain appears to bind cell surfaces. It has specificity towards [gal (β 1→3) gal NAc] and belongs to type II ribosome inactivating protein family (RIP II) with a protein synthesis inhibitory concentration (IC_{50}) of 0.469 μg/ml and a lethal dose (LD_{50}) of 5 mg/kg body weight in mice. The A chain has rRNA *N*-glycosidase activity and irreversibly inhibits protein synthesis in the eukaryotic ribosome while there are two galactose binding sites per molecule of the B that binds to cell surfaces (Bhutia et al., 2011b; Hegde et. al., 1991).

Here, we evaluated the anticancer efficacy of AGG through ATM-p73 pathway-dependent DNA-damage regulated apoptosis in FaDu cells. Moreover, we identified AGG-induced ROS accumulation as key mechanism in regulating its effect on apoptosis, DNA damage and DNA-damage response through inhibition of mitochondrial manganese-dependent superoxide dismutase (MnSOD). P53 being mutated in oral cancer (Reiss et. al., 1992; Partridge et. al., 2007), the focus of our study represented to p73, the sibling of p53, and its regulation in apoptosis in AGG-treated FaDu cells.

4.2. Material and methods

4.2.1. Chemical and reagents

N-Acetyl cysteine (NAC), Z-DEVD-FMK, Dihydroethidium or hydroedthidine (HE) and Cy3-labelled MnSOD were purchased from Sigma Aldrich, India. Fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), trypsin, antibiotic–antimycotic solution, Lipofectamine 2000 were purchased from Invitrogen, India. Caspase-Glo assay kit from Promega, USA were purchased. Antibodies against actin, phosphoATM and totalATM were purchased from Cell Signaling Technology, USA; p73 from BD Biosciences, USA. Addgene plasmids;

shp73 (#22978), shATM (#14542) and an empty backbone pcDNA3.1 plasmid (#10792).

4.2.2. *Abrus* agglutinin purification

AGG was isolated and purified from *Abrus precatorius* seeds by ammonium sulfate fractionation followed by lactamyl sepharose affinity chromatography and Sephadex G-100 gel permeation chromatography. The activity of isolated AGG was measured by a hemagglutination assay, and the purity of the protein was subsequently analyzed by SDS-PAGE, native-PAGE and gel permeation by HPLC (Hegde et. al., 1991; Mukhopadhyay et. al., 2014a).

4.2.3. Cell culture

FaDu cell (hypopharyngeal) was cultured in minimal essential medium (MEM), supplemented with antibiotic–antimycotic (1X) and 10 % fetal bovine serum.

4.2.4. Western blotting

FaDu cells were treated with AGG followed by extraction of proteins. Cell extracts in cell lysis buffer were prepared, and equal amount of proteins were resolved by SDS/PAGE, transferred to PVDF membrane, and protein level was evaluated using antibody against p73, phosphoATM, total ATM as described previously (Bhutia et al., 2016).

4.2.5. Caspase assay

After treatment, caspase activity was measured using Caspase-Glo assay following the manufacturer's protocol (Promega Corp., Madison, WI).

4.2.6. ROS analysis

AGG treated FaDu cells were incubated with Dihydrorhodamine 123 (2.5 µg/ml) for 30 mins. After incubation, the cell pellet was suspended in 500 µl PBS and analyzed through flow cytometry.

4.2.7. Determination of superoxide level

After AGG treatment, FaDu cells were incubated with the superoxide sensitive probe dihydroethidium or hydroethidine (DHE, 10 µM) in PBS solution with $Ca2^{+}/Mg2^{+}$. After 30 min incubation in CO_2 incubator, imaging was performed at 40X fluorescence microscope (Mukhopadhyay et. al., 2015).

4.2.8. Determination of superoxide dismutase activity

FaDu cells were treated with different doses of AGG. Quantification of the level of total and mitochondrial superoxide activity was performed by following the

instruction(s) of the superoxide dismutase assay kit from Cayman Chemical Company (Ann Arbor, MI, USA).

4.2.9. *In silico* study

The structures of MnSOD (1ZUQ) and AGG (2Q3N) were retrieved from pdb. Chimera 1.6.2 and Swiss PDB Viewer were used in intermediate steps in order to visualize the structures. The retrieved structures were used to analyze the receptor and the ligand interaction by Hex 8.0.0. Ligplus software was used to visualize interacting binding residues at the active site.

4.2.10. FITC labelled AGG for colocalization study with cy3-MnSOD

For colocalization study, FaDu cells were treated with FITC conjugated AGG for 2 h (10 μg/ml) and after treatment cells were incubated with anti-superoxide dismutase antibody for 6 h at room temperature. The cells were washed and colocalization study was performed at 40X fluorescence microscope. The colocalization was measured by using JACoP plugin in single Z-stack sections of deconvoluted images (Ghosh et. al., 2009).

4.2.11. Comet assay

AGG treated FaDu cells embedded in agarose were layered on the frosted slides that were precoated with 1 % agarose for both controls and treated cell suspensions. The protocol was revisited as described (Panda et al., 2014). The intensity of the comet tail relative to the head reflects the degree of DNA breaks. One hundred comets were analyzed for each concentration and time point by fluorescence microscopy (Olympus IX71) (Panda et. al., 2014).

4.2.12. Analysis of γ -H2AX foci formation

FaDu cells were treated with different doses of AGG onto chamber slides (Falcon; BD Biosciences, USA). After treatment, cells were incubated with anti-phospho-histone H2AX (Ser139) (1:500) for overnight at 4 $^{\circ}$C. Controls were incubated with only the secondary antibodies under the same experimental conditions. FITC-conjugated donkey anti-mouse IgG (1:500) (Molecular Probes) was used for visualization on a fluorescence microscope and quantified the foci formation.

4.2.13. Plasmids and transfections

FaDu cells were transfected following the manufacturer's protocol. The transfections were performed in the presence of shp73 (Addgene plasmid 22978), shATM (Addgene plasmid 14542) and an empty backbone pcDNA3 plasmid (Addgene

plasmid 10792) which was used for mock transfection. After 48 h of transfection, the cells were analyzed and used to study apoptosis.

Statistical analysis

All data were given as the mean ± SD. Experimental results were analyzed by Student's t-test. $P < 0.05$ was considered as the level of significance for values obtained for treated compound to control.

4.3. Results

4.3.1. AGG induced ROS-regulated DNA damage and apoptosis

Oxidative stress results in ROS production and is governed by mitochondria. The mitochondria are known to be one of the most important sites for the generation of ROS which act as biochemical mediators of apoptosis. To detect ROS production, AGG treated FaDu cells were incubated with DHR123 which was rapidly taken up by cells and converted to Rhodamine123 (Rh123) in the presence of ROS. Treatment with AGG increased the level of ROS in a dose-dependent manner as compared to control shown through flow cytometry analysis (Fig. 4.1A). Pre-treatment with a ROS scavenger, N-acetyl cysteine (NAC, 10 mM) for 2 h recorded reduction in ROS production compared to only AGG (10 µg/ml) treated FaDu cells (Fig. 4.1B).

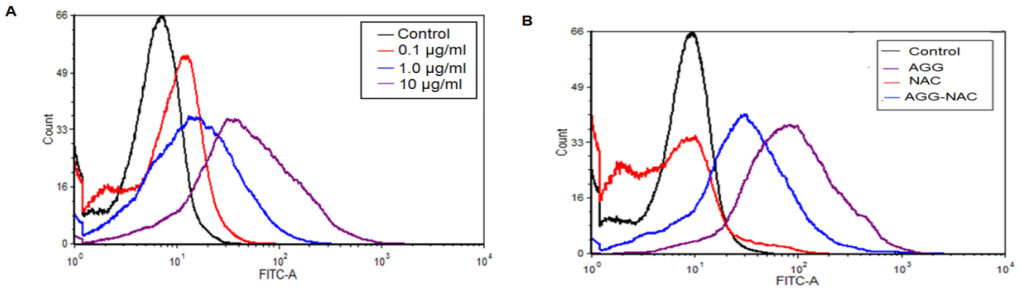

Figure-4.1. Analysis of ROS in AGG treated FaDu cells. AGG treated FaDu cells were incubated with dihydrorhodamine 123 (2.5 µg/ml) analyzed for ROS generation through flow cytometry (A). FaDu cells were pre-treated for 2 h with a ROS scavenger, N-acetyl cysteine (NAC, 10 mM) followed to AGG (10 µg/ml) and analyzed for ROS generation (B).

Furthermore, we monitored mitochondrial superoxide generation in AGG treated FaDu cells, and an increase in the dihydroethidium (DHE)-derived fluorescence, due to its reduced form 2-hydroxyethidium, was observed, which was significantly

reversed by NAC (Fig. 4.2A and 4.2B). Mitochondrial specific manganese-dependent superoxide dismutase (MnSOD) regulates superoxide production. We quantified

MnSOD activity in FaDu cells and observed downfall in its activity from 99 ±1 % in control to 95.5 ±1.2 %, 65.1 ±2.0 % and 47.3 ±1.3 % at the doses of 0.1, 1.0 and 10.0 µg/ml of AGG, respectively. Subsequently, a decline in the total SOD activity was observed from 99 ±0.6 % in control to 98.6 ±1.6 %, 76.3 ±2.7 % and 55.0 ±3.5 % at the doses of 0.1, 1.0, 10 µg/ml of AGG, respectively. The decreased MnSOD activity accounts for increased mitochondrial superoxide generation contributing to the accelerated apoptosis (Fig. 4.2C). Next, we performed *in silico* study to analyze the interaction between MnSOD and AGG (Fig. 4.3A). The docking operation was executed using Hex8.0.0 to gain the binding energy (ΔG) -73.12 kcal/mol, which showcased strong binding between the two proteins. The binding hot spots for MnSOD and AGG were also recognized; for MnSOD, the residues Asn71, Tyr166 and His163 provide significant contributions, and for AGG, Asn195, Tyr73, Tyr112 were recognized as hotspots basically from the active site region and adjacent areas of domain A1 of A chain in AGG (Fig. 4.3A). To further validate this *in vitro*, FaDu cells were treated with FITC labeled AGG (10 µg/ml) for 2 h followed by staining with anti-Cy3-MnSOD.

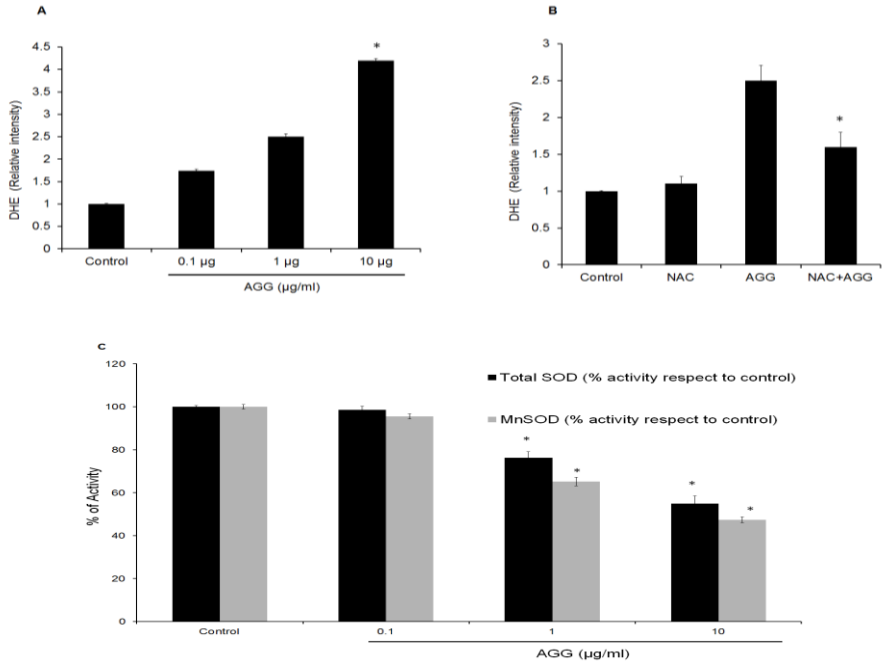

Figure.4.2: Analysis of superoxide level and superoxide dismutase activity. FaDu cells were treated with different concentration of AGG and superoxide anion was measured by Dihydroethidium staining (DHE, 10 µM) (A). *P value < 0.05 compared with control. FaDu cells were pre-treated for 2 h with NAC followed to AGG (10 µg/ml) and analyzed for superoxide anion accumulation (B). *P value <

0.05 compared with AGG treated group. FaDu cells were treated with different doses of AGG followed with the quantification of the level of total SOD and MnSOD activity (C).

We observed strong interaction between the two proteins with a dense yellow signal in the merged image (Fig. 4.3B). The Pearson's coefficient calculated from the fluorogram scatterplot appeared as 0.954 and 0.989, respectively (Fig. 4.3B).

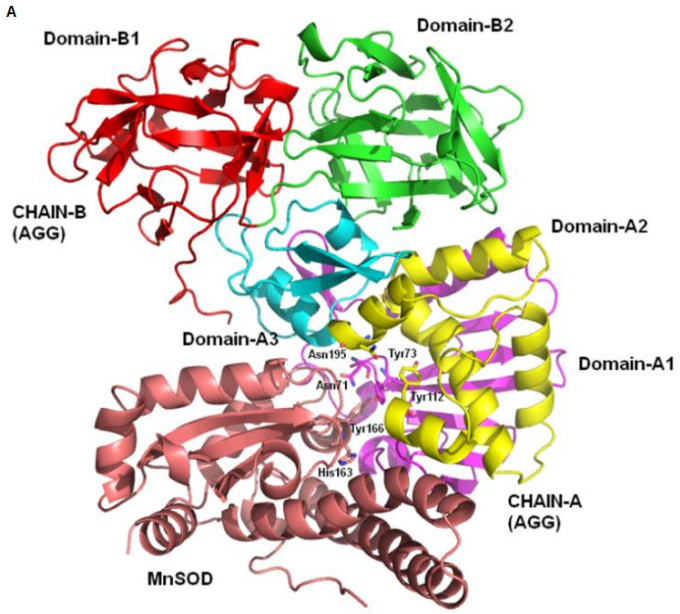

Figure-4.3. *In silico* study to analyze the interaction between MnSOD and AGG. The structures of MnSOD (1ZUQ) and AGG (2Q3N) were analyzed for the receptor and the ligand interaction by Hex 8.0.0, (ΔG) -73.12 kcal/mol. Ligplus software was used to visualize interacting binding residues at the active site. Asn71, Tyr166 and His163 provide significant contributions for MnSOD, and for AGG Asn195, Tyr73, Tyr112 recognized as hotspots basically from the active site region and adjacent areas of domain A1 of A chain in AGG (A). Cytofluorogram showcasing co-localisation study between MnSOD and AGG. The Pearson's coefficient and total overlap coefficient which was calculated from the fluorogram scatterplot appeared as 0.954 and 0.989 respectively. Scale bar denotes 20 μm (B).

Many plant lectins have been reported to have DNA-damaging activity apart from their RNA-N glycosidase action (Shrotriya et. al., 2012; Bhaskar et. al., 2008). To examine any direct DNA damage caused by AGG, comet assay and γH2AX staining were performed in FaDu cells. The data exhibited that AGG increased tail DNA length in a dose- as well as time-dependent manner in FaDu cell with maximum 6 h treatment (Fig. 4.3A and 4.3B).

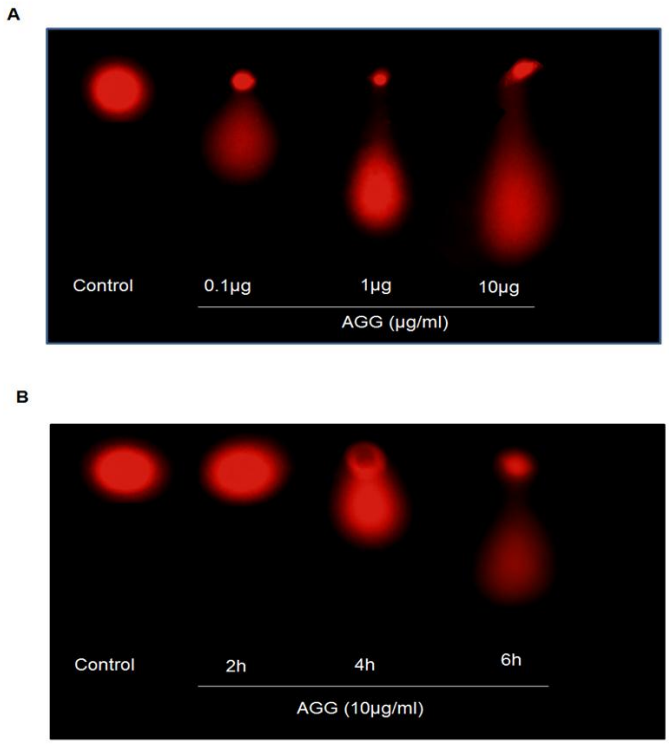

Figure-4.4. Comet assay in AGG treated FaDu cells. FaDu cells were treated for 6 h with different doses of AGG (0.1, 1, 10 µg/ml) and with the effective dose (10 µg/ml) for different time and followed by comet assay. After propidium iodide staining, photographs were taken in fluorescence microscope (Olympus IX71, 400X) (A and B).

In another independent experiment, γ-H2AX staining was executed; γ-H2AX is the phosphorylated form of histone H2AX (Ser 139) and histone H2AX phosphorylation occurs adjacent to double strand breaks (DSB) and is a very sensitive measure of double strand breaks. We observed that AGG increased the number of foci formation in a dose-dependent way in FaDu cells with the maximum effect at 10 µg/ml of AGG treatment (Fig. 4.4A). Similarly, AGG augmented foci formation in a time-dependent manner up to 6 h treatment with the effective dose (10 µg/ml) of AGG (Fig. 4.4B).

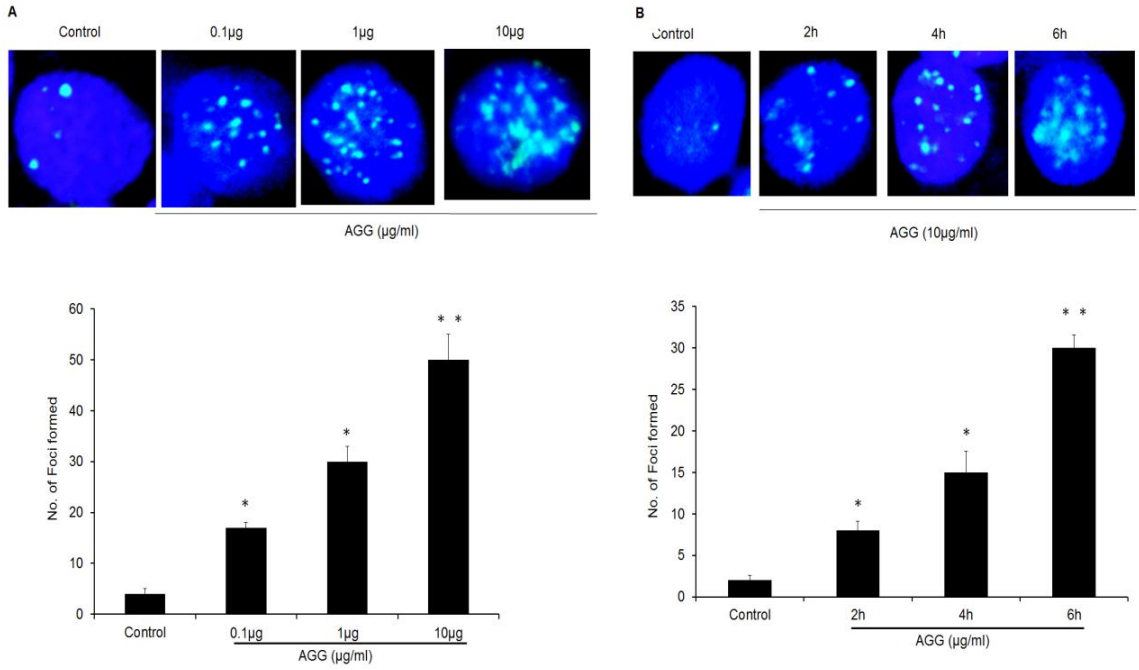

Figure-4.5. γH2X staining in AGG treated FaDu cells. FaDu cells were treated with AGG and γH2AX staining was performed to measure foci formation by fluorescence microscopy (Olympus IX71; 600X). Number of foci formation per nucleus was calculated and average was taken to plot the histogram (*P < 0.05; **P < 0.01 compared with control) (A and B).

