Evaluation of Role of Glyoxalase-1 in Radioresistant & Drug Resistant Cancer Cells

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CERTIFICATE

The research work embodied in this thesis entitled, "**Evaluation of Role of Glyoxalase-1 in Radioresistant & Drug Resistant Cancer Cells**" submitted for the award of degree of Doctor of Philosophy was carried out by **RAJ KUMAR** under the supervision of **Dr. Ashu Bhan Tiku**, at School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This work is original and has not been submitted so far, in part or in full, for award of any degree or diploma of any University.

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DEDICATION

This thesis is dedicated to my beloved mother, who taught me that "Best kind of knowledge is the one that is learned for its own sake". To my father, who taught me that even the impossible task can be accomplished if you have courage…… ✍

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Raj Kumar **20.07.2018**

ABBREVIATIONS AND SYMBOLS

AO	Acridine Orange
AGE	Advanced Glycation Endproduct
BSA	Bovine Serum Albumin
CMC	Carboxy Methyl Cellulose
DNPH	2-4 Dinitro Phenyl Hydrazine
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenylpicrylhydrazyl
DSBs	Double Strand Breaks
DMBA	7,12 Dimethylbenz[a]anthracene
DCFDA	Dichloro Fluorescin Diacetate
EDTA	Ethylene Diamine Tetraacetic acid
DMSO	Dimethylsulphoxide
Etbr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting
GSH	Glutathione
Gy	Gray
Glo-1	Glyoxalase-1
GLO-1	Glyoxalase-1 gene
H2O2	Hydrogen Peroxide
IR	Ionizing Radiation
IF	Immunofluorescence
ICD-O-3	International Classification of Diseases for Oncology-3
LMPA	Low melting point agarose
LDH	Lactate dehydrogenase
M	Molar
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ml	Milliliter
mg	Milligram
µg	Microgram

µl	Microliter
MDR	Multi Drug Resiatance
MMPs	Matrix metalloproteinase
MG	Methyl Glyoxal
RR	Radioresistance
NCD	Non Communicable Diseases
NG	Naringenin
NMPA	Normal point melting agarose
NO	Nitric oxide
Nrf-2	Nuclear factor Eerythroyed 2-related factor 2
PBS	Phosphate buffer saline
PL	Papilloma Latency
PAGE	Poly Acrylamide Gel Electrophoresis
PI	Propidium Iodide
ROS	Reactive Oxygen Species
R or Rad	Radiation
RT-PCR	Reverse Transription- Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
RAGE	Receptor for Advanced Glycation Endproducts
RR-HeLa	Radioresistant HeLa
SOD	Super Oxide Dismutase
SDS	Sodium Dodecyl Sulfate
SSBs	Single Strand Breaks
TCA	Trichloroacetic Acid

LIST OF GENE PRIMER USED

Name	Sequence
Glo-1 Forward	5'TGGGCGCTCTCCAGAAAA3'
Glo-1 Reverse	5'TTGTGGTAACTCTGGGTCTCATCA3'
Hif-1α Forward	5'ACAGAGCAGGAAAAGGAGTCATAGA3'
Hif-1αReverse	5'ACTCAAAGCGACAGATAACACGTT3'
β-Actin Forward	5'GGCACCCAGCACAATGAAG3'
β-Actin Reverse	5'GCCGATCCACACGGAGTACT3'

LIST OF CELL LINES USED

S.N	**Cell line names**
1	HeLa Human Cervical Cancer cell line
2	DU-145 Human prostate Cancer cell line
3	A-549 Human Lung Cancer Cell line
4	HT-29 Human Colon Cancer cell line
5	HEK Human Embryonic Kidney cell line
6	B16F10 Mice skin Cancer cell line

LIST OF ANIMAL USED

S.N	**Animals & Strain Names**
1	Swiss Albino Mice BALB/c strain
2	C57BL6 Mice

LIST OF FIGURE

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Chapter I

Introduction

Biologically cancer is uncontrolled growth of cells that can spread to nearby tissues or organ. Occurrence of cancers is dependent on many factors like age, dietary habits, genetics, environmental factors, racial etc. Thus cancer is not a standalone disease but is considered to be a group disease. Cancerous cells can move to any part of the body via circulatory systems i.e. blood and/or lymph. Many cancers are gender or sex specific. For example prostate cancer occurs only in men and ovarian cancer is specific to females. However breast cancer can occur in both but is more prevalent in females. Some cancers like skin cancer, lungs cancer, brain cancer, liver cancer are other types of cancers that are equally prevalent in males and females.

1. Classification of cancer:

Most of the cancers are named after the organ in which they originate. If a cancer spreads (metastasis) in the body, the new tumour bears the same name as the original tumour[1]. Thus cancers can be classified:

1.1. Based on original site of cancer: Based on the site of origin, cancers can be of many types like skin cancer, breast cancer, liver cancer, cervical cancer, lung cancer, prostate cancer, brain cancer etc [2].

1.2. Based on the tissue type: Based on tissue type as per the ICD-O-3 [2]. This system classifies cancer as –

1.2.1. Carcinoma

Carcinomas (malignancy of epithelial tissue) constitute about 80 to 90 % of all cases of cancer. Epithelial tissues are abundantly distributed in the body. Carcinoma can grow from the tissues as well as glands that are capable of secretion likes prostate, breast, colon and lungs.etc

Carcinomas can be further divided into two sub-types – squamous cell carcinoma (develops in squamous epithelium) and adenocarcinoma (organ or gland).

1.2.2. Sarcoma

Sarcomas develop from connective and supportive tissues such as bones, cartilage, fat and muscles. Bone cancer is an example of sarcoma and is also known as osteosarcoma based on site and tissue of origin. Other examples of sarcomas include mesothelial sarcoma or mesothelioma (membranous lining of body cavities), chondrosarcoma (of the cartilage),

mixed mesodermal tumor or Mesenchymous, Fibrosarcoma (fibrous tissue), Angiosarcoma or hemangioendothelioma (blood vessels), Glioma or astrocytoma, leiomyosarcoma (smooth muscles), rhabdomyosarcoma (skeletal muscles), Liposarcoma (adipose or fatty tissue), and Myxosarcoma (primitive embryonic connective tissue) [2].

1.2.3. Leukemia

Leukemia is a type of blood cancer. This cancer primarily affects the bone marrow, major site for blood cell production. The bone marrow produces large number of immature white blood cells that are unable to perform their normal function and the patient become sensitive to infection. Leukemias are of three main types:

Acute myelocytic leukemia (AML) – malignancy of the myeloid and granulocytic WBC, is dominant in childhood.

Chronic myelocytic leukemia (CML) – Predominantly seen in adult stage.

Acute lymphatic, lymphocytic, or lymphoblastic leukemia (ALL) – Malignancy of the lymphoid and lymphocytic blood cell manly occurs in childhood and young adults.

Chronic Lymphatic, lymphocytic, or lymphoblastic leukemia (CLL) – Occurs primarily in the elders.

Polycythemia vera or erythremia – Malignancy of erytherocytes and various blood cell products [2].

Myeloma- Develops within the bone marrow in plasma cells [2].

1.2.4. Lymphoma

Lymphoma occurs in the lymphatic system. Lymphomas are considered as “solid cancers”. They affect the lymph nodes of lymphatic system at specific sites like intestines, stomach, brain etc. The lymphomas are also called as extranodal lymphomas. Lymphomas are of two types – Non-Hodgkin’s lymphomas and Hodgkin’s lymphoma. The typical characteristic of Hodgkin lymphoma is the presence of Reed-Sternberg cells which are absent in Non-Hodgkin lymphoma [2].

1.2.5. Mixed types cancers

Mixed type cancers have more than two type of the cancer. Examples include mixed mesodermal tumor, carcinosarcoma (mixture of carcinoma and sarcoma), teratocarcinoma

(undifferentiated stem cells), adenosquamouscarcinoma (cancer of squmous cells and gland like cells) and blastomas (malignancies of precursor cells) [2].

1.3. On the basis of grade of cancer

The grade of cancer depends on the abnormality of the cancerous cells with respect to nearby normal tissues cells. Increasing abnormality of cancer cells increase the grade of cancer, ranging from 1–4.

Cells that undergo division and differentiation & closely resemble to normal cells, are considered as low grade tumors. While, cells which undergo perform division and remain undifferentiated showing high abnormality with surrounding tissues are considered as high grade tumors [2].

Grade 1 – Cells that undergo division and are well differentiated with slight abnormality.

Grade 2 – Moderately differentiated and slightly more abnormal cells.

Grade 3 – Poorly differentiated and very abnormal cells.

Grade 4 – Immature, primitive and remain undifferentiated cells.

1.4. Based on stages of cancer

Cancers are also classified according to their stages in an individual. There are many methods for classification of cancer, but the most frequently used method is TNM staging. This staging classifies the tumor according to their size (T), the degree of regional spread or node involvement (N), and metastasis (M). To determine the stage of cancer doctors use T, N, M data for each person. Base on this criteria cancers have four stages: starting from stages I to IV. Some cancers also have a stage 0 (zero).

Stage 0 - In this stage cancer is *in situ* (in place). Stage 0 cancers are situated at their initial point of origin. Cancer of this stage is highly curable, and can be removed by surgery [2].

Stage I - Small cancer or tumors that have not been spread to nearby tissues. This is called early-stage cancer.

Stage II and III - Cancers or tumors have grown deeply into nearby tissues, also may affect lymph nodes but not other body parts.

Stage IV - This is also called advanced or metastatic cancer in which cancer has spread to other organs or different body parts [2].

1- Global Cancer Burden

2.1 International cancer statistics

It is estimated that 1,735,350 new cancer cases will be diagnosed and 609,640 deaths will be from cancer in the United States in 2018 [3]. As per the estimates of European Union (EU) countries (France, Germany, Italy, Poland, Spain, UK) there will be a total of 1,382,000 deaths, due to cancer in 2018 [4]. Estimated number of cancer deaths in 2017 was approximately 378,000（222,000 males and 156,000 females) in Japan. The estimated lifetime probability of cancer diagnoses in men is 39.7% and 37.6% for women. The most commonly found cancers in men are prostate, lung, and colorectal cancers, the probability of prostate cancer alone occurs, 1 in 5 in men. The most common cancers in women are breast, lung, and colorectal cancers, breast cancer occurs in 30% of women [3]. Although mortality rate has decreased in many cancers e.g.: lung cancer death reduced to 45% (from 1990 to 2015) and 19% (from 2002 to 2015) in men and women respectively. Breast cancer death rate reduced to 39% (from 1989 to 2015) in women while, death from prostate cancer reduced to 52% from 1989 to 2015 in men. As per *American Cancer Society, Cancer Facts & Figures 2018*, ovarian cancer accounts for only 2.5% of all cancers in females, but causes 5% of death due to low survival rate. In 2018, 22,240 new cases of ovarian cancer were expected to be diagnosed and 14,070 ovarian cancer related deaths occur in the US "(*American Cancer Society, Cancer Facts & Figures 2018*).

2.2. Cancer Statistic in India

Cancer is one of the leading causes of mortality among adults in India also. In India, every year, over 7,00,000 new cancer patients are registered and 5,56,400 cancers related deaths occur [5], [6]. As per figures released by Indian Council of Medical Research, it is further going to increase to over 17 lakh new cases of cancer and over 8 lakh deaths by 2020.The top five cancers in men and women result in 47.2% of all cancer's death in India [6, 7]. Breast and cervix cancer in women and oral cavity and lungs cancer in men causes about 50% of all cancer's deaths [8] Out of every 2 women newly diagnosed with breast cancer, one dies in India [9-11].

Cervical cancer is the second most cancer in Indian women. Cervical cancer is the third prevalent cause of cancer mortality in India and accounts for 10% of all cancer's death in the country [12]. Estimated number of people having the disease is about 2,500,000 and one woman dies in every 8 minutes due to cervical cancer [5]. Rural women are more prone to cervical cancer as compared to urban women [13]. Survival depends on the cancer stage at the time of diagnosis. The chances of survival can be increased if the cervical cancer is diagnosed and treated at earlier stages.

2- Glyoxalase -1 and Cancer

The conventionally used treatment modalities for cancer are radiotherapy and chemotherapy. Besides this, surgery, immunotherapy, hormonal therapy etc are also now used for the cancer treatments. Although radiotherapy and chemotherapy are effective for treatment of cancer, these therapies have some drawbacks. One of the major problem is development of resistance to treatment. The resistance to therapy among other factors is attributed to hypoxia and over expression of enzyme glyoxalase-1. The review of literature suggested that most drug resistant cancer cells have high level of glyoxalase-1 activity as compared to normal and drug sensitive cancers [14].

Glyoxalase system is an ubiquitous cellular defence systems. It consists of two enzymes, Glyoxalase I (GLO1; E.C.4.4.1.5) and glyoxalase II (GLO2; E.C.3.1.2.6) and small amount of cofactor, reduced glutathione. It catalyzes the detoxification of methyalglyoxal (MG) produced in glycolysis to nontoxic D-lactate. Methylglyoxal is produced by the nonenzymatic degradation of triose phosphate products which are by-product of anaerobic glycolysis [15].

Intracellular methylglyoxal production is increased when glycolytic flux is high, and is connected with malignant transformation and tumor progression [16-19]. Indeed, various cancer cell lines over express Glo-1, which is a cellular response to high cellular stress associated with glycolytic adaptations of cancer cells [19-21]. Increased level of Glo-1 has been reported in various cancer cell lines and human tumor tissues like prostate cancer, breast cancer and invasive ovarian cancer and recently in gastric cancer [16, 20, 22-24]. Increased Glo-1 expression is also associated with resistance to chemotherapeutic agents and survival of cancer cell [14, 16, 25]. Recent reports suggest Glo-1 to be a valid molecular target for cancer chemotherapy.

The relationship between high level Glo-1 and drug resistance in cancer chemotherapy was reported by Sakomoto *et al* [14]. Over expression of glo-1 in gastric cancer enhanced the metastasises of tumor cells *in vitro* and *in vivo* [26]. It is observed that high expression of Glo-1 makes cells resistant to many anti-tumor agents like adriamycin and etoposide [14]. Hypoxia adapted Bcr-Abl^{+} cells have also been reported to have high Glo-1 activity & Glo-1 inhibitors effectively killed and eradicated these cells both *in vitro* and *in vivo* [27, 28]. In 1969 Vince and Wadd reported that glyoxalase inhibitors can works as anticancer agents by increasing the endogenous methyglyoxal concentration [29]. Thus Glo-1 can be used as a potential marker for the prognosis and detection of cancer [26].

The present study is based on the hypothesis that Glo-1 regulation plays a functional role in adaptations of cancer cells under adverse condition. Therefore modulation of Glo-1 enzyme can be an important strategy to overcome resistance in various therapeutic regimens. We studied the effect of hypoxia and phytochemicals on the modulation of Glo-1 so as to understand the mechanism of drug/ radioresistance in cancer cells. The phytochemicals selected for the present study were Naringenin and Galangin.

Naringenin

The flavanone naringenin is abundant in citrus fruits especially grapefruits, oranges and tomatoes. Grapefruit juice can provide much higher plasma concentrations of naringenin than orange juice [30]. *In vitro* studies suggest that naringenin protect body from reactive oxygen species such as alkoxyl, hydroxyl, or peroxyl radicals and also from aging, cancer, and cardiovascular diseases [31]. Naringenin has anti-proleferative effect on colon cancer cell line [32] & inhibits PI3K activity and uptake of glucose in 3T3-L1 adipocytes cell [33].

a) Grapefruit & Juice b) Tomatoes c) Cherry d) Beans

Figure1: Natural sources of Naringenin

Galangin

Galangin is a compound belonging to flavonol class of flavonoid. It is an active constituent of *Alpinia galanga*, a plant of the ginger family Zingiberaceae. It also occurs in roots of *Alpinia officinarum* and honey beehive product propolis. Galangin is a polyphenolic compound. Galangin was found to be an effective anti-proliferative, apoptosis inducer in Bcr-Abl overexpressing K562 and KCL22 cells and as well as in imatinib mesylate resistant K562-R and KCL22-R cells [34]. Galangin has inhibitory activity against cancer cells [35].

a) Galangal b) Helichrysum *thianschanicum* c) Propolis d) Honey

Figure 2: Natural sources of Galangin

3. Objectives:

Based on the hypothesis that over expression of Glyoxalase-1 may play a functional role in glycolytic adaptations of cancer cells the objectives of the present study were:

1- **To study the glyoxalase-1 activity in selected cancer & normal cells under hypoxic condition.**
2- **To study the modulatory effect of selected phytochemicals on glyoxalase-1 activity in cancer & normal cells.**
3- **To understand the mechanism of regulation of glyoxalase-1 in radio-resistant & drug resistant cancer cells & its modulation by selected phytochemicals.**

Chapter II

Review of Literature

Cancer is a group of diseases caused by deregulated division of normal cells. The ideal therapy for cancer involves the complete eradication of cancerous part from body. Sometimes this can be achieved by surgery but as cancer invades nearby and distant tissues by metastasis, which limits this treatment option. In majority of cases treatment modality is chemotherapy and radiotherapy but that also has associated side effects on normal tissues. The selection of therapy depends on the cancer type & grade of cancer as well as stage of cancer etc.

2. 1. Therapies for cancer treatment

2.1.1. Radiotherapy

Radiotherapy was developed in later half of 19^{th} century. The targeted tissue was exposed to radiation using external beam radiotherapy (EBRT) or by brachytherapy. The different types of radiation sources which are used for the treatment purpose are alpha, gamma, X-ray, neutron, etc. Radiotherapy is limited by radiation induced side effects like damage to normal tissues and cells as well as development of radio resistant cancers [1].

2.1.2. Chemotherapy

Chemotherapy is the use of chemicals to induce cell death in cancer cells. The chemotherapeutic drugs kill cancer cells by single or multiple mechanisms like DNA damage, apoptosis, cell cycle arrest etc. Chemotherapy is also associated with many side effects to normal tissue and sometimes other body parts [1].

2.1.3. Surgery

The localized and non-haematological cancer can be removed by surgery. But metastatic cancer cannot be eliminated completely. The surgery can be used to remove tumor or the whole organ. A single nearby cancer cell which escapes can regrow to form new tumour. So surgeries are always performed in combination with radiation and/ or chemotherapy [1].

2.1.4. Immunotherapy

The immunotherapy for cancer patients involves the strategy to boost and increase the immune system of patients to fight against their own tumor. In this therapy immune response is generated by using interferons, cytokines, cancer vaccine, patient specific vaccine, whole cell vaccine etc to fight against tumours. The autologous immune

enhancements therapies (AIET) are now a days used for the treatment of cancer. This involves the patient NK cell s and CT lymphocyte transplantation in combination with other therapies like radiotherapy, chemotherapy or surgery [1].

2.1.5. Hormonal therapy/ Targeted therapy

The targeted and hormonal therapy was developed in the last decade of 20th century. These therapies include the use of drug for specific proteins deregulated in cancer cells. Generally the drug binds with the domain mutated or over expressed in cancer cells. The growths of some cancers are hormone dependent like in prostate cancer. In such causes removing or blocking of estrogens or testosterone may be therapeutically beneficial [1].

Although these therapies are effective for treatment of cancer, yet development of resistance of cancer cells towards various treatments is one of the major problems.

2. 2. Mechanisms associated with -Therapeutic Resistance

Drug resistance causes 90 percent of failure in therapy at later or metastatic stage. Many factors play important role in development of drug resistance.

2. 2.1. Tumor heterogeneity

Genomic instability generates cellular heterogeneity or diverseness in different cancer types. Heterogeneity may arise due to epigenetic factors, intrinsic factors involving genotypic, stochastic variations in between cells, or hierarchical organization of cells [36-40].Hypoxia induced paracrine signalling interactions with stromal and other tumor cells, and pH are some more extrinsic factors [41, 42] responsible for drug resistance.

2.2.2. Tumor microenvironment

The tumor microenvironment includes extracellular matrix, normal stromal cells and other factors including cytokines and growth factors. Tumor stromal cell communication as well as tumor-ECM interface, contribute to drug resistance [43]. Also, GFs and cytokines produced provide additional environment for tumor cell growth and survival. Environment mediated-drug resistance, involves cell adhesion molecules (CAMDR) and soluble factor mediated drug resistance (SMDR), involve factors like VEGF (vascular endothelial growth factor); bFGF (basic fibroblast growth factor); SDF-1 (stromal cell-derived factor- 1); G-CSF (granulocyte colony stimulating factor); M-CSF (macrophage colony stimulating factor); IL-6 (interleukin-6); IL-3 (interleukin-3), GM-CSF

(granulocyte-macrophage colony stimulating factor); BAFF (B cell-activating factor of the TNF family); APRIL etc [43-45].

2.2.3. Inactivation of the anticancer drugs

In vivo interaction of drugs with different types of proteins present can alter the molecular feature of the drugs by reducing their activities to which cancer cells are resistant [46]. The acute myeloid leukemia, treatment with cytarabine after phosphorylations converted to cytarabine triphosphate [47]. Down-regulation or mutations in the proteins and enzymes involved in this pathway reduce the cytarabine activity resulting the cytarabine resistant cancer cells [48].

2.2.4. Releasing of drugs outside the cell

The ATP-binding cassette (ABC) protein plays an important role in acquiring the MDR in cancer cells. ABC family includes p-glycoprotein, multi-drug resistance-associated protein-1 and breast cancer resistance protein [49, 50]. The P-glycoprotein is a membrane transporter protein which also pumps the chloride out of the cells and transports a number of chemotherapeutic agents, like as Vinblastine, Doxorubicin and Taxol, by binding to hydrolyzed ATP and altering the p-glycoprotein. As a result, the chemotherapeutic agents are released into the extracellular space. The p-glycoprotein returns its basic structure after releasing the drugs outside of the cells [50, 51].

2.2.5. Decreasing the drugs absorption

The absorption of anticancer drugs into cancer cells takes place through passive transport (e.g., doxorubicin and vinblastine), facilitated diffusion and activate transfer [52]. The absorption of the drugs into the cells by towards high concentration occurs exclusively by the active transfer [53]. Reduction of the absorption of a drug occurs by two main ways: either by reducing the binding capacity of drugs or by reducing the numbers of transporter molecules [54]. Mutations in the transporter proteins result in the inhibition and reduction of the absorption of the drugs, e.g. resistance to drug methotrexate resulted due to the mutation in human folate carrier's gene in the patients with acute lymphoblastic leukemia. The substitution mutation of lysine by glutamic acid in the first transmembrane domain of human folate carrier's protein reduces the binding of the drugs for transporter [55].

2.2.6. Alteration in drug metabolism

The drugs are metabolized by different enzymes which plays important role in determining the concentration of the drug in cancer tissues. The phase I reactions (oxidation, reduction and hydrolysis of drug) and phase II reactions (consumption and conversion reaction of drug) play an important role in protecting the cells against anticancer drug. These reactions change the drug resistance either by increasing the enzyme activity or by reducing their activities. The important enzyme involved in phase I reactions detoxification is cytochrome P450 [56]. The DR against docetaxel in breast cancer is due to the increased level of cytochrome P450 [57]. The increased production of glutathione or increase in the phase II enzyme like glutathione transferases enhances the resistance to against alkylating anticancer drug and platinum conjugate anticancer drugs like cisplastin and doxorubicin [58].

2.2.7. Alteration in drug target

The effect of anticancer drugs also depends on the expression levels of their targets. The modification/mutation in target molecule may impart drug resistance. Doxorubicin is the drug of choice for the solid tumors in the breast and lungs act by inhibiting the topoisomerse II and thereby unwinding of the DNA. The cells with topoisomerase II mutations may result in the resistance against these particular drug [59].

2.2.8. Inhibition to cell death

Cell death in cancer cells takes place by different mechanisms like; necrosis, apoptosis, and autophagy which differ biologically from each other. Apoptosis induces cell death by activating both intrinsic and extrinsic pathways. In extrinsic mechanism, the external ligands/ death receptors like as FAS (first apoptosis signal) *receptor*, TNF-R (*tumor necrosis factor receptor)*, activates caspases-3, 6, 7 and 8 resulting in the lysis of actin protein, nuclear lamin proteins and ultimately resulting in apoptosis [60]. While the intrinsic pathway is mediated by mitochondria and many other proteins such as Bcl2, Akt act as the anti-apoptotic proteins and Bak, Bax and caspase-9 act as the preapoptotic proteins. The increased expression of antiapoptotic proteins (Bcl2, AKT etc) and decreased expression of the pre-apoptotic proteins (Bax, Bclxl etc) in cancer cells result in the developments of resistance against therapeutic agents [61].

2.2.9. Epigenetic modification

Epigenetic abnormalities include DNA methylation, histone modification and chromatin remodelling which regulate cell phenotype. In cancer cells epigenetic abnormalities affect the process of neoplasm. Epigenetic alternations also regulate radioresponse of cancer cells by hindering cellular processes involving pathways of DNA repair, cell cycles, apoptosis etc [62-66]. Epigenetic modifications like DNA mehylation is studied in many cancers, like as cervix, glioma, colon and breast cancers etc. [67-71]. Microanalysis study of radiosensitive H460 and radio resistant H1299 lung cancer cell lines disclosed that as many as 747 genes get hypermethylated at promoter CpG sites and 344 genes get hypomethylated [72]. Among all these genes, which were hypermethylated two genes i.e. SERPINB5 and S100A6 were found to be showing higher expression, in radiosensitive cells and their downregulation by siRNA showed significant increase in cell survival in response to IR.

2.2.10. Cancer stem cell population

Population of cells having capacity of self renovation within a tumor and possessing features of normal stem cells is defined as CSCs [73].Cumulative data suggest that CSCs show resistance in response to radiotherapy, thus influencing the overall outcome of patient treatment [74, 75]. CD133-positive glioblastoma (GBM) are considered as CSC cells, having strong STAT3 phosphorylation and increased tumorigenic and radio resistant properties [76]. Inhibiting STAT3 in GBM-CD133+ cells obstructs stemness property and allows its differentiation into GBM-CD133−cells. In these cells and also in HNSCC inhibiting STAT3 results in induction of apoptosis and radio sensitivity [77].Studies on various xenograft *in vivo* studies reveal that combination of STAT3 inhibitor and radiotherapy can act as a potential radio sensitizer by reducing tumorigenesis and metastasis, in anti-CSC therapy.

2.2.11. Radio-resistance

In the beginning of 20th century, radium was used as a radiation source via direct insertion in cancer tissues but it damaged nearby normal tissues as well. However with the technological advancements with time, radiotherapy (RT) has come a long way with improved technologies like Computed Tomography (CT), Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) and Intensity-modulated radiation therapy (IMRT) to locate the exact position of tumour and minimize the damage to normal

tissue. The proton beam therapy is the most advanced techniques to be introduced for RT. Although, these advanced techniques are very effective, but recurrence of tumour due to radioresistance in large number of cancer patients treated with radiotherapy is a major challenge. There are many intrinsic factors like deregulation of signalling pathways (e.g., HIF or NF-K/β, PI3K/AKT), activation of abnormal DNA damage response, epigenetic changes, cancer stem cells or oncogenic miRNA, which are known to be involved in inducing radioresistance.

2.2.12. Double-strand break repair

Ionizing radiation induces DNA damage by DSB which is very important as its repair is directly correlated to radio resistance. Two mechanisms have been reported so far i.e. homologous recombination and other one is non homologous end joining, based on their requirements for repair. Recent data suggested that IR-induced DSB activates many proteins for activating DNA repair complexes and for recovery of the damage like ataxia telangiectasia mutated (ATM) and BRCA1 [78-80]. In cells of breast cancer which are radioresistant, phosphorylated ATM gets hyperactivated and deubiquitylate CHK1 by interacting with ZEB1 and USP7, which induces HR response thus helping in acquiring radioresistance [81].

In pancreatic cancer cells G2/M arrest led by IR is removed by inhibiting ATM-Rad3-related (ATR) by VE-821 inhibitor, which increases DNA damage and inhibits HR [82]. In Non-homologous end joining NHEJ it directly recovers the damaged part and does not require homologus template, in S and M phases. NHEJ activation plays important role in radio resistance in many types of cancer, like esophageal cancer, cervical cancer, prostate cancer, lung cancer, glioblastoma cancer, oral squamous cancer [83-88]. DNA-PKcs, Ku70, and Ku86 over expression has also been reported in residual tumors relapse cancer samples obtained from cervical cancer patients treated with RT which has increased expression of NHEJ factors in comparison to the primary staged tumors [87].

2.2.13. Role of Hypoxia and HIF-1 pathways in resistance to therapy

The hypoxia is a condition in which the body or a part of the body is deficient in oxygen supply at the tissue level. The level of hypoxic condition depends on the type of tumor. It has been reported that level of oxygen in hypoxic tissues is between 1-2 % or lower than that as compared to the oxygenated normal tissues. The normal oxygenated condition of tissues is known as normoxia or physoxia. The concentration of oxygen in human varies

from tissue to tissue ranging from 9.5 % O_2 (renal cortex) to 4.6 % O_2 (brain) [89-92] . Hypoxia are of two types acute and chronic hypoxia both type of hypoxia play important role in developments of radioresistance both *in vitro* and *in vivo* [93-96] . However acute hypoxia induces metastasis of tumor and defines its aggressiveness [96-98].

HIF regulates the cellular adaptation to hypoxic condition. The HIF-α subunits i.e. HIF-1α, HIF-2α, HIF-3α is regulated by enzymes like Prolyl Hydroxylase Domains (PHDs) and Factor inhibiting HIF (FIH) along with oxygen sensor. The activity of PHD and FIH-1 depends on oxygen level [99]. Under normoxic condition, hydroxylation of PHDs and FIH-1, initiates the ubiquitination and proteasomal degradation of HIF- α [100]. Under hypoxic condition, HIF-1α hydroxylation gets inhibited as PHD enzymes get deactivated, which stabilizes the HIF-α and this non-hydroxylated subunit gets dimerized with HIF-β subunit leading to translocation into nucleus. Gene transcription gets initiated after it binds to DNA[99]. HIF pathways also get activated in hypoxia independent manner, for example by epigenetic changes or mutations which lead to loss or gain of functions [101-103]. Mitochondrial ROS [104, 105] and iNOS [106] also modulates the HIF-1α accumulation. The HIF pathways regulate hundreds of genes [107-109] including VEGF [110, 111], GLUT-1,GLUT-3, glycolytic enzymes [112-114], BNIP-3 [115], p53 [116, 117] TGF-β [118], E-cad, ZEB1, -2 and TCF3 expression [119], CXCR4, CAIX, LOX [120-122] MMP-2, and MMP-9 [107, 123, 124] which facilitate tumour growth by inducing angiogenesis, metabolism, regulation of cell apoptosis, cell survival. This also increases metastatic potential by modulating the cancer cell adhesion proteins and activating EMT, and invasion respectively.

2.2.14. Multi-drug resistance (MDR)

The characteristics of cancer cells to withstand a wide range of anticancer drugs is called as Multi-drug resistance (MDR) [47]. MDR mechanism may be due to the decreased accumulation/absorption of the drugs inside the cells by releasing the drugs outside the cells due to the functional or structural changes at the plasma membrane or within the cytoplasm, cellular compartments, or nucleus [125]. MDR cancer is a result of activation of more than two mechanism of resistance against therapy in cancer cells.

2. 3. The Glyoxalase system and cancer

2.3.1. Glyoxalase system

The glyoxalase system was discovered in 1913 as an enzymatic system and was reported to catalyse conversion of toxic methylglyoxal into nontoxic D-lactate inside the cytosol of cells [126, 127]. The system made of Glo-1 and Glo-II enzymes and catalyses two sequential steps. Glyoxalase is present in all mammalian cells. There are three types of glyoxalases that have been reported in living system, these include glyoxalase-I, glyoxalase- II and glyoxalase-III. The methylglyoxal produced in cells interacts with protein, nucleotides and lipids to form the advanced glycation end-products which play important role in the cellular activities. Abnormal accumulation of the dicarbonyl methylglyoxal occurs in the cells with high glycolytic rates which is associated with increased damage to proteome, genome and lipidome which in turn increases mitochondrial damage, DNA breaks and mutagenesis.

The glyoxalase activity in oxidative stress may cause increased glycation and tissue damage [128]. Methylglyoxal induces glycation of proteins by reacting with guanidino groups present in arginine and mostly form hydroimidazolone N (δ)-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1). The formation of MG-H1, damages the cellular functions. The proteins which are susceptible to methylglyoxal induced modifications related to impaired functions are called as "dicarbonyl proteome" (DCP). DCP are albumins, transcription factors, hemoglobin, extracellular matrix proteins, mitochondrial proteins, and other cellular proteins which are linked to dysfunction of mitochondria, anoikis, oxidative stress and apoptosis [129]. Free methyl glyoxal can also induce cell death by apoptosis or by anoikis or through necroptosis [130-132]. The role of advanced glycation end-products of phosphatidylethanolamine (AGE-PE) has been described in human erythrocyte and blood plasma [133]. The receptor that recognizes the AGEs in cells are called as receptor for advanced glycation end-products (RAGE), activation of RAGE is known to be involved in carcinogenesis [134]. The glyoxalase activity was observed to be high in both normal and cancerous tissues of albino rats [135, 136]. Glyoxalase inhibitors were found to be good anticancer agents as increasing the endogenous methyglyoxal concentration resulted in death of cancer cells [29]. The relationship between high glyoxalase-I level and drug resistance was reported by Sakomoto *et al* [137]. Recent reports have also shown that increased level of Glo-1 in many human tumors is because of the amplification GLO-1 gene [20, 138-140]. The more recent reports further suggest that Glo-1 activity and detoxifying capabilities are also linked with SNP in the GLO-1 gene [141].