Pre-treatment with NAC prevailed the AGG-treated FaDu cell from DNA damage, decreased tail length of the comet as well as reduced number of foci formation (Fig. 4.5A and 4.5B) confirming ROS production regulates DNA damage. We attempted to introspect the prospects of DNA damage as a direct phenomenon or consequence of DNA fragmentation by assessing the phosphorylation of H2AX in FaDu cells treated with AGG in presence of the broad spectrum pan-caspase inhibitor (Z-VAD.FMK). We found that AGG treatment did affect phosphorylation of H2AX (number of foci formation) in presence of Z-VAD.FMK (Fig. 4.5C) confirming ROS-induced DNA damage as an upstream event followed by apoptosis. To appraise the relationship between ROS, DNA damage and apoptosis, apoptotic activity of AGG was performed by annexin V staining and caspase-Glo assay in presence of NAC in FaDu cells. The AGG induced annexin V positive cells were declined from 29 ±3.5 % to 18 ±2.1 % with NAC introduction upon AGG treatment (Fig. 4.5D). Similarly, the caspase-Glo data indicated that caspase (3/7) activity of AGG was decreased in the presence of NAC-treated cells as compared to only AGG treated cells (Fig. 4.5E). These investigations uphold ROS to be the upstream signaling candidate that governs different cellular events including DNA damage followed by apoptosis.

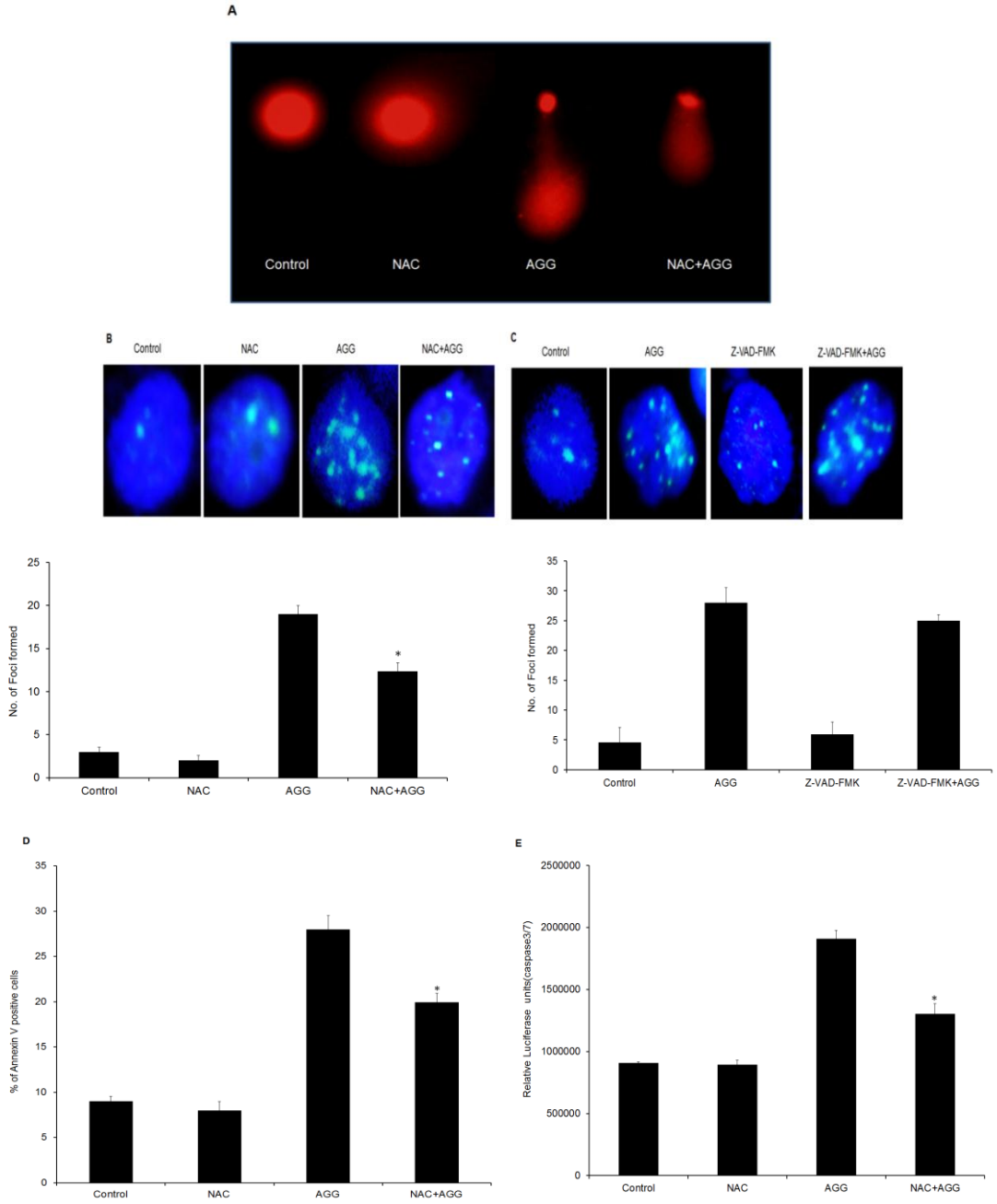

Figure-4.6. AGG facilitated DNA damage in FaDu cells. FaDu cells were treated with AGG in presence of NAC and analyzed for comet assay (A) and foci formation by γH2AX staining (B) through fluorescence microscopy. *P < 0.05, compared with AGG treated group. AGG induced DNA damage in presence Z-VAD-FMK was measured through foci formation by γH2AX staining with fluorescence microscopy in FaDu cells (C). FaDu cells were treated with effective dose of AGG (10µg/ml) in presence of NAC and analyzed for apoptosis induction by annexin V/PI staining through flow cytometry (D) and caspase activation by caspase Glo assay (E). Data reported as the mean ± S.D. of three independent experiments and *P value < 0.05 compared with AGG treated group.

4.3.2. AGG induced p73-mediated apoptosis

It has been demonstrated that plant lectins activate DNA damage signaling pathway and lead to apoptosis through activation of p53; however, majority of head and neck tumors witness p53 mutations and lack a functional p53 response. Henceforth, in our study we examined the older sibling of p53; p73 and its regulation in DNA damage-induced apoptosis in AGG treated FaDu cells. AGG treatment exhibited high expression of p73 protein as compared to control (Fig. 4.6A) which was reversed in presence of NAC (Fig. 4.6B). To appreciate the rationale of recruitment of p73 for caspase-mediated apoptosis, FaDu cells were transfected with shp73 and 48 h after transfection, AGG-induced apoptosis was quantified. The data showed the expression of p73 was decreased in p73 transfected cells as compared to pcDNA (Fig. 4.6C). The annexin V/PI staining analysis demonstrated that the apoptotic population induced by AGG was decreased from 38 ±4% to 24 ±3% in pcDNA and shp73 cells, respectively (Fig. 4.6D). We further performed caspase activity and found that there was a decrease in the activity of executioner caspases (3/7) in the p73 deficient cells as compared to controls (Fig. 4.6E). These data confer that AGG induced p73-mediated apoptosis.

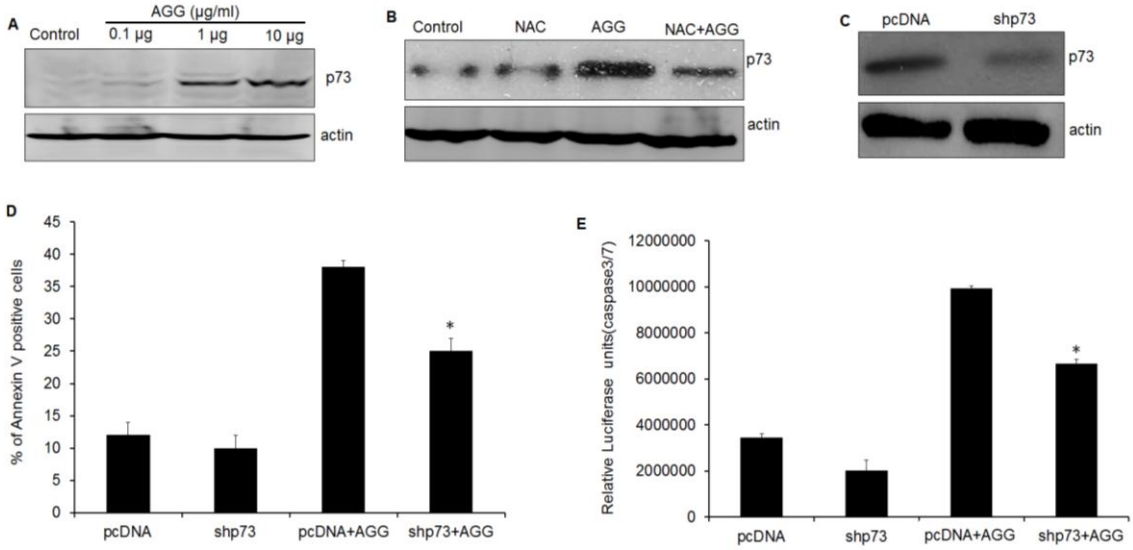

Figure-4.7. AGG induced p73 mediated apoptosis in FaDu cells. FaDu cells were treated with the variable doses of AGG and p73 expression was monitored by Western Blot (A). FaDu cells were treated with effective dose of AGG (10 µg/ml) in presence of NAC and p73 expression was evaluated by Western Blot (B). FaDu cells were transfected with shp73 for 48 h, p73 expression was analyzed by Western Blot (C). The transfected FaDu cells were treated with AGG for 24 h and apoptosis was measured by annexin V/PI staining (D) and caspase Glo assay (E). *P value < 0.05 values were considered significant against pcDNA-AGG treated group.

4.3.3. ATM-mediated p73 apoptosis in AGG treated FaDu cells

AGG treated FaDu cells showed DNA damage, like single and double strand breaks mediated by ROS generation. Double strand breaks activate ATM (ataxia telangiectasia mutated serine-protein kinase) and accumulates at the site of DNA damage (Roncuzzi et. al., 1996; Sancar et. al., 2004). To delineate the role of ATM expression in FaDu cells, we analyzed the expression of ATM phosphorylation after treatment with the effective dose of AGG (10 μg/ml). The level of phosphorylated-ATM (p-ATM^{ser1981}) expression was temporally increased in response to AGG, whereas total ATM levels remained unchanged (Fig. 4.7A). On introduction of NAC, AGG-induced DNA damage was minimized which eliminated the phosphorylation of ATM at serine 1981, and consistent with that suppression of AGG mediated p-ATM ^{ser1981} expression was observed (Fig. 4.7B). To investigate the role of ATM–p73 pathway and induction of apoptosis, we transfected FaDu cells with shATM (Fig. 4.7C) and studied the expression of p73 upon AGG treatment (Fig. 4.7D). At an effective dose of AGG, p73 expression was downregulated in shATM transfected cells compared to pcDNA control (Fig. 4.7D). Reduced activity of p73 resulted in a decreased number of apoptotic cells analyzed by annexin V staining and the apoptotic population dropped from 30 ±2.5 % in pcDNA to 18 ±2.6 % shATM treated cells (Fig. 4.7E). Further, quantification of apoptosis was done by analysing caspase activity of executioner caspases (3/7) which exhibited a noticeable decrease in the transfected cells (Fig. 4.7F) confirming AGG-induced ROS accumulation activates ATM, thus leading to ATM-mediated p73 apoptosis in FaDu cells.

4.4. Discussion

In the present study, AGG demonstrated to inhibit the proliferation of oral squamous cell carcinoma convincingly with no effect on the normal cell. To target cancer cells, one of the prosecutions can be alleviated by evoking DNA damage response, and recent reports have highlighted the DNA-damaging efficacy of plant lectins. *Sambucus nigra* bark lectin has been shown to induce DNA damage in Chinese hamsters in a study utilizing comet assay (Macewicz et. al., 2005). Similarly, *Abrus* abrin also caused DNA damage through oxidative stress which was reversed by antioxidant in human myeloleukemic cells (Bhaskar et. al., 2008). Our previous study showed that SBL induced DNA damage in both dose- and time-dependent manner quantified by

examining the length of the comet tail and the tail DNA percentage, and that ROS was the major switch which governs autophagy, apoptosis and DNA damage in HeLa cells.

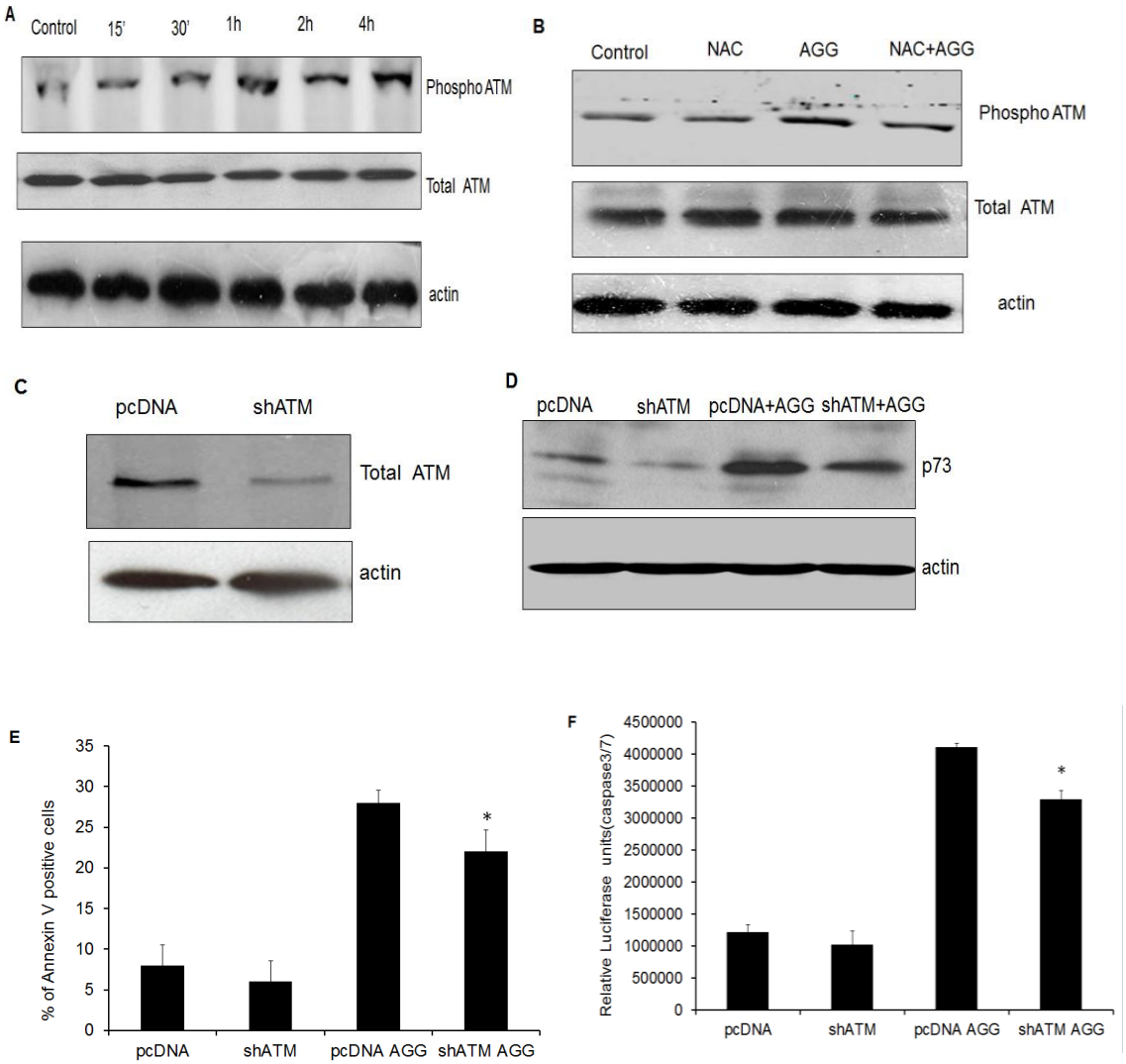

Figure-4.8. ATM-mediated p73 apoptosis in AGG treated FaDu cells. FaDu cells were treated with effective dose (10 µg/ml) of AGG for different time duration. The level of phosphorylated-ATM (p-ATM^{ser1981}) expression and total ATM expression were observed by Western Blot (A). FaDu cells were treated with the effective dose (10 µg/ml) in presence of NAC to evaluate the level of phosphorylated-ATM (p-ATM^{ser1981}) expression and total ATM expression by Western Blot (B). FaDu cells were transfected with shATM for 48 h and total ATM expression in pcDNA and shATM transfected cells were analyzed by Western Blot. The transfected FaDu cells were treated with AGG (10 µg/ml) for 24 h and p73 expression was analyzed by Western Blot (D) apoptosis induction by annexin V staining (E) and caspase Glo assay (F). Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P < 0.05 values were considered significant against pcDNA-AGG treated group.

In our results, AGG effectively induced ROS in FaDu cells triggering single and double strand breaks followed by apoptosis. Pre-treatment with ROS scavenger NAC reduced apoptosis and DNA damage, suggesting ROS as the central regulator of AGG-mediated DNA damage and apoptosis. In contrary, it was reported that *Abrus* abrin induced DNA damage in Jurkat cell was not the cause of apoptosis rather it was the consequence of DNA fragmentation in apoptosis (Mishra et. al., 2014). In response to DNA double-strand break (DSB), H2AX is recruited at the site of DSB and gets phosphorylated at Ser139 site by various kinases, including ATM, ATR and DNA-dependent protein kinase, allowing it to accumulate at the site of DNA damage (Zhao et. al., 2002). DNA damage signal from ATM/ATR as well as these kinases are largely inactive in cancer cells, but interestingly, we observed that AGG treatment temporally upregulated the phosphorylation of DNA damage sensor kinase ATM (at Ser1981) in FaDu cells, making it an important element in AGG activity.

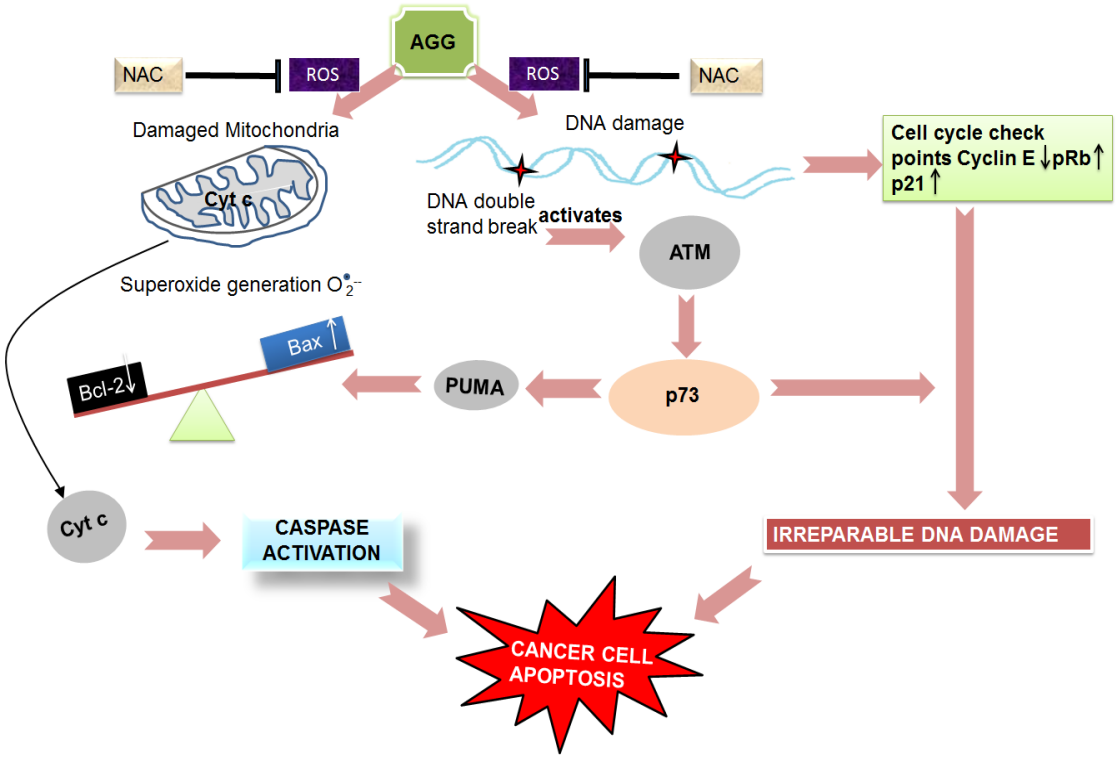

Figure-4.9. Flowchart demonstrating ROS mediated irreparable DNA damage induced by AGG leads to ATM-p73 dependent mitochondrial apoptosis.

ROS-mediated DNA damage can result in p53 and its family proteins accumulation inthe nucleus. In turn, the tumor suppressor p53 has been validated to regulate basal and DNA-damage-induced ROS levels (Seth et. al., 2005). However, most of head and neck cancers contain mutant or non-functional p53 limiting the benefit of targeting p53 in current cancer therapeutics. Therefore, the discovery of additional new factors that can induce apoptosis in a p53-independent manner is of great concern and utmost importance in targeting oral cancers. In this connection, p73 is a tumor suppressor that is rarely mutated, can compensate many of p53 target genes involved in cell cycle regulation and apoptosis and serve as the promising approach for oral carcinoma treatment (Engelmann et. al., 2015). Previous studies showed that different chemotherapeutic drugs induced p73-mediated apoptosis in response to DNA damage (Yuan et. al., 1999; Lunghi et. al., 2004; Leong et. al., 2007; Long et. al., 2015). Similarly, several different small molecules including p53-derived apoptotic peptide (Bell et. al., 2007), rapamycin (Wong et. al., 2011) and RETRA (Kravchenko et. al., 2008) have recently been reported to affect positively p73 apoptotic pathway. Here, we attempted to focus on p73, and its regulation of apoptosis in AGG-treated FaDu cells. AGG treatment efficaciously escalated the expression of p73, making it the protagonist of the outcome we observed. Our data showed that the percentage of annexin V-positive cells and caspase activity were declined in p73 deficient cells as compared to control. Melino et al, have demonstrated that p73-induced PUMA mediates Bax mitochondrial translocation and triggers mitochondrial apoptosis (Melino et. al., 2004). In support, our data showed that AGG augmented PUMA expression in a dose-dependent manner suggesting p73, induced by AGG, caused PUMA-mediated apoptosis. In conclusion, we established a connection among ROS, DNA damage, ATM and p73 in the efficacy of AGG against FaDu cells, which could have an association in improving therapeutic perspective of p53 deficient oral and other head and neck squamous cell carcinoma.

Chapter 5

p73 induction by *Abrus* agglutinin facilitates Snail ubiquitination to inhibit epithelial to mesenchymal transition in oral squamous cell carcinoma

Abstract

Epithelial-to-mesenchymal transition (EMT), a key step in cancer progression in oral cancer is associated with invasion, metastasis and therapy resistance, and targeting the EMT represents a critical therapeutic strategy for treating oral cancer metastasis. In the present study, *Abrus* agglutinin, a plant lectin inhibited EGF-induced EMT through p73 dependent pathway in FaDu cells. Our study showed that AGG increased expression of epithelial proteins and decreased the expression of mesenchymal proteins in dose dependent way in FaDu cells. Further, AGG found to inhibit EGF-stimulated invasiveness and stemness indicating the EMT inhibition as one of AGG's tumor suppressive mechanism. At molecular level, AGG-induced p73 found to suppress Snail expression leading to the EMT inhibition in FaDu cells. Importantly, AGG was shown to promote translocation of Snail from nucleus to cytoplasm of FaDu cells and triggered its degradation through ubiqutination. In this setting, AGG inhibited the interaction of Snail and p73 in FaDu cells resulting p73 activation and EMT inhibition. Moreover, in EGF-stimulated FaDu cells, AGG abrogated the upregulation of ERK1/2 that plays a pivotal role in upregulation Snail to regulate the EMT phenotypes. In immunohistochemistry analysis, FaDu-xenografts from AGG-treated mice exhibited decline in expression of Snail, SOX2, Vimentin and increase expression of p73 and E-cadherin as to control confirming inhibition of EMT as a part of its anti-cancer efficacy against oral cancer. In summary, AGG elicits p73 in restricting EGF-induced EMT, invasiveness and stemness by inhibiting the ERK/Snail pathway to facilitate the development of alternative therapeutics for oral cancer.

5.1. Introduction

Oral cancer mostly squamous cell carcinoma (SCC) is leading cause of cancer related death in India due to high incidence of consumption of tobacco product including Gutkha and Paan in young and middle-aged men. Moreover, it is one of the drastic human cancers due to its aggressiveness which has not been changed significantly during the last two decades with advances in current therapies. Interestingly, the oral SCC is associated with high rate of invasiveness and lymph node metastasis affecting the prognosis of the patients. During tumor metastasis, tumor cells acquire invasive property by activating epithelial-to-mesenchymal transition (EMT) program to endure phenotypic alternation to evade primary tumor and in oral cancer is associated with

invasion, metastasis and therapy resistance. Upon stimulation of epidermal growth factor receptor (EGFR) with EGF, SCC cells display increased motility, migration, invasion, metastasis, and resistance to apoptosis *in vitro* along with the down-regulation of E-cadherin and up-regulation of N-cadherin and vimentin (Zhu et. al., 2012). Moreover, activation of PI3K/Akt and MEK-1/2/ERK-1/2 or either of the two survival pathways is an important step for cancer cells to dwell in the metastatic sites and adapt to their new microenvironment (Li et. al., 2013; Jiao et. al., 2016; Mishima et. al., 1998). Further, the process of EMT is controlled by a group of transcriptional factors, zinc finger proteins and basic helix-loop-helix factors. Snail (snail1 or snai1) transcription factor with C2H2 zinc finger domain is a master regulator of EMT in several tumor progressions. Snail has a strong role in implicating the mitogen-activated protein kinase (MAPK) pathway. Snail and ERK1/2 activity was also found to be higher in breast cancer cells as compared to normal mammary epithelial cells. Snail overexpression in MCF-7 was shown to promote nuclear ERK1/2 activation (p-ERK), and poor prognosis (Smith et. al., 2014). According to report, chemokine (C-X-C) ligand 5 (CXCL5) could activate Raf/MEK/ERK, MSK1, Elk-1, and Snail leading to the downregulation of E-cadherin in breast cancer. Moreover, Snail induces EMT by potentially binding with E-box consensus sequence of epithelial gene E-cadherin leading to its transcriptional repression followed by decreased cell adhesion to the neighboring cells.

The transcription factors of the Snail family have been associated with EMT during both embryonic development and cancer metastasis. In mammalian neural precursors Snail promotes cell survival by antagonizing a p53-dependent death pathway as the coincident p53 knockdown rescues survival deficits caused by Snail knockdown. P53 knockdown rescued cells by altering cell death and/or proliferation (Zander et. al., 2014). Lee et al. showed that inhibiting the direct binding between Snail and DNA binding domain of p53 resulted in recuperation of p53 expression in K-Ras-mutated cancers including pancreatic, lung, and colon cancers. P73 or TAp73 repressed epithelial to mesenchymal transition (EMT) with an increase in E-cadherin and decrease in EMT transcription factors, but DNp73 accelerated Slug upregulation an EMT-like phenotype with loss of E-cadherin (Steder et. al., 2013).

Abrus agglutinin (AGG), a low-toxicity protein isolated from the seeds of *Abrus precatorius* inhibits protein synthesis followed by induction of mitochondrial dependent apoptosis and autophagic cell death documented in different cancer (Bhutia

et. al., 2008; Mukhopadhyay et. al., 2014a; Bhutia et. al., 2016; Panda et. al., 2016). Earlier we have validated pro-apoptotic potential of AGG in oral carcinoma by triggering ROS generation followed by ATM-p73 mediated apoptosis. In this study, our accumulated data prospered EMT inhibition by AGG treatment in EGF-stimulated FaDu cells. On introduction of PD 98059, an ERK inhibitor, AGG treatment resulted in synergistic aggravated inhibition of ERK/Snail, and propelled the upregulation of E-cadherin and p73 followed to reduced invasiveness and stemness. Since FaDu cell has non-functional p53, we hypothesized that in absence of functional p53, Snail might bind to p73 constraining its apoptotic function. We found that AGG act as a potent inhibitor in inhibiting the binding of Snail to p73 to rescue its apoptotic potential and hence thwarted EMT, invasiveness and stemness in EGF stimulated FaDu cells by inducing MET with upregulation of E-cadherin expression; the calculated response of AGG was mitigated in overexpressed Snail and shp73 cells, pronouncing AGG as a critical molecule in Snail degradation.

5.2. Material and Methods

5.2.1. Chemical and reagents

6-Diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO) was purchased from Sigma Aldrich, India. Fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), Dulbecco's modified Eagle's medium with high glucose (DMEM/high glucose), trypsin, antibiotic–antimycotic solution, Lipofectamine 2000 were purchased from Invitrogen, India. Antibodies against E-cadherin, p73, N-cadherin, Vimentin, SOX2, OCT4, Nestin, EGFR and Actin; growth factor EGF were purchased from BD Biosciences, USA. Involucrin from Santa Cruz Biotechnology, USA. ADAM17 from abcam, USA. Snail from Cell Signaling Technology, USA. PD98059 from Cell Signaling Technology, USA and MG-132 from Calbiochem. Addgene plasmids; shp73 (#22978), Snail ((Plasmid #16218), and an empty backbone pcDNA3.1 plasmid (#10792). Boyden chamber were procured from BD Biosciences, USA.

5.2.2. *Abrus* agglutinin purification

AGG was isolated and purified from *Abrus precatorius* seeds by ammonium sulfate fractionation followed by lactamyl sepharose affinity chromatography and Sephadex G-100 gel permeation chromatography. The activity of isolated AGG was measured

by a hemagglutination assay, and the purity of the protein was subsequently analyzed by SDS-PAGE, native-PAGE and gel permeation by HPLC.

5.2.3. Cell culture

FaDu (hypopharyngeal squamous cell carcinoma) cell line was cultured in minimal essential medium (MEM), supplemented with antibiotic–antimycotic (1X) and 10 % fetal bovine serum.

5.2.4. Immunofluorescence

After EGF (20 ng/ml) stimulation for 1 h, FaDu cells treated with AGG for 12 h, followed by fixation, permeabilization and primary antibodies incubation (1:100; E-cadherin, Involucrin, N-cadherin, Vimentin, β-Catenin, and p73 overnight. The cells were further incubated with the secondary anti-rabbit and/or anti-mouse antibodies conjugated with Alexa Fluor to study the fluorescence of our desired proteins. Imaging was performed at 40X fluorescence microscope (Olympus IX71).

5.2.5. Confocal Microscopy

EGF (20 ng/ml) induced FaDu cells were grown on chamber slides for 12 h. Cells were treated with AGG for 24 h, and then fixed in 10 % formaldehyde, washed with PBS, permeabilized with 0.2 % Triton X-100 for 20 min at RT along with primary antibody incubation of Snail overnight. Cells were then washed with PBS and incubated with secondary antibody for 6 h followed by DAPI counterstaining. Cell images were captured at 1000X magnification on a Leica TCS SP8, Wetzlar, Germany using 561/488/405 nm laser wavelengths to detect Snail (Red), and DAPI (Blue) emissions, respectively (Deep et. al., 2014).

5.2.6. Cell invasion assay

Matrigel was diluted to 1 mg/ml by 4°C precooled serum-free DMEM medium. 100 μL diluted Matrigel were added to the middle of the bottom of the 24-Transwell chamber, and incubated for 5 h at 37°C, in which 200 μL DMEM medium was added later. Cells were seeded into the upper compartment of the Transwell insert of pore size 8 μm (5000 cells/ml) in serum-free culture medium. Medium with 10 % FBS was added to the lower chamber as a chemo-attractant factor. Cells were cultured at 37°C, and then the non-invading cells on the upper surface of the membrane were gently removed with a cotton swab. After incubating for the indicated times (12 h), the cells were treated with AGG, and were fixed with 4 % methanol and stained with haematoxylin eosin for 10 min at 37°C. The fixed cells were then observed under a

bright field microscope at 10X bright field and the number of cells was counted per visual fields (Wang et. al., 2015; Bhutia et. al., 2016).

5.2.7. Western blotting and Immunoprecipitation analysis

The protein concentration of cell lysates from different experimental set up was quantified. 80 μg of protein was resolved on a 10–13 % SDS PAGE, followed by transblotting onto nitrocellulose membrane. The membranes were blocked in 5 % skimmed milk in PBST (Phosphate Buffer Saline with 0.1 % Tween-20), and subsequently incubated with diluted primary antibody in blocking buffer. After washing, the membranes were incubated in peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat secondary antibody, then washed, and visualized using ECL prime reagent. The membranes were stripped using stripping buffer prior to reprobe with a different antibody. For immunoprecipitation analysis, cell lysate was incubated first with primary antibody overnight at 4°C and then with protein-A/G-agarose for 2 hours. After centrifugation and washing five times, the precipitated complex was subjected to SDS-PAGE/Western blot analysis (Lee et. al., 2009; Henderson et. al., 2015).

5.2.9. Plasmids and transfections

FaDu cells were transfected following the manufacturer's protocol. The transfections were performed in the presence of shp73 (Addgene plasmid 22978), Snail ((Plasmid #16218), and an empty backbone pcDNA3.1 plasmid (Addgene plasmid 10792) which was used for mock transfection. After 48 h of transfection, the cells were stimulated with EGF (20 ng) for 1 h, and used to study the target protein expressions.

Statistical analysis

Data were presented as mean ± SD and evaluated with Student's t-test. $P < 0.05$ was accepted as statistically significant.

5.3. Results

5.3.1. AGG inhibits epithelial to mesenchymal transition and stemness in hypopharyngeal cancer cell

The EMT program is disruption of epithelial sheet and acquisition of mesenchymal phenotype with enhanced cell motility and is associated with cell migration, invasion, and stemness. To examine whether AGG inhibits EMT in oral cancer, we aim to determine expression of different EMT markers upon AGG treatment in FaDu cells. Our data showed that AGG was able to upregulate the expression of epithelial

markers- E-cadherin and involucrin, and downregulate expression of N-cadherin, and vimentin (Fig. 5.1A and B) as demonstrated by immunofluorescence and Western blot. Moreover, mesenchymal cells exhibit CSC-like feature with self-renewal property that can maintain the population of tumorigenic cells. Importantly, AGG effectively repressed the aberrant cytoplasmic expression of β-catenin evident from the immunofluorescence study (Fig. 5.1A). We identified through Western blotting that AGG curbed the expression of SOX2, OCT4 and Nestin that support plasticity of cancer stem cells (Fig. 5.1C).

Growth factor including EGF plays a complex role in EMT as well as modulating ECM degradation and facilitating the progression of OSCC. Here, we studied effect of AGG on EMT, stemness and invasive potential in EGF-stimulated FaDu cells. We stimulated FaDu cells with 20 ng/ml of EGF for 1 h followed to AGG treatment and expression of E-cadherin was observed in dose dependent manner FaDu cells (Fig. 5.2A). Inhibition of EMT was further shown as loss of mesenchymal markers including N-cadherin, β-catenin, and vimentin in EGF-induced FaDu cells (Fig. 5.2A). Interestingly, AGG significantly downregulated the expression of SOX2, OCT4 and Nestin in EGF induced FaDu cells thereby inhibited the pluripotency of cancer stem cells (Fig. 5.2A). Further, AGG arrested the tumor cell invasive property of EGF stimulated FaDu cells by downregulating the disintegrin and metalloprotease ADAM17 which plays an implicative role in cancer cells invasion (Fig. 5.2A). To accentuate our hypothesis, the invasive nature of cells were quantified by analyzing the number of HE stained cells entering into the lower chamber observed in Boyden chamber which decreased from 180 ±6.5 in control to 160 ±6.8, 120 ±7.6, 80 ±9 in instances of 0.1 μg, 0.5 μg and 1 μg respectively (Fig. 5.2B and C), confirming AGG as potent EMT inhibitor in oral cancer.

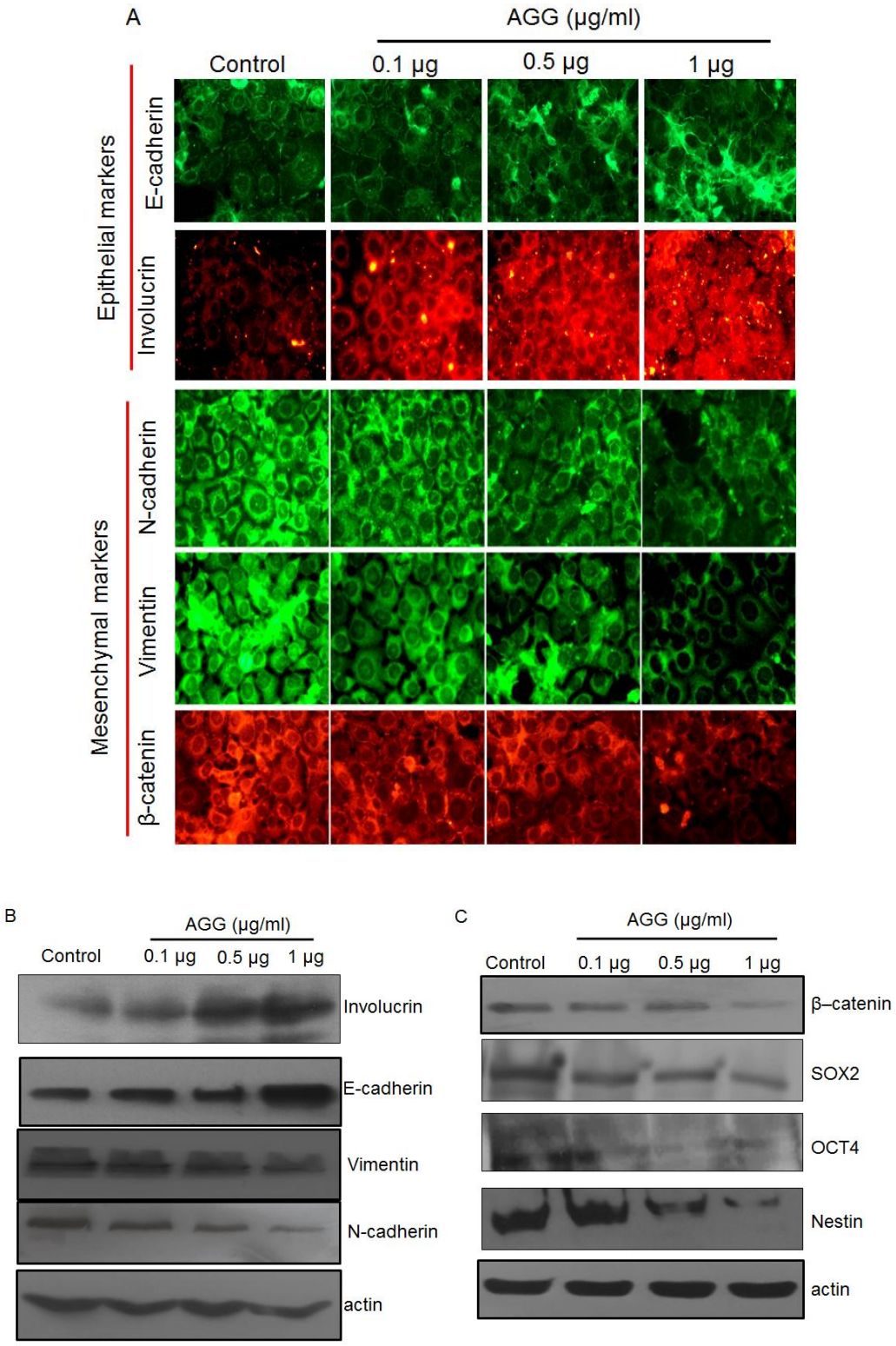

Figure-5.1. AGG inhibited epithelial to mesenchymal transition and stemness in hypopharyngeal cancer cell. FaDu cells were treated with different doses of AGG (0.1 µg, 0.5 µg and 1 µg/ml). The expression of epithelial (Involucrin and E-cadherin) and mesenchymal markers (N-cadherin, β-catenin, and Vimentin) were validated by immunofluorescence and Western blot studies (A and B). The expression of stemness markers (SOX2, OCT4 and Nestin) were analyzed by Western blot (C).