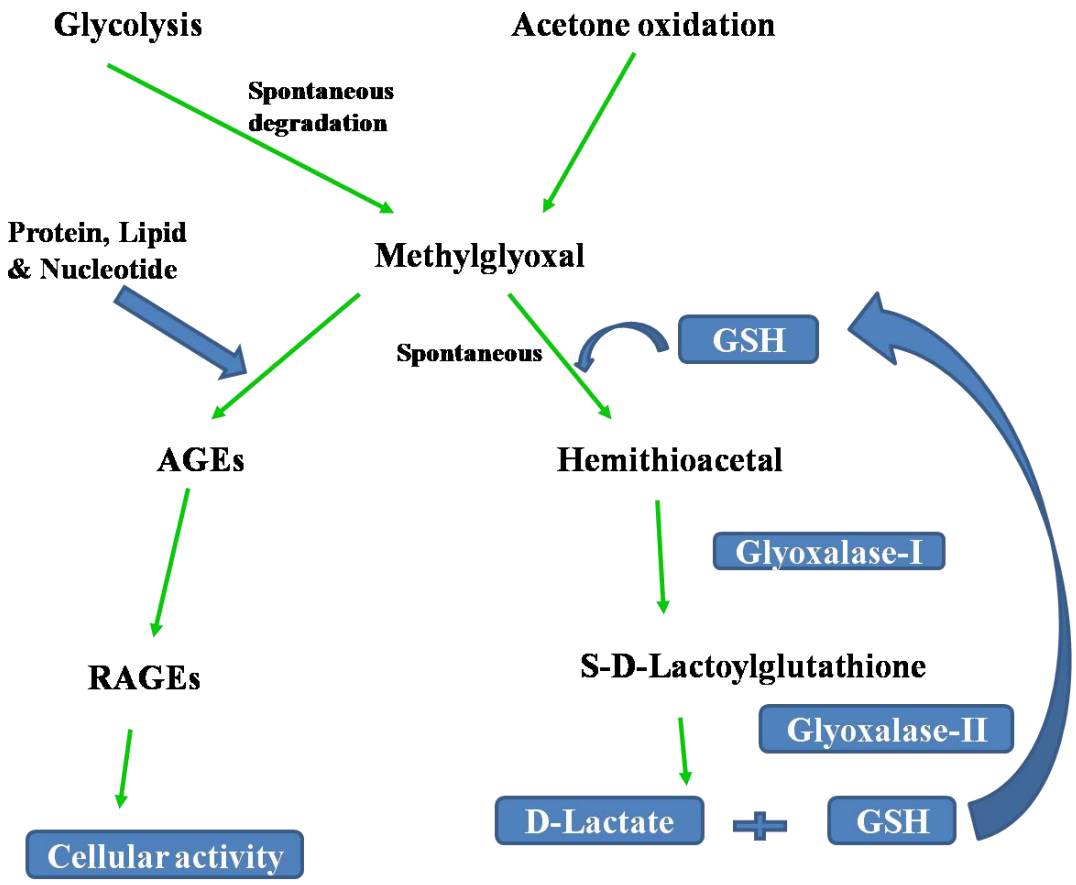

Figure 3: Methylglyoxal detoxification by Glyoxalase system

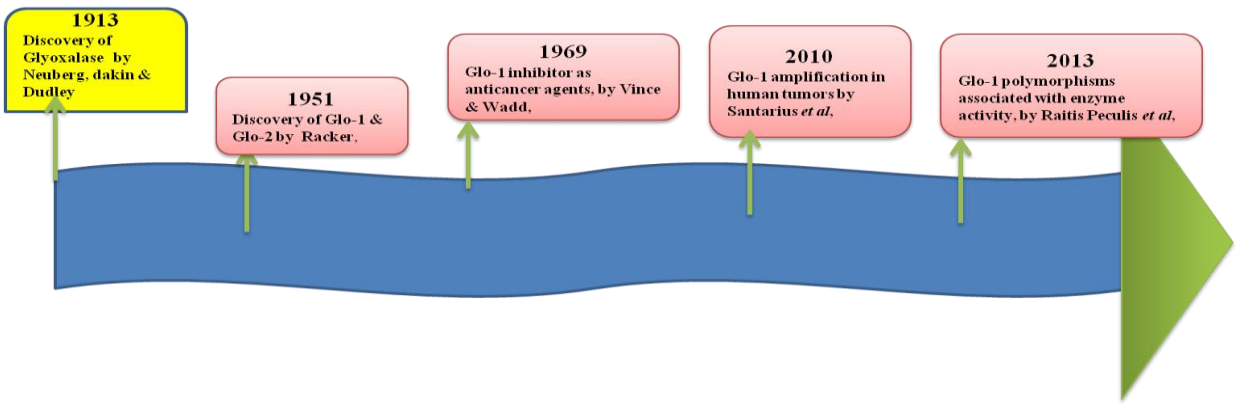

Figure 4: Milestones in discovery associated of Glyoxalase-1 and cancer

2.3.2. Glyoxalase-1 in different types of cancers

Blood cancer

It has been reported that the Glo-1 enzyme is selectively over expressed in the apoptosis-resistant human monocytic lukemia cells UK711 and UK 110 mutant cells and in K562/ADM, adriamycin-resistant leukaemia cells, in comparison of their parental cells

[14]. The over expressing Glo-1, in human JK cells inhibited by etoposide and adriamycin induced caspase mediated apoptosis which indicates the role of Glo-1 inhibition in apoptosis suppression. The co-treatment of cells with S-p-bromobenzylglutathione cyclopentyl diester, a inhibitor of Glo-1, selectively induced etoposide mediated apoptosis in resistant UK711 cells as compared to parental U937 cells, indicating that Glo-1 is a resistance factor in human leukaemia cells and the Glo-1 inhibitor could be a drug of choice for resistant cells [14].

Breast cancer

GC-MS metabolite profiling of selected breast cancer cell lines showed that glo-1 acts as marker for detection between triple negative and other breast cancer subtypes [143]. Proteomics approaches have led to the accelerated discoveries in the prognostic and diagnostic biomarkers. The proteomic analysis of breast tumors and normal human samples showed that out of 28 differentially expressed proteins, 21 proteins were up-regulated and 7 proteins were down-regulated in the samples. The over expression of one protein identified as glyoxalase 1, has been validated in paired normal and tumor breast samples through immunoblotting and tissue microarray 98 tumor and 28 normal samples, in clinical trials I-III. TMA analysis showed that GLO1 protein over expressed in 79% of samples of tumor. The over expression of Glo-1 are associated with advanced grade of tumor. Such reports showed the association of Glo-1 overexpression and he grade of tumor [144].

Hepatocellular carcinoma

GLO-1 gene aberrations are associated with progression of variuos cancers. GLO-1 genetic amplification and expression has been reported in hepatocellular carcinoma in Chinese patient. Glo-1 expression was found to be up-regulated in 48 % of tumor tissues in comparison of nearby non-cancer tissues [145]. Interfering/ or knocking-down GLO-1 gene expression with shRNA resulted in the inhibition of cellular growth and induction in apoptosis in cultured HCC cells which carry genetic amplified GLO-1 gene, while there were no inhibitory effects on proliferation of cells in HCC cells with normal copies of GLO-1 gene [145] [146].

Prostate cancer

The sample of prostate cancer showed a positive association between Hel pap-grading and the cytoplasmic /nuclear ratio of Glo-1 level. An evident positive correlation between Ki-

67 proliferation marker and the cytoplasmic Glo-1 was also observed [191]. The relation between Glo-1level with the pathological grade and proliferation in prostate cancer can be used to assign Glo-1 as a risk factor for prostate cancer grade. GLO-1 polymorphism has been descried to affect activity, oxidative stress and implications in prostate cancer [147]. The data of PCR/RFLP obtained from 571 prostate cancer patient and 580 healthy subjects showed that Glo-1 polymorphism causes a significant change in Glo-1 related pro-oxidant argpyrimidine , oxidative stress levels and cancer progression [147].

Colorectal cancer

Colorectal cancer (CRC), also known as bowel cancer, is the cancer of colon or rectum. Colorectal cancer is the third most common cancer in male and second in female world-wide. The amplification of Glo-1 in CRC is rather scare nevertheless previous studies showed a 2-fold increase in the activity of tumor vs. normal colonic tissue. AGE and AdipoR1 are involved in colorectal carcinogenesis [148]. Kuniyasu *et al* reported a close association between RAGE and amphotrein expression with metastasis of colorectal cancer [149]. Rage signalling induced development of intestinal tumours. The *ApcMin/+ Rage−/−* mice exhibit protection from tumour. Increased apoptosis is an indicator of a pro-survival function for RAGE ligands in intestinal tumourigenesis [150].

Renal cell carcinoma

The association of Glo-1 with the metastatic potential in parental SN12C cell line of human renal cell carcinoma (HRCC) and three clones developed from this parental SN12C cell showed that the level of Glo-1 is directly proportional to the metastatic potential of the SN12C clones. Glo-1 was found to be the only protein which consistently alerted according to the metastatic potentials of SN12C clones [151].

Gastric cancer

The role of Glo-1 in gastric carcinogenesis is well described [26]. The elevated expression of Glo-1 in gastric cancer tissue is linked with gastric wall invasion, metastasis and progression. Further up-regulation of Glo-1 in gastric cancer cell line increased cell proliferation and metastasis whereas down-regulation of Glo-1 with shRNA may cause reduction in metastatic potential of cells [38]. Glo-1 has been suggested to be a potential marker for the detection and prognosis of gastric cancer, as high expression of Glo-1 in

gastric cancer enhanced the metastasis ability of tumor cells both *in vitro* and *in vivo*, [26].

Pancreatic cancer

Pancreatic cancer (PC) is one of the highly malignant types of cancer, and the median survival period is less than 12 months, with an overall 5-year survival rate of less than 5%. The glo-1 was significantly over-expressed in pancreatic cancerous tissues compared with normal tissues [152]. Recently, it has been seen that RAGE is required for the activation of interlukin-6 mediated mitochondrial signal transducers and activators of transcription3 (STAT-3) signaling in pancreatic carcinogenesis in mouse by increasing autophagy [153].

Lungs Cancer

The glyoxalase-1 activity has studied in 38 different cancer cell line including the lung cancer cell line was detected. The lungs cancer line which over expressed Glo-1 activated caspase mediated cell death by Glo-1 inhibitor BBGC [20]. The Glo-1 inhibitor BBGC selectively shows antitumor activity *in vivo* with very little toxicity to the mice [20].

Skin cancer

The expression level in multiple human skin neoplasms show that Glo-1 was over-expressed by lesional tissues of squamous cell carcinoma, basal cell carcinoma, and verrucous carcinoma. However Glo-1 was weakly expressed in many benign neoplasms. Human papilloma virus 16 E6/E7– transfected keratinocytes shows high level of Glo-1 than normal keratinocytes. This was further validated showing that knockdown of Glo-1 by siRNA increased apoptosis in SCC-13 cells and inhibited cell invasion and migration *in vitro* and *in vivo* [154].

2.3.4. Regulation of glyoxalase in cancer cells

Accumulation of the dicarbonyl MG (methylglyoxal) occurs at abnormal levels in the cells exposed to high glucose concentrations and some abnormal health conditions like diabetes resulting in the increased damage to DNA and protein. The MG-mediated damage was countered by glutathione-dependent metabolism by Glo-1 enzyme. The human genome exhibits a functional ARE (antioxidant-response element) in the 5′-untranslated region of exon 1 of the mammalian GLO-1 gene [155]. Transcription factor Nrf2 binds with ARE, regulate the basal and inducible level of Glo-1. Activators of Nrf2 induce Glo-1 mRNA and protein activities. Increased expression of Glo-1 results in the

decreased cellular and extracellular level of MG, MG-derived protein adducts, mutagenesis and cell detachment. Hepatic, brain, heart, kidney and lung Glo-1 mRNA and protein level was found to reduced in Nrf2−/− mice, and urinary excretion of MG protein and nucleotide adducts increased by 2-fold [155]. Various iso-forms of human Glo-1and their functional assignment was done using Glo-1 isolated from human erythrocytes which showed that Glo-1undergoes four unidentified posttranslational modifications: (i) removal of the N-terminal methionine 1, (ii) N-terminal acetylation at alanine 2, (iii) a vicinal disulfide bridge between cysteine residues 19 and 20, (iv) a mixed disulfide with glutathione on cysteine 139. Glutathionylation of Glo-1 shows that both, N-acetylation and the oxidation state of Cys19/20, dose not impact/alter the Glo-1 activity. In contrast, glutathionylation inhibits Glo-1 activity *in vitro*. The mechanism of enzyme inhibition by glutathionylation was studied by molecular dynamics simulation. It was found to be regulated by an oxidative posttranslational modification. In the context of redox-dependent regulation of glucose metabolism in cells/ inhibition of Glo-1 by chemical modification with its co-factor and the role of its intramolecular disulfides is suggested to be an important factor [156].

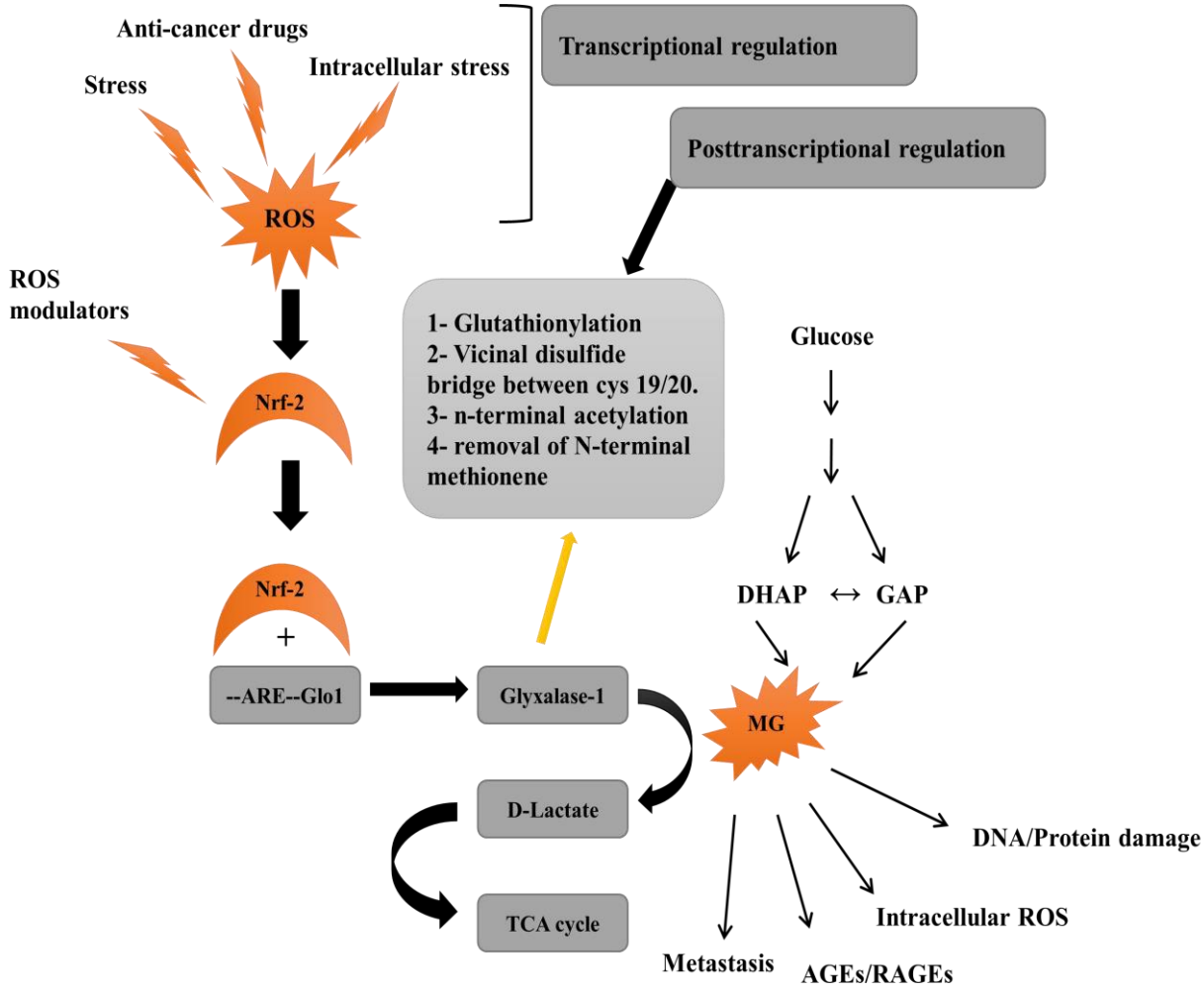

Figure 5: General mechanism of Glyoxalase-1 regulation in cancer cells

2.3.5. Role of Glyoxalase-1 in development of resistance

The oxidative stress and Glo-1 activity in dicarbonyl resistance cells shows that dicarbonyl toxicity is mediated by oxidative stress and Glo-1 activity. For example tamoxifen resistant cell line, generated from carcinoma cell line MCF-7 shows putative differences in the aldehyde defence system and accumulation of advanced glycation end products (AGE). These tamoxifen resistant cells are more sensitive to the dicarbonyl compounds glyoxal and methylglyoxal which suggests that glyoxalase and other aldehyde defence enzymes play an important role and can be a promising target for the therapy of tamoxifen resistant breast cancers [157].

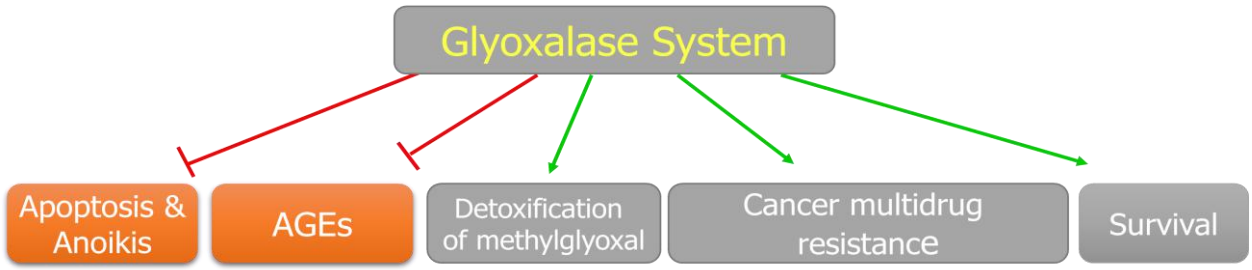

Figure 6: Role of Glyoxalase system in cellular activity

In 2010 Santarius *et al* screened 975 human cancer DNA sample using SNP microarray and qPCR, and found that 618 human cancer samples had GLO-1 gene amplification. The cell lines with GLO-1 amplification were more sensitive to Glo-1 inhibitors [158]. The over-expression of Glo-1 in murine NIH3T3 by Glo-1 cDNA shows increased tolerance for exogenous methylglyoxal and enhanced resistance to Glo-1 inhibitor as well as anti-cancer drugs mitomycin C and adriamycin [25]. The Glo-1 has been suggested to be a supplementary biomarker to differentiate between malignant and nonmalignant prostate cancer on the bases of data collected from prostate cancer patient. The level of Glo-1 was 233.3 ± 98.6 µmol/min in malignant while as it was 103.1 ± 19.7 µmol/min for the non-malignant patient [159].

The over-expression of Glo-II [hydroxyacylglutathione hydrolase (HAGH)] has also been linked with MDR. The transcription factor p63 and p73 regulate the expression of Glo-II by binding with specific response element in intron-1 of GLO-II gene. Overexpression of cytosolic Glo-II inhibits apoptosis induced by high concentration of methylglyoxal. The cells deficient in Glo-II were hypersensitive to apoptosis induced by methylglyoxal and anticancer drugs doxorubicin and cisplastin [160].

2.3.6. Therapeutic implication of glyoxalase-1 expression in cancer cells

There have been continuous efforts by the scientists to develop new valid targets for cancer treatment and to discover and develop new chemotherapeutic agents; but until today, there are no chemotherapeutic agents that have optimal characteristics for cancer treatment due to the lack of selectivity between normal cells and cancer ones. The search continues to find a candidate drug that will provide the best treatment and medical relief suffering from cancer [161, 162].

In this pursuit one of the valid targets known to be pivotal for cancer treatment is the glyoxalase system. The glyoxalase system is present both in normal and malignant cells,

however their expressions is elevated in tumor cells. Due to high glycolytic rate, the cancer cells have high methylglyoxal production and require high glyoxalase activity for their survival. Also up-regulation of Glo-1 confers resistance to anti-cancer agents such as adriamycin and etoposide [14, 163-165]. It has been proposed that inhibition of either Glo-1 or Glo-II results in an accumulation of toxic aldehydes, which can lead to self-destruction of cancer cells. Cancer cells are characterized by high metabolic rates; growing indefinitely. Therefore, inhibition of Glo-1 and/or Glo-II would result in accumulation of toxic aldehydes MG inside these cancer cells, can lead to death of cancer cells due to accumulation of toxic metabolite [128, 166, 167]. Glo-1 has received attention in the last decade as an anti-cancer target as many studies reported its importance as valid anti-cancer target.

Structurally, Glo-1 is a metalloenzyme, and required zinc metal as a cofactor in its active site, which plays a structural and catalytic role in the isomerization of the reactive and toxic aldehydes. It is made up of two identical polypeptide chains of homo dimer. The active site of Glo-1 is present in the interface of the two polypeptide chains and the zinc atom has a square pyramidal coordination with the adjacent amino acids [168, 169]. Various novel Glo-1 inhibitors of natural and synthetic origin have been developed by using the structure-based pharmacophore models of the Glo-1 and docking programs [170]. The simple S-aryl and S-alkyl GSH derivative are good competitive inhibitors of the Glo-1 & p-bromo benzyl-glutathione (figure 4) is one of the strongest inhibitors [171]. Thornally demonstrated that diethylester of S-(4-bromobenzyl) glutathione, undergoes de-estrification to give S-(4-bromobenzyl) glutathione and transition states of Glo-1 inhibitors have been developed. The diethyl ester of pro drug S-(4-bromobenzyl) glutathione and others display significant tumor selective toxicity *in vitro* [172]. Glo-1 inhibitors are frequently used in chemotherapy where other chemotherapeutic drugs fail to be effective. Takeuchi *et al*, suggested that Glo-1 could provide improved chemotherapy for MDR chronic myeloid leukaemia. The expression of Bcr-Abl gene induces expansion of malignant haematopoiesis, like as the leukemia stem cell pool and the suppression of non-malignant haematopoiesis. Abl tyrosine kinase inhibitors (TKI), imatinib and dasatinib are ineffective against Bcr-Abl^{+} leukaemia stem cells. Since hypoxia adapted Bcr-Abl^{+} cells have higher Glo-1 activity, glyoxalase inhibitors were quite effective in killing both *in vitro* and *in vivo*, and improving the survival of hypoxia adapted Bcr-Abl^{+} cell inoculated mice [27, 28].

2. 4. Phytochemicals in cancer

Plants provide a diverse array of phytochemicals that can be used to prevent treat and cure various cancers [173, 174]. However efficacy of a phytochemical depends on absorption, metabolism, bioavailability and availability at target site in body [175-177]. The phytochemicals may disturb the cellular process and induce cytotoxicity. They can initiate and modulate many signalling pathways [178].

Plants are provided with many metabolites or phytochemicals that are used for various health benefits[173, 174]. Among various phytochemical lavonoid are abundantly present in our diet, such as fruits, vegetables, teas etc & have strong antioxidative potentials, estrogenic regulatory and antimicrobial activities [179, 180]. In the present study we have studied the effect of flavonoids naringenin and galangin *in vitro* and *in vivo* and tried to understand the mechanism of action and specifically its effect on Glo-1.

2.4.1. Galangin: Review of literature

Galangin (GA, 3,5,7 -trihydroxyflavone), is an active constituent of *Alpinia galanga*, a plant of the ginger family Zingiberaceae. It is found in root of *Alpinia officinarum*, which is a common spice and active ingredient of many herbal medicines in Asia. Besides plants it is also present in propolis (bee hive material), produced by honey bees from gum of various plants & honey. Propolis has been reported to contain up to about 9% galangin.

Galangin belong to flavonol class of flavonoids. It is a flavonol derivative that contain hydroxyl group in the ring **(figure 7**). Galangin is the lipophilic compound due to presence of hydroxyl group and has similar flavonols structure such as kaempferol, morin, quercetin and myricetin, etc. Galangin has a wide variety of biological activities.

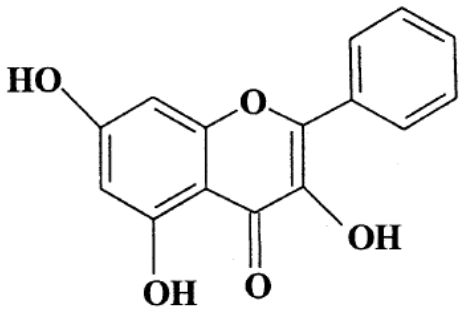

Fig 7: Chemical structure of Galangin

Toxicological studies:

The IC- $_{50}$ value of the galangin for different cancer cells has been reported between 25 μM to 150 μM [181-186] which suggests that toxicity profile of the galangin varies from cell to cells. However IC_{50} value for the HeLa was found to be 50 μM [187]. *In vivo* toxicity data, showed that medium lethal dose Oral LD mouse is >1500 mg/kg. Galangin oral administration reduces the plasma level of insulin, glucose and triglycerides. Galangin treatment reduces the expression of cytokines and increases the translocation of NF-kappa B into the nucleus in high fructose diet-fed animals [188].

Protection against DNA damage:

Galangin has found to reduced the ant-genotoxicity effect of alkylating agents , polycyclic aromatic hydrocarbons (PAH), different DNA cross-linking chemicals and radiomimetic agents by reducing the DNA adducts formation [189]. The data collected from *in vitro* and *in vivo* results also suggests that galangin reduces the mutagenicity and clastogenicity [190] [191]. Galangin is also a strong inhibitor of cytochrome P 450 1a1 (CYP1A1) and CYP1A2 and activates phase II enzymes which help in the detoxification of chemical carcinogens [192].

Protection against inflammation:

The propolis (a natural product having galangin) and pure galangin topical application reduces the inflammatory effects more than 50% in oedema induced by croton oil in mouse model within 3 hrs post application [193]. Galangin inhibits the human neutrophil degranulation by releasing the elastase a biomarker for degranulation [194].

Protection against various types of oxidative stress:

The galangin was found to be a strong antioxidant in *in vitro* and inhibited DPPH free radical [195]. Galangin reduced oxidative stress in rat liver induced by $Fe^{2+/}$citrate-mediated membrane lipid peroxidation. The 2, 3-double bonds and 4-oxo group present in flavonoid ring is responsible for the antioxidant activity of flavonoids [196]. Galangin preserve the protective effect of other antioxidants such as, vitamin E, and vitamin C, and reduces the lipid peroxidation in biological system [197].

Protect against the cancer:

Galangin showed inhibitory effects against various types of cancer cells [35]. Galangin *in vitro* showed the potent cytotoxicity and induced apoptosis-like morphology in human pancreatic cell line (PANC-1)[198].Many cancers like liver cancers and colorectal cancers show aberrant level of beta-catenin. Galangin treatments has found to reduce the intracellular level of beta-catenin by inactivating mutations of adenomatous polyposis coli and/or Axin in cancer cells [199]. Galangin promotes apoptosis with anticancer drugs like imatinib in imatinib sensitive as well as imatinib-resistant Bcr-Abl+ leukemias cell lines by reducing the level of Bcl-2 [34]. Galangin was also found to inhibits proliferation of human breast cancer cell line [200]. Recently galangin was found to induce cell death in HeLa cells via glyoxalse-1 mediated Nrf-2 axis [201].

Modulation of gene expression:

Galangin modulated the activity of many cellular enzymes like cytochrome P450 1A1 and inhibited cytochrome P450 1A1 in human intestinal epithelial cells (Caco-2) induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [202]. Several studies reported that among different flavonoids the galangin has highest acetylcholinesterase (AChE) inhibitory activity in brain *in vitro* and shows significant ATP synthase inhibitory activity [203]. Galangin inhibited the human butyrylcholinesterase (BChE, EC 3.1.1.8) reversibly and showed 12 times higher binding for BChE than human acetylcholinesterase enzyme (AChE, EC 3.1.1.7) [204].

2.4.2. Naringenin: Review of literature

Naringenin is a flavonoid mainly presents in the citrus fruits likes citrus paradise, orange (citrus sinensis), prunus davidiana, tomato and propolis. Grape fruits juice is a rich source of naringin and is present up to 800 mg/liter of juice [205]. In *Acacia farnesiana* it is also present in association with the Gallic acid [206]. It was shown to range from 68-302 mg/kg in Grape fruit juice [207, 208]. In citrus fruits it ranges from 11.6-20.1/100 gram [207, 208]. In Citrus paradisi it's predominant in the form of naringin (naringenin 7-neohesperidoside) followed by narirutin (naringenin 7-rutinoside) [209]. Whereas in case of tomato fruits it is presents in the free state or in the form of naringenin 7-rutinoside and naringenin 7-glucosides [209, 210]. It is abundant in tomato skin and level increases considerably during the maturation period. The level of the naringenin was found to range from 0.8 to 4.2 mg/100 g of tomato [210].

Naringenin is known by different names such as naringetol, pelargidanon, salipurpol, Salipurol, Asahina, 480-41-1, Naringenin, (S)-Naringenin, YSO1 (pubchem). Its molecular weight is 272.26 and the chemical formula is $C_{15}H_{12}O_5$ [(2S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one] **(Figure - 8**). It's insoluble in the water where as it is completely soluble in the organic solvent such as DMSO, alcohol etc. Unlike aglycone naringenin, naringin is fairly soluble in water up to 1 mg/ml in water at 40°C (Merck Index).

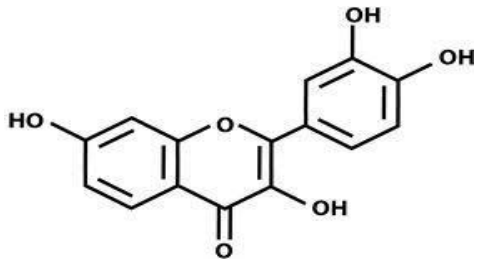

Figure 8: Chemical structure of Naringenin

Pharmacokinetics:

Studies using 3-[14C]-flavonoids in rats showed that the intestinal absorption of aglycone flavanones is more than 90% with respected its glycone [211]. The bioavailability of the naringenin and its glucoside was checked in rat after oral administration of naringenin (0.25%), naringenin-7-glucoside (0.38%) and naringenin-7-rhamnoglucoside. Glucurono and sulfo conjugated metabolites of naringenin had no relation with the type or form of naringenin administered. Naringenin-7-rhamnoglucoside showed a slow absorption in comparison to other 2 forms of naringenin. In one study it was observed that the different form of naringenin reached the peak in plasma at different time period and for naringenin (C_{max}:0.24 µmol/L) and naringenin glucuronide (C_{max}:0.09 µmol/L) it was 20-30 min (C_{max}:0.021 µmol/L) and for naringin it was 80 min [212]. In case of grape fruits, naringenin level reached up to 6 µM/liter in plasma after administration of the grape fruits juice equivalent of 200 mg of naringenin [30, 213].

In the distribution studies it was observed that naringenin is widely found in the body, starting from the liver at highest conc followed by stomach, small intestine, kidney, trachea, lung, testis, heart, ovary, fat, spleen, muscle, and brain in succession, however in brain its was almost negligible indicating that it can't cross the blood brain barrier [214]. Naringenin was also found to be excreted from the urine in the range from 8.5-8.8%

[213]. However a study done by Fuhr *et al.*, (1995) in human subject, the presence of naringenin was detected after 8 hours of administration of naringenin. Most administrated part was found to be excreted in urine, which was 57% molar equivalent of administrated naringin. Urine and bile is the most preferred route for its elimination.

Aglycon are readily absorbed in to the body in comparison to their glycone counterpart since latter needs to be first processed by gut flora before being absorbed in the body. The grape juice is a rich source of naringin (glycone) and it was observed that the Cmax value for naringenin in plasma was highest after 5.5 hours in comparison to 4-4.8 hours when pure compound (naringin) was administrated [213] [210]. Bugianesi *et al.*, (2002) showed that Cmax value for naringenin in plasma reached within 2 hours of oral ingestion of tomato paste, which is a rich source of aglycone naringenin. Its bioavailability was found to be enhance by associating it with the cyclodextrin [215]. Similarly its effectiveness could be also be enhanced by coating over the nanoparticles [216].

Toxicological studies:

Tundis and coworker (2011) found that IC-$_{50}$ value of the naringenin was 2.2, 7.7, and 33.4 μM for C32, LNCaP, and COR-L23 cell lines respectively [217]. However IC50 value for human hematoma cell lines Hep G2, MA 104 and A 549 was >1 mM which suggested that toxicity profile of the naringenin varies from cell to cells. However IC_{50} value for the Vero cells line was found to be high (0.319 mM) [187]. *In vivo* toxicity data, showed that medium lethal dose LD50 for mice and rat is >5000 mg/kg [218]. While as toxicity of narengenin for embryonic tissue of mice was 300 μM [219].

Protection against DNA damage:

Naringenin reduced the DNA damage induced by ferrous sulphate in human prostate cancer cells (LNCaP) by inducing the level of the DNA polymerase beta, 8-oxoguanine-DNA glycosylase 1, and apurinic/apyrimidinic endonuclease [220]. Naringenin induced the melanin production in mice melanoma cells, B16F10 cells by modulating the Wnt/β-catenin pathway and could be helpful in protecting against radiation induced DNA damage and stimulating melanin production [221, 222]. Narengenin protect the DNA damage in rat liver in alloxan-induced diabetic mice by lowering the oxidative stress [223].

Protection against inflammation:

Naringenin inhibits the inflammation in various biological systems by reducing the level of the main inflammatory mediator NF kB and iNOS [224-227]. Naringenin also reduced the severity of colitis by modulating the level of pro-inflammatory mediators like intercellular adhesion molecule-1 (ICAM-1), monocyte chemo-attractant protein-1 (MCP-1), cyclo-oxygenase-2 (Cox-2), inducible NO synthase (iNOS), IL-6, TLR4, NF-kB and TNF-α level in the colon mucosa [225]. The main cause of obesity mediated inflammation and insulin resistance in the body is the expression of TLR2 in adipocytes. In this context narangenin was found to reduce the level of inflammatory mediators like. TLR, c jun NH2 terminal kinase and TNF α in adipocytes after co-culturing it with the macrophage [228]. Narengenin also inhibited the release of inflammatory cytokines from the macrophage via down regulation of AP-1 [229, 230]. Narengenin inhibited the LPS induced proinflammatory cytokines by inactivating the NF-kB and MAPK pathway in the BV2 microglia cells. Some studies conducted on glial cells showed that naringenin protect cells against the LPS induced inflammation by inhibiting the iNOS and p38 expression [231]. In *in vivo* treatment with naringenin (50 mg/kg) decreased the level of NF-kB, proinflammatory cytokine, and iNOS in the pancreatic tissue of streptozotocin nicotinamide treated Wistar rats and increased the count of RBC, WBC and platelets [232]. Narangenin treatment *in vivo* at a dose 50 mg/kg reduced the inflammation induced by ethanol in mice by decreasing the level of NF-κB, COX-2, TNF-α, IL-6, MIP-2, iNOS protein and CD-14.