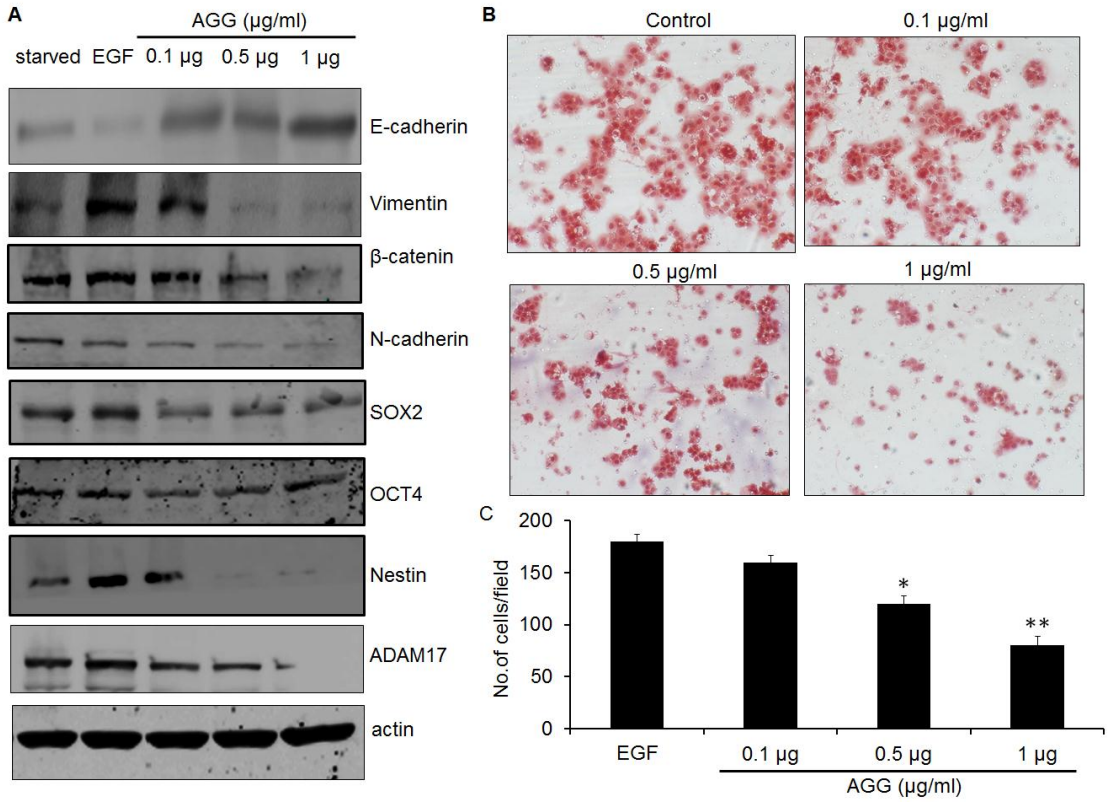

Figure-5.2. AGG inhibited EGF stimulated epithelial to mesenchymal transition and stemness in hypopharyngeal cancer cell. FaDu cells were stimulated with 20 ng/ml EGF for 1 h followed with AGG treatment, the expression of E-cadherin along with mesenchymal (N-cadherin, β-catenin, and Vimentin) and stemness markers (SOX2, OCT4 and Nestin) were analyzed by Western Blot. The invasiveness was studied through monitoring the expression of ADAM17 by Western Blot (A). Further, the invasive nature of cells were quantified by analyzing the number of HE stained cells entering into the lower chamber observed in Boyden chamber and the number of cells/field were counted and quatified (B and C). Data reported as the mean ± S.D. of three independent experiments and *P value < 0.05 compared with AGG treated group.

5.3.2. Snail inhibition by AGG lead to p73 dependent E cadherin mediated EMT regulation

Snail promotes cell migration and invasion through repression of E-cadherin tumor suppressor by binding to E-boxes located in the promoter region. Since Snail has a short half-life period and gets degraded quickly, we found that in presence of MG132, EGF stimulated FaDu cells exhibited decreased expression of Snail in presence of AGG treatment (Fig. 5.3A). Downregulation of Snail was inversely correlated with expression of p73 in presence of AGG (Fig. 5.3B). Our previous findings confirmed that AGG effectively upregulated the expression of p73. Here, we found altered p73

expression in Snail overexpressed EGF stimulated FaDu cells upon AGG treatment with the effective dose (1 µg/ml) (Fig. 5.4A and B).

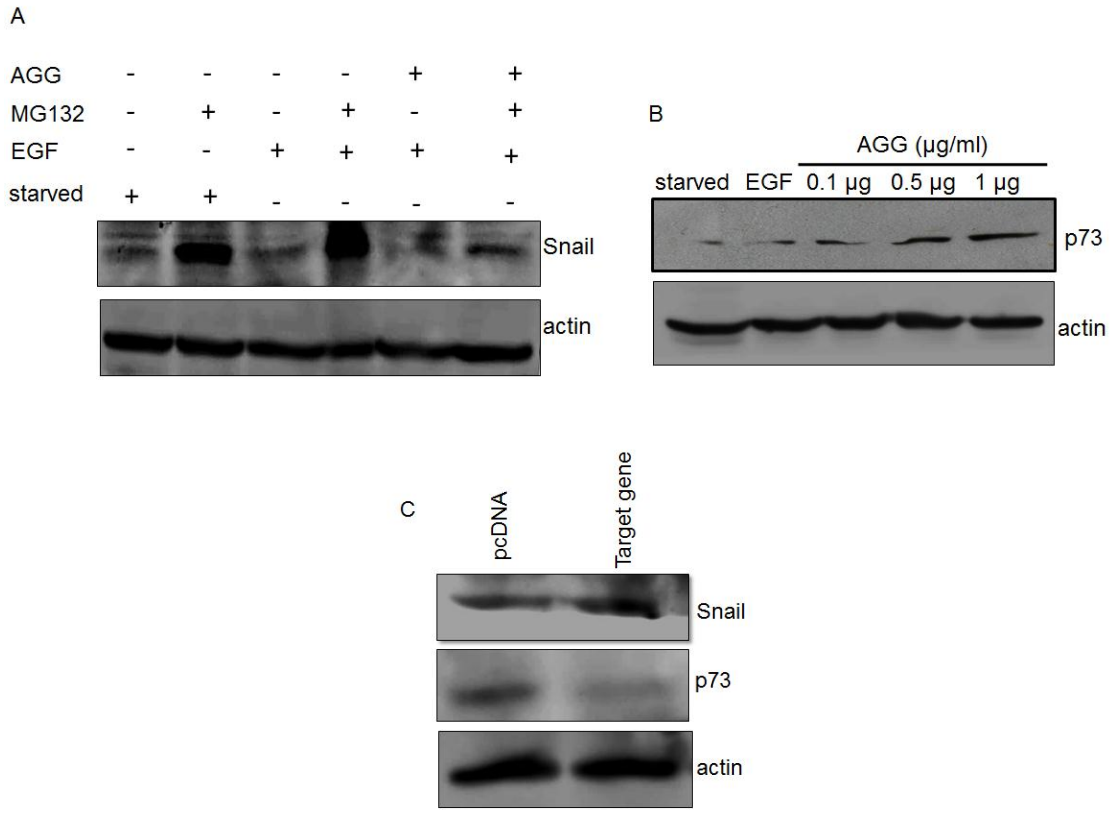

Figure-5.3. Snail inhibition by AGG in FaDu cell. Snail has a short half-life period and gets degraded quickly; therefore the FaDu cells were pre-treated with MG132 for 3 h, stimulated with EGF (20 ng/ml) for 1 h followed with AGG treatment, the Snail expression was evaluated by Western Blot (A). The p73 expression was checked by Western Blot (B). FaDu cells were transfected with the target genes and the expression of the target proteins were analyzed by Western Blot (C).

Overexpression of Snail inhibited cell–cell contacts and decreased the expression of E-cadherin (Fig. 5.4A). The invasive capacity of Snail overexpressed FaDu cells increased with the upregulated expression of ADAM17 compared to mock cells upon EGF stimulation, implying that Snail could be considered a master gene in the EMT leading to its invasive properties that occurred due to decreased cell polarity, cell dedifferentiation, changes in connections between cells and strengthened cells invasive ability (Fig. 5.4A). Moreover, the overexpression of snail was associated with the stemness of FaDu cells, decoded by the upregulated expression of transcription factors SOX2 and OCT4 responsible for the maintenance of the cancer stem cell (CSC)-like phenotype.

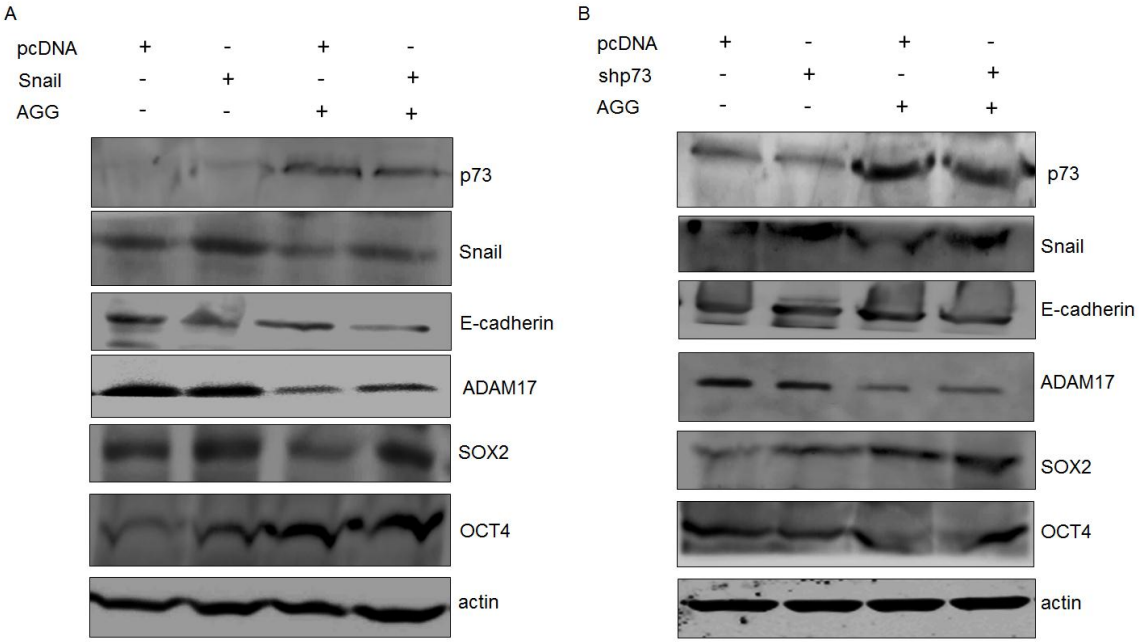

Figure-5.4. Snail inhibition by AGG lead to p73 dependent E cadherin mediated EMT regulation. FaDu cells after being transiently overexpressed with Snail and transfected with shp73 were stimulated with EGF (20 ng/ml) for 1 h followed with treatment of varying doses of AGG. Cell lysate were subjected to check the protein expression of different EMT modulators by Western Blot (A and B).

The cancer cells undergoing an EMT have been found to show increased resistance to apoptosis and chemotherapeutic drugs with the potential to attain the traits of cancer stem cells. P73 induces apoptosis through its ability to bind p53 DNA target sites. An interesting feature we checked by transiently transfecting FaDu cells with shp73 (Fig. 5.3C). The shp73 transfected FaDu cells were EGF stimulated and after AGG treatment with the effective dose (1 µg/ml) the p73 expression reduction restored the Snail expression compared to the mock cells with the reduced expression of E-cadherin (Fig. 5.4B). Compared to the wild type, blocked p73 expression contributed to the invasiveness, evident from ADAM17 expression in AGG treated shp73 cells (Fig. 5.4B). Increased Snail expression upon AGG treatment (1 µg/ml) in shp73 cells corroborated with upregulated transcription factors; SOX2 and OCT4 responsible for the plasticity of cancer stem cells (Fig. 5.4B). Henceforth, disruption of E-cadherin and p73 expression with enhanced Snail expression plays a crucial role in tumor progression.

5.3.3. Snail degradation activated p73 mediated EMT inhibition

AGG treatment resulted in Snail downregulation; further confocal study revealed that Snail was translocated to the cytosol followed by its ubiqitination evident from immunoprecipitation data. To escape apoptotic cell death, we hypothesize that Snail binds to p73, masking its apoptotic candidature. Establishing a relationship between apoptosis and EMT (Lee et. al., 2015); double immunofluorescence analysis was performed using inverted fluorescence microscopy, we observed that Snail interacted with p73 in EGF stimulated FaDu cells due to its nuclear localization, visible by the overlap of the two proteins in the merged image showing dense yellow signal, depicting the Pearson's coefficient 0.933, in the cytofluorogram scatter plot; while in the presence of AGG, EGF stimulated FaDu cells showed decline in the Pearson's coefficient of 0.889 respectively (Fig. 5.6). To further validate our immunofluorescence data; coimmunoprecipitation was performed, equal amount of Snail protein and p73 protein were pulled out from the lysates of the two sets of the experiment to study the expression of either antibody anti-Snail or anti-p73 for immunoprecipitation followed by immunoblotting with anti-p73 or anti-Snail analyzed by the Western blot. The results showed that stimulation with EGF in FaDu cells enhanced the propensity of interaction between Snail and p73 while AGG hindered the interactome leading to the Snail translocation, ubiquitination and degradation in the cytosol followed by activation of p73 (Fig. 5.5A and 5.6A). Henceforth, we hypothesize that AGG inhibits the interaction of Snail-p73 during EGF induced EMT, stemness and invasiveness, and results in MET through switching back to upregulation of E-cadherin expression.

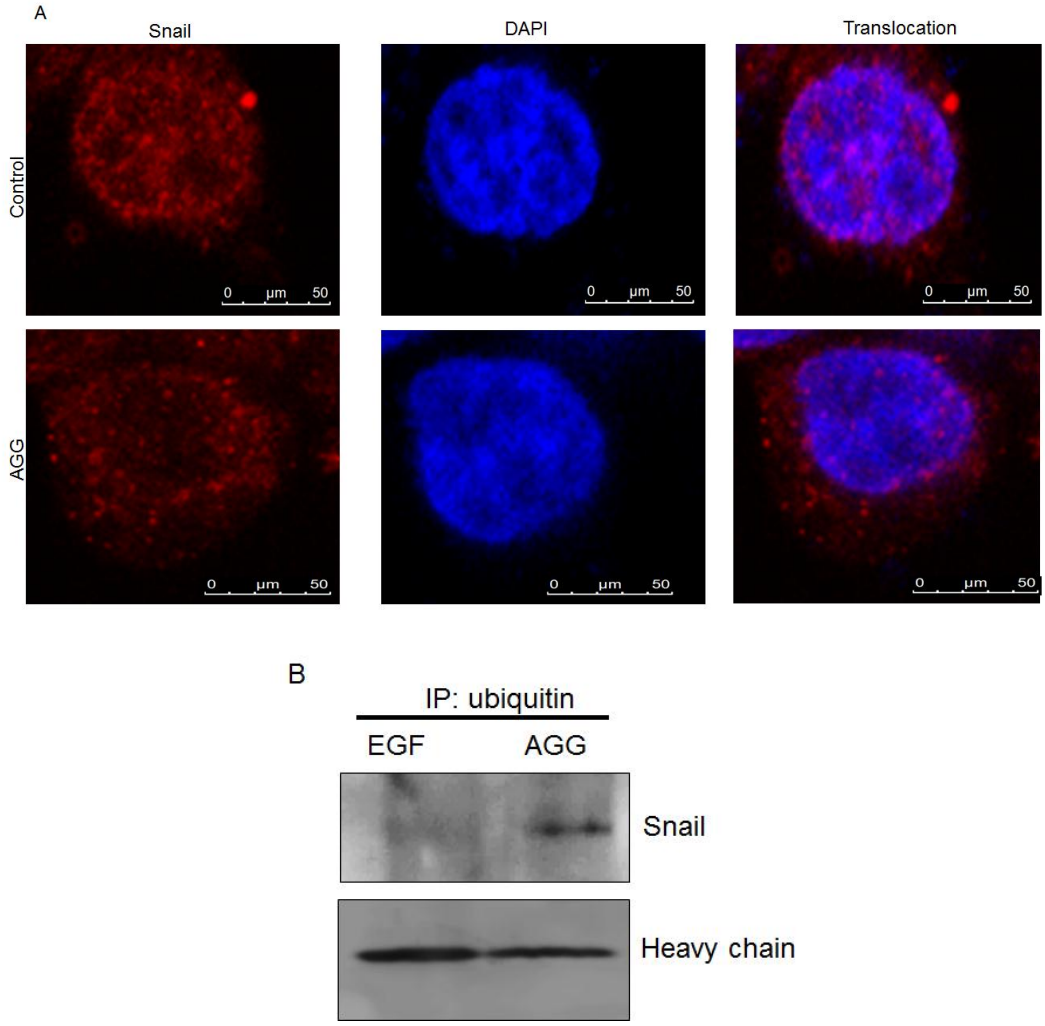

Fig-5.5. AGG induced Snail translocation to cytosol. EGF-induced FaDu cells were grown on chamber slides for 24 h. Cells were treated with AGG for 12 h, fixed and permeabilized followed to incubation of primary antibody, Snail. Cells were then washed with PBS and incubated with secondary antibody followed by DAPI counterstaining. Cell images were captured at 1000X magnification (A). Snail degradation was studied by immunoprecipitation study with Ubiquitin (B).

5.3.4. AGG regulated EMT, stemness, invasiveness through ERK inhibition

Cytoplasmic/nuclear β-catenin expression has also been found to significantly correlate with EGFR expression in OSCC. EGFR overexpression has been demonstrated in approximately 90 % of head and neck cancers, our data exuded the downregulation of EGFR in AGG treated EGF induced FaDu cells in a dose dependent manner (Fig. 5.7A). OSCC might employ an alternative signaling pathway, like the PI3K/Akt, MEK/ERK or Wnt/β-catenin pathway. In presence of EGF, Snail expression is modulated via extracellular signal-regulated kinase (ERK), serine/threonine kinases resulting in increased cell spreading and greatly enhanced cell

survival. Our study showed that AGG downregulated the activated ERK1/2 in response to EGF stimulation temporally (Fig. 5.7B).

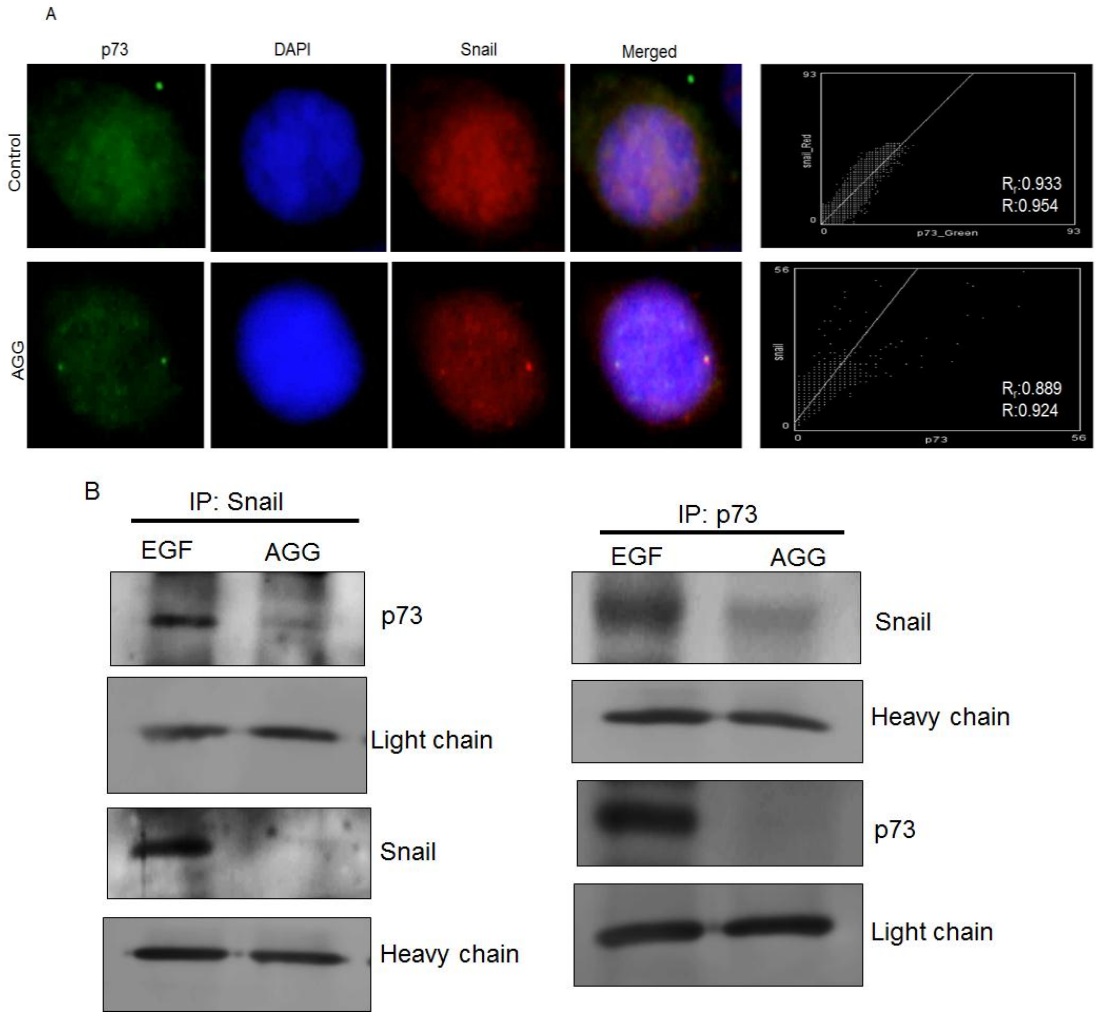

Figure-5.6. Double immunofluorescence and coimmunoprecipitation studies to evaluate Snail-p73 interaction. Double immunofluorescence analysis was performed to study Snail interaction with p73 in EGF stimulated FaDu cells due to its nuclear localization using fluorescence microscopy. Equal amount of Snail protein and p73 protein were pulled out from the lysates of the two sets of the experiment to study the expression of either antibody anti-Snail or anti-p73 for immunoprecipitation followed by immunoblotting with anti-p73 or anti-Snail analyzed by the Western Blot.

On introduction of PD98059, a highly selective flavonoid that specifically binds to inactive forms of MEK1, and inhibits both MEK 1 and MEK2 activation *in vitro* with varying IC_{50} values; ameliorated ERK inhibition in presence of AGG (Fig. 5.7C). The downstream molecule of ERK, Snail expression also decreased. Following the downregulation of Snail, E-cadherin as one of the structural protein regarded as caretakers of the epithelial phenotype was upregulated shunting the expression of

SOX2 and OCT4, transcriptional factors responsible for the stemness, and invasive marker ADAM17 in EGF stimulated FaDu cells (Fig. 5.7D). The molecular mechanisms involved in ERK1/2-mediated cell death in oral cancer are largely unknown. Here our data showed that in presence of PD98059, AGG deactivated ERK1/2, and prompted increased p73 expression in EGF induced FaDu cells. Thus, p73 acts downstream of MEK activation, and play an important role in snail inhibition.

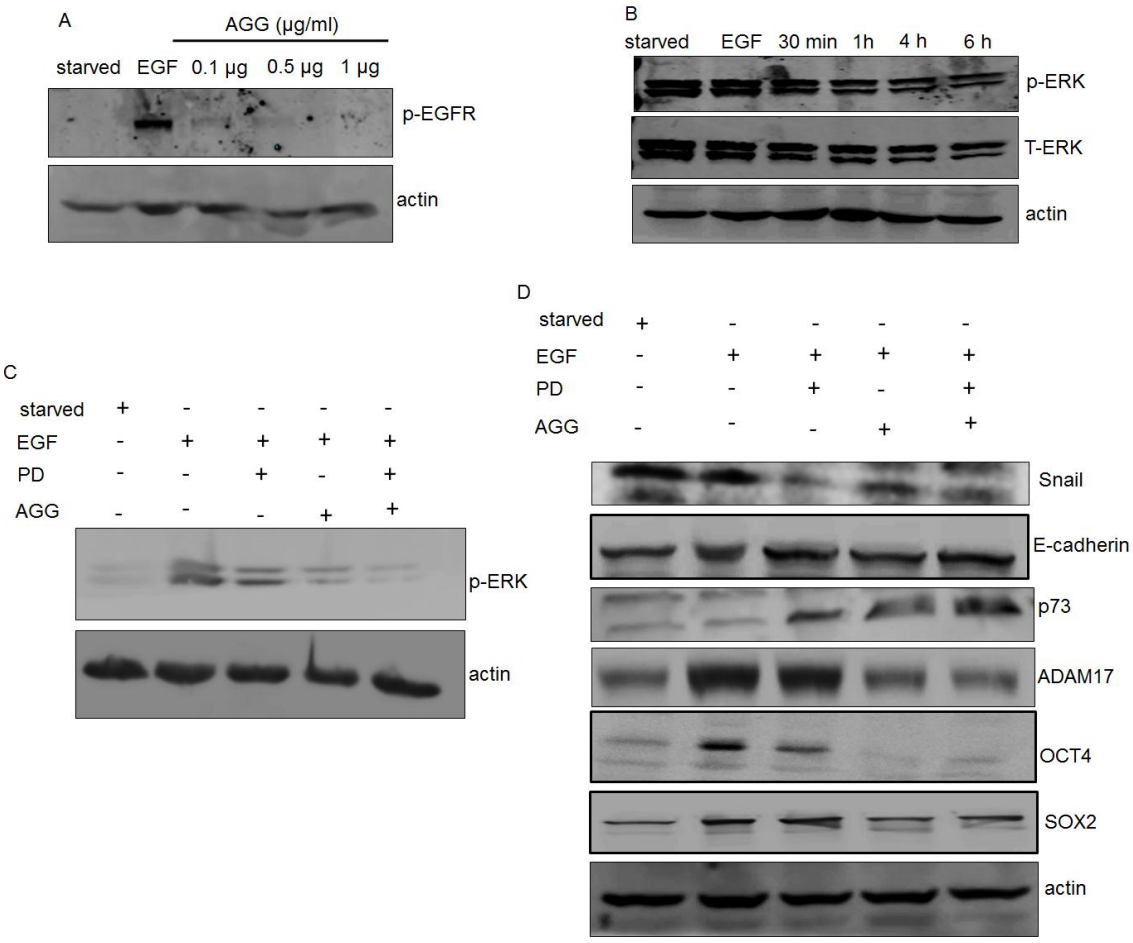

Figure-5.7. AGG regulated EMT, stemness, invasiveness through ERK inhibition. EGFR expression was monitored in EGF induced FaDu cells in a dose dependent manner by Western Blot (A). Activated ERK1/2 expression in response to EGF stimulation was monitored temporally by Western Blot on treatment with AGG (1µg/ml) (B). On introduction of PD98059, a highly selective flavonoid that specifically binds to inactive forms of MEK1, and inhibits both MEK1 and MEK2 ameliorated ERK inhibition was studied by Western blot in presence of AGG (1 µg/ml) (C). Upon treatment with AGG (1µg/ml), the downstream molecule of ERK, Snail expression followed with the expression E-cadherin and p73, ADAM17, SOX2 and OCT4 were investigated by Western Blot (D).

5.4. Discussion

Despite progress in the available sequential treatments, oral cancer is associated with high morbidity and poor prognosis due to local invasiveness and distant metastasis. The progression in metastasis is due to the aggressive phenotypical change supported by the cancer cells known as epithelial-to-mesenchymal transition. *Abrus* agglutinin (AGG) being a natural molecule has shown its anticancer activities both *in vitro* and *in vivo* with minimal side effect towards non-cancer cells. Our previous studies have been greatly ascribed to its effects on multiple signaling pathways related to ROS generation, apoptosis, and autophagy; making it a novel catalogue of chemotherapeutic agents for the clinical trials. Natural molecules like curcumin, plumbagin, pterostilbene, and resveratrol, have successfully established their candidature as anti-invasive and anti-metastatic (Li et. al., 2013; Lee et. al., 2015; Pan et. al., 2015). AGG treatment in EGF induced FaDu cells inhibited EMT progression with reestablishment of E-cadherin expression following with snail inhibition, and rescued apoptotic potential of inactivated p73. Mishima et al. showed overexpression of ERK/MAP kinases in the poorly differentiated cells of OSCC patients. In response to mitogens; EGF, HGF, IGF1, PDGF, TGF-α, MAPKMEK1/2-ERK1/2 activation occurs enhancing cell proliferation and survival pathway in hepatocytes by activating receptor tyrosine kinases (RTKs) such as; EGFR, c-MET, FGFR. In FaDu cells upon EGF stimulation, our data revealed that AGG inhibited the cells undergoing EMT, characterized by upregulation of E-cadherin, Involucrin, and downregulation of N-cadherin, Vimentin, and β-catenin. AGG restricted the expression of EGFR by blocking the tyrosine kinase signaling with decreased expression of downstream molecule; that is ERK1/2 in a time dependent manner. Snail, a labile protein and a key transcription repressor of E-cadherin confers resistance to the lethal effects of serum depletion or TNF-α by activating the MAPK and PI3K survival pathways; henceforth, increasing the propensity of cells to have increased invasive and migratory traits. Gkouveris et al, showed ERK1/2 inhibition resulted in a dose-dependent reduction in OSCC cell growth and viability; AGG administration along with PD98059 resulted in inhibition of activation of ERK1/2 in EGF induced FaDu cells. Furthermore, AGG validated its efficiency by inhibiting the expression of snail and invasive marker, ADAM17 in presence of MEK1/2 inhibitor. EGF stimulus in head and neck cancers has been associated with regulation of glycolysis/EMT/CSCs axis through

downstream EGFR/PI3K signaling pathways. Moreover, we have also showed that in EGF stimulated FaDu cells, AGG in upregulated the expression of E-cadherin and p73 with concomitant decline in the OCT4 and SOX2. This data encircles the unexplored, intriguing role of p73 in EMT. Loss of E-cadherin expression is influential due to the overexpression of Snail or Twist that plays a distinct role in tumor progression identified in various kinds of epithelial tumors; including gastric cancer, prostate cancer, breast cancer, and head and neck cancer. Baritaki et al, predicted that overexpression of Snail in the non-metastatic LNCaP cell line resulted in EMT induction and the acquisition of more mesenchymal phenotype compared to the wild-type cells. Furthermore, Snail a key transcription repressor of E-cadherin, its overexpression induced apoptosis resistance in breast cancer cells. We witnessed the deregulated E-cadherin, and p73 expression in EGF induced Snail overexpressed FaDu cells. AGG restrained the Snail-mediated survival, and thus halted the ability of tumor cells to invade and metastasize with decline in the expression of ADAM17. Snail being a labile protein has short half-life period. Proteasome inhibitor, MG132 treatment stabilizes Snail in a hyperphosphorylated form and AGG inhibited its expression in presence of MG132. Interaction between the Notch1 intracellular domain (NICD) and Snail resulted in MDM2-dependent degradation of Snail. Our confocal data provoked its cytoplasmic export from nucleus upon AGG treatment for ubiquitin-mediated proteasome degradation manifested from immnoprecipitation. The degradation of Slug is controlled by p53 and MDM2; though p73 has a role in EMT, its interaction with Snail has not been described previously. Here we have shown that p73 plays a role in Snail translocation and degradation. The Snail-mediated survival may thus enhance the ability of tumor cells to invade and metastasize.

P53 plays important roles in controlling cancer invasion and metastasis. Fan et al. examined OSCC tumor derived from patients, showed low E-cadherin expression coevolved with low p53 expression. Zhang et al, found that upon knockdown of p73 or TAp73, but not ΔNp73, compelled MCF10A cells to undergo EMT via down-regulation of E-cadherin coupled with up-regulation of β-catenin and laminin V. P53 being non-functional in FaDu cells, AGG treatment upregulated E-cadherin, and p73 expression in EGF induced FaDu cells dose dependently and FaDu cells transfected with shp73, attenuated E-cadherin expression with increase in invasiveness and stemness markers.

Hereafter, AGG substantiates its EMT inhibitory role in p53 deficient oral cancer cell carcinoma via inhibition of ERK/Snail pathway.

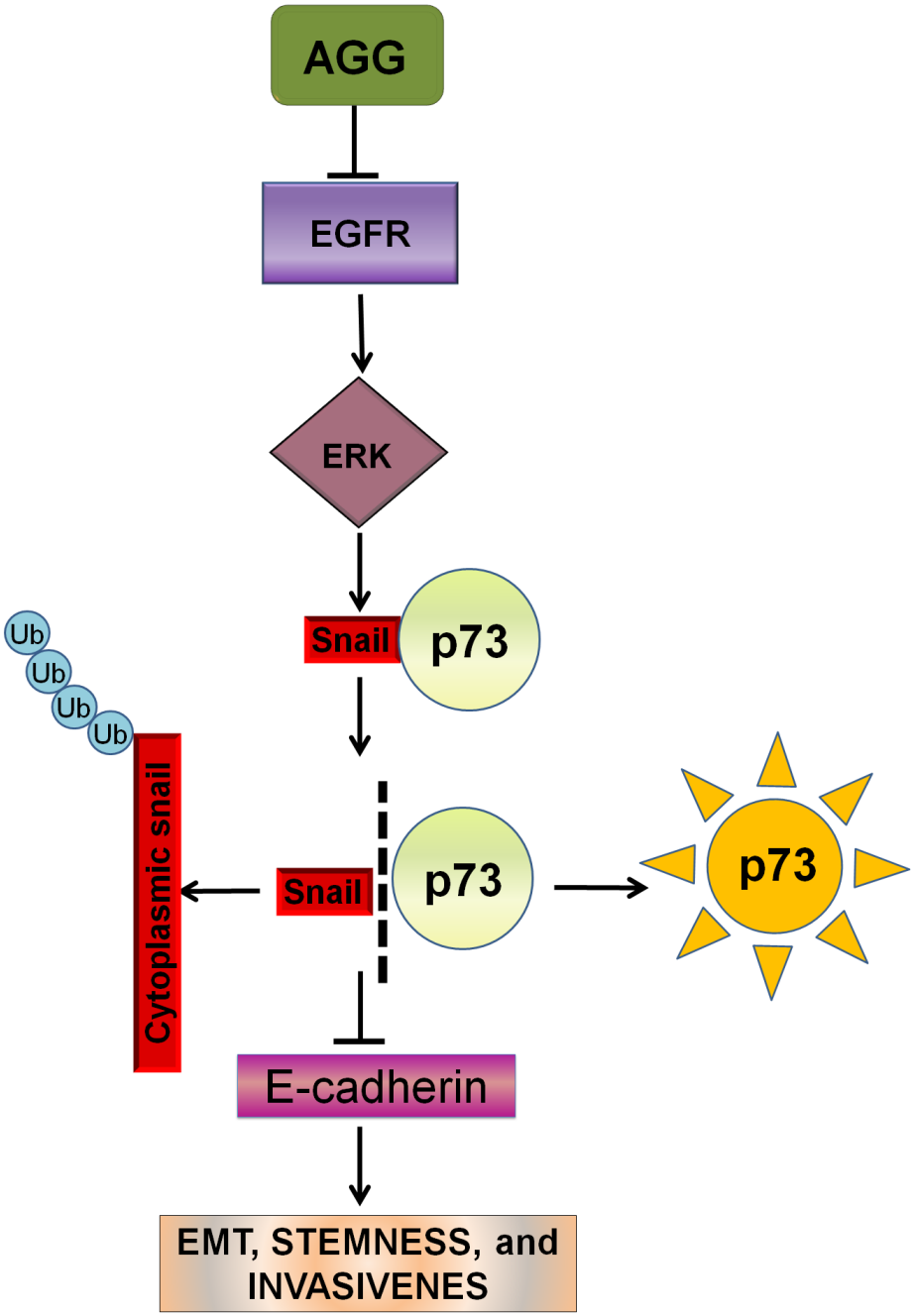

Figure-5.8. Flowchart demonstrating influence of AGG in Snail degradation and p73 activation followed to inhibition of EMT, stemness, and invasiveness.

Chapter 6

Abrus agglutinin targets cancer stem-like cells by eliminating self-renewal capacity accompanied with apoptosis in oral squamous cell carcinoma

Abstract

The accumulating evidences show that *Abrus* agglutinin (AGG), a plant lectin demonstrates an extensive assortment of anticancer activity including cancer-specific induction of apoptosis; however the underlying molecular mechanism of AGG induced oral cancer stem cells (CSCs) elimination remains elusive. Our data documented that AGG effectively downregulated the $CD44^+$ expression with the increased $CD44^-$ population in different oral cancer cells. After 24 h AGG treatment, FaDu cells were quantified for orosphere formation in ultra-low attachment plates and data showed that AGG inhibited the number and size of orosphere in a dose dependent manner in FaDu cells. Further, AGG hindered the plasticity of FaDu orospheres as supported by reduced sphere formation, and downregulated the self-renewal property via inhibition of Wnt-β-catenin signaling pathway. Introduction of LiCl, a GSK-3β inhibitor rescued the AGG stimulated inhibition of β-catenin and pGSK-3 β in FaDu cells derived orospheres, confirming importance of Wnt signaling in AGG mediated inhibition of stemness. In this connection, our data showed that AGG restrained proliferation and induced apoptosis in FaDu derived CSCs in dose dependent manner. Moreover, Western blot data demonstrated that AGG increased the Bax/Bcl-2 ratio with activation of PARP and caspase-3, favoring apoptosis induction in orospheres. AGG induced reactive oxygen species (ROS) accumulation in orospheres and pretreatment of N-acetyl cysteine, a ROS scavenger inhibited caspase-3 activity upon AGG treatment and β-catenin expression indicating ROS as a prime controller of Wnt signaling and apoptosis. In conclusion, AGG has a budding role as an integrative therapeutic methodology for fighting oral cancer through targeting self-renewability of orospheres via ROS mediated apoptosis.

6.1. Introduction

Head and neck squamous cell carcinoma (HNSCC) comprising the cancer of several distinct subsites (e.g. nasopharynx, oropharynx, hypopharynx, larynx, lip, tongue) ranks as the sixth most frequently occurring cancer globally. The Indian subcontinent is the global epicenter of HNSCC where it is the most common cancer of males and the fifth most common in females (Joshi et. al., 2014). Tobacco smoke, excessive alcohol consumption, betel quid chewing, radiation exposure, HPV infection are the main risk factors for developing the disease (Chen et. al., 2013). Despite the advancement in the present treatment modalities; though the quality of life of oral

cancer patients has improved, the five year endurance rate has not improved in the past decades because of treatment resistance, loco-regional recurrences and distant metastasis (Whang et. al., 2015). Recent report has ascertained the phenotypic heterogeneity of HNSCC tumors that supports the existence of rare subset of intratumoral cells called as Cancer Stem Cells (CSCs) (Costea et. al., 2006). Prince et al, were the first to accomplish the isolation of a pool of HNSCC cells with high CD44 expression that exhibited stem cell-like characteristics like self-renewal, generation of differentiated progeny, lack of differentiation markers, and expression of immature cell markers. These $CD44^{high}$ cells were shown to have exclusive tumorigenic capacity when introduced in immunosuppressed mice (Prince et. al., 2007).