Narangenin protect against the renal damage in diabetic mice by decreasing the plasma level of glucose, creatinine and blood urea, by upregulation the expression of insulin and inhibiting the inflammatory cytokines like IL-6 and TNF-α and inflammatory proteins like Cox-2, NF-kB & microphase inflammatory protein-2 (MIF-2) [233, 234]. Beside these narengenin also decreases the level of the hepatic injury marker in serum aspartate and alanine transaminases [234].

Protection against various types of oxidative stress:

Naringenin decreased the oxidative stress induced by anticancer drug doxorubicin in mice by inhibition of ROS formation and iNOS inhibition [235]. Naringenin has been reported to work as a prooxidants in some studies and this could be due to its ability to oxidize NADH and production of phenoxyl radicals in the presence of hydrogen peroxide and

peroxidases [236, 237]. However some studies shown that narengenin works as an antioxidant [238, 239] and no prooxidant activity was found in mice at a dose up to 120 mg/kg [240].

In vivo oral dose of the naringenin (50 mg/kg) neutralized the oxytetracycline (200 mg/kg for 15 days) induced nephrotoxicity in rat by decreasing the level of hepatic markers in serum likes urea and creatinine with increasing levels of antioxidant enzyme [241]. Naringenin also protects against liver damage from carcinogens like dimethylnitrosamine (DMNA) and reduces the level of malondialdehyde formation at a dose 20 mg/kg [242]. It also reduced the ROS level in animal model system for alzheimer's disease and ameliorated the scopolamine induced amnesia in the mice at a dose of 4.5 mg/kg [243]. Reduction of the carbonyl mediated neurotoxicity in the mouse neuroblastoma cell lines (Neuro-2A), was attributed to reduction of the oxidative stress, apoptosis and maintaining the proper mitochondrial potential for the functioning of the system [244].

Protection against the cancer and tumor:

Naringenin was reported to be cytotoxic to a number of cancer cell lines i.e. stomach (KATOIII, MKN-7), and colon (Caco-2), liver (HepG2, Hep3B, Huh7), breast (MCF-7, MDA-MB-231), leukemia (HL-60, NALM-6, Jurkat, U937) cervix (Hela, Hela-TG) as well as pancreas (PK-1) [245]. Narengenin blocks cell cycle and induce apoptosis in the liver carcinoma cells (Hep G2) by modulating the expression of Bax and p53 [246] and increase apoptosis in human lung cancer A549 cells through TRAIL pathway [247].Naringenin induce cell death by apoptosis in the gastric cancer cells (AGS) by inhibiting the expression of β catenin/Tcf pathway, which has an important role in carcinogenesis [248]. Narenginin enhances the cell death in human leukemia HL-60 cells by activating the NF-kB pathway which subsequently causes the depletion of intracellular ATP and mitochondrial dysfunction [249]. Narenginin suppress the carcinogenesis in colorectal cancer by inhibiting the Cox-1 enzyme involved in carcinogenesis [250]. The naringenin was found to inhibits the synthesis of polyamines, which is essential for regulation of many cellular activities like cell proliferation, RNA and DNA synthesis, DNA- protein interactions which disrupt certain cellular functions in *in vitro* and *in vivo* [251-253]. Naringenin at the dose rate of 50 mg/kg inhibits the growth of C6 glioma cells in rat brain by decreasing the expression of PK-C, nf-Kb, cycline D1 and CDK4 [254]. Narengenin was found to decrease the no of metastatic cancer cells in lungs and extend

the life of the tumor resected mice by immunosuppressing the regulatory T cells [255]. Oral administration of Narengenin for 5 days in mice reduced the size of implanted sarcomas-180 in *in vivo* [245]. The Ehrlich ascites carcinoma tumor model showed that there was 80% reduction in the implanted ascites carcinoma cells at 50 mg/kg dose. This effect was due to the inhibition of the expression of VEGF, HIF 1α, HSP 90 and pAKT [256]. Further naringenin shows anticancer activity in animal models, as well as in human epidemiological trials [255, 257-259]. The cumulative results of *in vitro* and *in vivo* studies shows that naringenin selectively induces apoptotic cell death and arrests the cell cycle in cancer cell as compared to normal cells [260-262].

Naringenin reduced the DMBA induced oral carcinogen in Golden Syrian hamsters [216, 263]. Similarly it also inhibited the carcinogenesis induced by the N nitrosodiethylamine in mice [246]. Epidemiological studies suggested that naringenin helped in prevention of breast cancer in the women's eating high proportion of citrus fruits [264, 265].

Radiomodulatory potential of naringenin:

Narringenin showed radioprotective effect against gamma radiation *in vitro* and *in vivo* [266]. Naringenin protects the human skin cancer cells (HaCaT) against the UV radiation-induced damage through by activating DNA repair [221] and increase melanin level in mice melanoma (B16−F10) cell lines [222]. Naraingenin protects mice against radiation-induced genotoxicity [266].

Modulation of gene expression:

Naringenin modulates the expression of many gens likes NF kB, p21, p53, COX-2, iNOS etc. [216, 227, 267] and affects many signalling pathways likes: Akt/PI3K, β-catenin/Tcf, TGF-β1/Smad3, STAT-1, c-jun pathway mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) pathway etc. *in vitro* in cell lines and *in vivo* [224, 229, 248, 268, 269]. Besides this it also reduce the level of various inflammatory cytokine like IL-1, IL-6, IL-8 in various model systems [229]. Nareringenin shows weak estrogenic activity, and induce apoptotic cascade in cells after interaction and promotes the apoptosis by induction of the P38/MAPK signalling and up-regulate the Bcl 2, Cx43, p21 etc [257, 261, 262]. Narengenin was found to induce the DNA repair in LNCap prostate cancer cell lines [220]. Naringenin activates apoptosis in cancer cells by down regulation the Akt/PI3K pathway in cancer cells. Narengenin in normal cells, reduces the apoptosis by lowering down the expression of the AIF/endo G [260].

Chapter III

Methods & Material

3.1. Chemicals

RPMI medium 1640, antibiotic solution (100X) and FBS were purchased from HiMedia Lab Ltd, India. Naringenin, Galangin, primary monoclonal Ig-G cyto-c, agarose, Acridine orange (AO), Ethidium bromide (EtBr), trypan blue, fluoroshield, Reduced glutathione (GSH), DPPH, DAPI (4',6-diamidino-2phenylindole), DC-FDA (2',7'–dichlorofluorescein diacetate), bovine serum albumin (BSA), Triton X-100, Tween 20, IGEPAL-CA 630® and MTT [3(4,5-dimethyl thiozol-2-yl] were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Primary Ig-Gs (Nrf-2, glyoxalase-1and β-actin) and secondary antibodies (anti-mice-HRP, anti-rabbit-HRP) were purchased from Santa Cruz Biotechnology, Inc. USA. Freezing solution was purchased Leica Microsystems (Japan). Proteinase K, folin ciocalteu reagent and RNase-A purchased Bangalore Genei, 100 bp DNA marker and prestained protein markers were purchased from Biorad.

3.2. *In vitro* experiments

3.2.1. Typan Blue assays

Dye exclusion test is used to observe the no of viable cells. It is solely based on the theory that, live cells exclude the dye due to intact membrane dead cells do not. The Trypan blue dye helps to make a differentiation between viable (glowing) and nonviable cells (blue colour) under a microscope. Cell suspension was mixed with trypan blue dye (0.4% in PBS) in an equal proportion and counted manually within 3-5 minutes after mixing. Cells counted by use of hemocytometer under microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan) at 10X magnification.

3.2.2. Cell proliferation by MTT assays

The MTT assay is carried out to check the cell proliferation which is based on the ability of the cellular mitochondrial dehydrogenase enzyme of live cells to form dark blue formazan crystals by cleaving the tetrazolium rings of the MTT. The treated cells were seeded in a flat bottomed 96-well Elisa plates at a density of 5 x 103 cells/200 µl of cell culture medium. After requisite time period, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well followed by 4 hour incubation in CO_2 incubator. After incubation media was removed from plate with the help of pipette and DMSO (200 µl) was added to each well of plate. Plate was incubated at 37°C in dark for 5 minutes with intermittent

shaking. The optical density of each well was measured by using an automatic Elisa plate reader (Molecular Devices, USA) at 570 nm.

3.2.3. Clonogenic assay

After exposure to radiation/or drug treatment a fixed number of cells (1x 10^3) were seeded in six well plates having 2 ml of complete RPMI- 1640 media per well. The plate was then incubated in CO_2 incubator at 37^OC for one week and media was changed twice a week. After one week the cells were washed twice with PBS and then fixed for 10 minutes in fixative solution and stained with crystal violet solution for 10 minutes. After staining the plate excess stain was washed with tap water and colonies were observed by naked eye.

3.2.4. Morphological changes

To observe changes in morphology, equal no of cells were seeded in the 60 mm plates and after attachment the cells were treated with different concentrations of drug/radiation. After 24 hrs of incubation the morphology of cells was observed and images were captured using the microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan) at 10X magnification.

3.2.5. Doubling times

Doubling time of cancer cells was determined by counting of cells at different time interval and putting the value using the following formula.

Formula:

***Doubling Time* = *duration* * log (2) / log (*Final Concentration*) − *log* (*Initial Concentration*)**
Where "log" is the logarithm to base 10 or 2 or any other base

3.2.6. Wound healing assay

Equal no of cell were seeded into each well of 6 well plate and when cells reached 80% confluence a starch was made with 200µl tip in each well of plate and media replaced with the drug containing media. The wound healing was monitored at different time points 0 to 24 hrs under a microscope and images was analyzed by NIS elements Nikon analysis software.

3.2.7. Micro drop migration assay

2 µl (40000 cells/2 µl in RPMI having 0.3 % agarose) of cell suspension was seeded in each well in duplicate in 96 well plate. Then 100 µl of media having different concentration of drug was added and incubated for different time intervals. The images were captured using microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan) at 10X magnification. The images were analyzed by NIS elements Nikon analysis software.

3.2.8. Speroid formation assays

The effect of drug on tumor growth was evaluated using spheroid formation assay. In brief 50 µl 1.5% agarose was coated in the 96 well plate and was solidified at room temperature Then 100 µl media having 1000 cells was seeded in the wells containing different concentration of drug. Wells contained no drug ware used as control. Then 200 µl PBS was added in the outer well of 96 well plate to create an evaporation barrier. Plate were incubated for 24 hrs time periods in CO_2 incubator and images were captured using light microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan) at 10X magnification.

3.2.9. Gelatin zymography for MMPs

Equal no of cells were seeded in 6 well plate and at 80% confluence cell were treated with different concentration of drug. After 24 hrs of treatment the media was collected from each well and MMPs activity was checked by Native PEGE. After native electrophoresis, the gels were washed with 2.5% of Triton X-100 twice for 20 min each and then incubated in Tris-HCl (50 mM, pH 8.4) twice for 5 min each. The gels were then incubated overnight or 20 hrs at 37 °C in incubation buffer Tris-HCl (50 mM, pH 8.4) having 5 mM $CaCl_2$ and 1 µM $ZnCl_2$. After incubation, the gel was stained with 0.25% CBB for 3 hrs and after washing image of gel was taken.

3.2.10. Cell cycle distribution analysis by PI staining

Cells were seeded in 60 mm dish and left for attachment. After treatment cells were treated with drug/radiation for required time. There after cells were harvested and washed with PBS. Then cells were fixed with 70% ethanol. Further washing of cells was done using PBS then stained with 500 µl of PI solution and 25 µl of RNAase-A and incubated in dark for 30 min at 37°C. Fluorescence emission of PI-DNA complex was measured using FACS Caliber instrument (Becton Dickinson, Franklin Lakes, NJ, and USA). Each

sample taken for examination consisted of at least 20,000 cells. The distribution pattern of cell cycle was expressed as subG1, G1, S, and G2/M phases using BD software.

3.2.11. Measurement of free radical scavenging activity in a cellular system

To elucidate the free radical scavenging capacity of the compounds, DPPH assay was performed. In brief 50 µl of different concentrations of compounds were mixed with 1.5 ml of methanol. 75 µl of DPPH solution in methanol (4.3 mg DPPH in 3.3 ml of methanol) was added to the reaction mixture followed by 15 min incubation. Optical density was measured at 517 nm against methanol as reference.

3.2.12. Detection of intracellular ROS by DCFDA assay

The ROS (Reactive oxygen species) in HeLa cells was measured using the 2',7'-dichlorofluorescein-diacetate dye. 5×10^5 cells were seeded on cover-slip in 6-well plate and incubated overnight for attachment. Next day cells were treated with fresh medium having required concentration of drug. The cells were incubated at 37°C for required time (4-6 hr). At the end of incubation cover-slip was removed from the culture plate and stained with 40 µM DCFDA for 30 min. After washing with 1X PBS the cover-slip was mounted on glass slide and observed under a fluorescence microscope.

3.2.13. EtBr/AO staining for apoptosis detection

The morphological changes that occur in the cell during apoptosis were studied using fluorescent DNA and RNA binding dyes, Ethidium bromide and acridine orange respectively. After the requsite treatment, cells were collected and washed once with PBS. The cell pellet was resuspended in 50 µl of PBS and mixed with 10 µl of fluorescent dye mixture [Acridine orange (1 mg/ml): Ethidium bromide (1 mg/ml) in 1:1 proportion] followed by incubation for 2 minutes in ice. After incubation the cells were observed using the fluorescent microscope (Olympus BX51, Olympus Microscopes, Tokyo, Japan). The cells were differentiated as live (green), apoptotic (yellow) and dead/necrotic cells (red).

3.2.14. Annexin V/ Propidium iodide (AV/PI) staining assay for apoptosis

Annexin-V-FITC/propidium iodide staining kit (CST) was used for analysis of apoptotic cell death. Six-well plate containing cells were treated with drug/ radiation for required time. Cells were then harvested and after washing with cold PBS and resuspended in 100 µl binding buffer, mixed and incubated for 15 min on ice containing annexin V-FITC

and PI as per the instruction of manufacturer. Cells were then analyzed through flow cytometry.

3.2.15. Mitochondrial membrane potential by JC-1 dye

Mitochondrial membrane potential was measured by the JC-1 (5,5',6,6'-Tetrachloro1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide).The measurement of mitochondrial potential is based on the formation of aggregates (emission: 590 nM) from monomers (emission: 530 nM). In the present experiment we measured the Mitochondrial Membrane Potential by using Kit (Caymanchem Chemicals, USA). Instructions were followed as described by manufacturer. The level of mitochondrial potential in cells was observed using fluorescence microscope.

3.2.16. Induction of Hypoxia

Hypoxia was induced by the treatment with cobalt chloride. The required number of cells ($4x10^5$ cells/ml, counted using haemocytometer) were suspended in RPMI-1640 media and treated with different conc. of cobalt chloride. Cells were then incubated in CO_2 incubator at 37 ^{0}C for required period of time.

3.2.17. Development of radio resistant cancer cell line

The HeLa cells were exposed to radiation in gamma chamber (dose rate of 2.11 Gy/Min) and exposed to 6 gray radiation at a time. The cumulative dose of 60 gray radiations was given in fraction of 6 gray each. The cells that survived 60 gray of cumulative dose of radiation treatment the cells were considered as radio resistant cancer cells.

3.2.18. Protein isolation for biochemical assays

The protein for biochemical assays was isolated in phosphate buffer (pH.7.0) having 0.1% tritonX-100. The cell lysis was done by sonication (Barson, USA). In brief the cell suspension was sonicated at 25% magnitude for 40 second at 10 second interval. After sonication the lysate was centrifuge at 12000 rpm for 20 minute and supernatant was used for biochemical assays.

3.2.19. Glyoxalase-1 activity

Cells were lysed in PB buffer containing 1 mM EDTA by freez/thaw and sonication and then centrifuged at 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction. The glo-1 assay was performed in 0.2 % MG, 2% glutathione in 0.1 M phosphate

buffer (pH 6.8). Increase in absorption at 240 nm, attributable to the formation of S-D-lactoylglutathione, was measured with each cytosolic fraction.

3.2.20. Cytochrome-c in cytosolic fraction

All steps were carried out at 4 °C. After treating the cells with drug, the cells were trypsinized and washed with 1X PBS and resuspended into 500 µl fractionation buffer [Buffer HEPES (pH 7.4) 20 mM, KCI 10 mM, MgCl2 2 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM protease inhibitors cocktail (50 µl/10 ml) buffer] and incubated for 15 minute on ice. Using 1 ml insulin syringe and 27 gauge needle the cells suspension was passed 10–15 times and further incubated for 20 minute on ice. After this the lysate was centrifuged at 3000 rpm for 5 minute. Supernatant was taken in another eppendroff tube again centrifuged at 8000 rpm further for 5 min. The pellet was discarded and supernatant used as cytosolic fraction. The protein in cytosolic fraction was measured by Bradford dye. The cytosolic protein was separated in 10% SDS-PAGE for western blotting.

3.2.21. Western blotting assay

20 µg of protein was mixed with equal volume of Laemmli buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, (pH 6.8). Sample was denatured by heating it at 90 ^{0}C for 5 minutes. Proteins were separated in polyacrylamide gel (PAGE) applying a 40 volt potential difference across the electrophoresis unit. The gel was taken out of the assembly once the dye front reached near to the other end of gel. Protein was transferred to nitrocellulose membrane (NCM) by applying 65 volt across the terminals for 65 minutes at room temperature. After transferring the protein to NCM, it was blocked by 5% BSA (PBST, 0.1% Tween 20) for 1.5 hours at room temperature. After blocking, the membrane was incubated overnight in primary antibody solution (diluted in PBST) at 4 ^{0}C. After incubation membrane was washed 5 times (10 minutes each) by PBST (PBS, 0.1 % Tween 20). Membrane was again incubated for further 1 hour at room temperature in HRP conjugated secondary antibody solution (diluted in PBST). After incubation membrane was washed 5 times (10 minutes each) using PBST and the protein bands were captured on x-ray films using FemtoLUCENT™ PLUS-HRP Chemiluminescent reagents (G Biosciences. Singapore).

3.2.22. Immunofluorescence assays

For immunofluorescence studies the HeLa cells were grown on cover slips in 6 well plate with drug for 24 hr. Then cells were fixed with 4% formaldehyde in 1X PBS for 15 min at RT. After washing with PBS and blocking was done with 1% BSA in 0.1% PBST, the cells were then incubated with primary antibodies at 4 ℃ overnight. After washing three times with PBST, the cells were incubated with Alexa Fluor 488–conjugated secondary antibodies for 1 h at RT and counterstained with DAPI. Antibody–antigen complexes were detected using fluorescence microscope (Nikon ECLIPSE Ti, Tokyo, Japan), using a 60X objective lens.

3.2.23. RNA isolation and c-DNA synthesis

Cells were collected and RNA was isolated using TRIzol (Invitrogen). First-strand cDNA was synthesized using 1μg of total RNA [270] in the presence of 5×RT buffer, 2 μL of 10 mM dNTPs, 1 μL of oligo-(dT)18 primer, 0.5 μL RNasin, 2 μL Moloney murine leukemia virus reverse transcriptase (MMLV) (Gibico, BRL), and volume for adjustment to 20 μL according to the manufacturer's instruction.

3.2.24. Real-time RT- PCR

Human GLO-1 and HIF-1 alpha m-RNA were quantified by real-time RT-PCR SYBR green method on ABI 7500 fast real-time PCR system. Glo1 was amplified using the (forward) RT primer- 5'TGGGCGCTCTCCAGAAAA3' and (reverse) RT primer-5'TTGTGGTAACTCTGGGTCTCATCA3'. For human HIF-1 alpha, the following primers were used: Hif1 RT primer-5'ACAGAGCAGGAAAAGGAGTCATAGA3' (forward) and RT primer-5'ACTCAAAGCGACAGATAACACGTT3'(reverse).The human beta actin gene primer was used as reference gene [270].

3.2.25. Estimation of total carbonyl content

Total carbonyl content was measured as per Kumar [201, 271], with some modification. In brief the protein/ cells were precipitated with cold trichloroacetic acid (TCA, 15% final concentration). The precipitate was then collected by centrifugation and 10 mM DNPH (Dinitrophenyl hydrazine) solution in 2 N HCl was added to the precipitated protein pellet of each sample. 2 N HCl was added to corresponding sample aliquot reagent blanks. Samples were allowed to stand in the dark for 1 h with vortexing every 10 min at room temperature; they were then precipitated with 15% TCA (final concentration) and

centrifuged for 5 minute. The supernatants were discarded; the protein pellets was washed once with 15% TCA, and further washed three times with 1 ml mixture of ethanol/ethyl acetate (1:1, v/v) to remove trace of DNPH. Samples were then suspended in 6 M guanidine hydrochloride (in 2 N HCl) at 37 °C for 15 min with vortex mixing. Then Carbonyl contents were measured at 366 nm spectrophotometarically.

3.3. *In vivo* experiments

3.3.1. Preparation of Drugs

The Naringnin has suspended in 0.5% CMC solution in autoclaved DD water.

3.3.2. Animals used

Random-bred male Swiss albino mice (6–8 week old) were used in present study. Animals were maintained in centralized air-conditioned animal house facility at Jawaharlal Nehru University, New Delhi 110067 with a 12-hr light/12-hr dark cycle and provided with standard food pellets and water ad libitum. The experimental protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals and guidelines of Institutional Animal Ethics Committee.

3.3.3. Experimental Design

DMBA plus croton oil induced skin papillomagenesis was done according to method described by Kumar et al [272]. This experiment was divided into two parts: control groups and treatment groups. The animals were housed 6 animals per cage in propylene cages and were acclimatized for 1 week before use. Two week after oral treatment with Naringinin, hair on dorsal skin of mice were shaved off in an area of $6cm^2$ approximately. Mice showing nicks or cuts were removed from the study. Suitable animals were randomized by weight into control groups and treatment groups, and duration of these experiments was 16 week.

Control Groups

There were three control groups.

Group I (Negative control): Mice were kept on a normal diet and given water ad libitum.

Group II (Vehicle control): Mice were kept on a normal diet and given water ad libitum. This group of mice was topically treated with 100 µl acetone twice a week.

Group III (Positive control): Mice were kept on normal diet and given water ad libitum. On Day 14, a single dose of DMBA (100 µg in 100µl acetone) was topically applied on shaved dorsal area of skin of mice as papilloma initiator according to standard protocol. One week later, 1µl Croton oil in 100µl acetone was applied twice a week till termination of experiment. Topical application of croton oil acts as tumour promoter.

Treatment Groups

Two types of treatment regimes were followed:

Anti initiation treatment (Group-IV and V): Mice were kept on normal diet and given 50 mg/kg and 100mg/kg dose of Naringinin in 0.5% cmc starting one week before application of DMBA and discontinued just before first application of croton oil. A single dose of DMBA was topically applied followed by croton oil application.

Promotion treatment (Group VI and VII):

Mice were kept on normal diet and given 50 mg/kg and 100 mg/kg dose of Naringinin in 0.5% CMC starting from first application of DMBA and continued till termination of experiment. A single dose of DMBA was topically applied followed by croton oil application. Mice were observed on regular basis for various parameters like mortality and weight gain/loss.

3.3.5. Preparation of mice skin tissue homogenate

The skin tissue was separated and washed with cold saline. The skin tissue (approximately 250 mg) was carefully chopped into fine pieces. The minced skin was homogenized in cold phosphate buffer (pH 7.4) having 0.2% triton-100 in a homogenizer. Briefly, the homogenization was done at a speed of 1000-1500 rpm, for 4-5 times at 4°C to until complete homogenization of the skin tissue. The resulting homogenate was centrifuged at 12000 rpm for 15 minute at 4°C and the supernatant was collected and stored at -20°C until further use.

3.3.6. Estimation of catalase activity

The catalase activity was estimated in the skin/tumor tissue supernatant, in brief 50 µl supernatant was added to 1.0 ml of 50 mM phosphate buffer (pH 7) and 0.1 ml of 30 mM hydrogen peroxide and mixed. The O.D. was taken at 240 nm for 3 min in spectrophotometer. The activity of catalase was expressed as U/mg of protein.

3.3.7. Estimation of superoxide dismutase activity

The superoxide dismutase activity was estimated in the skin/tumor tissue supernatant in brief 100 µl supernatant was added to 2.85 ml of phosphate buffer (pH 8.4, 0.1 M) and 50 µl of 7.5 mM pyrogallol mixture. The O.D. was taken using spectrophotometer at 420 nm for 3 min. The enzyme levels were expressed as U/mg of protein.

3.3.8. Estimation of glyoxalase-1 activity

The glyoxalase-1 assay was performed in skin/tumor homogenate supernatant using 0.2% MG and 2% glutathione in 0.1 M phosphate buffer (pH6.8).Change in absorption was recorded at 240 nm for 3min attributable to the formation of S-D-lactoylglutathione.

3.3.9. Histopathological studies

The normal and papilloma-bearing skin tissues from different treatment groups were fixed in 10% formalin. The tissues were dehydrated through ascending grades of alcohol and cleared in toluene and embedded in low melting point paraffin. Five micron thick sections were cut, put serially on glass slides and then de-parraffinized using descending grades of alcohol & dehydration done by toluene. Sections were prepared from each tissue and stained with haematoxylin and eosin for histopathological analysis.

3.3.10. *In vivo* study in B16F10 cell tumor Xenofraft

Random-bred Female C57BL6 mice (6–8 week old) were used in the Xenograft study [273]. Animals were maintained in centralized air-conditioned animal house facility at Jawaharlal Nehru University, New Delhi-110067 with a 12-hr light/12-hr dark cycle and provided with standard food and water ad libitum. The experimental protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals and guidelines of Institutional Animal Ethics Committee.

3.3.11. Experimental Design

The animals were divided in to three groups

Negative control group – Vehicle only (PBS)

Positive control group – B16F10 cells

Treatments group – Naringenin + B16F10 cells

3.3.12 Isolation & culturing of B16F10 cells from Xenograft tissue

The cells from xenograft tissues were isolated by cold tripsinization technique. In brief the xenografted tissue were tripsinized by tripsin and after incubation the debris etc was removed from the cell suspension and after subsequent washing with media cell were sub cultured in RPMI-1640 media.

3.4. Statistical analysis

Experimental values are given as mean ±SD. various groups were tested for normal distribution, and statistical significance of difference between control and experimental groups was determined through one-way analysis of variance. A value of $P < 0.05$ or more was considered significant.

Chapter IV

Results

Hypoxia and Glyoxalase System

4.1. Glyoxalase-1 activity in different cell lines and tissues

In the present study different cell lines Du-145 (prostate cancer cell line), A-549, (lung cancer cell line) HeLa (Cervical cancer cell line) and 293T (normal kidney cell line) are used for the base line glyoxalase activity. Different cancer cell lines show variation in glyoxalase-1 activity. Data shows that Du-145 has highest glyoxalse-1 activity among the four cell lines tested while as HeLa had lowest **(Figure -9).** Therefore further experiments were carried out with HeLa cells to study the effect of induced Hypoxia. Besides cell lines glyoxalase-1 activity was determined in different mice tissues. The results show liver tissue has highest glyoxalase-1 activity in comparison to other tissues including the kidney and lung **(Figure -10).**

4.2. Effect of Cobalt chloride induced Hypoxia in HeLa Cells:

Cells morphology, proliferation and viability

The cobalt chloride was used as a hypoxia mimic agent, so we first standardized the dose of cobalt chloride for induction of hypoxia in HeLa cells. The MTT assay shows that the toxicity of cobalt chloride was dose dependent and at 100 μM concentration there was not significant effect on cell proliferation as compared to untreated cells **(Figure -11 A)**.Where as higher concentration (200 μM to 500 μM) of cobalt chloride show toxic effect on HeLa cells. Trypen blue assay further confirmed cobalt chloride induced cell death in a dose dependent manner in HeLa cells. Cell viability was not much affected at 100 μM. However beyond 100 μM there was significant decrease in cell viability. At 500 μM, only 25% cell could survive **(Figure-11 B)**. Cobalt chloride treatment resulted in significant change in cell morphology. The morphological changes observed were cells shrinking, cell death, apoptosis and detachment. The extent of these morphological changes increased with increasing concentration of cobalt chloride **(Figure -12)**.

4.2.1. Hypoxia inducible factor 1(HIF-1α)

HIF regulates the cellular adaptation to hypoxic condition. In order to validate the induction of hypoxia the HIF-1 α mRNA was detected by real time RT-PCR and results show that cobalt chloride at 100μM concentration increases the level of HIF-1 α mRNA significantly at 4 hrs post exposure however at 6 and 12 hrs the level decreased again **(Figure -13)**.

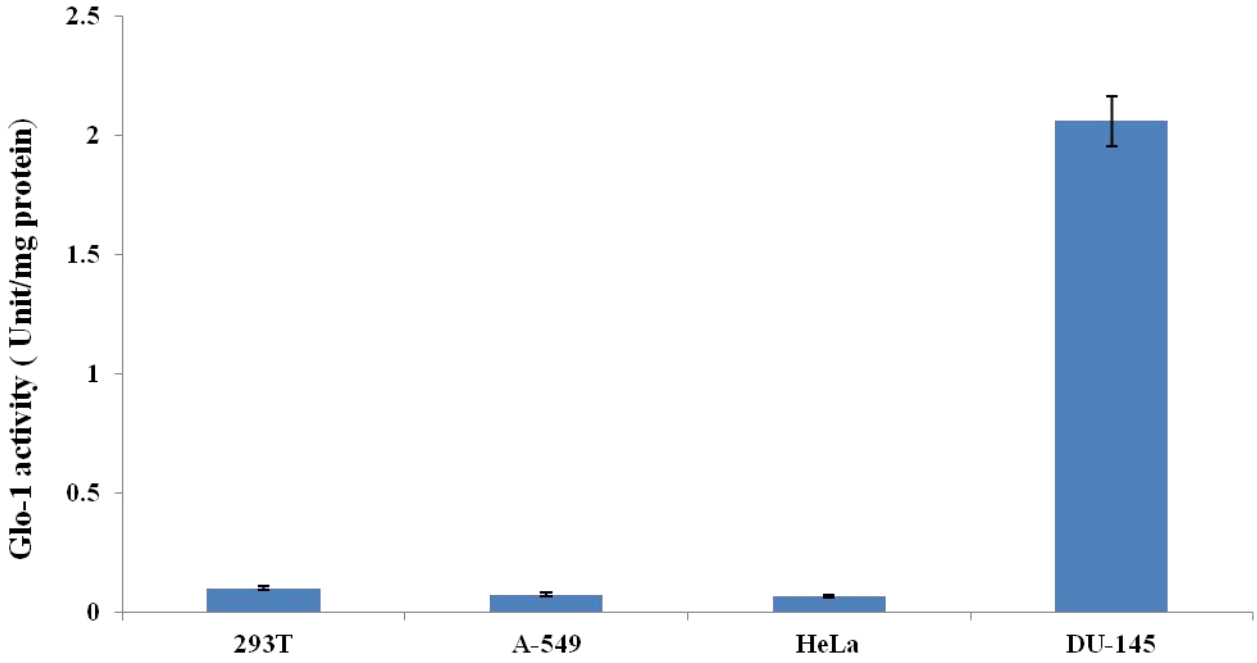

Figure 9: Glyoxalase-1 activity in *in vitro* in four human cancer cell lines, human normal kidney (293T), lungs cancer cells (A-549), cervical cancer cells (HeLa), prostate cancer cells lines (DU-145).

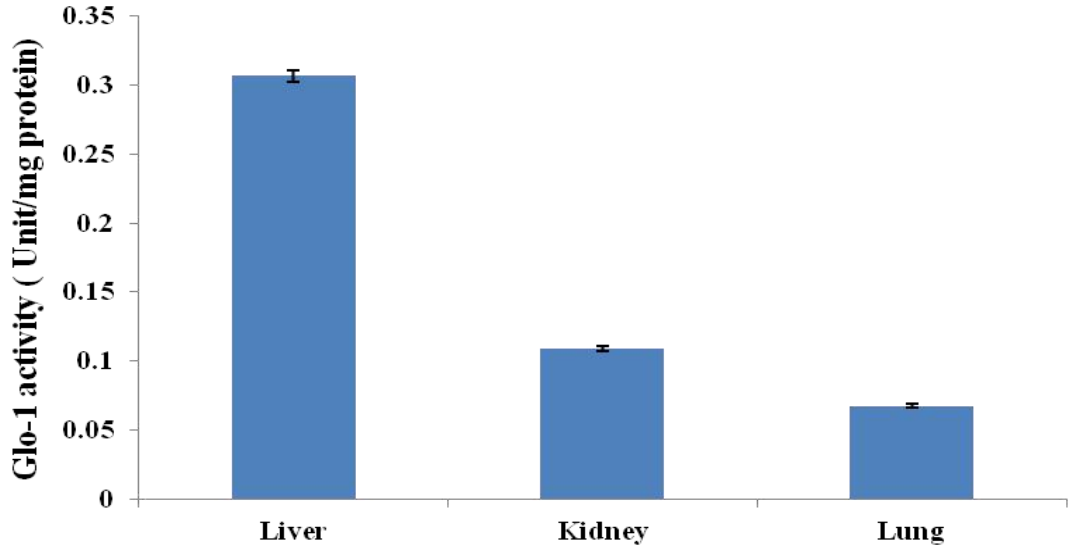

Figure 10: Glyoxalase-1 activity *in vivo* in different tissues of mice.

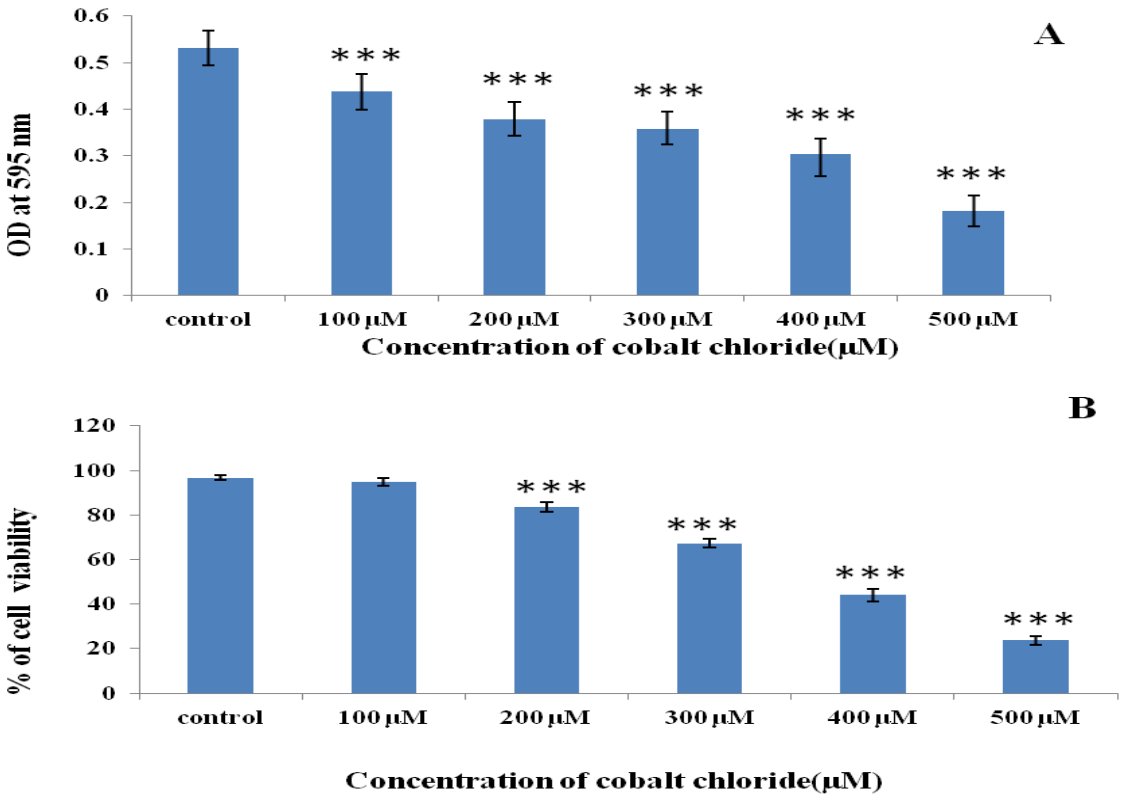

Figure 11: Effect of different concentrations of cobalt chloride on HeLa cells; A- Cell proliferation by MTT assay, B- Cell viability by Trypan blue assay. Data are presented as mean ± S.D. of three separate experiment ***p< 0.001 with respect to control.

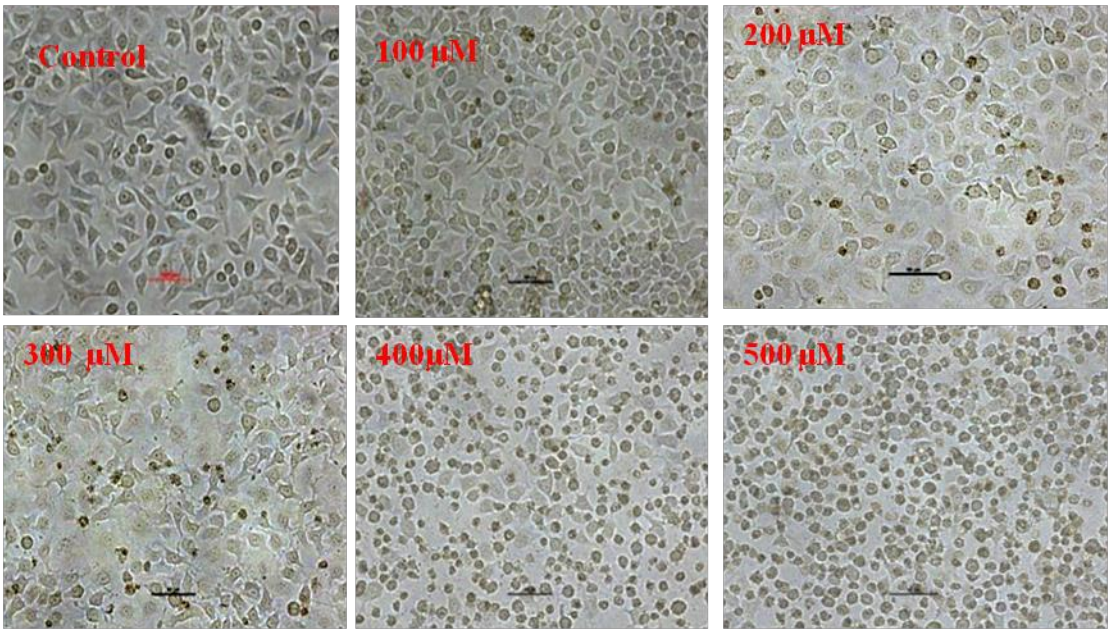

Figure 12: Effect of different doses of Cobalt chloride on HeLa cells, morphological changes like spikes formation, detachment, floating cells in medium 24h post treatment (Images were captured by Nikon inverted microscope at 10X magnification)**.**

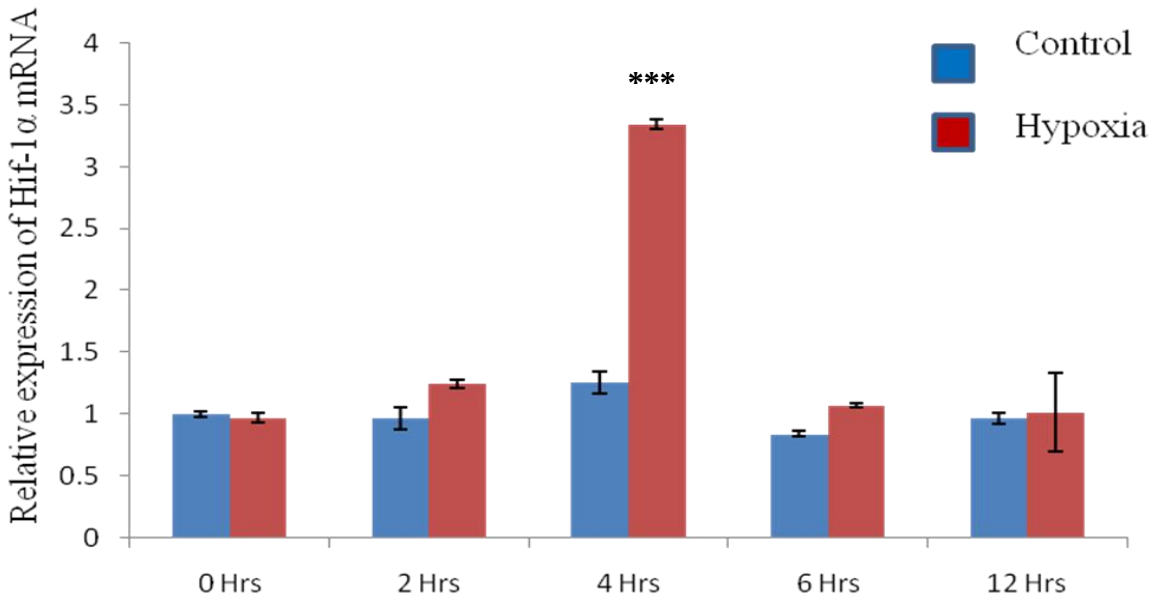

Figure 13: HIF-1 m-RNA expression in hypoxic HeLa cells at different time point by RT-PCR.

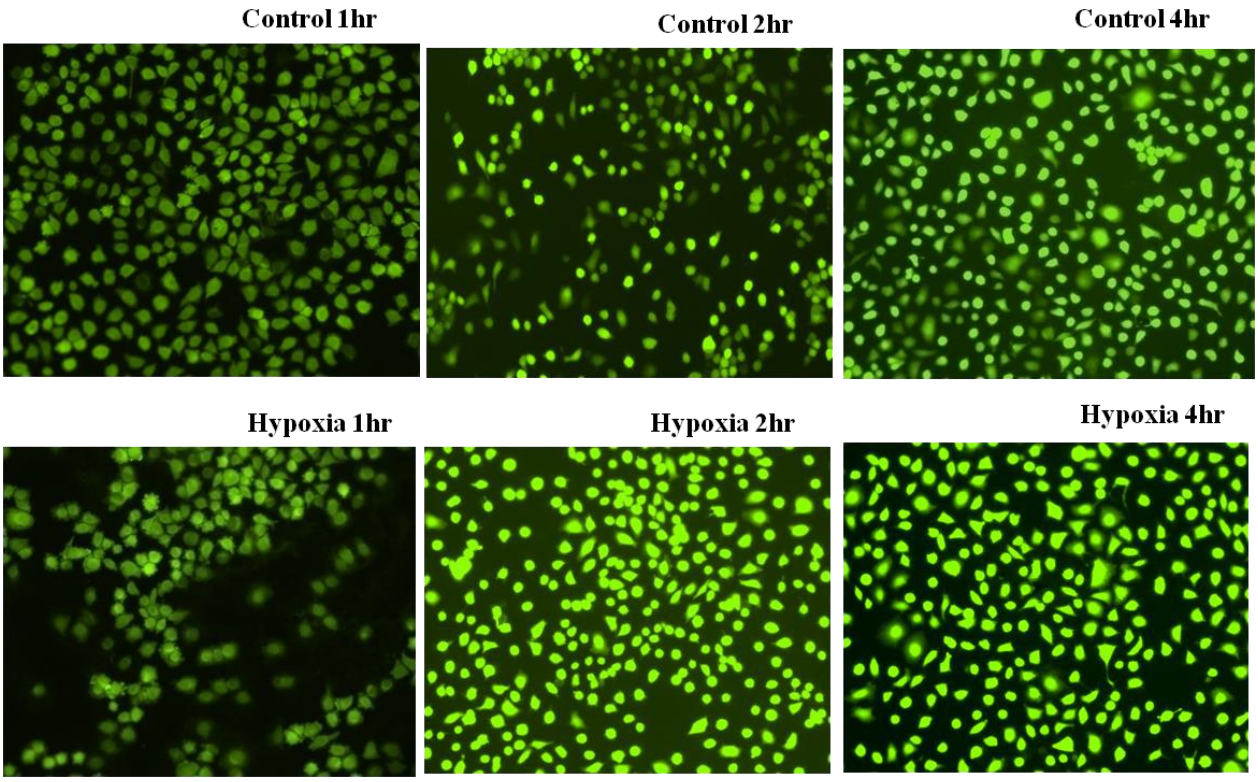

Figure 14: Effect of cobalt chloride induced hypoxia on ROS level in HeLa cells. Images show increase in fluorescence with increasing concentration of cobalt chloride.

4.2.2. ROS levels

ROS were measured as change in fluorescence intensity of DCFDA in cells exposed to $CoCl_2$ .The level of ROS increased in a time dependent manner. The level of ROS induced by the cobalt chloride was highest at 4 hrs post hypoxia as compared to other time intervals **(Figure -14)**.

4.2.3. Glo-1 enzyme activity, m-RNA and protein

The hypoxia induced by cobalt chloride at 100 µM concentration increased the glyoxalase-1 activity in HeLa cells. The level of glyoxalase-1 increased in hypoxic condition at 1 and 4 hours whereas remained constant in normoxic condition **(Figure -15 A)**.The Glo-1 mRNA was detected by real time RT-PCR and results show that level of Glo-1 mRNA significantly increased at 4 hrs post exposure and decreased thereafter reaching to normal control levels by 24 hrs **(Figure -15 B)**.The western blotting results further show that glyoxalase-1 protein level also increases under hypoxic condition **(Figure -15 C)**.

4.4. Discussion

Hypoxia refers to decreased oxygen concentration (below optimum) in any organ, tissue or cells. It may be physiological or pathologic in nature [274-276]. Hypoxia can be induced *in vitro* using certain chemicals, called "hypoxia mimetics", such as desferrioxamine (DFO) and the transition metal cobalt as well as hypoxia chambers [277]. The use of cobalt chloride as hypoxia mimic agents is cost effective and fast as compare to hypoxia inducing hypoxic chambers. It is reported that DFO and cobalt stabilize HIF-1α in cells by inhibiting the PHD activity or by Fe^{2}+ depletion or substitution [278]. Literature suggested that cobalt stabilizes the HIF-1 by substituting the Fe^{+2} in active centre of prolyl hydroxylase [278]. Cobalt generates ROS which may also help in stabilization by two ways first by oxidizing Fe^{2+} to Fe^{3+} in active sites of pHD and produce inactive pHD [279, 280] second by activating the ERK1/ERK2 and PI-3K signalling pathways, that induce HIF-1 pathway [281]. In the present study the toxicity of cobalt chloride was dose dependent in Hela cells. The data shows that 100 µM concentration of cobalt chloride was non toxic for HeLa cells but induced cell stress and could be used to study the effect of hypoxia. Accumulation of ROS in oxidative stress has been associated in the pathogenesis of many diseases including cancer. In the present study also we found an increase in the level of ROS in Cobalt chloride treated HeLa cells.

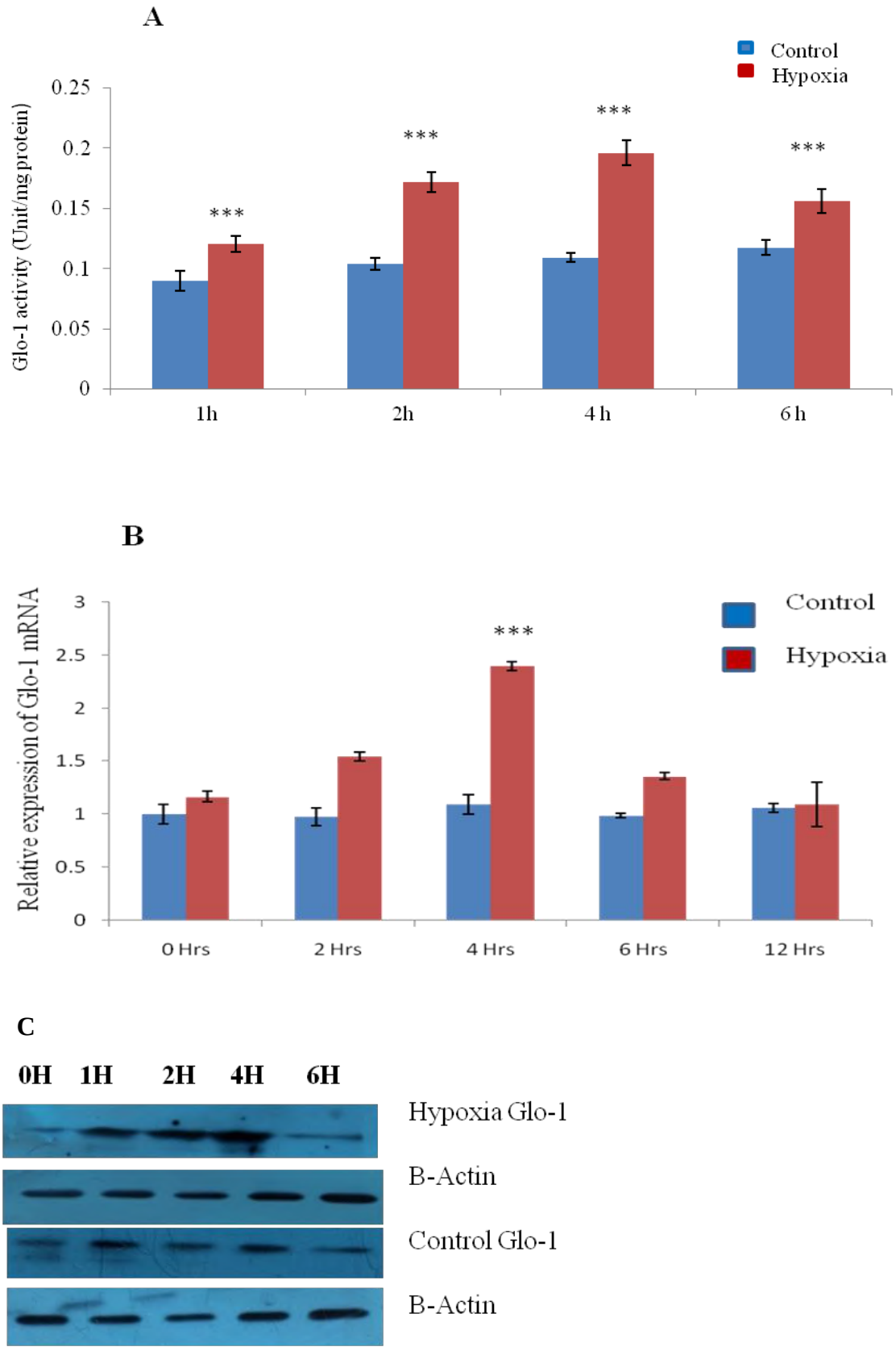

Figure 15: Effect of hypoxia on A) Glyoxalase-1activity B) m-RNA level by RT-PCR and C) protein expression by Western Blotting. Data are presented as mean ± S.D. of three separate experiment ***p< 0.001 with respect to control.

The cobalt chloride has been reported to induces HIF-1in HeLa cells that is ROS mediated [279]. In our studies also an increase in HIF-1α levels was observed in cells treated with 100uM Cobalt chloride further validating that this concentration is able to induced hypoxic response in Hela cells. Similar observations were made in Hep3B and Mum2B cell lines [104, 282] where HIF-1α levels and ROS levels were increased in response to cobalt chloride treatment [281].

We also observed an increase in the glyoxalase-1 activity in hypoxic HeLa cells in comparison to cells growing in normoxic conditions. The level of glyoxalase-1 increased up to 4 hrs and decreased thereafter. Ours results suggest a correlation of glyoxalase-1 activity in hypoxia mimetic condition. Hypoxia and glyoxalase-1 have been reported to play important role in many disease conditions e.g. kidney disease [283-285], neurodegenerative diseases, aging, obesity, cardiovascular disease, anxiety, depression, autism, chronic renal disease etc [286, 287]. Further under hypoxic condition the HIF1 alpha is stabilised and modulates the genes involved in angiogenesis and transfer of oxygen [288] which can result in the aggressive phenotype of various cancers. A correlation between increased glyoxalase activity and aggressiveness of cancer is already reported. HIF is up regulated in cancer cells by multiple means and can modulate the metabolism in cancer cells. Many of the enzymes involved in cancer metabolism are targets of HIF. There are multiple effects of HIF activation on metabolism in cancer. For instance, activation of PI3K/PTEN/AKT signaling activates HIF and enhances, indirectly activates transcription of genes encoding multiple glycolytic enzymes. Since HIF is primarily a transcription factor, a number of studies have shown its effects on gene expression. There are relatively few studies that show effect of HIF on specific metabolic enzymes. However, key enzyme activities are regulated at many levels other than gene expression, and altered metabolism requires coordinated changes in the function of many enzymes. In the present study it can be speculated that the activation of HIF could be directly or indirectly effecting the regulation of glyoxalase enzyme. Thus it is an important, but as yet incompletely answered question, that how HIF activation can lead to better survival of cancer cells & regulation of glyoxalase-1 could probably be one of the mechanisms.

Chapter V

Results

Radiation Resistance and Glyoxalase System

5.1. Effect of radiation on HeLa cell survival and proliferation:

The effect of radiation on HeLa cells was observed by clonogenic assay and it is clear that the number and size of colonies is inversely proportional to dose of radiation **(Figure -16)**.The MTT assay further confirmed that radiation induce cell death in a dose and time dependent manner **(Figure -17)**.

5.2. Effect of radiation on Glo-1 activity:

The effect of radiation on glyoxalase-1 activity in HeLa cells was studied 24 hrs post radiation treatment and results show that there was an increase in the glyoxalase-1 activity as compared to untreated cells. From **(Figure -18)** it is clear that the glyoxalase-1 activity was highest at 6 gray radiation dose as compared to other radiation doses**.**

5.3. Development of Radio resistant HeLa cells:

In order to check the role of glyoxalase I in induction of radioresistance, radiation resistant cell line was developed in house. The radioresistant HeLa cells were developed by giving a total gamma radiation dose of 60 gray in ten 6 gray fractions. All fractions of radiation were given at the 60-70% confluence. The radio resistant HeLa cells were obtained after giving the cumulative dose of 60 gray of gamma radiation were termed as RR-HeLa cells and were used for further studies.

5.4. Characterization of radio resistant cells using Colonogenic assays:

Morphologically parental and RR-HeLa cells did not show any drastic change. Both were comparable with respect to shape, size and other features **(Figure -19)**. However coloney formation studies showed that RR cells formed more number of colonies in comparison to parental cell line on exposure to 6 Gray of radiation. The size of the colony was also bigger **(Figure -20)**.

5.4.1. Doubling time and cell cycle study in RR HeLa cells:

Doubling time is the average time taken by the cell population to become double and is a measure for the proliferation of cells. The cell doubling time of RR- HeLa cells was found to be increased by 4 hrs as compared to parental HeLa cells. The Cell cycle studies in parental HeLa and RR-HeLa done using FACS shows difference in G1 phase. The G1 phase was increased in RR-HeLa cells as compared to parental HeLa cells **(Figure -21)**.

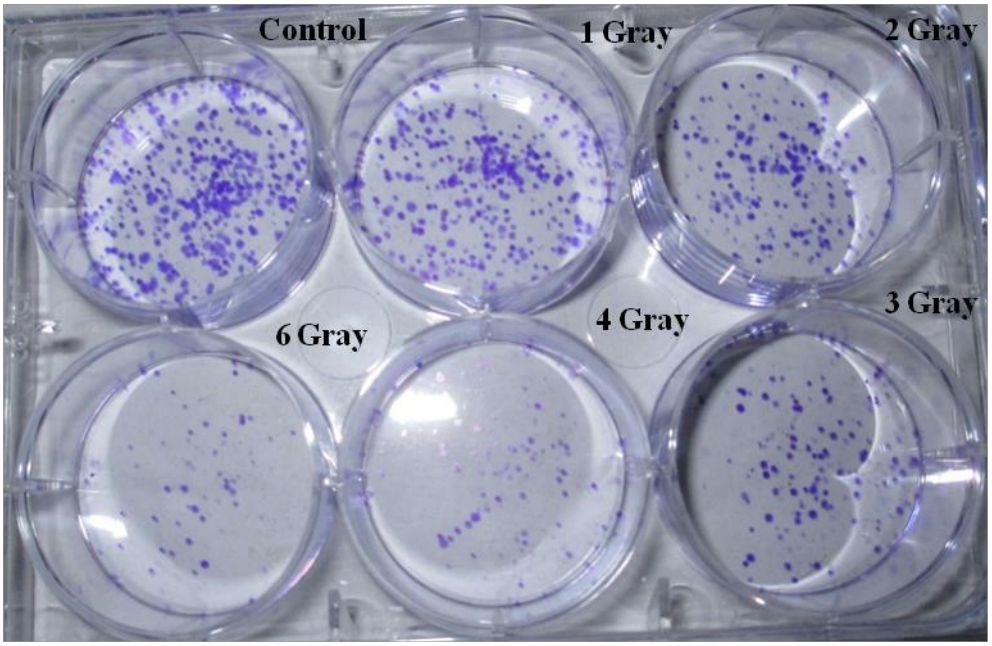

Figure 16: Effect of 1-6 Gy Gamma radiation on colony forming ability of HeLa cells.

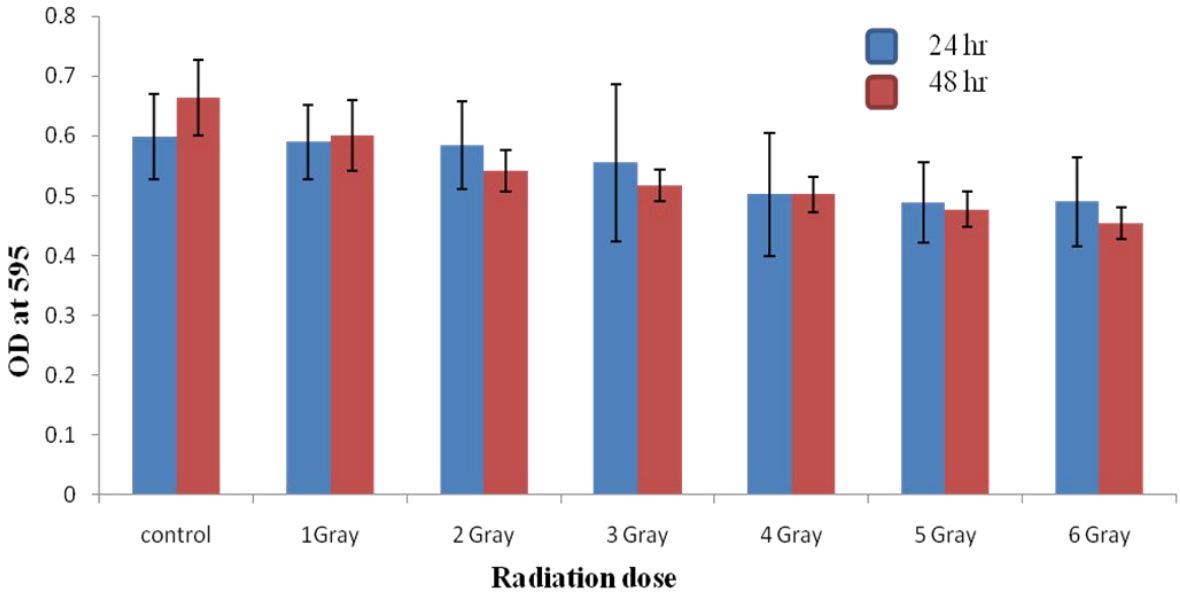

Figure 17: Radiation dose response in HeLa cells at 24 hrs and 48 hrs using MTT assay.

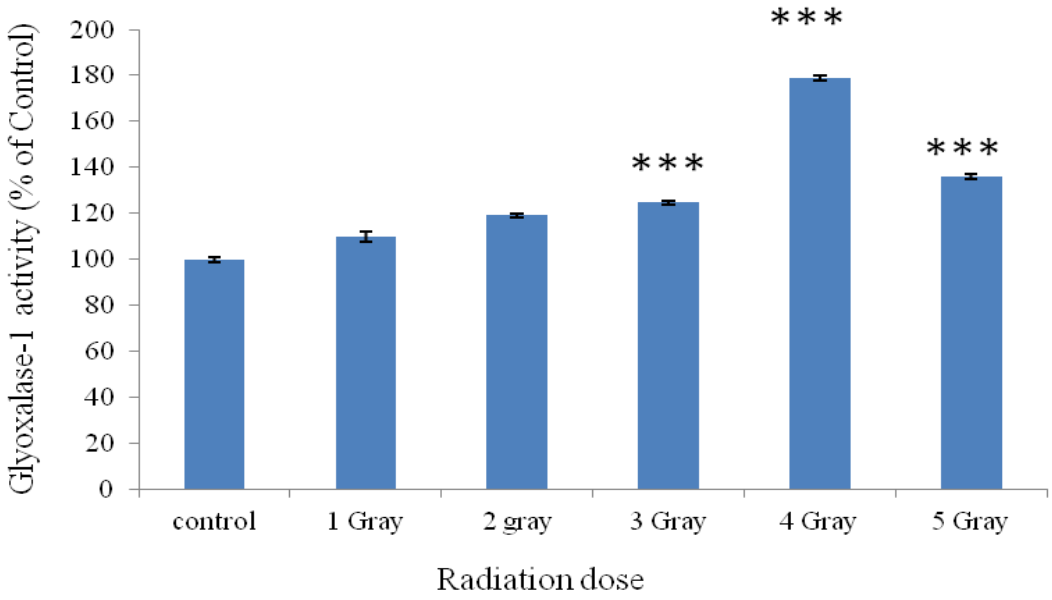

Figure 18: Effect of different doses of radiation on Glyoxalase-1 activity in HeLa cells. Data are presented as mean ± S.D. of three separate experiments ***p< 0.001 with respect to control.

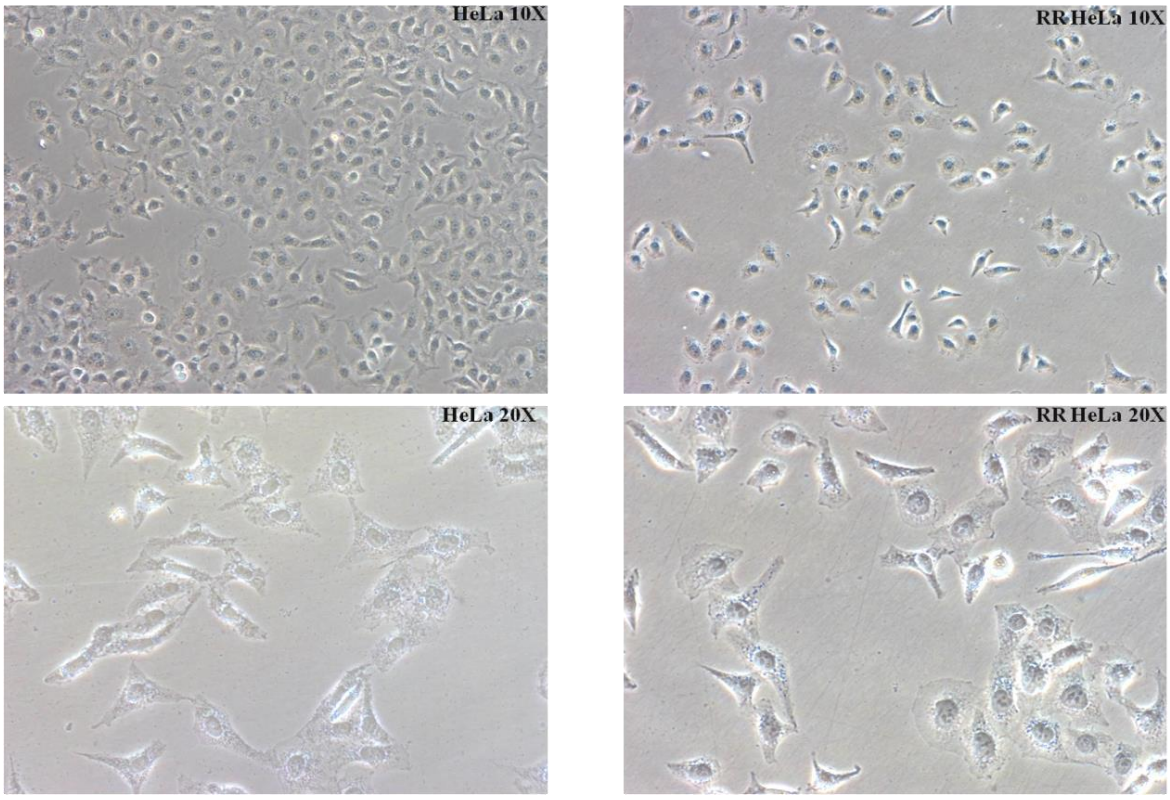

Figure 19: Morphology of parental HeLa & Radioresistant HeLa cells (Images were captured by Nikon inverted microscope at 10X & 20X magnifications)**.**

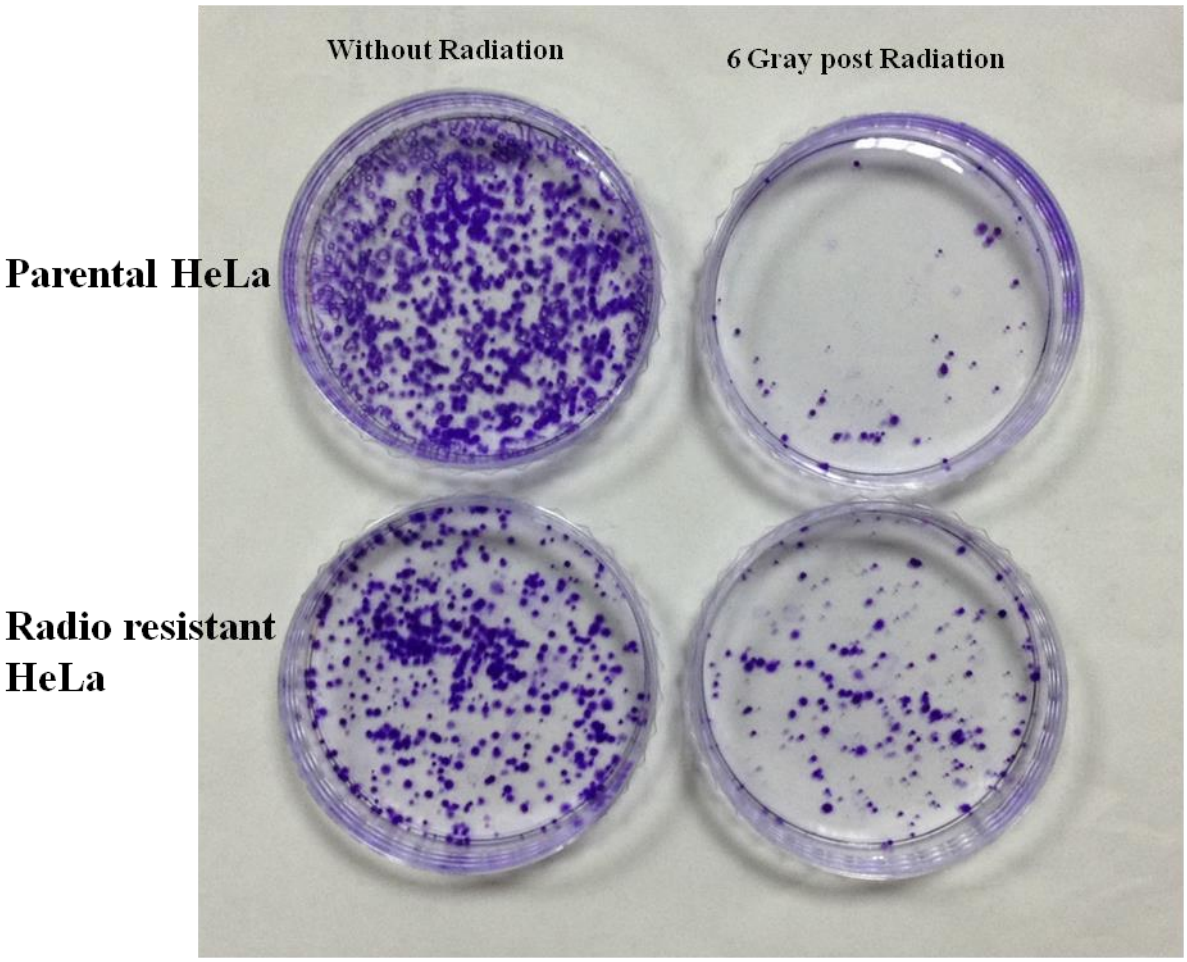

Figure 20: Effect of radiation on parental & RR-HeLa cells by colonogenic assays

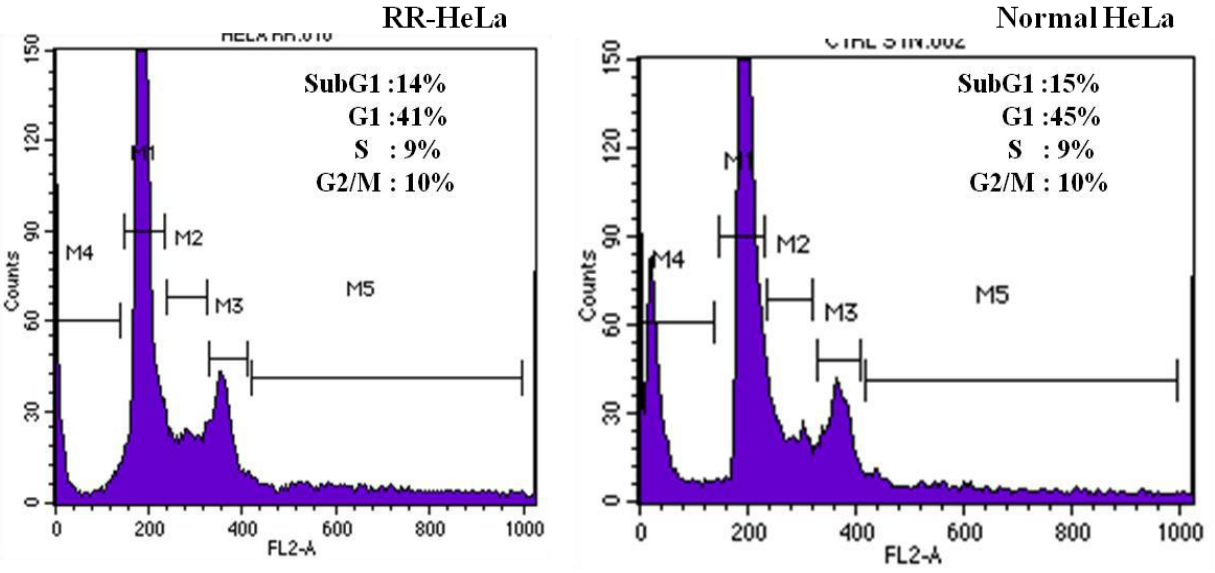

Figure 21: Cell cycle analysis of parental HeLa & RR-HeLa by FACS

5.5. Radiation response of parental HeLa & RR-HeLa:

5.5.1. Effect of radiation on cell morphology

Significant morphological changes were observed in normal HeLa as compared to RR-HeLa cells on exposure to 6Gray radiation. The morphological changes were time dependent. With increase in time post irradiation time, cells became more and more deformed in shape, there was formation of stress granules and spike like structures and by 72 to 96 hrs the normal HeLa cells lost its characteristic morphology **(Figure -22)**. However no such changes were observed in RR HeLa cells.