Like normal stem cells, CSCs are also anticipated to possess intrinsic and exclusive self-renewal propensity that allows sustained repopulation (Verga et. al., 2012). The canonical Wnt/ β-catenin signaling is a crucial developmental pathway involved in self-renewal, differentiation, migration, proliferation and tumorigenesis (Kikuchi et. al., 2009). The significance of Wnt/ β-catenin signaling in controlling the tumorigenesis and self-renewal of CSCs in HNSCC is well established. It has been documented that overexpression of β-catenin in HNSCC leads to dedifferentiation to stem-like state whereas its downregulation inhibits self-renewal capacity, stemness-associated gene expression, and *in vivo* tumorigenicity (Lee et. al., 2014). Apoptosis resistance, one of the key hallmarks of cancer has been demonstrated prominently in CSCs. The side population (SP) cells isolated from Ho-1-N-1 oral squamous cell carcinoma cell line are highly resistant to 5-fluorouracil and carboplatin as compared to the non-SP owing to the enhanced expression of antiapoptotic protein CFLAR, BCL2 and BCL2A1 (Yajima et. al., 2009). Moreover, Wnt/ β-catenin signaling was reported to induce apoptotic resistance by inhibiting not only tumor necrosis factor (TNF)/c-Myc-mediated apoptosis, but also cell detachment-mediated apoptosis (anoikis) which is dependent on the death receptor signaling pathway (Yang et. al., 2006). Moreover, HNSCC $CD44^{+}$ cells showed upregulation of Bcl-2 and inhibitor of apoptosis (IAP) family genes compared with $CD44^{-}$ cells (Lim et. al., 2012; Chikamatsu et. al., 2012).

Abrus agglutinin (AGG), a heterodimeric plant lectin of 134-kDa was isolated from the seeds of Abrus precatorius, an Indian medical plant. It has two 30 kDa toxic A-chain and two 31 kDa B-chains linked through disulfide bridges; the B chain that binds to the cell surface while A chain has rRNA *N*-glycosidase activity and

irreversibly inhibits protein synthesis through inactivation of ribosomes. It has specificity towards [gal (β 1→3) gal NAc] and belongs to type II ribosome inactivating protein family (RIP II) with a protein synthesis inhibitory concentration (IC_{50}) of 0.469 μg/ml and a lethal dose (LD_{50}) of 5 mg/kg body weight in mice. The anticancer effects of AGG have been elucidated in several tumor models at sublethal doses by direct killing of tumor cells through extrinsic and intrinsic apoptosis. In addition, AGG showed immunostimulatory properties and has a strong mitogenic activity to lymphocytes. It exhibits both humoral and cellular immunity with propensity to stimulate the innate effector arms like macrophage and natural killer cells by activating splenocytes leading to Th1 response. Moreover, heat denatured and tryptic digested AGG show potent antitumor and immunomodulatory activities in normal as well as in tumor bearing mice (Bhutia et. al., 2008; Bhutia et. al., 2016; Mukhopadhyay et. al., 2014; Panda et. al., 2014). Our previous study showed that AGG induced potent anticancer activity through p73 dependent pathway in oral cancer. However, its efficacy in eliminating CSCs in oral cancer has not been yet deciphered. Here, we investigated the growth inhibitory potential of AGG in orospheres present in oral cancer cells. Our findings showed that AGG could inhibit the growth of orospheres via the ROS mediated induction of caspase-dependent apoptosis in FaDu cells. Moreover, AGG found to regulate the plasticity of orospheres by inhibiting the canonical Wnt signaling pathway in oral cancer cells.

6.2. Material and Methods

6.2.1. Chemical and reagents

6-Diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO), and N-Acetyl cysteine (NAC) were purchased from Sigma Aldrich, India. Fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12), trypsin, antibiotic–antimycotic solution were purchased from Invitrogen, India. Annexin V from Immunotools, Germany was purchased. Antibodies against β-catenin, pGSK3β, Bcl-2, Bax, PARP, cleaved caspase 3, and actin were purchased from Cell Signaling Technology, USA; CD44 from BD Biosciences, USA.

6.2.2. *Abrus* agglutinin purification

AGG was isolated and purified from *Abrus precatorius* seeds by ammonium sulfate fractionation followed by lactamyl sepharose affinity chromatography and Sephadex G-100 gel permeation chromatography. The activity of isolated AGG was measured by a hemagglutination assay, and the purity of the protein was subsequently analysed by SDS-PAGE, native-PAGE and gel permeation by HPLC.

6.2.3. Cell culture and sphere culture

Hypopharyngeal cells (FaDu) was cultured in minimal essential medium (MEM), supplemented with antibiotic–antimycotic (1X) and 10 % fetal bovine serum. The tongue squamous cell carcinoma (SSC4, SSC25) were cultured in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 *(DMEM/F-12)* supplemented with 400 ng/ml hydrocortisone. For sphere-forming culture, FaDu cells were cultured in serum free medium supplemented with 1 % N_2 supplement (Gibco), 2 % B27 supplement (Gibco), 20 ng/mL bFGF-2 ((basic fibroblast growth factor, BD Biosciences), and 20 ng/mL EGF (Epidermal growth factor, BD Biosciences) and plated in ultra-low attachment plates (Corning). Cells were grown in these conditions as non-adherent spherical clusters of cells (usually named spheres or orospheres). All the cultures were maintained at 37^0C in a humidified 5 % CO_2 incubator. After 14 days of culture, the number of orospheres after AGG treatment was counted under a microscope.

6.2.4. Quantification of $CD44^-$ population

Bulk populations of SCC25, SCC4 and FaDu cells were treated with the different doses of AGG. After 24 h treatment, cells were washed with 0.1 % 1X PBS-F. Cells were stained with 200 µl of CD44 and were suspended in 500 µl PBS-F for further analysis by FACS Calibur.

6.2.5. Quantification of Cell viability by trypan blue exclusion method

About 2000 cells/well were cultured in an ultra-low adherent 6-well plate at 37 °C, and exposed to different concentrations of AGG for 24 h. All the experiments were performed in triplicate, and the relative cell viability was expressed as the percentage relative to the untreated control cells.

6.2.6. Expression of CD44 and β-catenin by confocal immunofluorescent staining

The orospheres were plated in chamber slide, and were treated with different doses of AGG. After 24 h, the expression of CD44 and β-catenin were analyzed by double immunofluorescence imaging by confocal laser microscope (Leica TCS SP8).

6.2.7. Apoptosis analysis by Annexin V/PI staining

The oral cancer-initiating/stem cells were seeded in six-well plates and were treated with AGG for 24 h and processed and evaluated for Annexin V binding as described.

6.2.8. ROS analysis

AGG treated orospheres were incubated with Dihydrorhodamine 123 (2.5 μg/ml) for 30 mins. After incubation, the cell pellet was suspended in 500 μl PBS and analyzed through flow cytometry.

6.2.9. Western blotting

The treated orospheres were subjected to the preparation of whole-cell lysates followed by SDS/PAGE for β-catenin, pGSK3β, Bcl-2, Bax, PARP, cleaved caspase 3, and actin (1:1000, mouse monoclonal, BD Biosciences) which were further incubated with HRP-conjugated secondary antibody (1:5000, mouse monoclonal, BD Biosciences) to examine the protein levels as described.

Statistical analysis

Data were presented as mean ± SD and evaluated with Student's t-test. $P < 0.05$ was accepted as statistically significant.

6.3. Results

6.3.1. AGG significantly downregulateed CD44 expression and preferentially targeted self-renewal potential in orospheres

The CSC population in oral cancer comprises $CD44^+$ cells with aggravated proliferation and higher tumorigenic potential than the $CD44^-$ cells. Henceforth, CD44 is an important biological marker to screen oral cancer stem cells in bulk tumor cell population (Li et. al., 2010; Grimm et. al., 2015; Kokko et. al., 2011; Kim et. al., 2014). To explore the significance of AGG in inhibition of $CD44^+$ population, we treated bulk cell populations of FaDu, SCC4 and SCC25 with different doses of AGG for 24 h. The flow cytometry analysis corroborated our hypothesis with increased $CD44^-$ cell populations in FaDu cells from 9 ±1 in control to 10 ±1.5, 13 ±1, 18 ±2.5 in respective doses of AGG. The $CD44^-$ cell populations in SCC4 increased from 4 ±1 in control to 13 ±1.5, 17 ±1.5, 18 ±1.5 while SCC25 cells displayed from 27 ±1.2 in control to 58 ±1, 60 ±1.04, 61 ±1 in dose dependent manner (Fig. 6.1A and 6.1B). Since the oral stem/progenitor cells are enriched in non-adherent spherical clusters, in order to evaluate the effect of AGG on the formation of orospheres, FaDu cells (2000 cells/well) were enriched in a 6-well plate for 14 days after 24 h treatment with

various doses of AGG. Our results demonstrated that AGG treatment could decrease the number of spheres as well as reduced sphere size in FaDu cells (Fig. 6.2A and 6.2B).

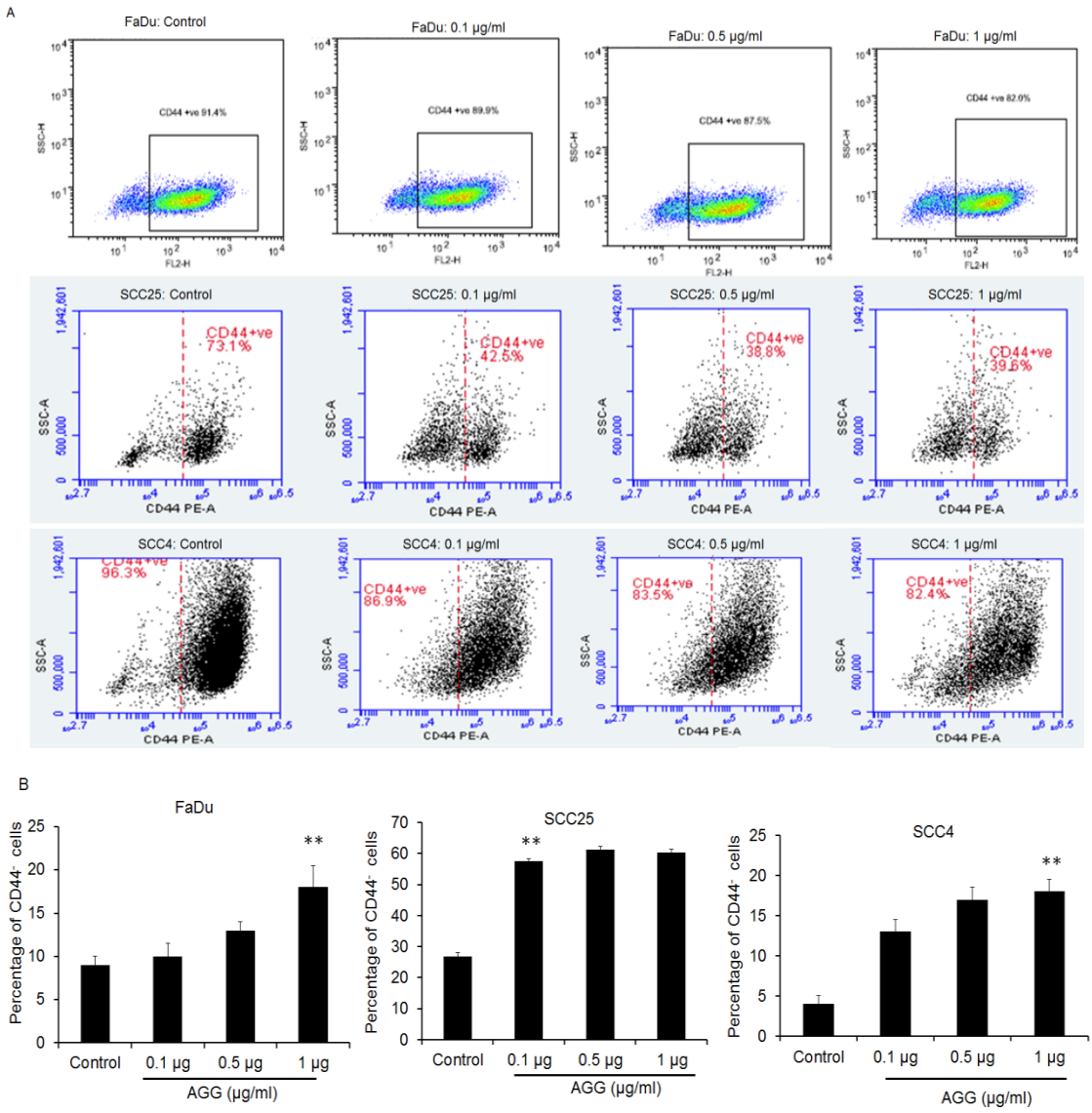

Figure-6.1. AGG significantly downregulated CD44 expression. The oral squamous cell carcinoma (FaDu, SCC4, and SCC25) were treated with different concentration of AGG for 24 h and flow cytometry was performed to check the $CD44^{+}$ and $CD44^{-}$ populations (A and B). Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05 was considered significant as compared with control.

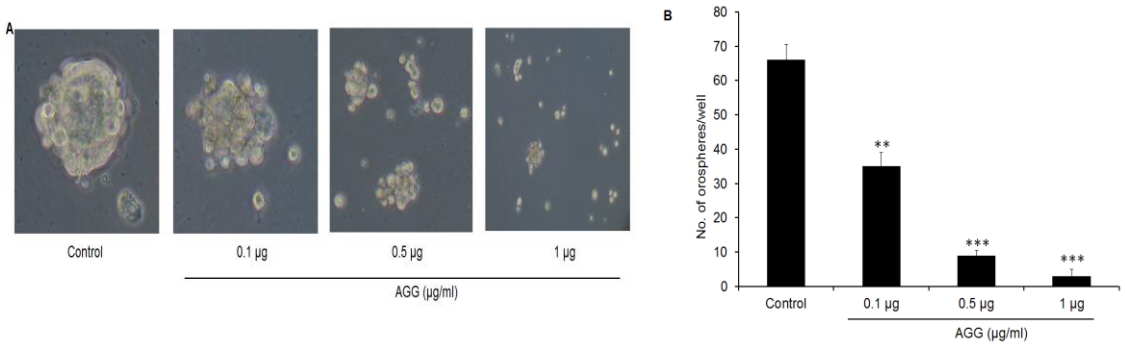

Figure-6.2. AGG inhibited formation of FaDu orospheres. FaDu cells were treated with different concentration of AGG for 24 h and FaDu cells (2000 cells/well) were cultured in serum free media in a 6-well ultra-low attached plate. After for 14 days the orospheres were observed in microscope, photographed and quantified (A and B). Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01; ***P value < 0.001 were considered significant as compared with control.

The plasticity of CSCs is maintained by self-renewal pathways including Wnt signaling, Hedgehog signaling and Notch signaling that play a provoking role in a cancer cell transformation contributing to tumorigenicity and its progressive metastasis. Our confocal microscopy data showed that AGG treatment significantly decreased the expression of β-catenin along with CD44 expression in FaDu- derived orospheres (Fig. 6.3A). To evaluate the activation Wnt canonical pathway, FaDu orospheres were treated with AGG for 24 h and the expression of β-catenin, the transcriptional activator of Wnt signaling was analyzed. β-catenin expression decreased in a dose dependent manner in presence of AGG (Fig. 3B and C). The cytoplasmic β-catenin level is regulated by a multifaceted kinases including glycogen synthase kinase3β (GSK3β). The GSK3β at its dephosphorylation state promotes the ubiquitin-proteasome degradation of β-catenin through phosphorylation at Ser33/Ser37/Thr41 residues. Our data showed that AGG inhibited the expression of pGSK-3β without altering expression of total pGSK-3β and could manifest its role in destruction complex preventing aberrant accumulation of β-catenin (Fig. 6.3B and 6.3D). LiCl, an activator of Wnt pathway inactivates GSK-3β through Ser9 phosphorylation which in turn diminishes β-catenin degradation. The orospheres from FaDu cells when treated with AGG in presence of LiCl showed increased expression of β-catenin as compared to only AGG treated group (Fig. 6.3E-G). Henceforth, AGG effectively restrained the Wnt/β-catenin canonical pathway which is important for self-renewability of cancer stem cells and tumor development.

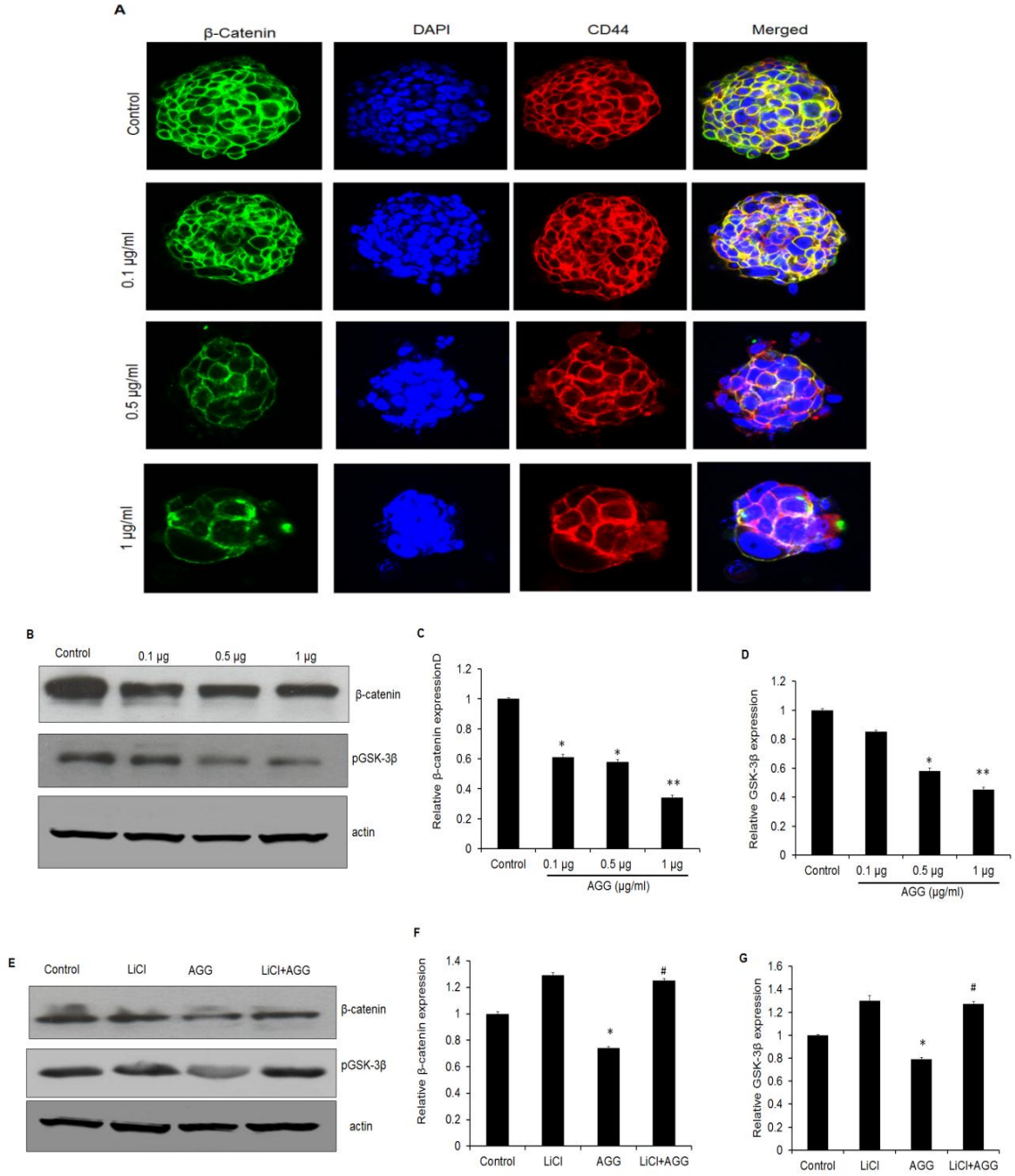

Figure-6.3. AGG preferentially targets self-renewal potential in orospheres. Orospheres derived from FaDu cells were treated with AGG for 24 h and analyzed for the expressions of β-catenin along with CD44 by confocal microscopy (A). Cell lysates obtained after AGG treatment in presence or absence of LiCl were analyzed for expression of β-catenin, and GSK-3β (B and E) by Western Blot. Densitometer of proteins is represented as histogram plotted on the basis of actin to the target protein ratio (C and D, F and G) by ImageJ. Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01were considered significant. # indicates data compared against AGG treatment.