5.5.2. Effect on Radiation induced apoptosis

HeLa and RR-HeLa cells were exposed to 6 Gy of radiation, The percentage of live cell in parental HeLa after radiation treatments at different time point 24h,48h,72h and 96h was found to be 47%, 30%, 14% and 3% and high amount of apoptotic cells were observed. On the other hand the percentage of live RR-HeLa cells was much higher 55%, 40%, 22% and 20% respectively **(Figure -23)**. The number of apoptotic cells was much less. With increasing time after radiation significant increase in no of viable cells was seen in RR HeLa. At 92H the survival percentage was significantly high, 20 % in RR HeLa cells only 3% in parental cells.

5.5.3. Effect of radiation on mitochondrial membrane potential ($\Delta\Psi$)

Member integrity is an important parameter in cell survival. Therefore effect of radiation was studied on the Mitochondrial membrane potential ($\Delta\Psi$) of both parental and RR-HeLa cells. The membrane potential ($\Delta\Psi$) measured by JC-1 dye shows that membrane integrity of parental HeLa cells was significantly altered on exposure to radiation while a RR-HeLa cells did not show much change. This further substantiates that RR-HeLa are more resistant to radiation induce stress and have better survival **(Figure -24)**.

5.5.4. Effect of radiation on Glyoxalase-1, Nrf-2 and total Carbonyl content

The base line glyoxalase-1 activity was found to be almost similar in both the RR-HeLa and Parental HeLa cells. However the two cell lines differed with reference to their response to radiation. Glyoxalase-1 activity was found to be significantly increased in RR-HeLa cells in response to radiation in comparison to Parental Hela cells **(Figure -25 A).** On the other hand there was a significant increase in total carbonyl content in parental

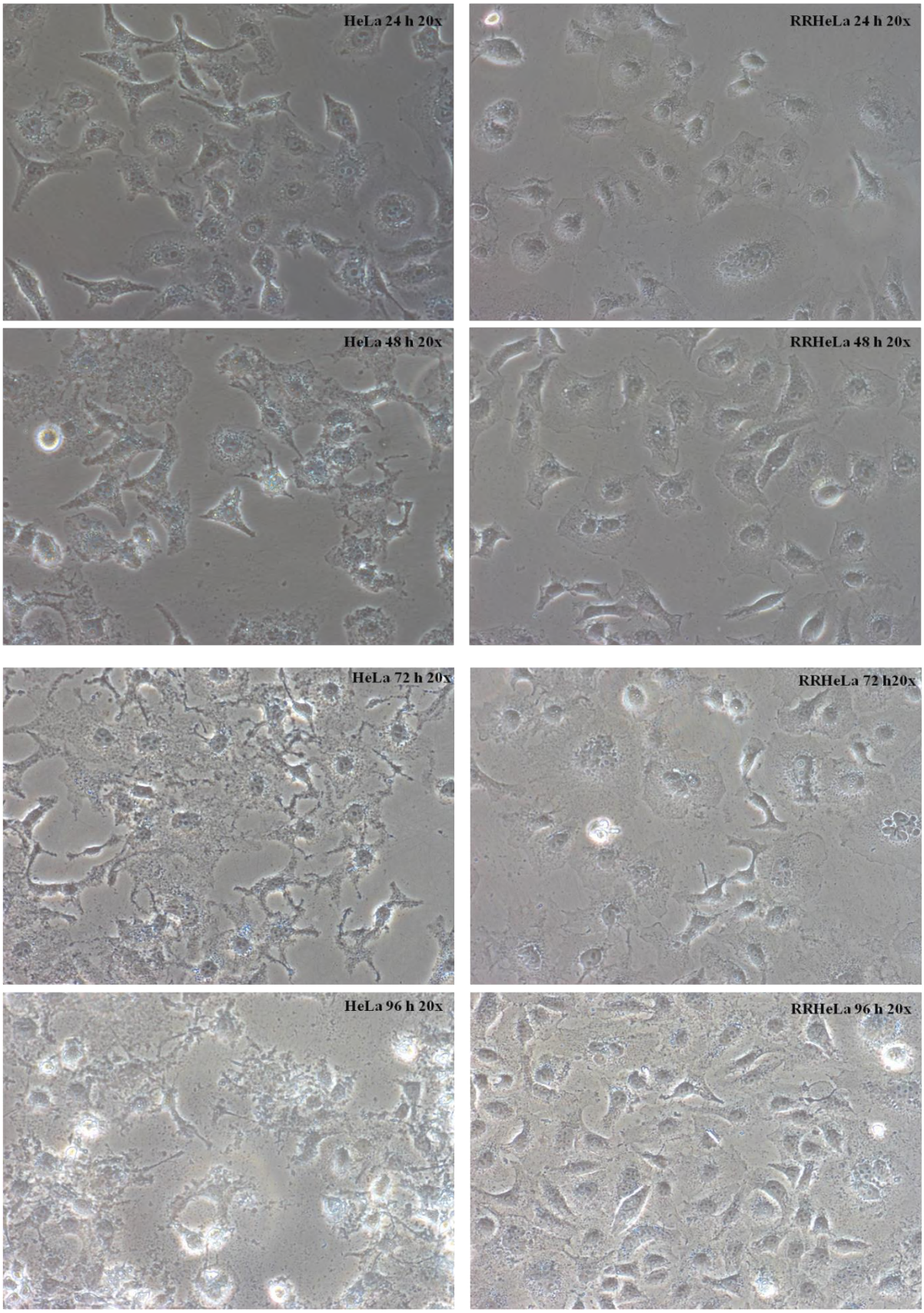

Figure 22: Effect of 6 gray radiation on morphology of parental HeLa & RR-HeLa at different time interval (Images were captured by Nikon inverted microscope at 20X magnifications)**.**

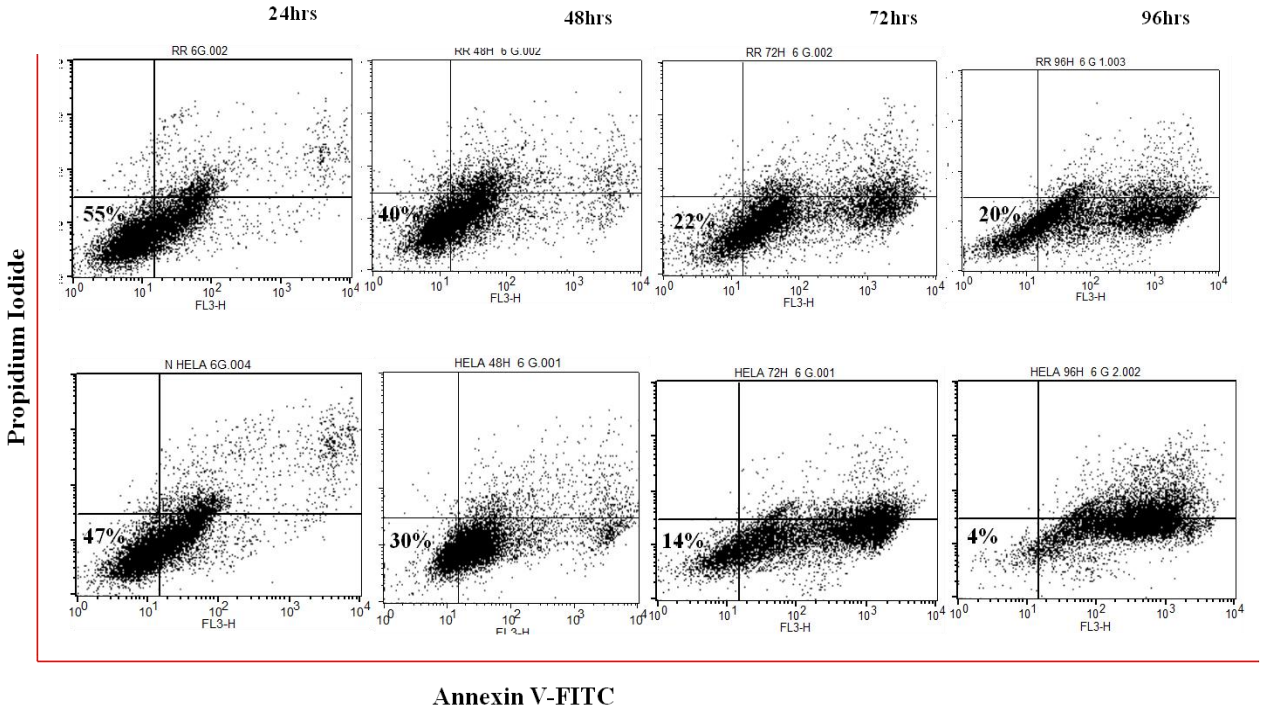

Figure 23: Effect of radiation (6 Gy) on apoptosis by AnnexinV/PI at different time points in parental HeLa & RR-HeLa.

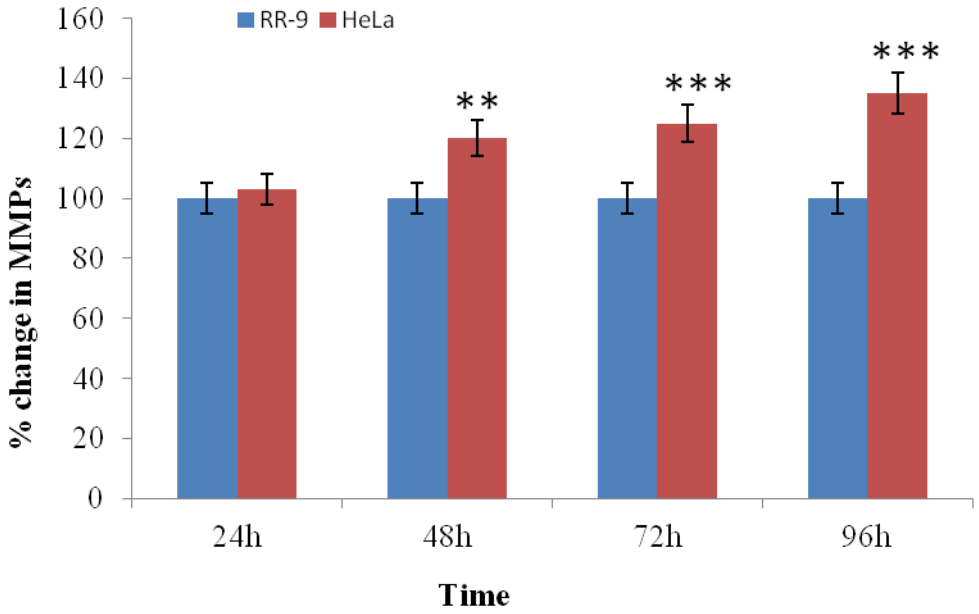

Figure 24: Effect of 6 gray radiation on mitochondrial membrane potential in parental HeLa & RR-HeLa. Data are presented as mean ± S.D. of three separate experiment ***p< 0.001, **p< 0.05 with respect to parental control.

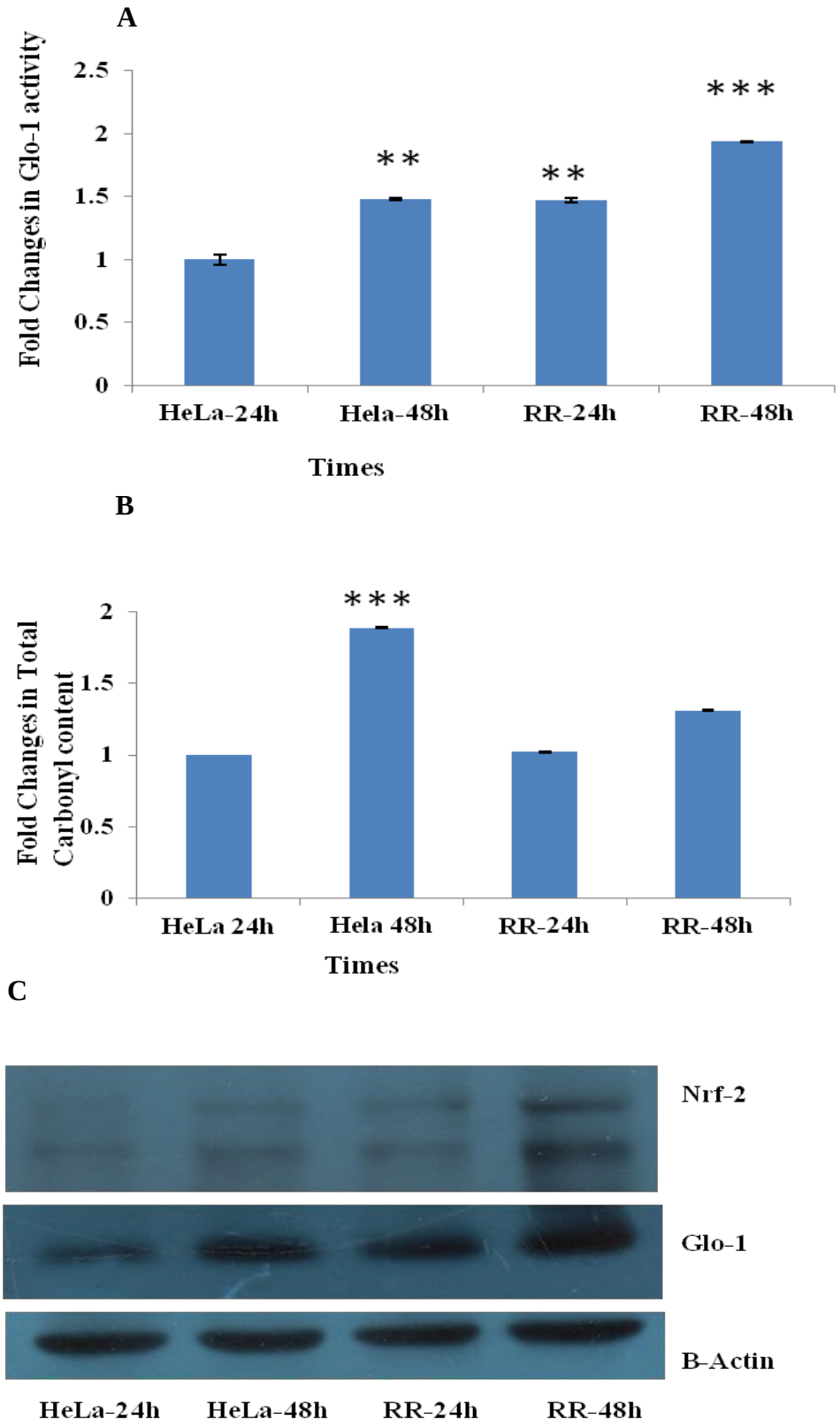

Figure 25: Effect of 6 gray radiation on parental and radio resistant HeLa cells at different time points (24 h & 48h) A) Glyoxalase-1 activity B) Total carbonyl content C) Nrf-2 & Glo-1 protein expression by western blotting. Data are presented as mean ± S.D. of three separate experiments ***p< 0.001, **p< 0.05 with respect to parental control.

HeLa cells as compared to RR-HeLa. The total carbonyl content is the measure of oxidative & carbonyl stress in cells. The increased glo-1 activity could be correlated to the decreased carbonyl content and better survival response against radiation in RR-cells **(Figure -25 B)**.

The western blotting results also showed that Glyoxalase-1 protein level was also increased after 48 hrs of radiation treatments in both parental HeLa and RR-HeLa as compared to 24 hrs post treatments **(Figure -25 C)**. However RR-HeLa cells show high level of glo-1 expression as compared to normal HeLa Cells. The Nrf-2 protein is a transcription factor which regulates the basal and inducible level of glyoxalase-1. An increased level of Nrf-2 protein was also observed in RR-HeLa cells after radiation treatment as compared to normal HeLa cells **(Figure -25 C).**

5.6. Discussion

Radiation therapy is still the main stay of cancer treatment and 50-60% of cancer patients stil undergo radiation therapy during cancer treatments regimens. However development of radio resistance is an important limiting factor. Various factors that play an important role in development of resistance include tumor microenvironments, decreased drug uptakes, induction of some emergency response genes, alteration of drug targets, overexpression of certain receptor proteins or increased expression of some enzymes like catalase, peroxidases etc. The role of many of these enzymes has been reported in radioresistance like increased superoxide dismutase (SOD) scavenge the ROS produced by radiolysis of water and protect tumor tissue form ionizing radiation and catalase or PPx [289, 290[291-293]]

The involvement of glyoxalase-1 in radioresistance is not clearly known. The glyoxalase-1 enzyme that is associated with carbonyl stress generally increases in response to various stress conditions [155].The ionization radiation exposure results in induction of ROS responsible for inducing oxidative and carbonyl stress. The main molecule responsible for carbonyl stress is methyl glyoxal which is a dicarbonly moiety.

The basal level of glyoxlase-1 enzyme was approximately same in both parental HeLa as well as RR-HeLa but was found to be increase selectively in RR-HeLa after radiation exposure. As a result of which the total Carbonyl content was also found to be decrease in RR-HeLa cells as compared to parental HeLa cells **(Figure -25 B)**. Nrf-2 is a protein that works as a transcription factor which regulates the basal and inducible level of glyoxalse-

1 in cells. MG, Glyoxalase-1 & dicarbonyl stress have emerged as the targets of Nrf-2/keep system [155]. The level of Nrf-2 was found to be selectively increased in RR-HeLa as compared to Parental HeLa after radiation exposure **(Figure -25 C)**. Therefore radioresitance in RR HeLa cells is a function of increased Nrf-2 induced upragulation of Glyoxalase enzyme resulting in decreased carbonyl stress.

Chapter VI

Results

Phytochemicals and Glyoxalase System

6.1. Galangin: Effect of Galangin on Hela cells

6.1.1. Free radical scavenging activity of Galangin *in vitro*

DPPH assay is an *in vitro* assay to detect the antioxidant property of a drug in a cellular system. Galangin was found to scavenge the DPPH free radicals in a concentration dependent manner. *In vitro* the IC50 value of galangin for DPPH was observed approximately 75 µM **(Figure -26)**. This data shows that galangin can be a strong antioxidant *in vitro*.

6.1.2. Effect of Galangin on morphology, proliferation and cell survival

The galngin induced changes in morphology of HeLa cells. The treated cells showed cell shrinkage, spindle shape, detachment, and spike formation. The extent of change in morphology as well as number of cells showing changes was concentration dependent (**Figure -27 A)**. Besides this the effect of galangin on HeLa cell survival was also evaluated by colonogenic assay **(Figure -27 B)**. The colonogenic assay showed that galangin significantly decreased the number and size of colonies in a concentration dependent manner Galangin inhibited *in vitro* proliferation of HeLa cells up to 50% at 50 µM **(Figure -28)**. The EtBr/AO dye staining showed that galangin treatment for 24 h induced apoptosis in dose dependent manner. The number of apoptotic and dead cells in galangin treated samples increased significantly **(Figure -29)**.

6.1.3. Effect on the metastatic potential

Galangin at concentration of 25–100 µM demonstrated a clear dose dependent anti migratory effect in wound healing assays of human cervical cancer cell line **(Figure -30)**. In untreated controls 50% migration took place while as cells treated with 25 µM of galangin only 20% cells migrated, after 24 H. This was further confirmed by microdrop migration/ invasion assay. Galangin inhibited the migration of HeLa cells in concentration dependent manner (IC50 ~ 12.5 µM) & at 75 µM cell invasion in to agerose gel was completely inhibited **(Figure -31 A & B)**.

6.1.4. Effect on reactive oxygen species

Galangin treatment was able to increase the intracellular ROS level in treated HeLa cells as compared to untreated control cells as measured by fluorescence microscopy **(Figure -32)**. From the fluorescence images of cells it is clear that the intracellular fluorescence

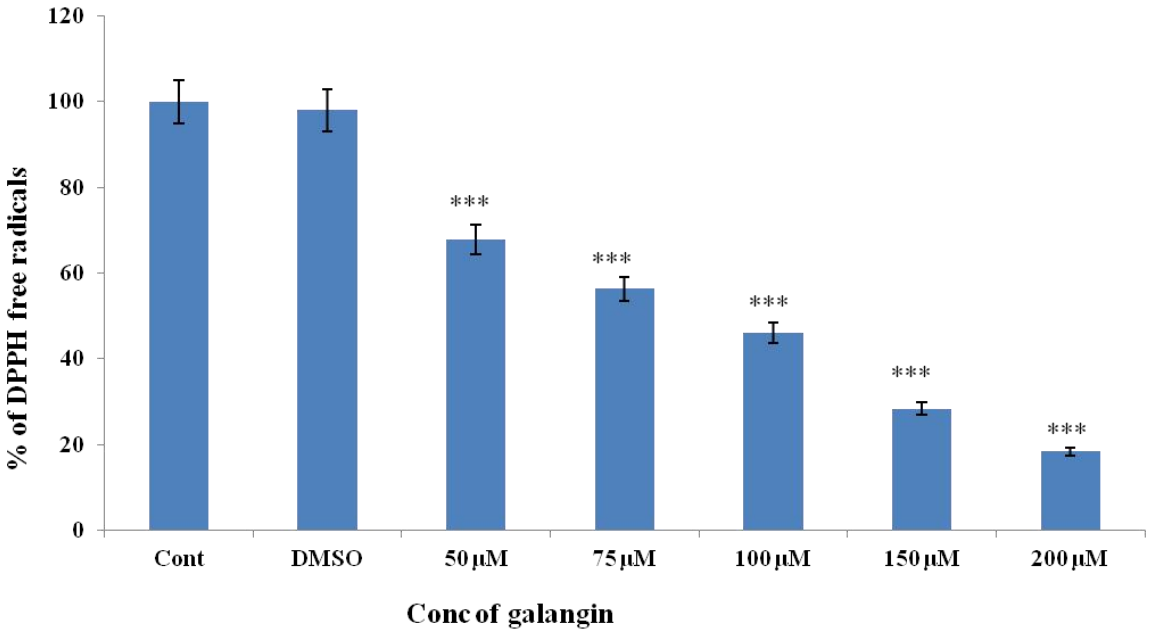

Figure 26: Effect of Galangin on DPPH free radicals. Data are presented as mean ± S.D. of three separate experiments ***P< 0.001 with respect to control.

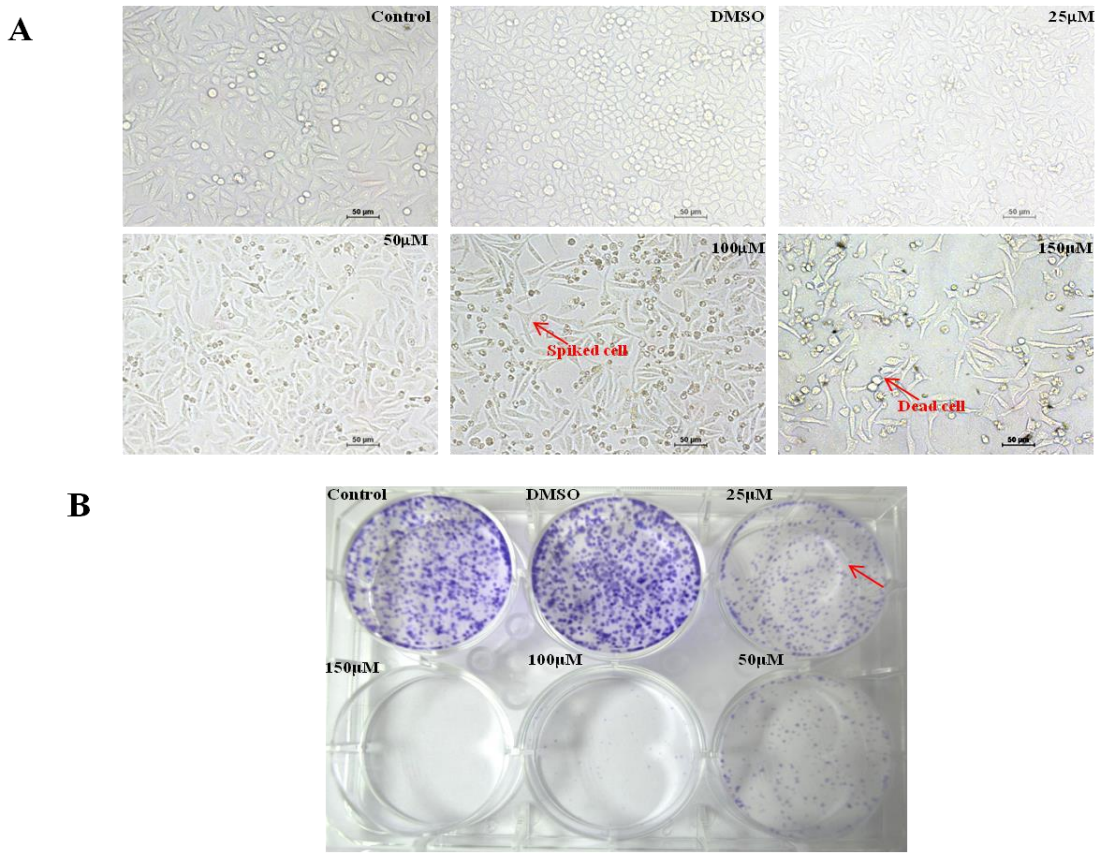

Figure 27: Effect of different concentration of Galangin on HeLa cells (A) Morphological changes like spikes formation, detachment, floating cells in medium after 24h of treatment (Images were captured by Nikon inverted microscope at 10X magnification) **(B)Colony formation.** (Arrow shows decrease in size and number of colonies in treated groups).

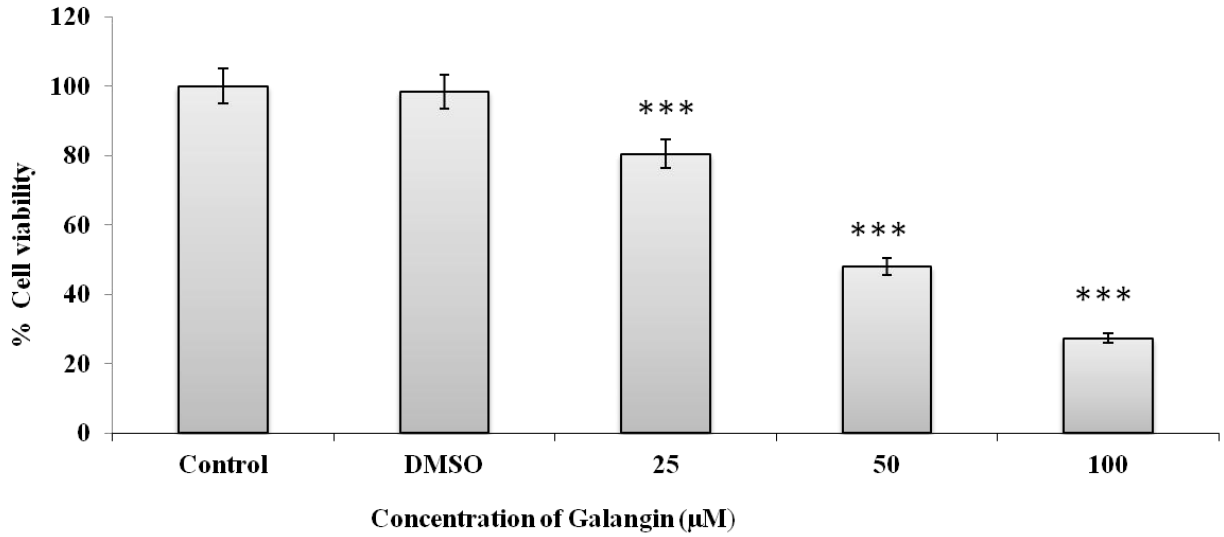

Figure 28: Effect of Galangin on cell viability by MTT assay. Data are presented as mean ± S.D. of three separate experiments ***P< 0.001 with respect to control.

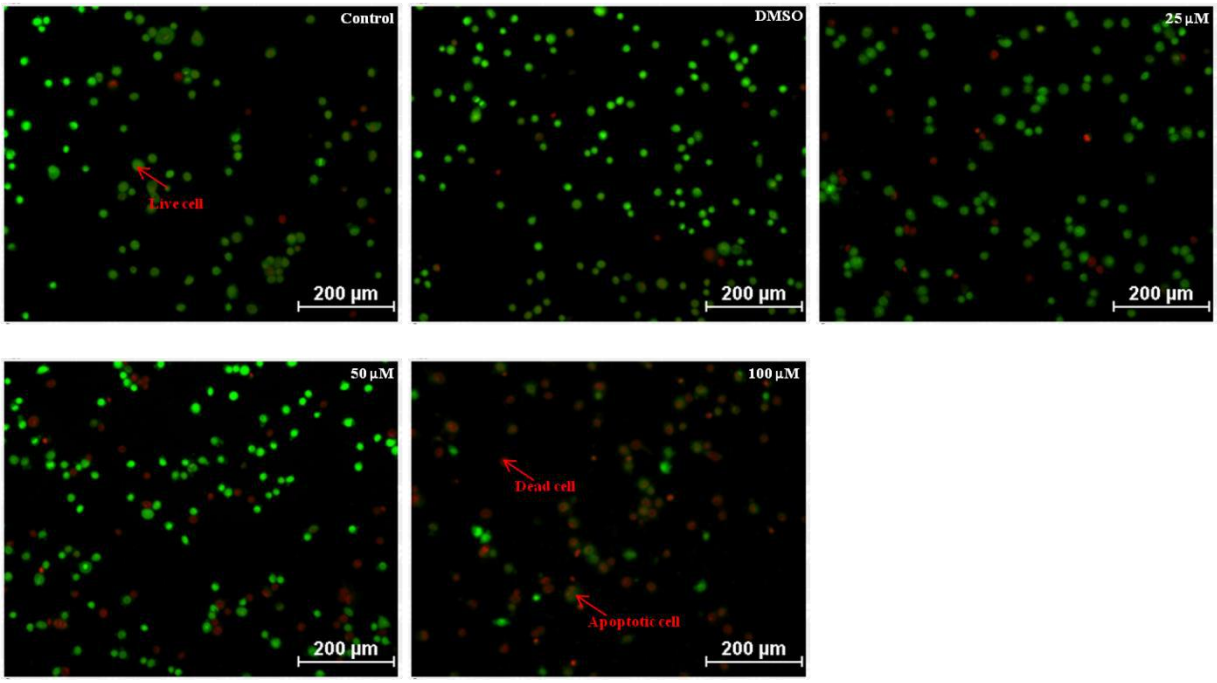

Figure 29: Galangin induced apoptosis in HeLa cells 24h after treatment as detected by EtBr/AO staining .Images were analysed by NIS elements Nikon analysis software. Green, orange and red fluorescence represents live, apoptotic and dead cells respectively.

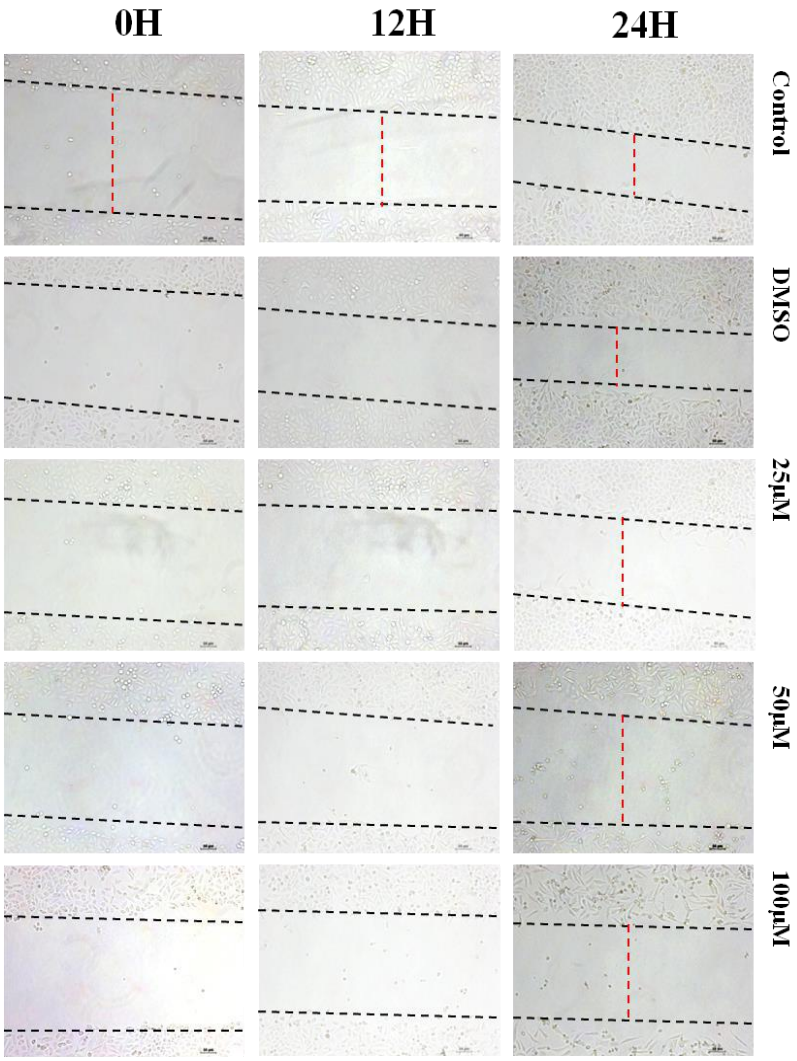

Figure 30: Effect of Galangin on HeLa cells migration by monolayer scratch assay at different time points. Mark narrowing gap with time in control at 24h but very little migration in galangin treated samples.

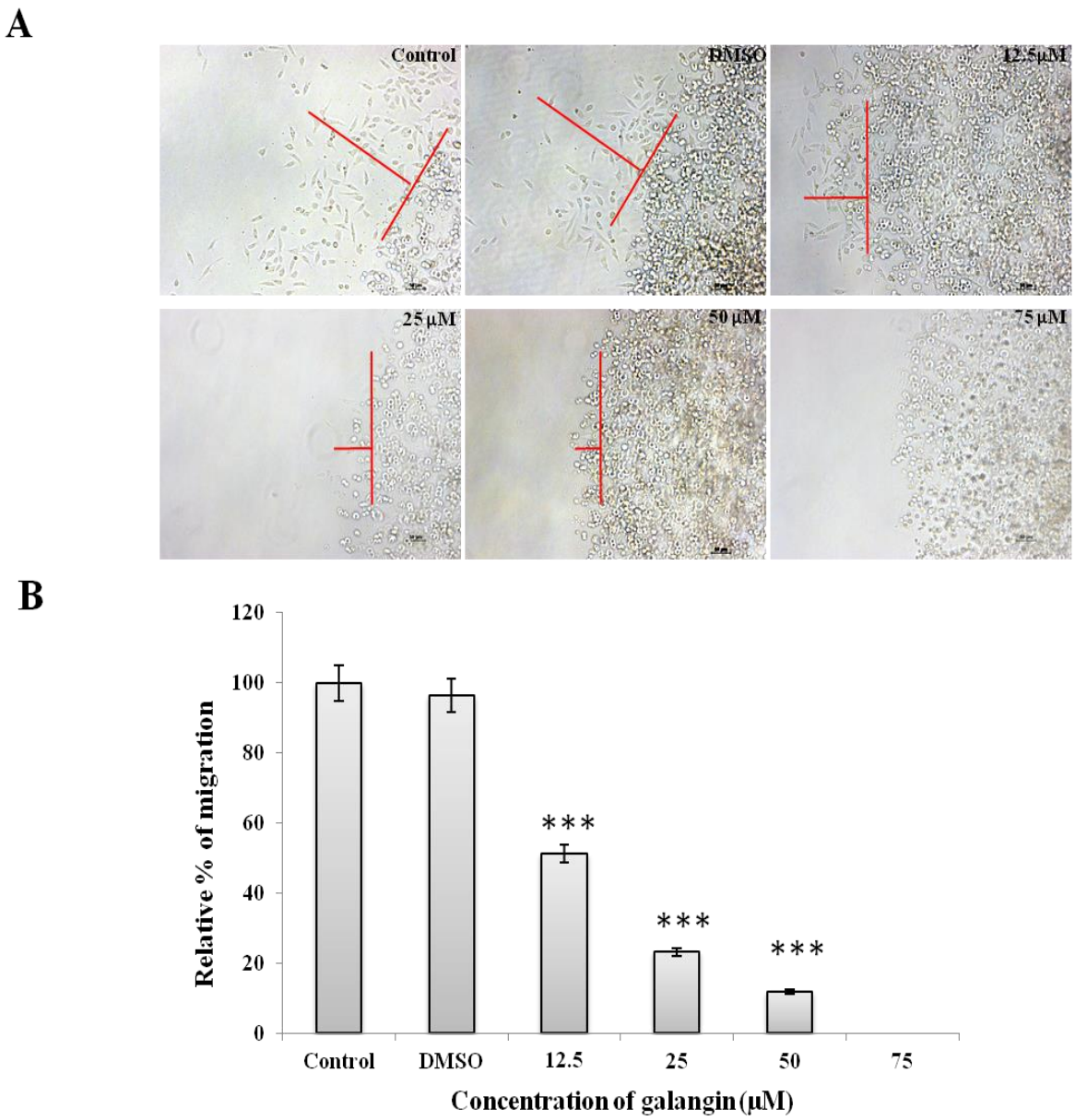

Figure 31: Effect of Galangin (12.5µM - 75µM) (A) Migration of HeLa cells out of agarose microdroplet (B) Depicting % migration of HeLa cells (***P< 0.001, with respect to control).

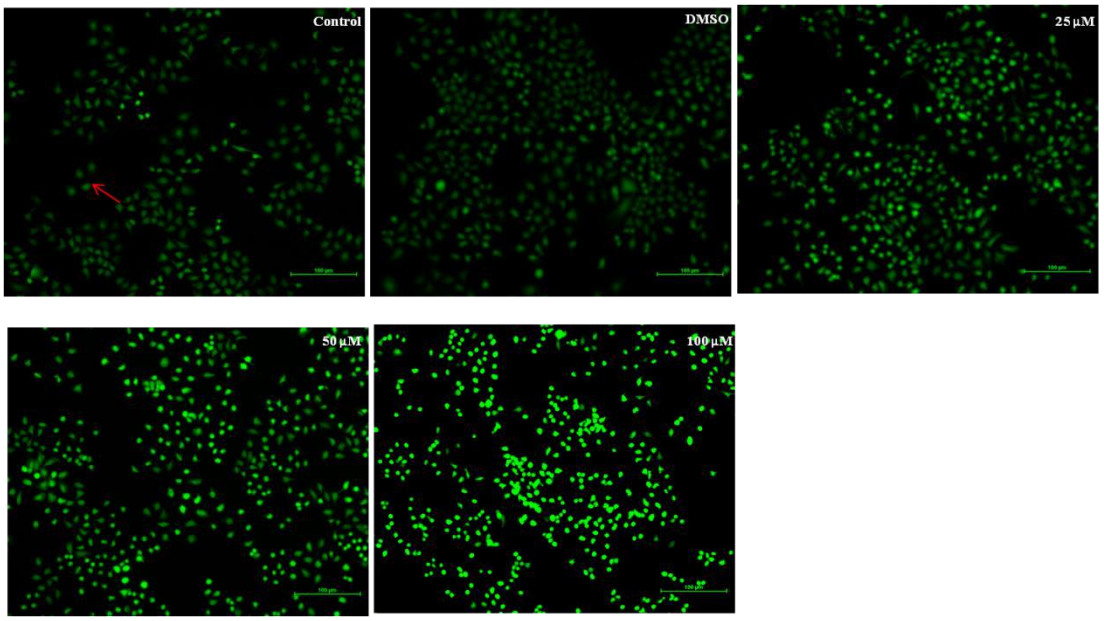

Figure 32: Effect of different concentration of Galangin on ROS level in HeLa cells after 4h of treatment. Fluorescence intensity of dye increased with increasing concentration of Galangin.

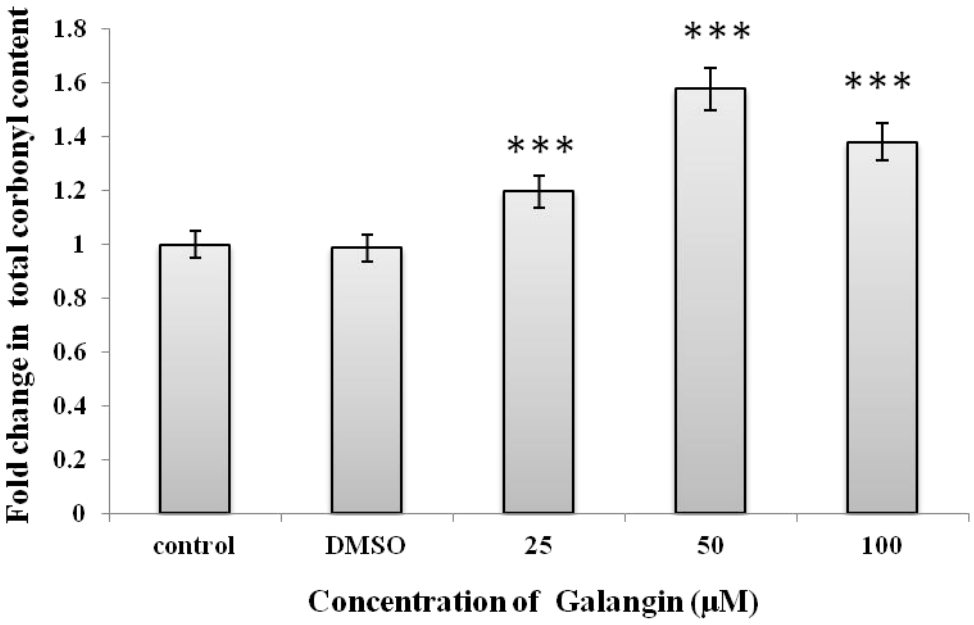

Figure 33: Effect of Galangin on total carbonyl content in HeLa cell 24h post treatments. (Data are presented as mean ± S.D. of three separate experiments ***P< 0.001)

intensity of individual cells markedly increased and positively correlated with increased galangin concentration.

6.1.5. Effect on Total carbonyl content

The total carbonyl content (an indicator of oxidation damage) in galangin treated and control cells were measured using DNPH spectrophotometeric methods. Galangin treatment increased the total carbonyl content as a function of dose (**Figure -33)**. Treatment of cells with 50 μM of galangin increased the carbonyl content by about 1.5 fold in comparison to untreated controls cells.

6.1.6. Modulation of Expression of Glyoxalase-1 and Nrf-2 in HeLa cells by Galangin

To understand the molecular mechanism of cell death associated with galangin the level of glyoxalase-1 protein was studied. As can be seen in (**Figure -34)** the galangin treatment decreased the glyoxalase-1enzyme activity in a concentration dependent manner which could be correlated with a significant increase in carbonyl content. Further to understand, glyoxalase-1 regulation, the level of Nrf-2 transcription factor was also checked. The result showed that the galangin decreased the Nrf-2 protein in a dose dependent manner after 24 h of galngin treatment both in western blotting & immunoflurescence studies **(Figure -35)**.

6.1.7. Discussion

Present studies investigated the mechanism/s associated with cell death induced by galangin in cervical cancer cell line (HeLa). The morphological changes in cells clearly indicate that galangin is cytotoxic and induces cell death in a concentration and time dependent manner. Within 24 hrs of treatment, cells undergo apoptosis and show decreased proliferation. This was further confirmed by colony formation assay that showed decrease in size and number of colonies as compared to control. Cytotoxicity of Galangin has been reported in a number of cancer cell lines like HCT-15, HT-29, (human colon cancer cells), B16F10 (melanoma cells) and Caki-1 and 786-0 (renal carcinoma cells) [181-183].The IC-50 value for different cancer cells has been reported between 25 μM to 150 μM [181-186]. In the present case also the IC 50 value for HeLa cells was approximately 50 μM **(Figure -28)**. Galangin has been reported to decrease the antioxidant defence *in vitro* and *in vivo* in murine models by modifying the xenobiotic and

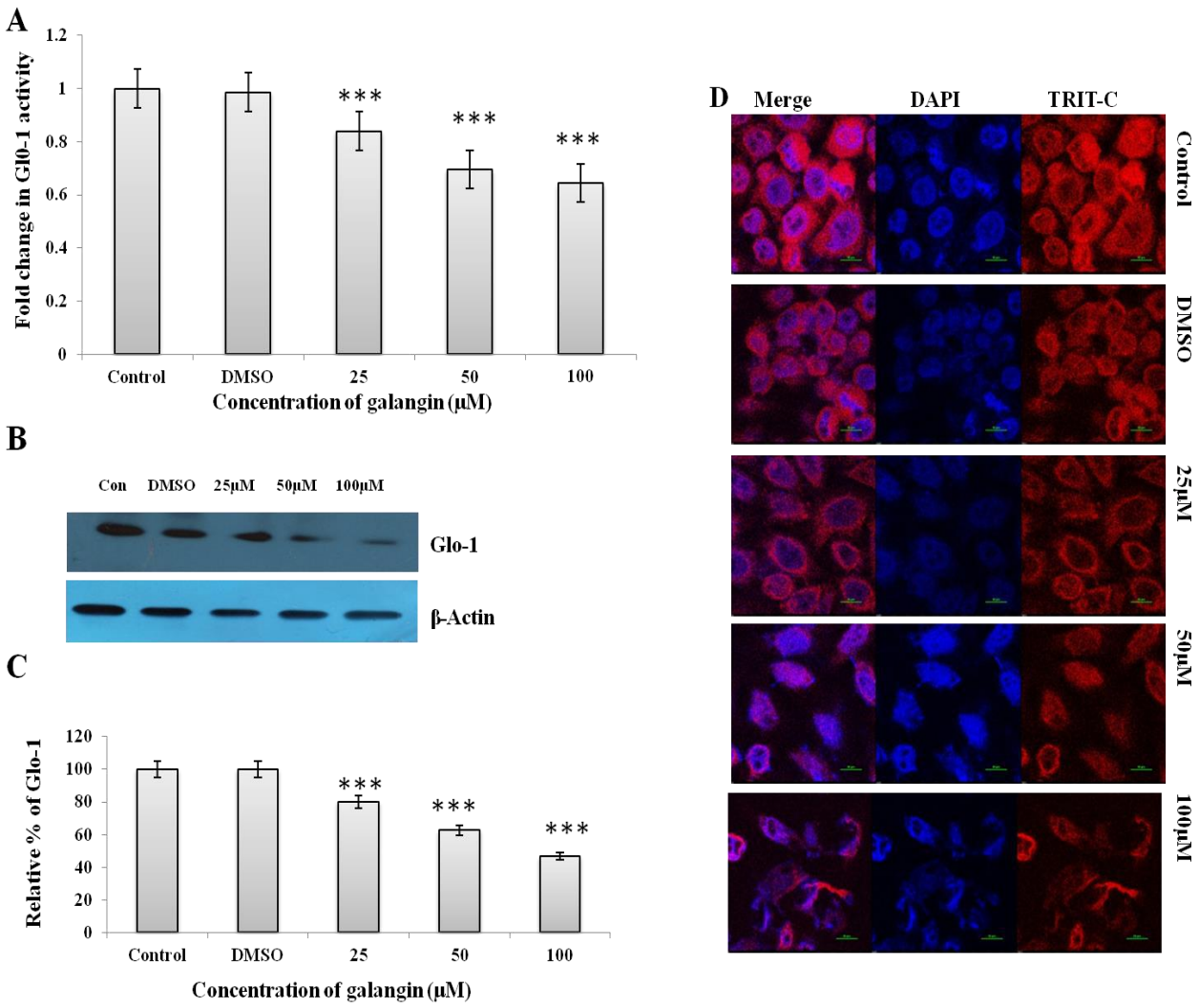

Figure 34: Effect of 24h treatment of Galangin (25µM – 100µM) on Glyoxalase-1 (A) Enzyme activity (B) Protein expression using western blotting (C) % Relative expression level of glyoxalase-1 corresponding loading control β-Actin (D) Immunofluorescence images (Nikon ECLIPSE Ti) at 60X magnification (Data expressed as mean ± SEM of triplicates.***P < 0.001 significant vs. control).

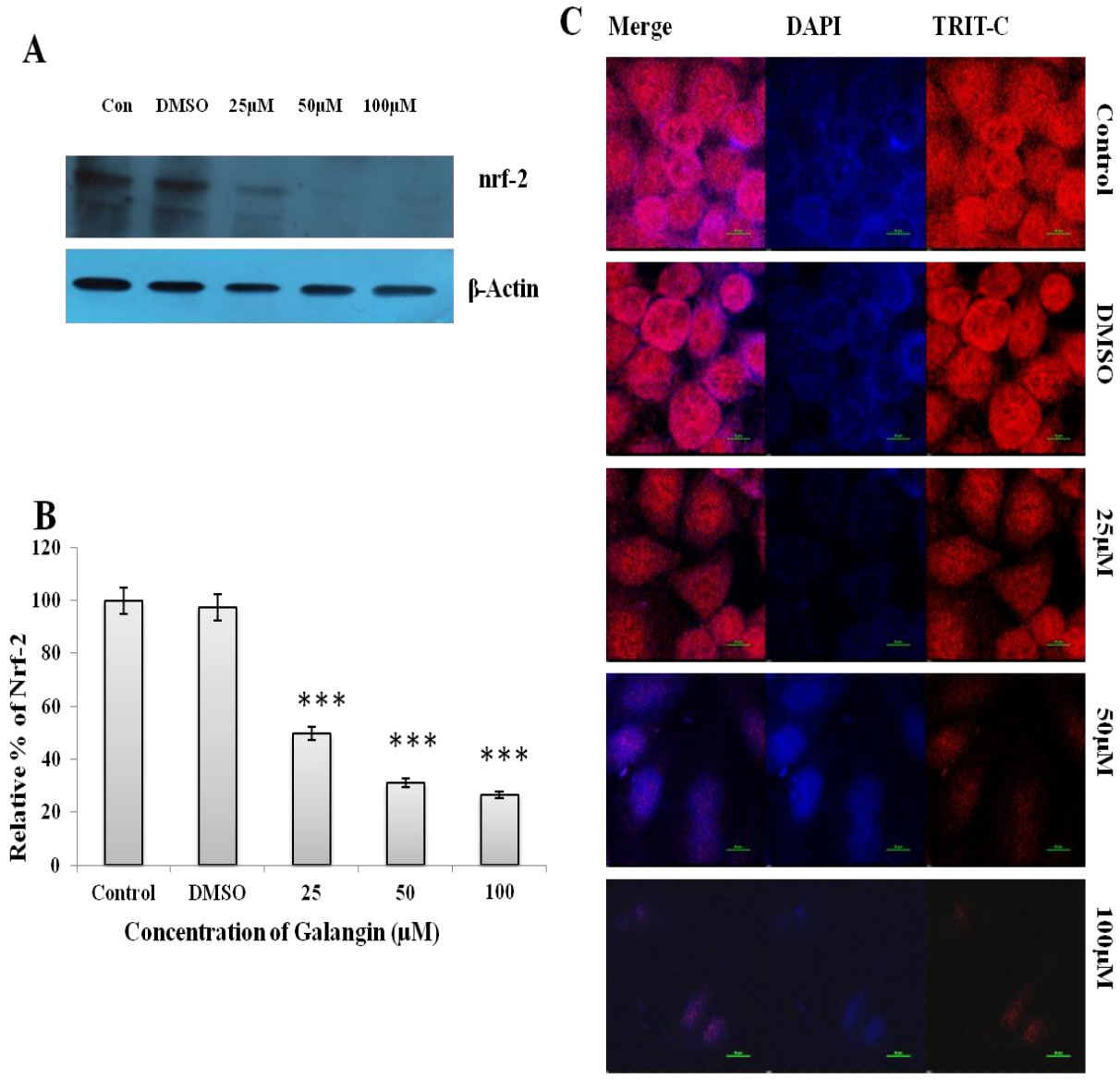

Figure 35: Effect of 24h treatment of Galangin (25µM – 100µM) on Nrf-2 (A) protein expression using western blotting (B) % Relative expression level of Nrf-2 corresponding to loading control β-Actin (C) Immunofluorescence images. (Nikon ECLIPSE Ti) at 60X magnification. (Data expressed as mean ± SEM of triplicates.***P < 0.001 significant vs. control).

anti oxidant enzyme and decreasing the total antioxidant levels resulting in accumulation of intracellular ROS [181-183, 300]. Our results also indicate that the intracellular ROS level is significantly increased in galangin treated HeLa cells **(Figure -32)**. Increase of ROS beyond a threshold results in DNA damage that could result in the activation of cell death via various mechanisms including apoptosis. Galngin could induce apoptosis in Bcr-Abl expressing blood cancer cells (both imatinib mesylate sensitive as well as resistant cells K562 and KCL22) by arresting cell cycle and decreasing pRb, cdk-4, cdk-1 and cycline B levels [34]. Galangin has also been reported to induce apoptosis and cell death in human colon cancer cells and B16F10 melanoma cells by intrinsic mitochondrial pathway through altering mitochondriyal membrane potential and dysfunction by activating the caspase-3 and -9 in a dose dependent manner [182].

In most cancer cells Glyoxalase-1 is highly expressed and inhibition of glyoxalase-1 can selectively induce apoptosis [20]. Galangin treated samples also showed a decrease in Glyoxalase-1 activity. In the present study we further observed that galangin treatment led to an increase in the total carbonyl content indicating an increase in oxidative damage **(Figure -33)**. Total carbonyl content is a general indicator for protein oxidation and is used as a measure of oxidative injury [271]. The decreased glyoxalase-1 activity can result in accumulation of dicarbonyles like methylglyoxal (MG) and other derivatives including AGEs, DNA and protein derivatives resulting in cell death [155, 301-303]. Since Nrf-2 is known to transcriptionally regulate glyoxalse-1, to provide defence against dicarbonyl stress, we also checked the level of the transcription factor Nrf-2. A decrease in the levels of Nrf-2 was observed in protein expression studies that were further confirmed by immunofluorecence studies. Previous studies have also shown that modulators of nrf-2 modulate Glyoxalase-1 expression [155]. Nrf-2 binds at ARE site of GLO-1 gene to regulates the expression of glyoxalase-1 enzyme in response to stress. Besides glyoxalase-1, Nrf-2 also regulates expression of antioxidant enzymes resulting in reduced antioxidant defence. In the present study also we observed a decrease in Nrf-2 as well as Glo-1 levels in galangin treated samples.

From the above discussion it can be speculated that the galangin modulates Nrf-2 levels resulting in decreased expression of glyoxalase-1 leading to decreased detoxification of MG. Increased MG levels can lead to increased levels of oxidative stress resulting in cell death. MG, Glyoxalase-1 & dicarbonyl stress have emerged as the targets of Nrf-2/keep system [155]. Thus Galangin can induce cell death in HeLa cells by multiple mechanisms. Inhibition of Nrf-2 can be linked to decrease in glyoxalase-1 expression &

increased accumulation of MG and oxidative stress in combination with carbonyl stress produced by MG, thus could induce cell death in the HeLa cells.

Besides cell death galangin was able to decrease the metastatic potential of the Hela cells *in-vitro*. Both migratory and invasive potential were found to decrease in a dose and time dependent manner. A significant inhibition of Hela cell migration was observed *in vitro* **(Figure - 30 & 31)**. Galangin has been reported to efficiently inhibit TPA-induced invasion and migration of HepG2 cells through a protein kinase C/extracellular signal-regulated kinase (PKC/ERK) pathway [184]. Galangin and kaempferol have also beeen reported to play a role in decreasing invasive potential by decreasing MMP-9 expression by blocking activation of NF-κB and AP-1 [185]. Knockdown of GLO1 is associated with reduced tumor-associated properties such as migration and proliferation in cancer cells [294]. On the basis of the present studies, it can be inferred that galangin induced inhibition of metastatic potential can be correlated with the decrease in the glyoxalase-1 enzyme.

6.2. Naringenin:

6.2.1. Modulatory potential of Naringenin on DMBA+ Croton oil induced Papillomagenesis in BALB/c mice

Naringinin consumption for a period of 16 week did not shows any health effects like changes in weight gain/loss pattern or mortality, suggesting that there was no toxic effect of naringinin on long-term consumption **(Table-6.1)**. There was no evidence of developments of spontaneous papillomas observed in negative control and vehicle control groups of mice. Whereas DMBA plus croton oil treated positive control group showed 100% incidence of papillomas. Naringinin treatment reduced papillomas incidence particularly at higher doses. It is evident from the animals of pre-treatments group 100 mg/kg of naringinin, was able to decrease papilloma incidence by 20% **(Table 6.1 & Figure -36).**

Table - 6.1 Chemopreventive effects of Naringinin on DMBA induced skin cancer

Group	Body weight		PN	TI
	Initial	Final		
Control	34 + 2.16	46 + 2.7 0	----	----
Vehicle control	32.4 + 2.64	43.8 + 1.70	----	----
DMBA- control	31.9 + 1.88	46.2 + 2.5	17	100%
Pre-treatment 50mg/kg	31.5 + 2.75	43.2 + 2.21	3.2	100%
Pre-treatment 100mg/kg	32.1 + 1.82	46 + 2.70	2.25	80%
Post-treatment 50mg/kg	32.3 + 4.75	43 + 2.58	9.5	100%
Post-treatment 100mg/kg	31.5 + 2.75	45 + 2.87	10.8	100%

Values are shown as mean ± SD of 5-6 animals. PN= Average no of papilloma per mouse. TI= % of Tumour Incidence.

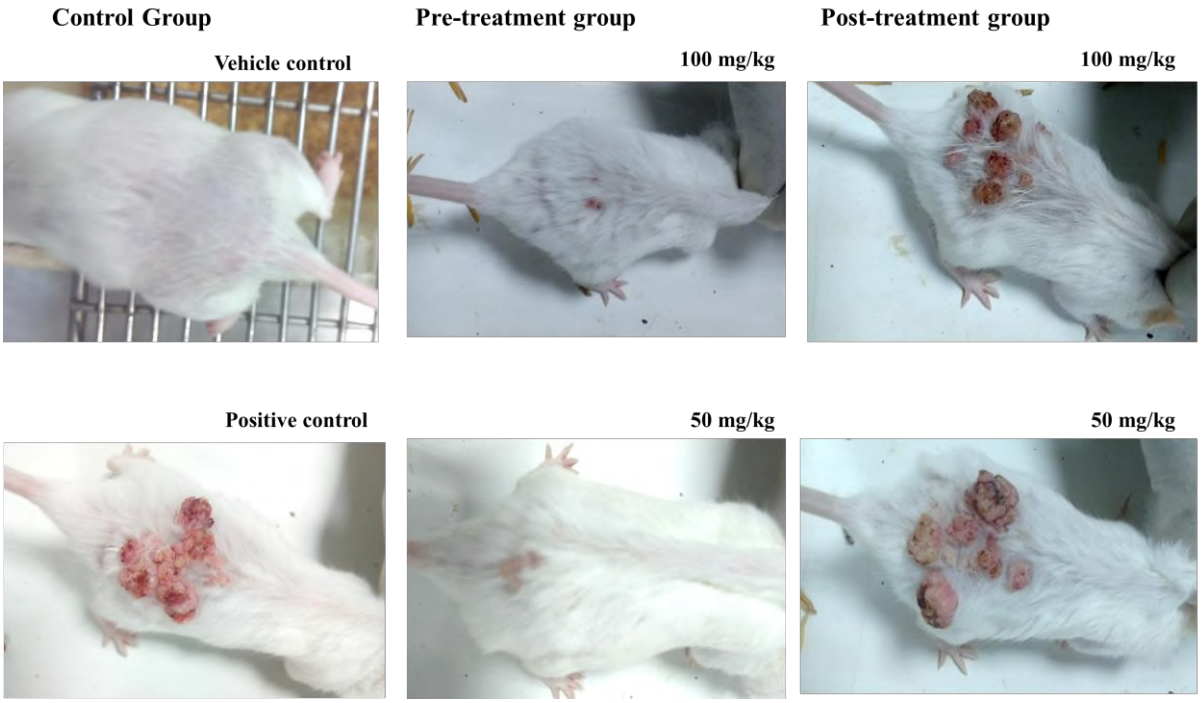

Figure 36: Images showing tumour in different groups of mice after 16 weeks of DMBA + Croton oil application.

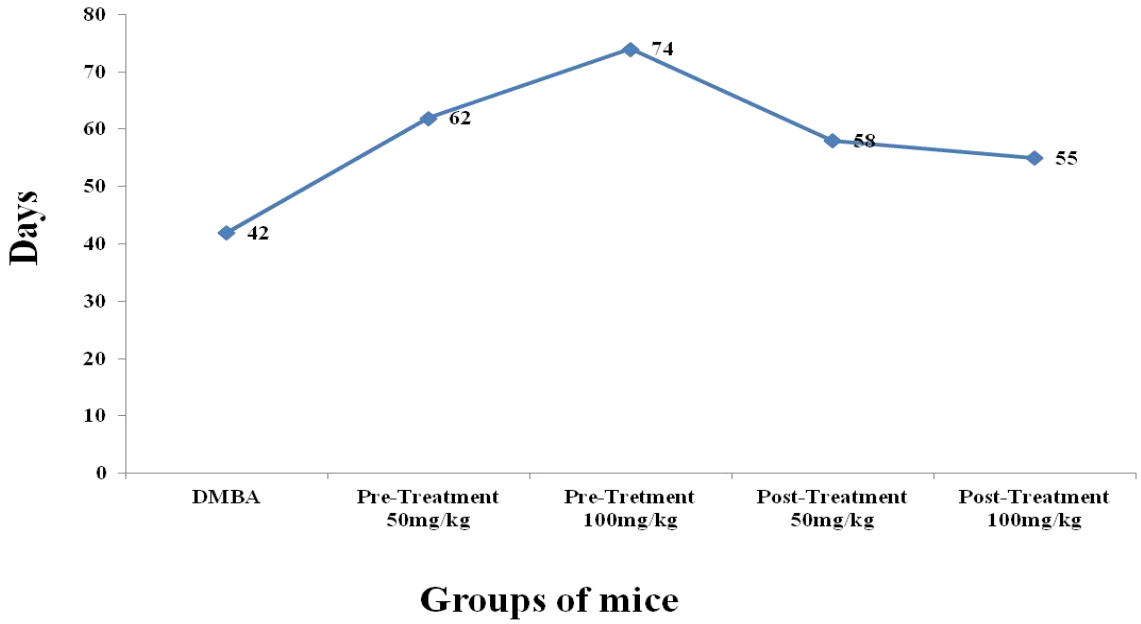

Figure 37: Effect of Naringenin treatment on papilloma latency in days in different groups of mice. Number of animals per group: 6.

The naringinin treatments increase papillomas latency. The latency of papillomas in treatments groups was found to be increased in both pre-treatment & post treatments groups **(Figure- 37)**. It was observed that pre-treatment of naringinin increase the latancy more as compared to post treatments. The papilomas were observed in pre-treatment at 74 day whereas in post treatments groups it was on 55 days at higher dose 100 mg/kg.

Naringinn treatments very effectively reduced the size of papilloms. The naringinin treatments reduced the size of papillomas in both pre-treatment and post treatments groups as compared to positive control groups. The pre-treatment of naringinin was observed to be more effective as compared to post treatments **(Figure -38)**. The average number of papillomas was found to be reduced in post treatment group & further reduced in pre-treatment groups in a dose dependent manner in comparison to positive control group. The papillomas number observed was low in pre-treatment group with respect to positive control group and post treatment group **(Table-6.1 & Figure -38)**.

6.2.2. Carbonyl and oxidative stress in tumour tissues

Naringinin treatments was found to reduce the glyoxalase-1 activity in skin tumour tissues. The glyoxalase-1 activity was found to be 3 fold higher in papilomas tissues as compared to normal tissues whereas in pre-treatment and post treatments groups the activity was found low in comparison to positive control **(Figure -39)**. The total carbonyl content was also found to be less in DMBA treated positive control skin tissues as compared to naringenin treated and negative control groups **(Figure -40)**. The level of peroxidise enzyme was not significantly affected by naringinin treatment but catalase activity was reduced in naringinin treated groups of mice tissues **(Figure -41 A & B)**.

6.2.3. Histopathological studies of skin papillomagenesis

Animals without any treatment and treated with only acetone showed normal histological characteristic of epidermis (E) and dermal (D) layers **(Figure -42)**. In DMBA plus croton oil treated positive control group, skin was showing papillomas arising from the epidermal surface. The basal layers were extending into the dermis. The loss of normal stratification of the epidermal cells was seen in positive control and treatment group. The epithelial cells were seen pushing into the adjacent dermis **(Figure -42)**. The histopathological analysis in naringenin treated groups, showed a significant reduction in pathological

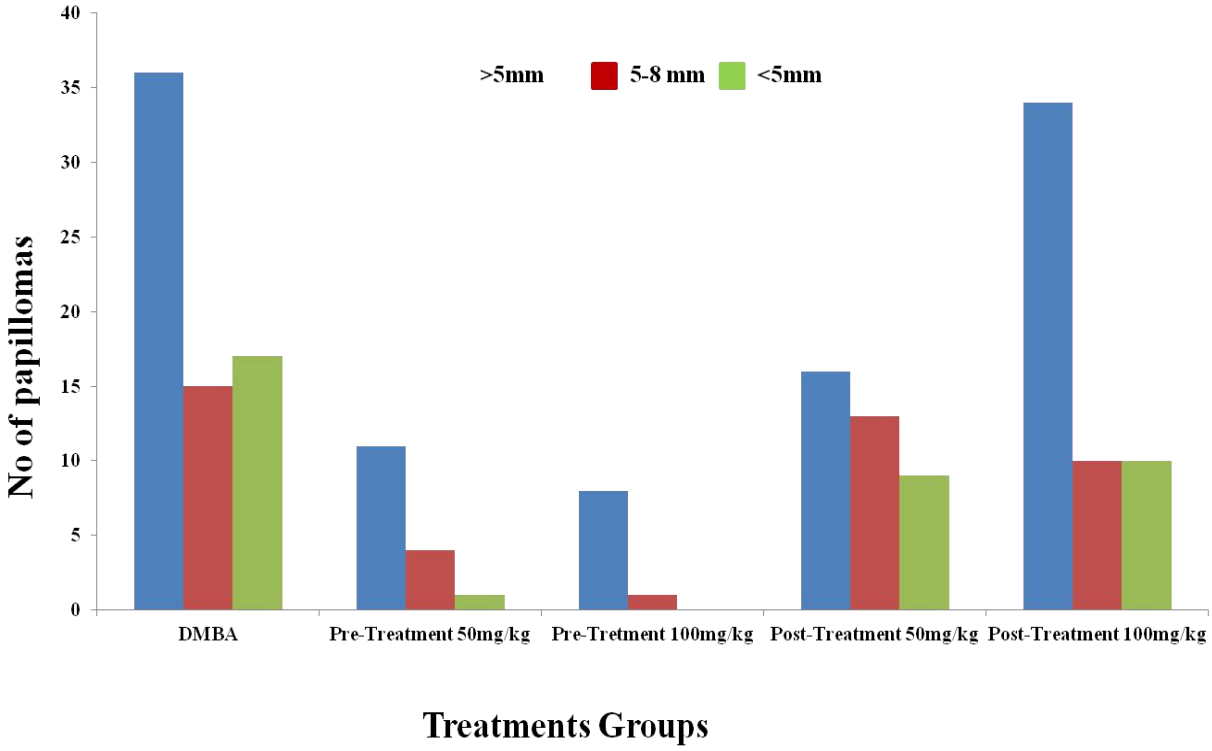

Figure 38: Change in number of different tumour size range in mice treated with different dose of Naringenin. Number of animals per group: 6.