6.3.2. AGG inhibits cell proliferation and induces apoptosis in orospheres

To target the proliferative propensity of CSC in FaDu, we studied the cell viability assay of orospheres upon treatment with different doses of AGG for 24 h. The data showed AGG decreased the average number of viable cells from 94 ±2 to 71 ±4, 50 ±5, 13 ±3 in FaDu derived orospheres (Fig. 6.4A). To decipher the mechanism of action for apoptosis caused by AGG, we performed annexin V/PI staining analysis through flow cytometry after 24 h of AGG treatment. The data showed that there was an increase in apoptotic population from 20 ±1 in control to 24 ±1.5, 34 ±1, 41 ±1.5 in the instance of 0.1, 0.5, and 1 µg/ml of AGG respectively (Fig. 6.4B and 6.4C).

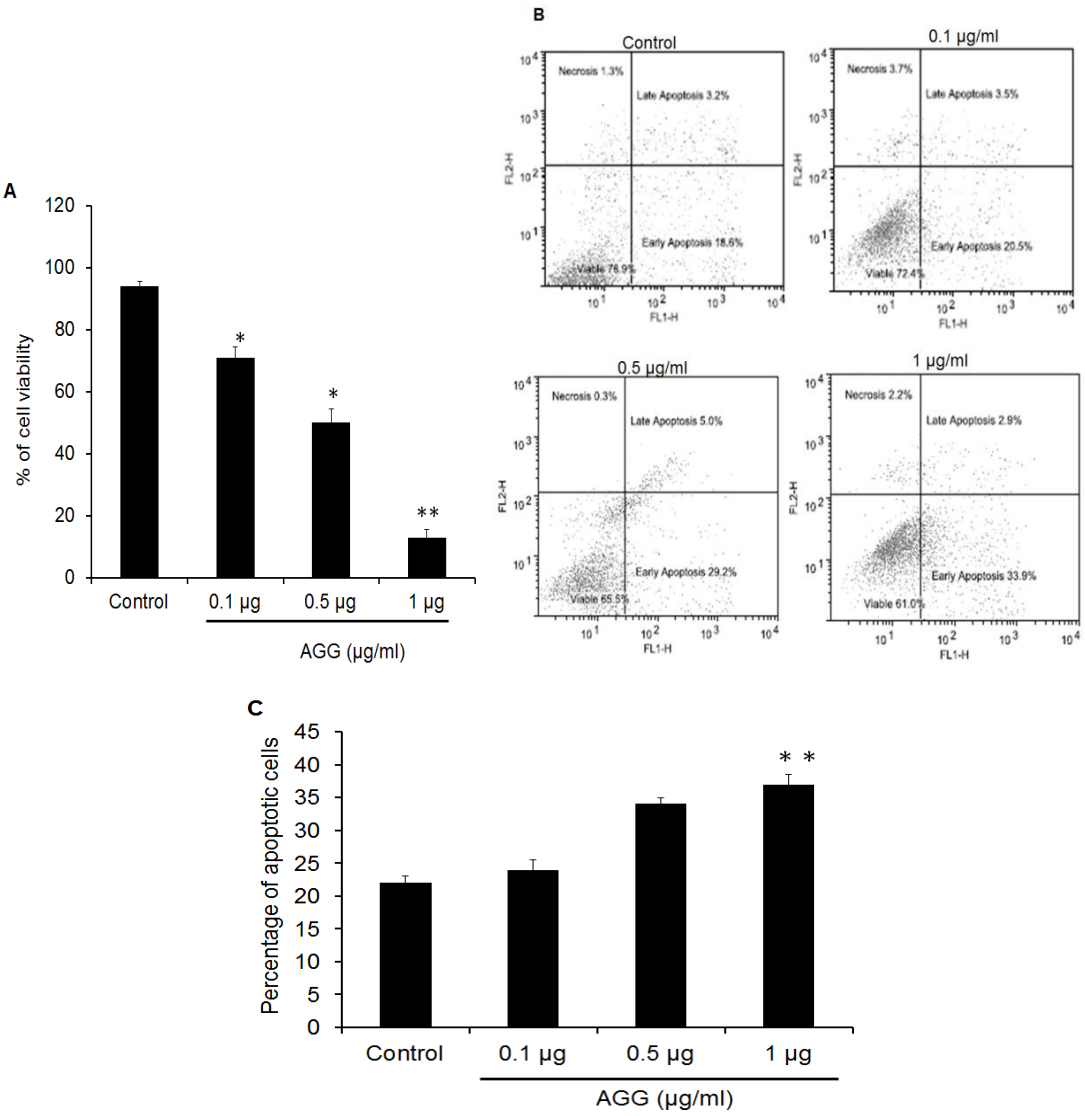

Figure-6.4. AGG inhibits cell proliferation and induces apoptosis in orospheres. Orospheres were treated with different concentrations of AGG for 24 h and the cell viability assay through trypan blue was evaluated (A). The Annexin V/PI staining analysis was performed through flow cytometry after 24 h of AGG treatment (B and C).

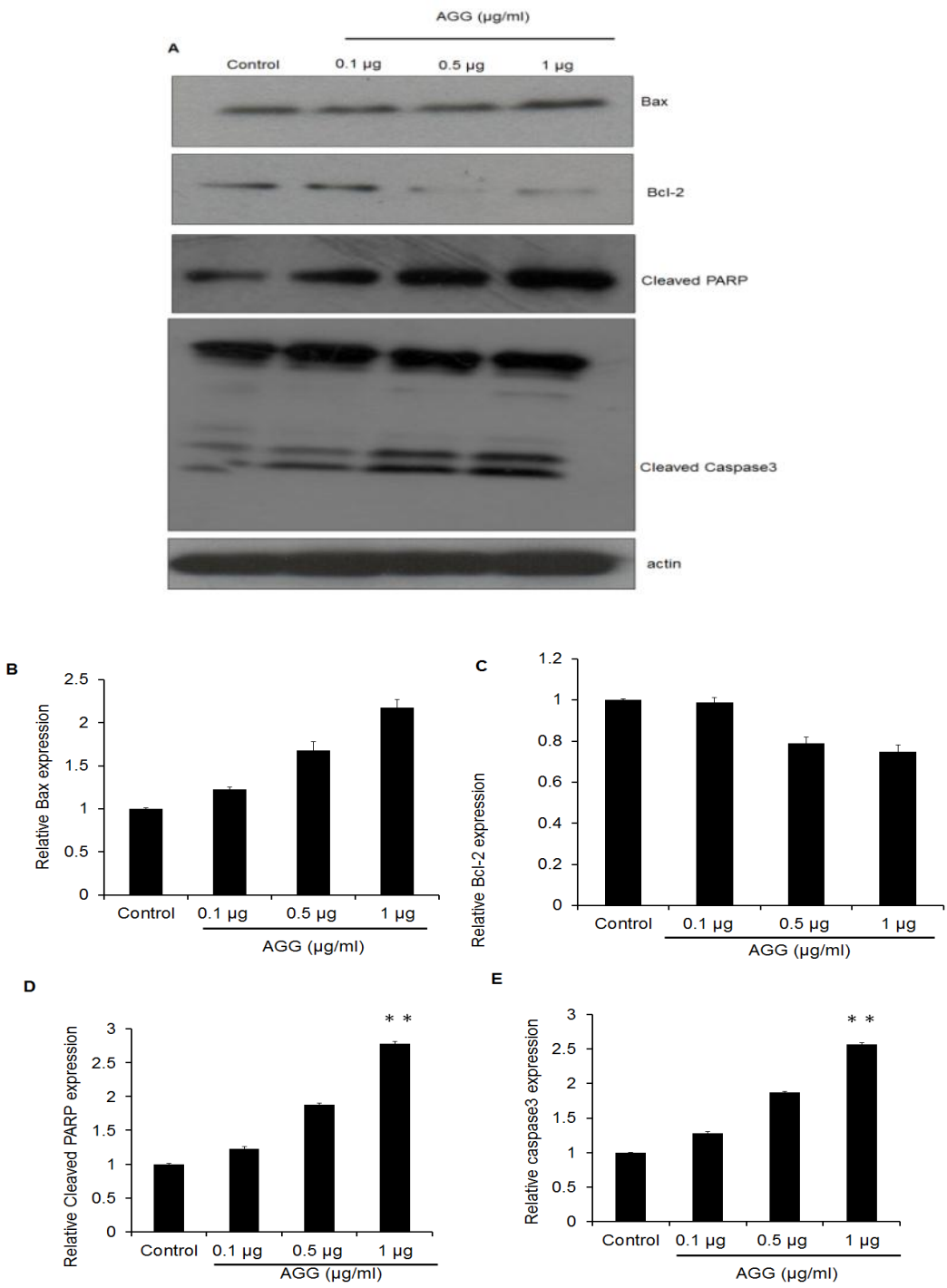

Figure-6.5. AGG upregulates apoptotic molecules in orospheres. Cell lysates obtained after AGG treatment for 24 h were analyzed for expression of Bax, Bcl-2, cleaved PARP, and cleaved caspase-3 (A) by Western Blot. Densitometer of proteins is represented as histogram plotted on the basis of actin to target protein ratio (B-E) by ImageJ. Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01 were considered significant.

We further examined the changes in expression of the Bcl-2 and Bax proteins in FaDu cancer sphere cells that regulate the intrinsic apoptosis pathway. AGG decreased the anti-apoptotic Bcl-2 while increased the pro-apoptotic Bax protein in a dose dependent manner (Fig. 6.5A-C). Increased Bax to Bcl-2 protein ratio led to the activation pro-caspase 3 to activated caspase 3 (Fig. 6.5A and 6.5E). The FaDu derived orosphere showed increased expression of cleaved PARP with increased dose of AGG (Fig. 6.5A and 6.5D). These results suggest the pivotal role of AGG in mitochondria-dependent (intrinsic) apoptosis in AGG-mediated elimination of CSC in FaDu cells.

6.3.3. ROS induced by AGG regulates apoptosis and Wnt signaling

Cancer stem cells have low levels of ROS and possess an anti-oxidant expression profile to maintain quiescence and self-renewal. ROS generation by anticancer therapy may disrupt the redox stability and selectively induces apoptosis in cancer cells without significant toxicity to normal cells. To define the role of ROS in apoptosis, orospheres were treated with AGG for 24 h and analyzed for ROS generation. The cells were incubated with DHR123 which was rapidly taken up by the cells and converted to Rhodamine123 (Rh123) in the presence of ROS. Flow cytometry analysis showed increased level of ROS after AGG treatment as compared to control. Pre-treatment with a ROS scavenger, N-acetyl cysteine (NAC, 10 mM) for 2 h recorded reduction in ROS production compared to only AGG (1 μg/ml) treated orospheres (Fig. 6.6A). Importantly, AGG induced caspase activity was declined in presence of NAC in FaDu orospheres. In addition, AGG modulated β-catenin expression, was rescued in NAC pretreated group as compared to AGG in orospheres, suggesting the implication of AGG induced ROS in caspase-dependent apoptosis and self-renewal activity in oral CSCs (Fig. 6.6B-D).

6.4. Discussion

In recent years advanced HNSCC tumors contain a rare small subset of CSCs, which is characterized by therapeutic resistance and frequent tumor relapse. Treatment failures resulting in the development of a second primary tumor, have urged for the novel non-toxic natural approaches to target CSCs for treatment of cancer. Lectins have undergone advanced research in Asia comprising of China and India and Europe as an alternative tumor therapy, but more work has to be done to target CSCs. The present work underpins the antineoplastic potential of AGG, a RIP II family member that inhibits proliferation and plasticity of orospheres and triggers ROS mediated

caspase dependent apoptotic type-I programmed cell death. Here, we report for the first time the novel *in vitro* and *in vivo* effects of AGG on oral cancer stem cells.

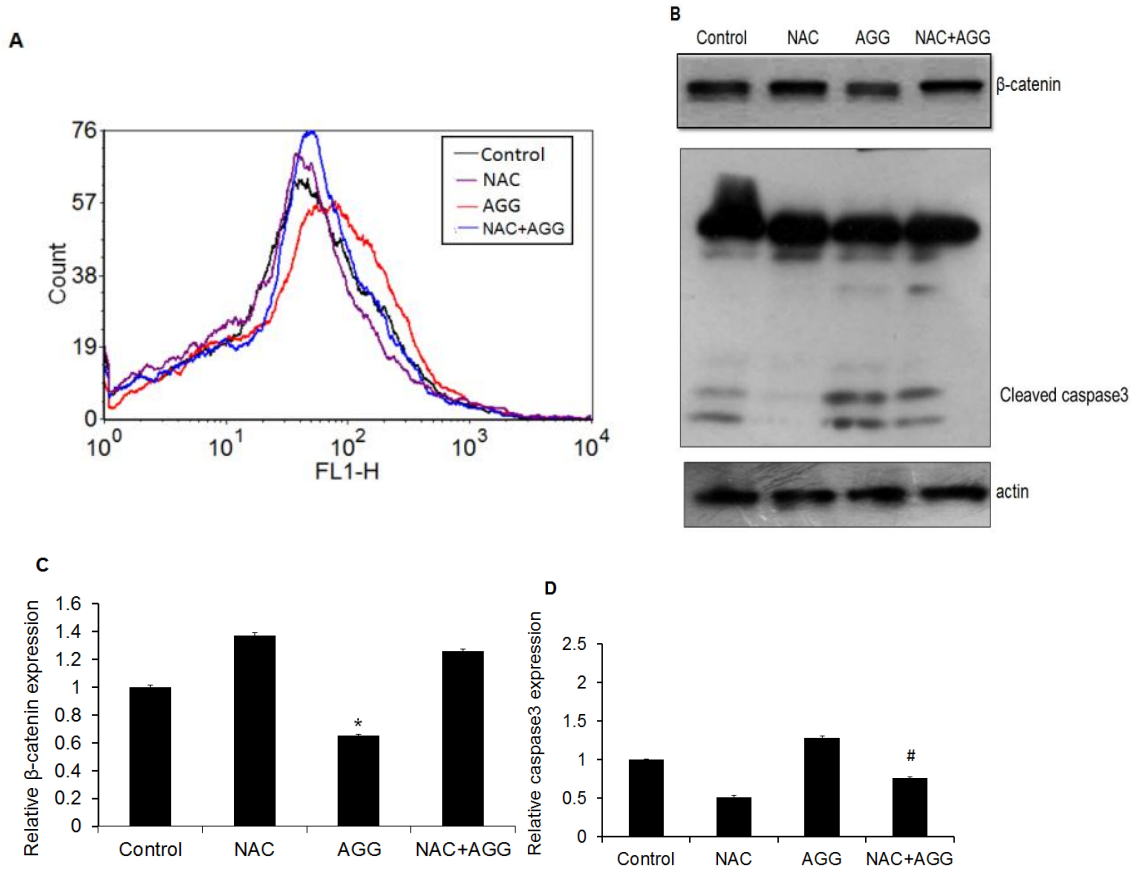

Figure-6.6. ROS induced by AGG regulates apoptosis and Wnt signaling. Orospheres treated with AGG in presence of N-acetyl cysteine (NAC, 10 mM) were incubated with dihydrorhodamine 123 (2.5 µg/ml) analyzed for ROS generation through flow cytometry (A). Orospheres were pre-treated N-acetyl cysteine (NAC, 10 mM) followed to AGG treatment (1 µg/ml) and analysed for expression of cleaved caspase-3 and β-catenin (B) by Western Blot. Densitometer of proteins is represented as histogram plotted on the basis of actin to target protein ratio (Fig. 6C and 6D) by ImageJ. Data reported as the mean ± S.D. of three independent experiments and compared against PBS control. *P value < 0.05; **P value < 0.01 were considered significant. # indicates data compared against AGG treatment.

CD44 expressions appear to exhibit CSC properties in many cancers, including HNSCC. CD44 is a family of transmembrane receptors found on a number of different benign and malignant cells. Although CD44 alone is not sufficient for precisely isolating CSC in head and neck cancer cells, the CD44 cells show elevated self-renewal, proliferation, and differentiation, thus expressing enhanced tumorigenicity. Palagani et al. showed significant decrease in the expression of CD44 after

chemotherapy independent of any drug combination or tumor type. Zerumbone inhibited the EGF-induced CD44 expression through inhibition of the STAT3 pathway (Grimm et. al., 2015; Kokko et. al., 2011; Kim et. al., 2014; Chen et. al., 2010; Palagani et. al., 2012). Similar to these results, our research work showed regressed CD44 expression by AGG treatment in a dose dependent manner. Downregulation of $CD44^+$ cells restricted the aberrant CD44 expression in FaDu, SCC25, and SCC4 cells. Further, our confocal data showed that CD44 expressed in orosphere decreased in dose dependent manner with AGG treatment, indicating a positive correlation between AGG and decline in $CD44^+$ populations in oral squamous cell carcinoma.

Wnt/β-catenin signaling pathway has a wide range prospects in developmental biology including maintenance of stem cell compartments in adult tissue. The signaling pathway is derailed in CSCs contributing to its plasticity and therapy resistance. The underlying mechanism behind these two salient properties of CSCs is very little known. Honokiol, an active compound of *Magnolia officinalis*, has come up strongly with its potential to eliminate human oral cancer stem cells by inhibiting Wnt/β-catenin signaling axis and apoptosis induction (Yao et. al., 2013). It has also reciprocated its potential in suppressing the proliferation as well as angiogenesis in the xenograft growth of oral CSC-like cells. A natural polyphenolic compound abundantly found in plant foods, resveratrol inhibited the proliferation of breast cancer stem cells (BCSCs) followed by induction of autophagy via suppressing Wnt/β-catenin signaling pathway (Fu et. al., 2014). Wnt/β-catenin pathway is responsible for the clonogenic propensity involved during survival and development of MLL leukemic stem cells (LSCs). Cordycepin selectively reduced β-catenin stability by regulating GSK-3β to eradicate leukemia via elimination of LSCs. In this study, we proposed that the activated Wnt/β -catenin pathway might be critically responsible for the development and aggressiveness of oral cancers and could potentially be excellent candidates for targeting cancer-initiating/stem cells (Ko et. al., 2013). Our molecule successfully restricted the self-renewal property of CSCs as evidenced by the reduced orosphere formation by inhibiting the wnt canonical pathwaythrough downregulation of β-catenin and GSK-3β expression. The confocal data further supported our hypothesis with decline in the β-catenin-CD44 interaction in orospheres with AGG treatment.