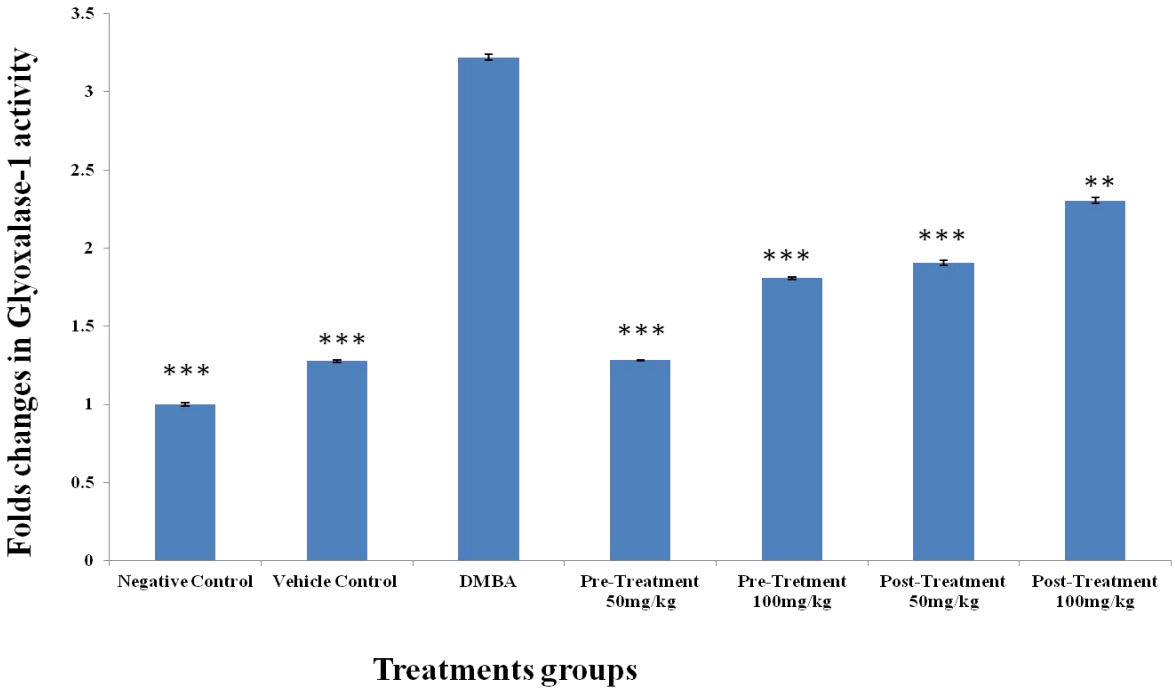

Figure 39: Glyoxalase-1 activity in tumour tissues of different group of mice. (Data are presented as mean ± S.D. of three replicate ***P < 0.001 and **<0.01 with respect to DMBA treated positive control)

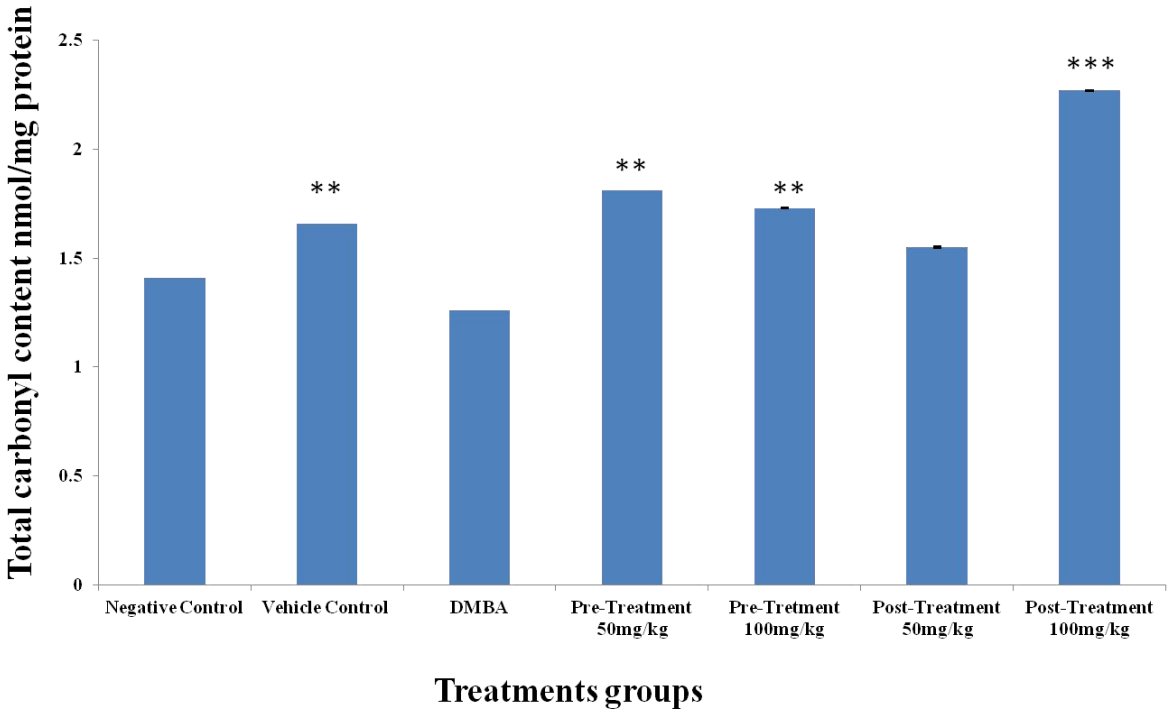

Figure 40: Total carbonyl content in tumour tissue of different group of mice. (Data are presented as mean ± S.D. of three replicate ***P < 0.001 and **<0.01 with respect to DMBA treated positive control)

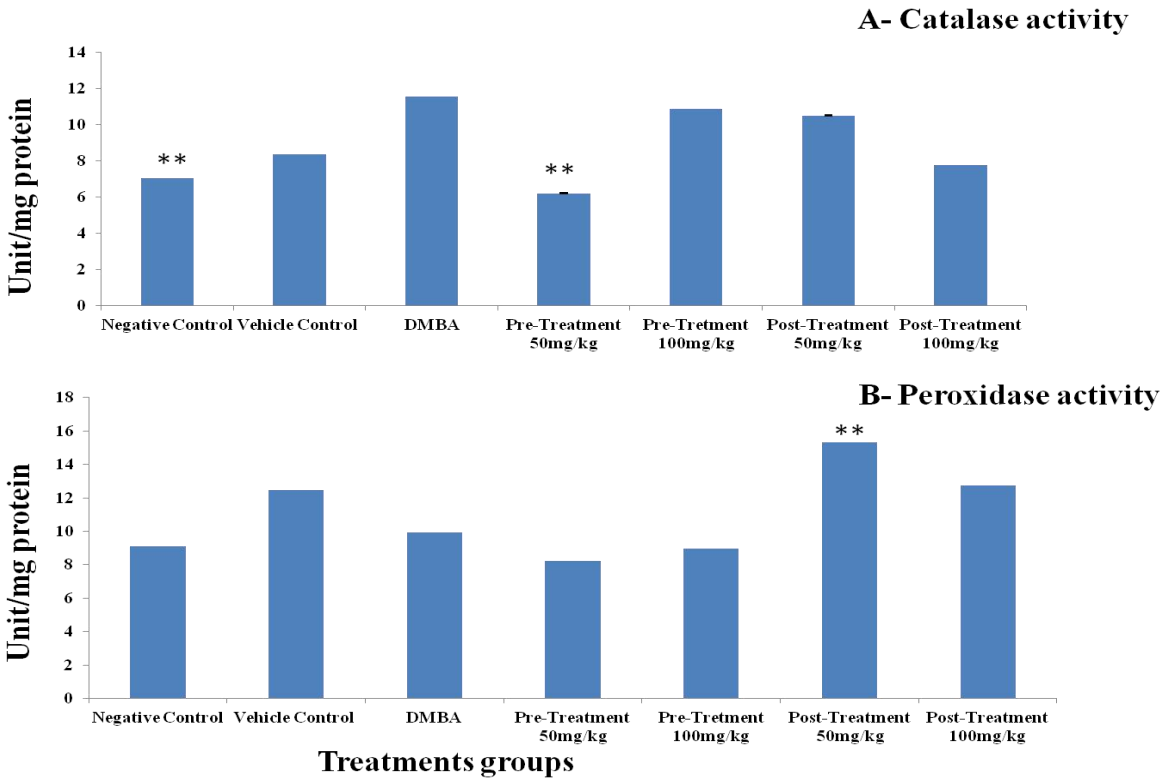

Figure 41: The oxidative enzymatic activity in different group of mice. (Data are presented as mean ± S.D. of three replicate **P < 0.01 with respect to DMBA treated

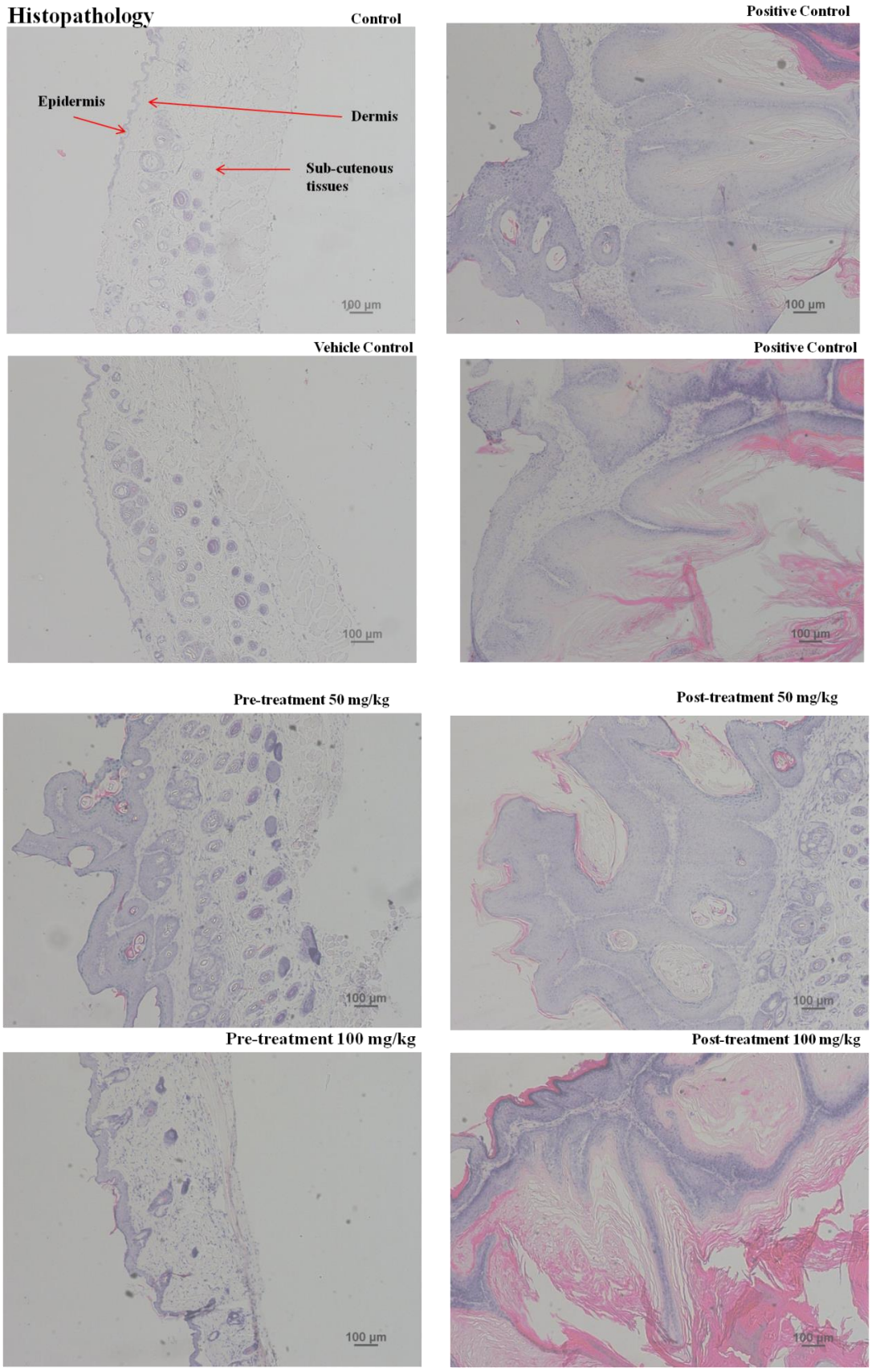

Figure 42: Hematoxylin & Eosin stained section of all groups of mice skin (Normal and tumor bearing mice) treated with naringenin at 5X magnification.

features of papillomas such as branching and layering. In the anti-initiation groups, the loss of stratification was reduced in 50mg/kg dose of the treated group whereas 100mg/kg of the dose treated group did not show any loss of stratification. However, in anti-promotion groups minimal loss of stratification was observed **(Figure -42)**. Low-dose treated groups had minimal loss of basal aspect of epidermis and there was little dermal infiltration. However, with higher doses, the basal aspect of epidermis was smooth and there was not any dermal infiltration **(Figure -42)**. Therefore, the higher dose was showing better chemoprevention. There were apparent nuclear differences, such as enlargement, hyperchromasia only in carcinogen treated group as compared to phytochemical treated groups **(Table 6.2 & Figure -42)**.

Table- 6.2 Histopathological analysis of the skin of tumour bearing Mice

Group	Histopathological Finding					
	Papillomas		Dermal	Loss of	Nuclear Changes	
	Branching	Layers	infiltration	stratification	Enlargment	Hyperchromasia
Control	----	2-3	---	---	---	---
Vehicle control	----	2-3	---	---	---	---
DMBA- control	+++	8-9	+++	+++	+++	+++
Pre-treatment 50mg/kg	+++	5-7	+	+/-	+++	+++
Pre-treatment 100mg/kg	+	3-4	-	---	+	+
Post-treatment 50mg/kg	+++	8-9	+++	+++	+++	+++
Post-treatment 100mg/kg	+++	8-9	+++	+++	+++	+++

6.2.4. Discussion

The food rich in fruits and vegetables have large amounts of antioxidant components like polyphenols that reduced the risk of cancer [304, 305]. In the present study we found that naringenin inhibits the carcinogenesis in pre-treatment as well as promotion stage. Many phytochemicals from natural sources have shown papilomas suppressing effects [272]. Naringenin has been reported to modulate the expression of enzyme involving DNA repairs [266]. Naringenin has also been reported to protect against oxidative stress and inflammation [266]. The pathway activated by Nrf-2-ARE has been found to be modulated by naringenin.

In positive control group the carbonyl content in the tumour tissue was found to be decreased as compared to the treatment and negative control group. The increased activity of glyoxalase-1 enzyme is responsible for carbonyl stress. There was decreased

glyoxalase-1 activity in treatment groups as compared to positive control groups. Phytochemical have been reported to decrease the glyoxalase-1 activity and increase the carbonyl contents in cancer cells [201].

Many phytochemical from natural sources have been shown to have papilloms suppressing effect against skin cancer developments [306]. Naringenin has been reported to reduce the DMBA induced oral carcinogenicity in hamsters [216, 263] and carcinogenesis induced by the N nitrosodiethylamine in mice [246]. Recent research supported that naringenin shows anticancer activity against different types of cancer including liver, stomach, prostate, lung, breast, colon, and other sites [216, 245, 260]. Papilloma suppression has also been linked to anti-inflammatory effects of phytochemicals. Narengenin has been reported to works as an antioxidant [238, 239]. Naringinin treatment groups showed the reduction in pathological features of papilloma such as branching and layering. This was due to the anti proliferative properties of naringinin [307]. Nuclear differences, such as enlargement, hyperchromatroa etc were observed in positive control & treatments groups. Naringinin has been reported to protect against the DNA damage *in vitro* & *in vivo* [266].

The present result suggested that naringenin shows chemo preventive effect against skin cancer which was reflected as decrease in papillomas number, incidence, latency, number & size, which was supported by histopathological study and biochemical analysis of skin tumour tissues. From this *in vivo* data it can be inferred that naringenin might be valuable as an antitumor & chemo preventive phytochemical for DMBA induced chemical carcinogenesis.

6.3 *In vitro* & *in vivo* effect of Naringenin on B16F10 cells & tumor xenograft in C57BL/6 mice

6.3.1. Effect on cells survival, proliferation and apoptosis

The naringenin treatment for 24 hours induced changes in morphology of B16F10 cells. The treated cells showed cell shrinkage, spindle shape, detachment, and spike formation. The extent of morphological changes and number of cells was found to be concentration dependent **(Figure -43)**.Naringenin inhibits the proliferation of B16F10 cells and the IC50 value of naringinen was observed to be approximately 200 µg/ml. At 200µg/ml about 50% cells survived whereas at 300µg/ml only 10% cells survival was observed **(Figure - 44 A)**.The cell survival was further evaluated by colonogenic assay. The colonogenic

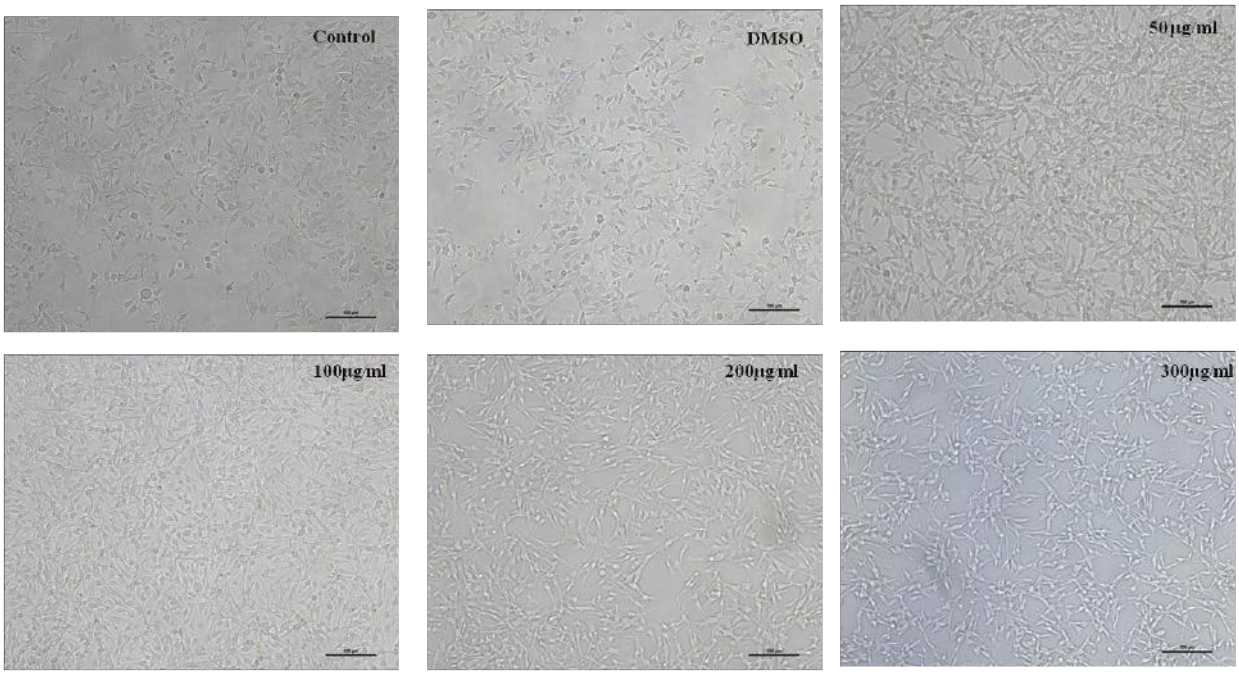

Figure 43: Morphological changes induced by Naringenin in B16F10 cells at different concentrations, morphological such as spike formation, detachment, floating cells in medium 24h after treatment (Images were captured by Nikon inverted microscope at 10X magnification).

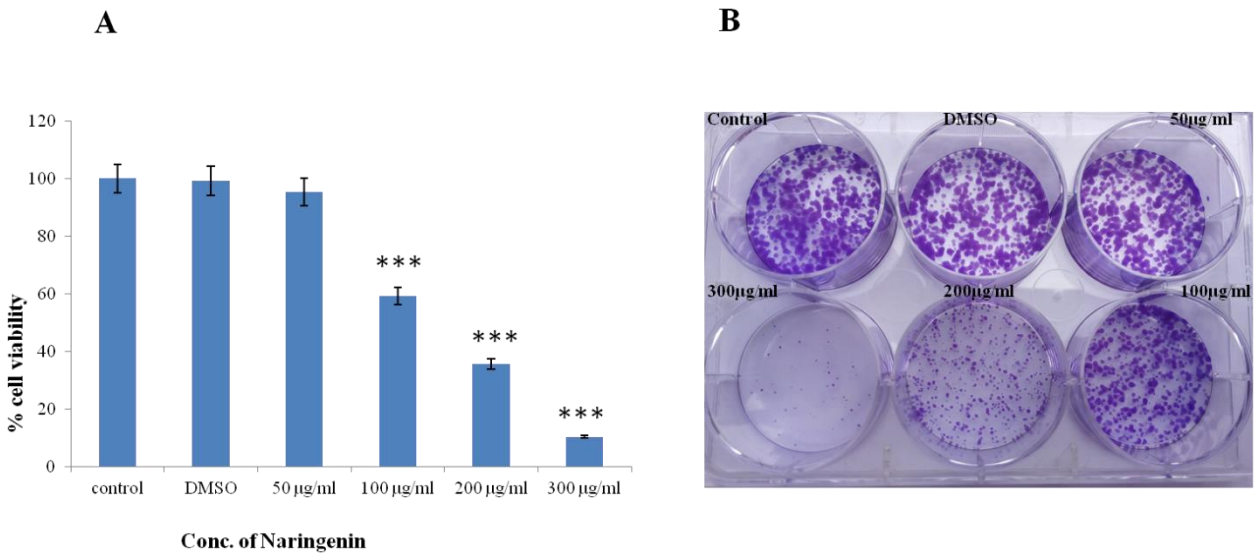

Figure 44: Effect of Naringenin on B16F10 cells A) Cell proliferation by MTT assay B) Colonogenic assay (Data are presented as mean ± S.D. of three separate experiments ***P< 0.001 with respect to control).

assay showed that naringenin significantly reduced the number and size of colonies in a dose dependent manner **(Figure -44 B)**.

The EtBr/AO staining showed that naringenin treatment for 24 hours induced apoptosis in concentration dependent manner in B16F10 cells. The number of apoptotic and dead cells increased significantly in naringenin treated cells **(Figure -45 A)**. The apopototic cell death was further confirmed by annexinV/PI assay **(Figure -45 B)**. Naringenin treatment also affects the intracellular ROS level in B16F10 cells as compared to untreated controls, as measured by fluorescence microscopy. From the fluorescence images of cells it is clear that there is an increased intracellular fluorescence in samples with increasing naringenin concentration **(Figure -46)**. The naringenin treatment alters the potential of mitochondrial membrane which was found to be dose dependent in B16F10 cells. The fluorescence images showed that there is a decrease in red aggregates in naringenin treated sample and increase in green florescence in a dose dependent manner **(Figure -47 A)**. The apoptosis associated protein caspsase-3 was observed in the total cellular protein to confirm the apoptosis. The western blotting result clearly show that there was increased level of caspase-3 in naringenin treated cells **(Figure -47 B)**.To understand the molecular mechanism of cell death associated with naringenin the cytochrome-C in cytosolic fraction was studied by western blotting which was found to be increased in treated sample as compared to untreated control sample **(Figure -47 C)**.

6.3.2. Effect on metastatic potential of B16F10 cells *in vitro*

Naringenin at a concentration of 50-300 µg/ml clearly showed a dose dependent anti-migratory effect as seen in wound healing assays in B16F10 cells **(Figure -48 A)**. The untreated controls showed 50% migration after 24 hours, however in cells treated with 200 µg/ml of naringenin, migration was completely inhibited. The inhibition of metastasis by naringenin was further confirmed by spheroid formation assay which showed that there is a dose dependent effect on inhibition of spheroid formation **(Figure -49)**. The spheroid formation was found to be decreased at 100 µg/ml which was completely inhibited at 200 µg/ml. Many molecular mechanisms may be responsible for inhibition of metastasis. The naringenin was found to inhibit MMP-9 in a dose dependent manner **(Figure -48 B)** a protein involved in metastasis.

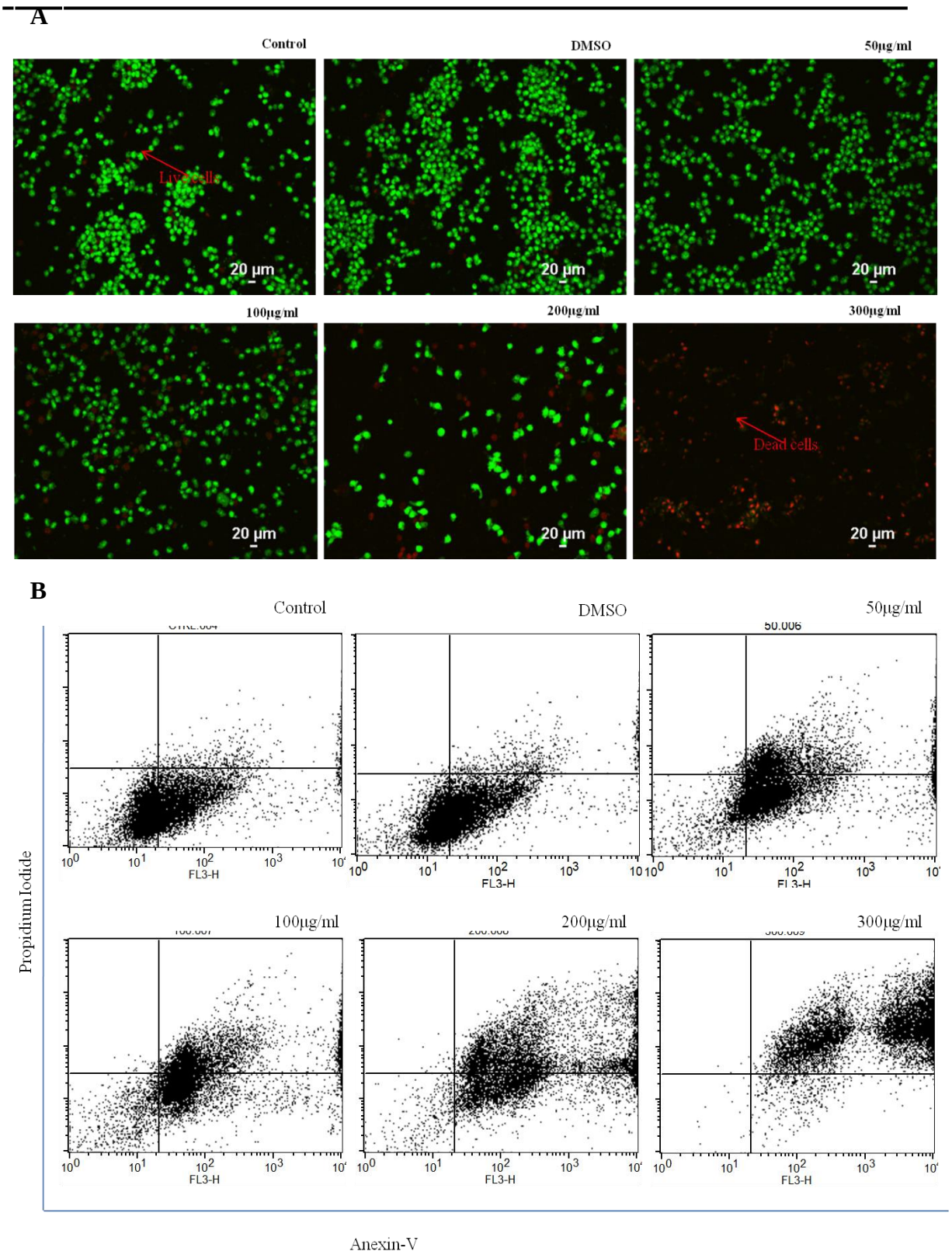

Figure 45: Naringenin induced apoptosis in B16F10 cells 24h after treatment A) EtBr/AO staining .Images were analysed by NIS elements Nikon analysis software. Green, orange and red fluorescence represents live, apoptotic and dead cells respectively. **B) Annexin-V/PI staining by FACS assay.**

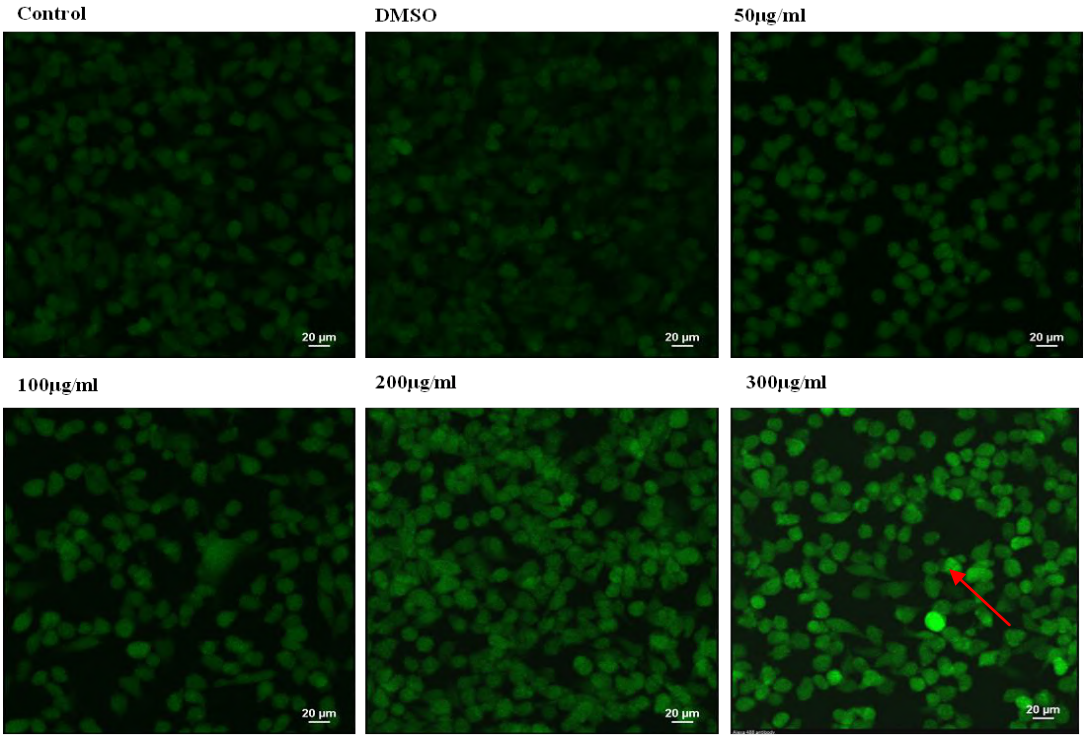

Figure 46: Effect of different concentration of Naringenin on ROS level in B16F10 cells after 4h of treatment. Fluorescence intensity of dye increased with increasing concentration of Naringenin (indicated by arrows).