There are several natural compounds that have restored cell sensitivity to death stimuli, suppressing CSC self-renewal and tumor metastasis. Recently, sulforaphane,

Cucurbitacin I, resveratrol, quercetin, and curcumin have been reported to suppress the cancer stem cells by inducing apoptosis (Li et. al., 2010; Chen et. al., 2010; Fu et. al., 2014; Chen et. al., 2012; Huang et. al., 2016). The proliferation of colon CSCs was eliminated by 20 (s)-ginsenoside Rg3 by induction of apoptosis through caspase-9 and caspase-3 pathways. In $CD133^{+}$ rectal CSCs, curcumin was found to induce apoptosis and significantly increased its radiosensitivity. Earlier our group has shown that AGG treatment in different cancer cells triggered ROS generation followed by programmed cell death. Here, we observed that AGG treatment in orospheres succinctly generated ROS in a dose dependent manner. Orospheres have high Bax/Bcl-2 ratio and increased annexin V positive cells that correlated with the low caspase 3 cleavage in presence of NAC. ROS mediated apoptosis targeted the orospheres since AGG disturbed the redox balance that directs the selective killing of cancer cells. Flow cytometric distribution explained about the increase in the $CD44^{-}$ population in different oral cancer cells upon AGG treatment in dose dependent manner. Thus, eradicating the orospheres by phytoproducts like *Abrus* agglutinin has emerged as a valued complementary strategy in successful treatment of oral cancer.

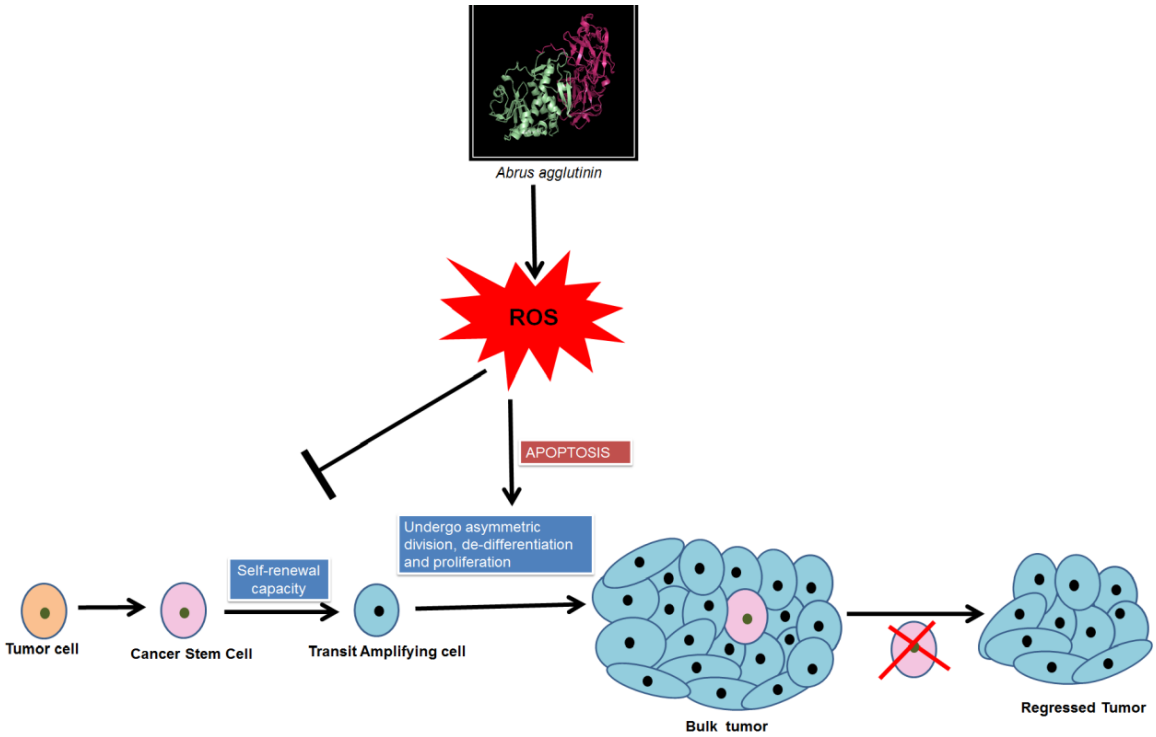

Figure-6.7. Flowchart demonstrating AGG inhibits cancer stem like cells (CSCs) in Snail degradation and p73 activation followed to inhibition of EMT, stemness, and invasiveness.

Chapter 7

Conclusion

7.1. Summary and conclusion

Abrus agglutinin (AGG), a heterodimeric glycoprotein of 134-kDa is a low-toxicity protein isolated from the seeds of *Abrus precatorius* induced antitumor activity in different oral squamous cell carcinoma. The efficiency of AGG has been already established in different type of cancer, but it's for the first time AGG treatment has been explored in oral squamous carcinoma. P53 being mutated in oral cancer, the focus of our study represented to p73. P73 is rarely mutated in cancers. We demonstrated that AGG treatment regulated in DNA damage mediated programed cell death in AGG treated FaDu cells. AGG effectively inhibited the cell proliferation of different oral squamous cell carcinoma cell lines *in vitro* and *in vivo*, with IC_{50} values 1-10 µg/ml. The molecular mechanism of ROS accelerated DNA damage followed by p73 mediated apoptosis in FaDu cells remain unexplored. Our data showed inhibition of ROS generation by NAC suppressed the apoptosis and DNA damage induced by AGG, indicating a critical role of ROS in AGG mediated apoptosis and DNA damage. The upregulation of p73 protein was observed in the presence of the different doses of AGG in FaDu cells, and data showed a decrease in the percentage of annexin V positive cells and caspase activity in p73 deficient cells. To delineate the ATM expression with ROS generation and p73 in FaDu cells, we found reduced activity of p73 which resulted in decreased number of apoptotic cells in shATM cells with the most effective dose of AGG. In conclusion, we deciphered the role of AGG in connecting the ROS, ATM and p73 molecules. There are no reports showing natural molecule in Snail degradation. The novelty of research is the p73 molecule and its unknown connection with the epithelial-mesenchymal-transition (EMT) regulator Snail. There are several manipulations a tumor cell does in a cell to escape the therapy. Cancer cells undergo metastasis in which epithelial cells endure alterations in cellular architecture (loss of their characteristic epithelial polarity), adhesion (disassemble of cell-cell junctions), morphology (assuming a fibroblastoid mesenchymal morphology). Immunofluorescence and western blot study prove that AGG efficaciously upregulates the expression of epithelial proteins (E-cadherin, Involucrin) and downregulates the expression of mesenchymal proteins (N-cadherin, Vimentin, β-catenin) in AGG treated EGF induced FaDu cells. Mesenchymal characteristic enhances the migratory and invasive properties of the cancer cells. The migration ability of AGG treated FaDu cells were monitored by wound healing assay in absence or presence of EGF. The

intriguing stemness was also found to be abrogated with downregulation of OCT4, SOX2, and Nestin expressions. In a Snail overexpressed FaDu cell, E-cadherin and p73 expression decreased on AGG treatment when compared to mock cells while the ADAM17, SOX2 and OCT4 expression were higher. Similar results were observed in shp73 cells. There are restricted reports governing the interaction between p53 with snail. Lee et al, showed that there is direct interaction between snail and p53. The degradation of Slug is controlled by p53 and MDM2. With this clue we expected p73 to play an intriguing role in EMT inhibition. Once cancer cells are detached from ECM (extracellular matrix), to remain viable in the circulatory system, they resist anoikis and consequently avoid death receptor signaling. To establish a relationship between apoptosis and EMT; *in vitro* coimmunoprecipitation study showed AGG treatment inhibited snail-p73 intereaction and prompted its cytosol translocation and ubiquitination; *in silico* studies were performed, that revealed that snail binds to p73 leading to the suppression of p73 activity while AGG binds to snail inhibiting the snail-p73 interaction. Further work demonstrated the efficiency of AGG on cancer stem like cells. It's an important affair to target CSCs, as they remain occult during the therapy, thus play a pivotal role in recurrence of cancer. The molecular mechanisms by which AGG induces apoptosis in FaDu CSCs have not been appraised. This study monitored the key molecules involved in the maintenance of cancer stem like cells; β-catenin and GSK-3β. Our data further documented inhibition of proliferation of CSCs with the reduced cell viability of $CD44^+$ cells in a dose dependent manner by triggering ROS mediated apoptosis. The above interesting results suggest that AGG has a potential role as an integrative therapeutic approach for combating oral cancer. Unraveling the molecular biology of oral cancer cells will facilitate the development of apoptosis-based therapeutic intercessions for oral cancer.

7.2. Scope of future work

The future study focuses to elucidate the novel signal transduction pathway of AGG *in vitro*. The *in vivo* antitumor study in murine model with detail mechanism of action may give a platform for therapeutic utility of the AGG. A nonspecific immunostimulatory response of tumor targeting peptides in tumor-suppressed hosts will provide an insight about possibility of attacking tumor via indirect immunostimulation. We have studied Snail degradation and have found the residues involved in Snail-p73 binding through in silico approach. Further single nucleotide mutation could be performed to understand the intricacies and importance of L3 and

L2 loop of p73 that binds to snail. Does AGG treatment leads to its nucleus translocation and results in its direct effect on snail or p73, or there is an intermediate molecule that propels the inactivation or activation respectively. The detailed study of p73 mechanism could have implication in enhancing the therapeutic potential of p53 deficient oral squamous cell carcinoma. The *in vivo* antitumor study in murine model with detail mechanism of action may give a platform for therapeutic utility of the AGG. A nonspecific immunostimulatory response of tumor targeting peptides in tumor-suppressed hosts will provide an insight about possibility of attacking tumor via indirect immunostimulation. The detailed study of p73 mechanism could have implication in enhancing the therapeutic potential of p53 deficient oral squamous cell carcinoma.

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CURRICULUM VITAE

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OBJECTIVES AND CAREER SUMMARY:

I am an enthusiastic, dynamic and determined girl. My focus is to study the mechanism by which a medicinal plant lectin, *Abrus* agglutinin showcases its anti-neoplastic activity in oral cancer. I have worked on different cancer cell lines; like, HeLa, MDA-MB 231, KB, PC3, SCC9, SCC4, SCC15, SCC25, H357, FaDu, HEp2, RPMI2650. I have endeavored to explore the anti-cancer properties of *Bryonia* lasciniosa, Concanavalin A.

AREA OF INTEREST: Oncology, Cancer Stem Cell, Molecular biology, Protein biology

PERSONAL DATA:

Date of Birth	9th May, 1987
Gender	Female
Nationality	Indian
Marital Status	Single

EDUCATIONAL QUALIFICATIONS:

2011- November 2016 (continuing)	Graduate studies (PhD) at Department Of Life Science, National Institute of Technology, Rourkela, under the supervision of Dr. S.K BHUTIA, Head of the Department at Life Science Department. Thesis title: "Inhibiting carcinogenicity and the escaping mechanism in oral squamous cell carcinoma (OSCC) using *Abrus* agglutinin (AGG)
2009-2011	Master Of Science in Biotechnology at Department Of Biotechnology, Rajiv Gandhi Institute Of Biotechnology, Bharati Vidyapeeth University. Subjects covered: Molecular Bio & Genetic Eng. Immunology, Animal Tissue Culture, Plant Tissue Culture, Env.Biotech, Nanobiotech **Percentage scored 62.10%. Division First Class**

2006-2009	Bachelor Of Science in Biotechnology at Department Of Biotechnology, Amity School Of Biotechnology, Amity University. Subjects covered: Animal Biotech, Plant Biotech, RDT, Env-Industrial Biotech, Genomics & Proteomics **CGPA scored 8.01. Division First Class**
2005	Senior Secondary, Class XII. Devsangha National School. CBSE. Subjects covered: Bio, Chem, Phy, Eng, Eco **Percentage scored 70.20%. Division First Class**
2003	High School, X. Saint Francis School. ICSE.Subjects covered: Sci, S.St, Maths, Hindi, Eng, Eco. **Percentage scored 72.16%. Division First Class**

RESEARCH EXPERIENCE:

M.Sc research project done on anti-cancer property of *Bryonia* lasciniosa in different cancer cell lines at Bharati Vidyapeeth University, Pune. Currently, working as **Senior Research Fellow** (SRF) at National Institute of Technology, Rourkela; under the supervision of Dr. Sujit Kumar Bhutia, Head of the Department, Life Science Department, NIT Rourkela.

TECHNIQUES HANDLED:

- ***Protein Biology***
  Isolation of plant proteins, familiar with techniques such as dialysis and ammonium sulfate precipitation, well acquainted with the techniques for quantitation of protein such as Lowry assay and Bradford assay, handling and storage of proteins, purification of proteins by Gel filtration, Affinity chromatography, SDS PAGE, Native PAGE
- ***Cell and Cancer Biology***
  Maintaining and freezing of several mammalian cancer cell lines, in vitro anti-proliferative study, transfection of mammalian cancer cells, colony forming assay, cell cycle analysis and apoptosis by FACS, cell study by fluorescence and confocal microscopy, in vivo antitumor study in different murine model
- ***Immunology***
  Isolation of B and T cell by MACS, immunophenotypic by FACS, ELISA
- ***Molecular Biology***
  Isolation of nucleic acids (genomic DNA and RNA) from eukaryotic cells, Reverse Transcriptase-PCR, agarose gel electrophoresis, Western blot analysis using Chemidoc

FELLOWSHIP AND HONOURS:

- ❖ NIT ROURKELA Institute PhD fellowship: July 2011 – present
- ❖ Qualified Graduate aptitude test in engineering (GATE-BT): March 2011, 96 percentile

Scholarly Publications: International peer-reviewed, SCI-indexed with Impact factor (IF)
h-index: 9

Publications from Thesis:

Sinha N, Mukhopadhyay S, Das DN, Panda PK, Bhutia SK. Relevance of cancer initiating/stem cells in carcinogenesis and therapy resistance in oral cancer. Oral Oncol. 2013 Sep;49(9):854-62. **IF:4.2**

Sinha N, Panda PK, Das DN, Naik PP, Maiti TK, Bhutia SK.
***Abrus* agglutinin targets cancer stem-like cells by eliminating self-renewal capacity accompanied with apoptosis in oral squamous cell carcinoma**
Tumor Biology. **IF: 2.9**

Communicated manuscripts:

Sinha N, Panda PK, Das DN, Mukhopadhyay S, Naik PP, Maiti TK, Bhutia SK. ***Abrus* agglutinin induces irreparable DNA damage via ROS generation followed to p73 mediated apoptosis in FaDu cells**
under review in Molecular Carcinogenesis- MC-16-0176. **IF: 4.8**

Sinha N, Panda PK, Naik PP, Maiti TK, Bhutia SK. Snail ubiquitination facilitates p73 upregulation by *Abrus* agglutinin to inhibit epithelial to mesenchymal transition in oral cancer.
Oral Oncol. **IF:4.2**

Manuscripts under preparation:

Sinha N, Panda PK, Naik PP, Maiti TK, Bhutia SK. Phytohemagglutinins: an earnest endeavor to propel the cancer cell undergo programmed cell death (under preparation)

Publications outside Thesis work:

Das DN, **Sinha N**, Naik PP, Panda PK, Mukhopadhyay S, Mallick SK, Sarangi I, Bhutia SK. Mutagenic and genotoxic potential of native air borne particulate matter from industrial area of Rourkela city, Odisha, India. Environ Toxicol Pharmacol. 2016 Sep;46:131-9. **IF:2.1**

Mukhopadhyay S, **Sinha N**, Das DN, Panda PK, Naik PP, Bhutia SK. Clinical relevance of autophagic therapy in cancer: Investigating the current trends, challenges, and future prospects. Crit Rev Clin Lab Sci. 2016 Aug;53(4):228-52**. IF:4.1**

Sahu S, **Sinha S**, Bhutia S, Majhi M and Mohapatra S. Luminescent magnetic hollow mesoporous silica nanotheranostics for camptothecin delivery and multimodal imaging. J. Mater. Chem. B 2014; 2: 3799-3808. **IF:4.8**

Bhutia SK, Mukhopadhyay S, **Sinha N,** Das DN, Panda PK, Patra SK, Maiti TK, Mandal M, Dent P, Wang XY, Das SK, Sarkar D, Fisher PB. Autophagy: cancer's friend or foe? Adv Cancer Res. 2013;118:61-95. **IF:4.7**

Mohapatra S, Sahu S, **Sinha N**, Bhutia SK. Synthesis of a carbon-dot-based photoluminescent probe for selective and ultrasensitive detection of Hg(2+) in water and living cells. Analyst. 2015 Feb 21;140(4):1221-8. **IF:4.1**

Mukhopadhyay S, Panda PK, **Sinha N**, Das DN, Bhutia SK. Autophagy and apoptosis: where do they meet? Apoptosis. 2014 Apr;19(4):555-66**. IF:3.5**

Mukhopadhyay S, Naik PP, Panda PK, **Sinha N**, Das DN, Bhutia SK. Serum starvation induces anti-apoptotic cIAP1 to promote mitophagy through ubiquitination. Biochem Biophys Res Commun. 2016 Oct 28;479(4):940-946. **IF:2.2**

Das DN, Panda PK, Mukhopadhyay S, **Sinha N**, Mallick B, Behera B, Maiti TK, Bhutia SK. Prediction and validation of apoptosis through cytochrome P450 activation by benzo[a]pyrene. Chem Biol Interact. 2014 Feb 5;208:8-17. **IF:2.6**

Mukhopadhyay S, Panda PK, Das DN, **Sinha N**, Behera B, Maiti TK, Bhutia SK. Abrus agglutinin suppresses human hepatocellular carcinoma in vitro and in vivo by inducing caspase-mediated cell death. Acta Pharmacol Sin. 2014 Jun;35(6):814-24. **IF:3.1**

Mukhopadhyay S, Das DN, Panda PK, **Sinha N**, Naik PP, Bissoyi A, Pramanik K, Bhutia SK. Autophagy protein Ulk1 promotes mitochondrial apoptosis through reactive oxygen species. Free Radic Biol Med. 2015 Dec;89:311-21. **IF:5.7**

Mukhopadhyay S, Panda PK, Behera B, Das CK, Hassan MK, Das DN, **Sinha N,** Bissoyi A, Pramanik K, Maiti TK, Bhutia SK. In vitro and in vivo antitumor effects of Peanut agglutinin through induction of apoptotic and autophagic cell death. Food Chem Toxicol. 2014 Feb;64:369-77. **IF:3.5**

Panda PK, Mukhopadhyay S, Behera B, Bhol CS, Dey S, Das DN, **Sinha N**, Bissoyi A, Pramanik K, Maiti TK, Bhutia SK. Antitumor effect of soybean lectin mediated through reactive oxygen species-dependent pathway. Life Sci. 2014 Aug 28;111(1-2):27-35. **IF:2.6**

Bhutia SK, Behera B, Nandini Das D, Mukhopadhyay S, **Sinha N,** Panda PK, Naik PP, Patra SK, Mandal M, Sarkar S, Menezes ME, Talukdar S, Maiti TK, Das SK, Sarkar D, Fisher PB. Abrus agglutinin is a potent anti-proliferative and anti-angiogenic agent in human breast cancer. Int J Cancer. 2016 Jul 15;139(2):457-66. **IF:5.5**

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Panda PK, Mukhopadhyay S, Das DN, **Sinha N**, Naik PP, Bhutia SK. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. Semin Cell Dev Biol. 2015 Mar;39:43-55. **IF:5.1**

CONFERENCE PROCEEDINGS:

A conference proceeding at 34th Annual Convention of Indian Association for Cancer Research (IACR) & International Symposium on "Cancer Research: from Bench to the Bedside", Jaipur, 2015. DNA damage by *Abrus* agglutinin induced p73 mediated apoptosis through ROS generation in oral squamous cell carcinoma.

Niharika Sinha, Sujit K Bhutia

A conference proceeding at 2nd International Conference on Fronteirs In Biological Sciences (InCoFIBS-2015), NIT Rourkela. AGG induced p73 mediated apoptosis in FaDu cells.

Niharika Sinha, Sujit K Bhutia

A conference proceeding at 32nd Annual Convention of Indian Association for Cancer Research (IACR) & International Symposium on "Infection & Cancer", New Delhi, 2013. Autophagic cell death induction with *Abrus* agglutinin.

Prashanta Kumar Panda, Subhadip Mukhopadhyay, Durgesh Nandini Das, **Niharika Sinha**, Sujit K Bhutia