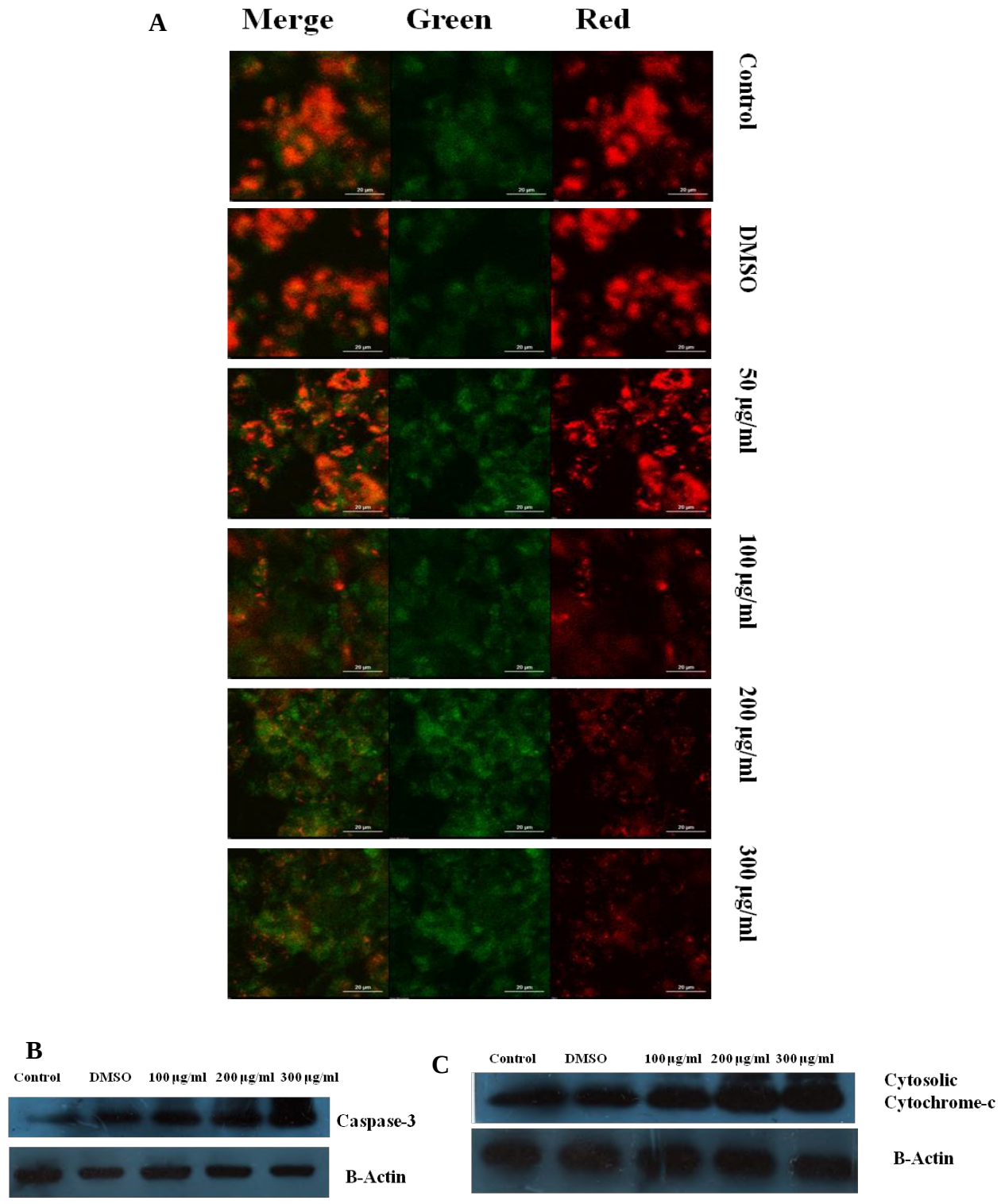

Figure 47: Effect of Naringenin on B16F10 cells 24 hrs post treatment. A) Mitochondrial membrane potential changes by staining with JC-1 dye. Increase the expression of apoptosis related proteins B) Western blotting of Caspase-3 in whole cell protein C) Cytochrome-C in cytosolic fraction of cells.

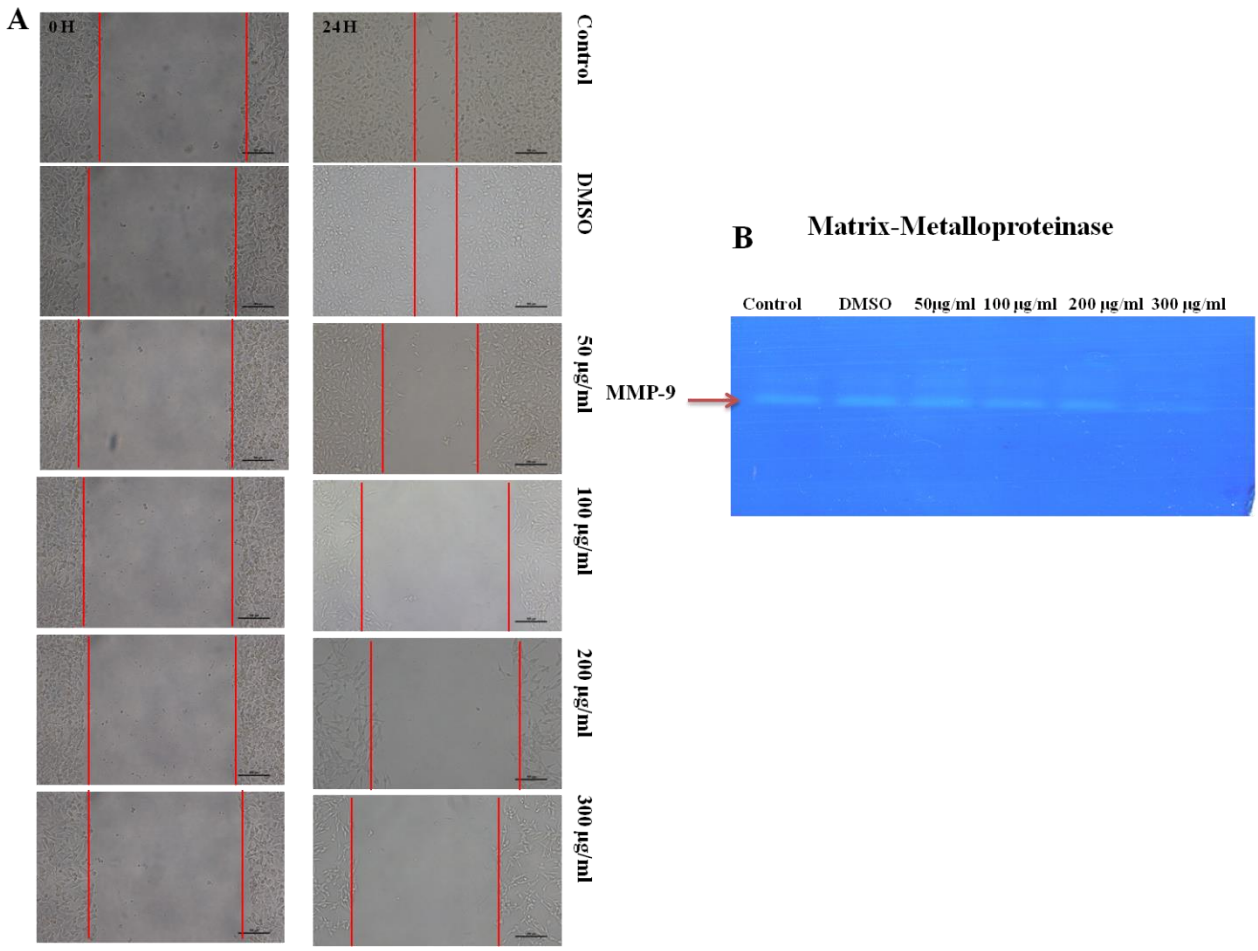

Figure 48: Naringenin inhibits metastatic potential of B16F10 cell A) Wound healing assay B) Gelatin zymography

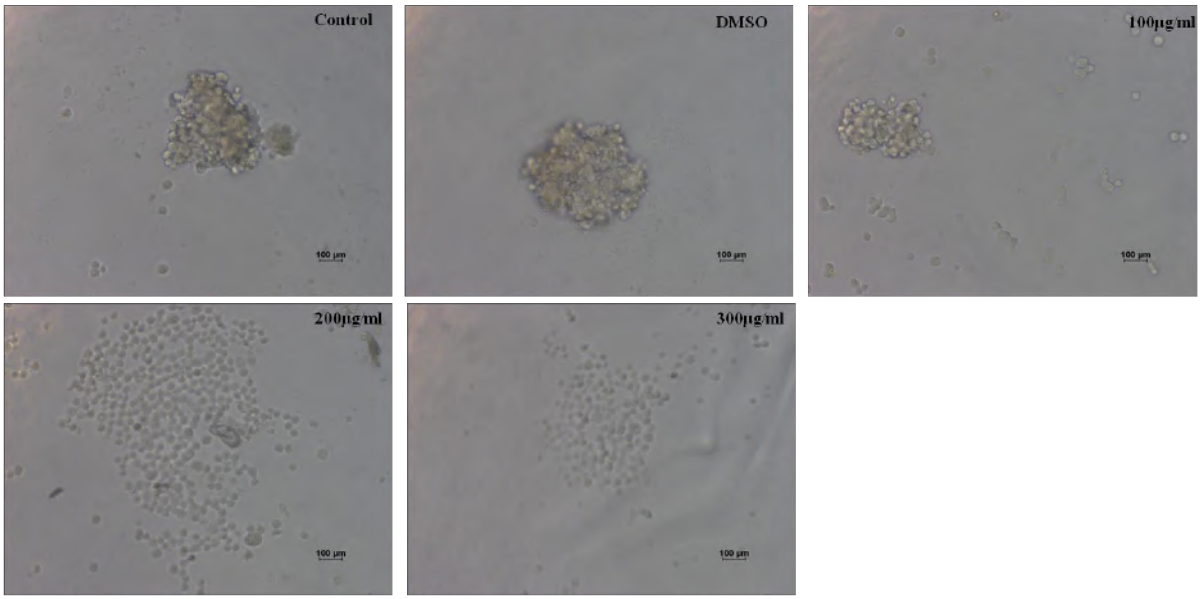

Figure 49: Naringenin treatment inhibits spheroid formation in B16F10 cells.

6.3.3. Effect on growth of tumour in xenograft mice model

Naringenin treatment at 100 mg/kg significantly decreases the xenograft tumour size 50% and volume 40% in treatment group as compared to positive control group **(Figure -50 & Figure -51)**. In addition the size and weight was significantly more reduced in pre-treatment group as compared to post treatment group.

6.3.4. Discussion

Present studies investigated the mechanism associated with cell death induced by naringenin in mice skin melanoma cancer cells (B16F10) *in vitro* and C57BL-6 mice *in vivo*. The *in vitro* data showed that naringenin induced morphological changes **(Figure -43)** in cells which clearly showed that naringenin is cytotoxic and induces cell death in a dose and time dependent manner. Within 24 hrs of treatment, cells shows signs of apoptosis and decreased cell proliferation. Which was further confirmed by colonogenic assay.

Cytotoxicity of naringenin has been studied in a number of cancer cell lines like Hep G2, MA 104 and A 549 and have IC50 approximate >1 mM which suggest that toxicity of the naringenin can vary. The IC_{50} value of naringenin was found 2.2, 7.7, and 33.4 μM for C32, LNCaP, and COR-L23 cell lines respectively [217]. In the present case the IC 50 value for B16F10 cells was approximate 200 μg/ml **(Figure -44 A)**.

Naringenin has been found to have anticancer activity in a number of cell *in vitro* stomach (KATOIII, MKN-7), liver (HepG2, Hep3B, Huh7), leukemia (HL-60, NALM-6, Jurkat, U937), breast (MCF-7, MDA-MB-231), cervix (Hela, Hela-TG), pancreas (PK-1), and colon (Caco-2), [245]. Naringenin has been found to decrease the total antioxidant levels resulting in accumulation of intracellular ROS. We also found the naringenin induced the ROS in B16F10 cell in a dose dependent manner **(Figure -46)**. The increased levels of ROS modulate to the mitochondrial membrane permeability in B16F10 cells. Our data confer already published reports. The altering mitochondrial membrane potential alters the permeability of mitochondria resulting in release of cytochrome-C to cytosol. Increased ROS beyond a threshold level induce DNA damage and could result in activation of cell death via various mechanisms including apoptosis.

Naringenin has been reported to induce apoptosis and cell death in human cancer cells lines by various mechanisms. Narengenin activated apoptosis and blocked cell cycle in the liver carcinoma cells (Hep G2) by altering the expression of Bax and p53 [246] and

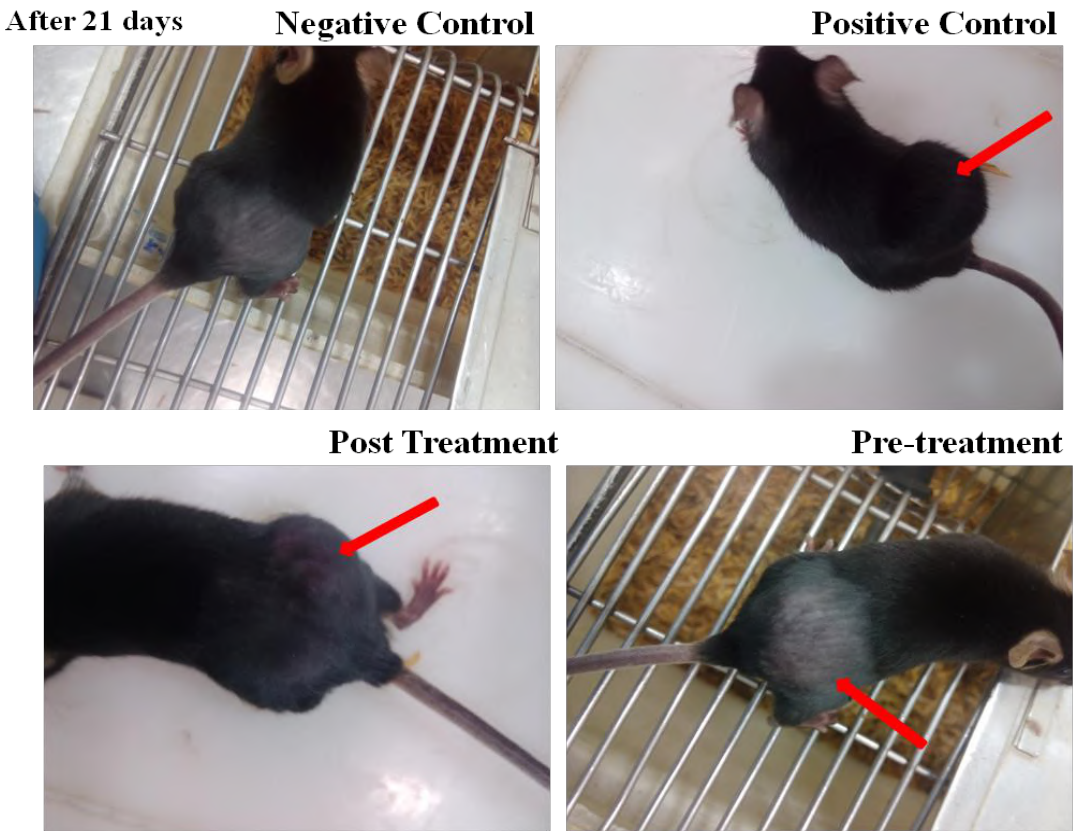

Figure 50: Effect of Naringenin treatment on B16-F10 cells tumor xenograft growth in C57BL6 mice.

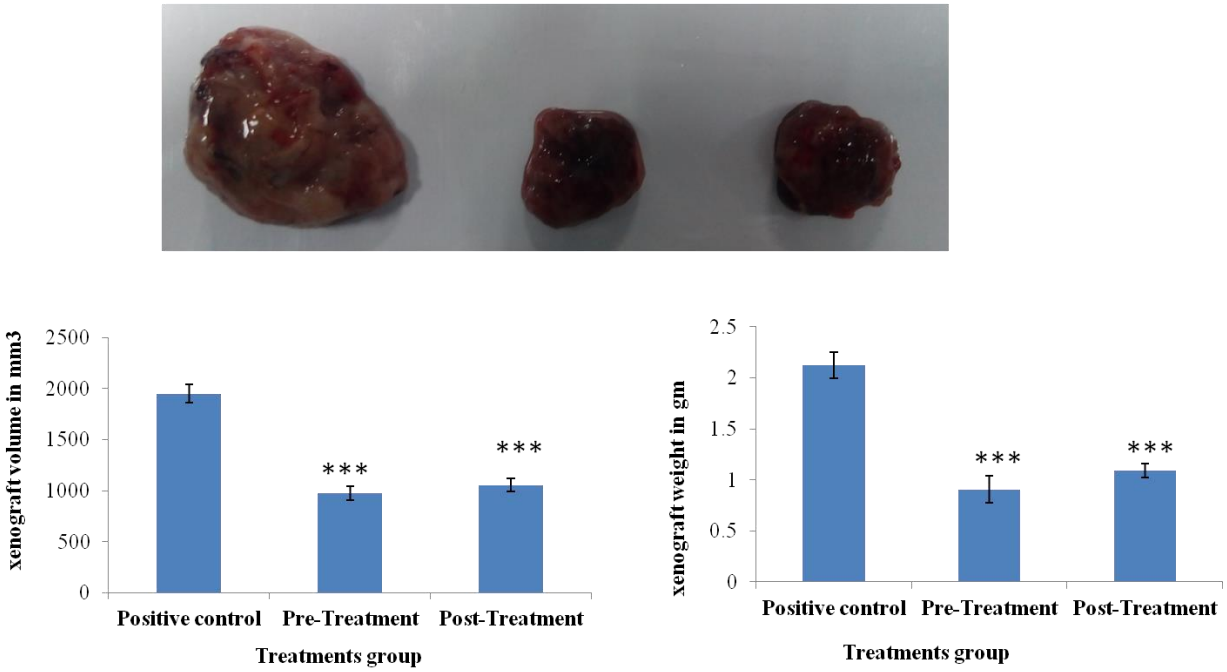

Figure 51: B16F10 cells tumor xenograft growth (volume and weight) inhibition upon oral gavages pre and post treatment, with Naringenin at 100 mg/kg body weight.

increased TRAIL dependent apoptosis in human lung cancer cells A549 [247].Naringenin induced apoptosis in the gastric cancer cells AGS by decreasing the expression of β catenin/Tcf pathway, which play an important role in carcinogenesis [248]. Narenginin enhanced the cell death in human pro myelocytic leukemia HL-60 cells by increasing the expression of NF- kB which subsequently caused the intracellular ATP depletion and mitochondrial dysfunction [249]. Our data shows that narenginin induced cell death in the B16F10 melanoma cells in time and dose dependent manner. As shown by EtBr/AO assay the number of apoptotic and dead cell increased in naringenin treated cell as compared to control **(Figure -45 A)**. The results were also confirmed by FACS using annexin V/PI staining **(Figure -45 B)**. Our result also shows that naringinin activates the intrinsic apoptotic pathway in B16F10 cancer cell by activating the ROS mediated caspase-3 activation.

In vivo toxicity data, showed that medium lethal dose LD50 of naringenin for mice and rat is >5000 mg/kg [218]. Narengenin was found to decrease the no of metastatic tumor cells in lungs and extended the life span of the tumor resected mice by immunosuppressing the regulatory T cells [255]. Oral administration of Narengenin for 5 days in mice reduced the size of implanted sarcomas-180 in *in vivo* [245]. Naringenin at the dose rate of 50 mg/kg inhibited the proliferation of C6 glioma cells in brain of rat by decreasing the expression of PK-C, nf-Kb, cycline D1 and CDK4 [254]. The cumulative results of *In vitro* and *in vivo* studies show that naringenin selectively arrested the cell cycle and induced cell death by apoptosis in cancer cell as compared to normal cells [260-262]. The Ehrlich ascites carcinoma tumor model showed that there was 80% reduction in the implanted ascites carcinoma cells at 50 mg/kg dose. This effect was due to the inhibition the expression of HIF 1α, VEGF, HSP-90 and pAKT [256].

Ours result also show that naringenin treatment inhibits the B16F10 cancer cell xenograft tumor volume and weight in mice C57BL6 mice **(Figure -50 & 51)**. Naringenin inhibits the migration of melanoma cell in wound healing assays and the decreases spheroid formation in a dose dependent manner. The inhibition of matrix mattaloprotease-9 (MMP-9) by naringenin could be one of the important targets involved in inducing decrease in metastatic potential.

Conclusion

Conclusions: From the present study we conclude and purpose

A. Glyoxalase I has role to play in the Radioresistance of HeLa cells

- 100 µM concentration of cobalt chloride induced hypoxia which was confirmed by observing the expression of HIF-1 α mRNA in HeLa cells.
- The cobalt chloride at 100 µM concentration also increased ROS level and Glo-1 activity, Glo-1mRNA and protein.
- HeLa cells exposed to different doses of radiation induced inhibition of proliferation and colony formation however the expression of glyoxalase-1 was increased.
- The parental HeLa & RR-HeLa shows difference in G1 phase of cell cycle and doubling time by 4 hrs.
- The RR-HeLa cells were resistant to radiation induces morphological changes, mitochondrial membrane potential and apoptosis as compared parental HeLa cells.
- The RR-HeLa cells selectively showed higher level of Glo-1 activity, protein and Nrf-2 and reduced level of total carbonyl content as compared to parental HeLa cells.
- The RR-HeLa cells showed increased glyoxalase-1 activity in response to radiation.

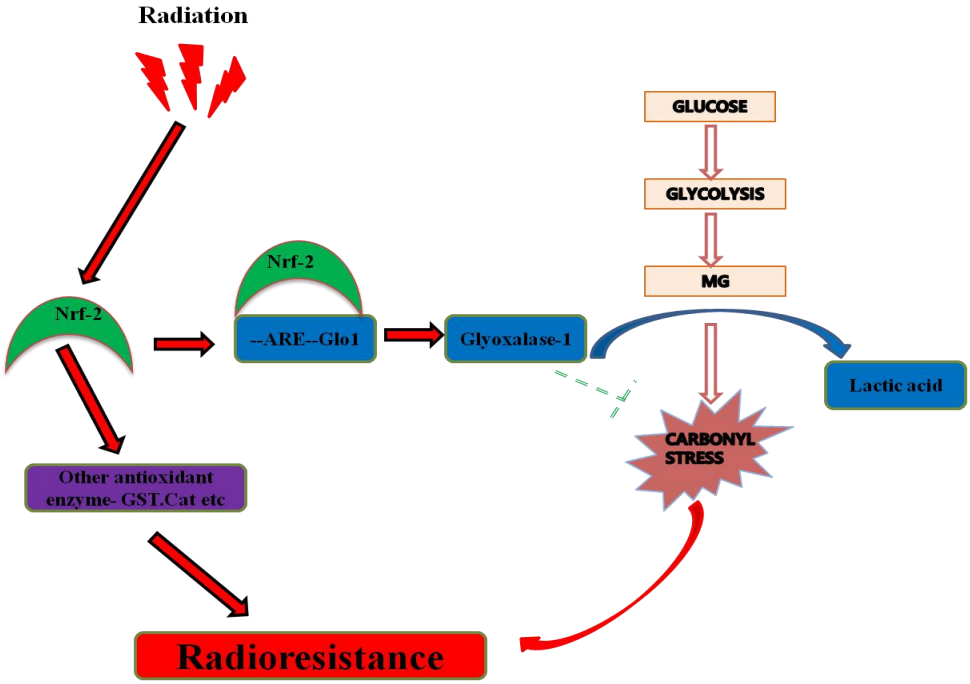

Figure 7A: Proposed mechanism of role of Glyoxalse-1 in radioresistance in HeLa cells

B. **Galangin induces cell death in HeLa cells by modulating Glyoxalase I**

- The toxicity of galangin is dose and time dependent as indicated by MTT and colonogenic assay.
- Galangin shows antioxidant activity in a cellular system.
- Galangin showed cell inhibition and migration in Hela cells *in-vitro*.
- Galangin induces ROS formation on concentration dependent manner & increased total carbonyl content in HeLa cells.
- Galangin inhibits Glyoxalase-1 in Hela cells by Nrf-2 mediated mechanism & induces apoptosis.

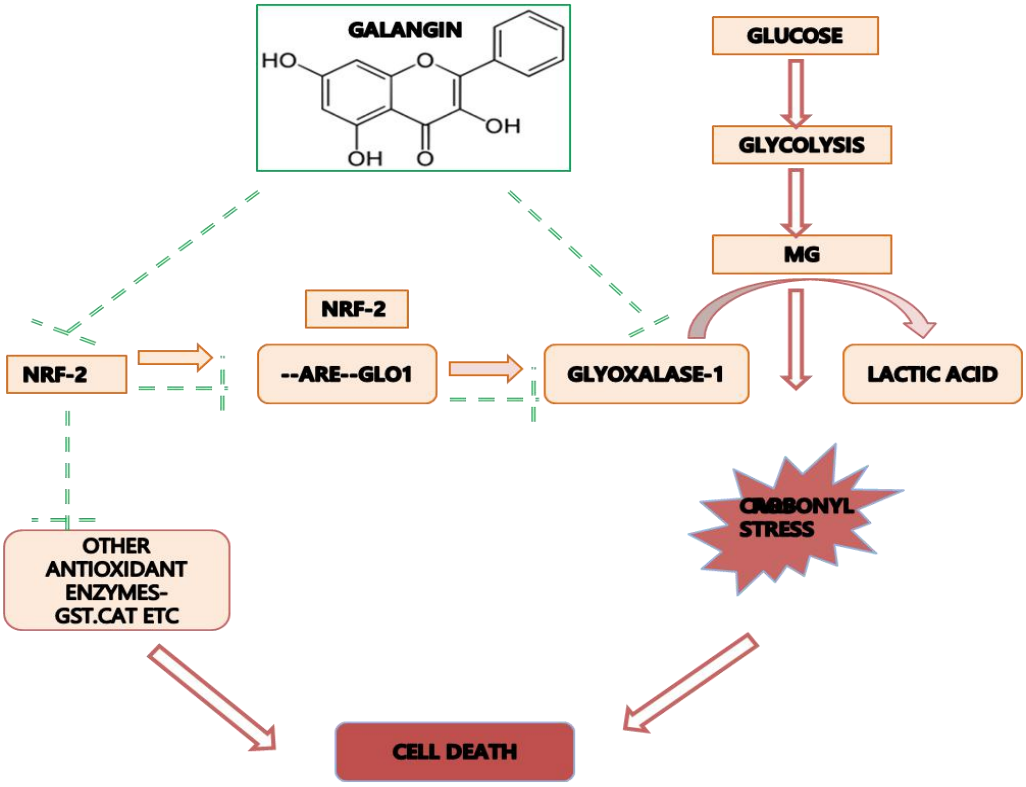

Figure 7 B: Proposed mechanism of cell death induced by Galangin in HeLa cells.

C. Naringenin Inhibits metastasis and induces apoptosis by decreasing the Glyoxalase I *in Vivo*.

- Naringenin long term consumption has no adverse effect on BALB/c mice.
- Naringenin treatment decreases Glo-1 activity and increase total carbonyl content in treatment groups of mice.
- Naringenin shows chemo-preventive effect on DMBA + croton oil induced papillomagenesis by decreasing the papilloma number, incidence, latency and size which was supported by histopathological analysis.
- Naringenin treatments in B16F10 cells induced morphological changes and decrease the cell survival and increase apoptosis in dose dependent manner.
- The naringenin inhibits migration of B16F10 cells in wound healing assay and spheroid formation.
- Naringenin inhibits MMP-9 in dose dependent manner.
- Naringenin induced apoptosis in B16F10 cells by activation of intrinsic apoptosis pathway by increasing release of mitochondrial cytochrome-c to cytosol and increasing caspase-3.
- Naringenin oral treatments decreased the B16F10 xenograft tumor in C57BL-6 mice.

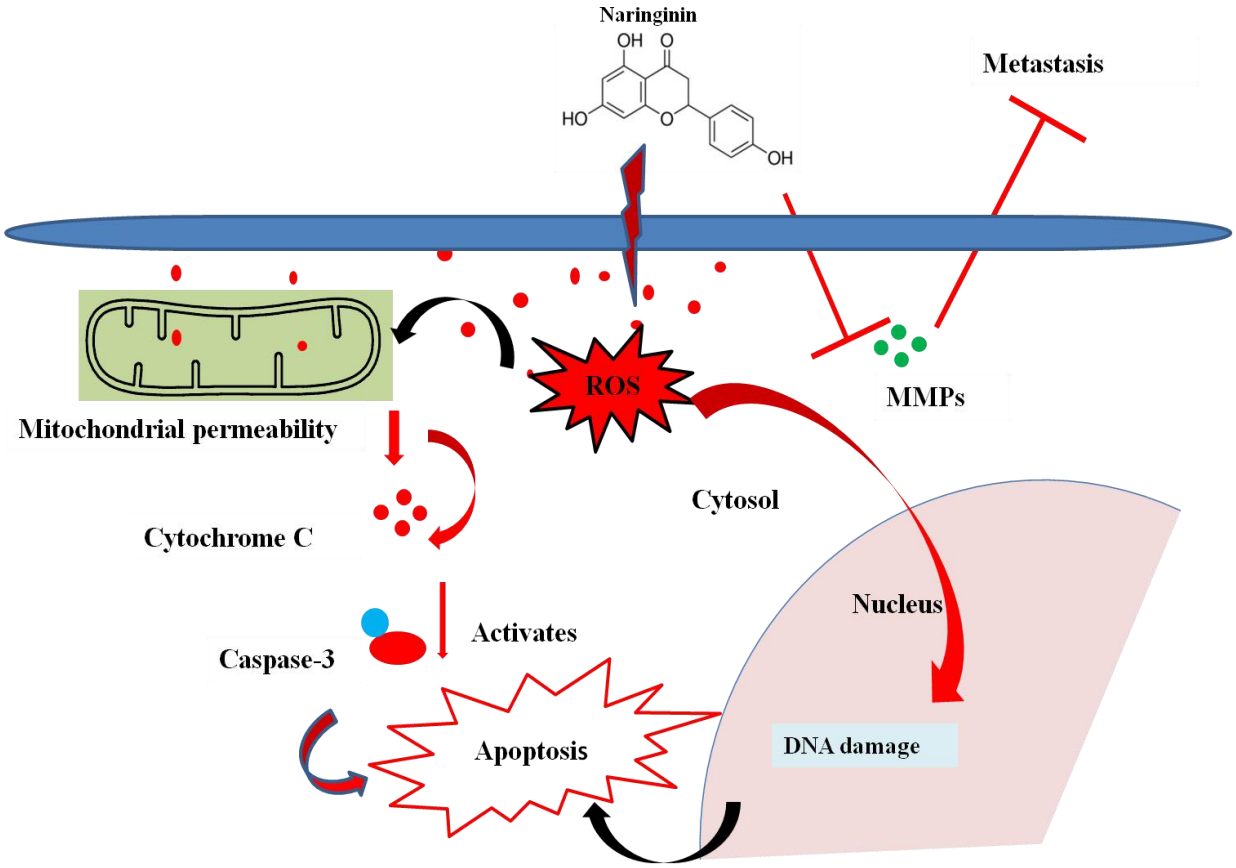

Figure 7 C: Proposed model of molecular mechanism for inhibition of metastasis and apoptosis induction by Naringenin in mice B16F10 melanoma cancer cells.

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Publication and poster presentation

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Poster presentation

1- **Raj Kumar** and A.B.Tiku ; **Role of glyoxalase-1 in development of radio resistance in cancer cells.** (28 Feb 2018*) Science Day*, JNU, New Delhi-110067
2- **Raj Kumar,** Shikha Bharati & A.B.Tiku.; **Chemopreventive effect of Naringenin a flavonoid from citrus fruit against chemical induced skin papillomagenesis.** International Symposium on Cancer Prevention and Treatment, (Feb 9-10, 2018) School of Life Sciences, JNU, New Delhi-110067
3- **Raj Kumar,** and A.B.Tiku; **Inhibitory effect of Galangin on Glyoxalse-1 & NF-κB in Human Cervical cancer cell line (HeLa).** International symposium on Role of Herbals in Cancer Prevention and Treatments (Feb 9-10, 2016) School of Life Sciences, JNU, New Delhi -110067
4- **Raj Kumar**, Rishi Kumar Jaiswal, P.K.Yadav, and A.B.Tiku.; **Effect of chemically induced hypoxia on Glyoxalse-1 in HeLa cells.** International symposium on Current Advance in Radiobiology, Stem Cells and Cancer Research, (Feb 19-21, 2015), School of Life Sciences, JNU, New Delhi-110067
5- **Raj Kumar** & A.B.Tiku.; **Effect of Cobalt chloride on metastatic potential in human cervical cancer cell line (HeLa)**. *Biosparks*, (March 21-22, 2014) School of Life Sciences, JNU, New Delhi-110067

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Galangin induces cell death by modulating the expression of glyoxalase-1 and Nrf-2 in HeLa cells

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ABSTRACT

The present study was designed to understand the anticancer property and molecular mechanisms associated with chemo preventive effects of galangin. The anticancer effect was evaluated *in vitro* using human cervical cancer cell line (HeLa). Galangin was found to be effective in inducing cell death and inhibiting proliferation & migration significantly. The inhibitory effect of galangin could be correlated with the increase in ROS production & induction of apoptosis. Besides this the activity of glyoxalase-1, an enzyme important for the detoxification of cytotoxic metabolite methy glyoxal and Nrf-2 (a trascription factor), involved in redox signalling were found to be decreased. We concluded that galangin exerts its chemo preventive effect via redox signalling by inhibiting glyoxalase-1 & increasing oxidative & carbonyl stress.

1. Introduction

A number of studies have shown that glyoxalase system can be an important molecular target for cancer prevention and treatment as elevated level of glyoxalase-1 has been observed in many tumor tissues and cancer cells [1–6]. Glyoxalase system is a defence pathway against dicarbonyl stress produced by methylglyoxal (MG) in actively dividing cells. The detoxification system consists of two enzymes, glyoxalase-1 and glyoxalase-2 and catalytic amount of cofactor, reduced glutathione (GSH). It converts toxic methylglyoxal, produced by the spontaneous degradation of triose phosphate intermediate in glycolysis, to nontoxic D-lactate [7]. Under conditions of high glycolytic flux, a condition encountered by all cancer cells, the formation of MG is increased which is counter balanced by Glyoxalase system & gives these cells an adaptive advantage [3]. Over expression of glyoxalase-1has been associated with resistance to various therapeutic treatments also. Although pharmacological inhibition of glyoxalase system can be an important strategy in cancer therapy yet due to toxicity of chemical inhibitors their clinical use has been elusive [8–10]. Phyto chemicals present in various plants especially the ones which are consumed by humans as part of their diet have been gaining attention for the past few decades as sources for various drugs. The important features being time tested usage and low toxicity. Therefore, in the present study we have evaluated the chemo preventive effect of Galangin, an active principle of galangal,a spice used in Asia & an important ingredient of many traditional medicines. It is an important flavone present in honey, propolis, *Helichrysum* & *Alpinia officinarium*. It has been reported to have many health promoting benefits ranging from free radicals scavenging to potential cancer preventive effects [11,12]. *In vitro* and *in vivo* data indicate that galangin has antioxidant properties and is capable of suppressing the genotoxicity of chemicals by modulating the activity of a number of enzymes [13]. It has also been shown to produce synergestics effects with other anticancer drugs to induce apoptosis and inhibit proliferation in various cancer cells [14].

Despite various biological effects galangin is one of the less explored flavonoids and mechanism of action has not been studied in detail. In the present work we have tried to explore the role of glyoxalase-1 in cell death induced by this phytochemical.

2. Methods and materials

2.1. Chemicals and antibodies

RPMI-1640, FBS and antibiotics were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai- 400086, India. Galangin, Bovine Serum Albumin (BSA), Ethidium bromide (EtBr), Acridine orange (AO), Triton X-100, and 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Primary mice β-actin, primary rabbit Nrf-2, primary rabbit Glo-1 and secondary anti-mice Ig-G-HRP and anti-rabbit Ig-G-HRP were purchased from Santa Cruz Biotechnology, Texas, USA. All other chemicals used were of molecular biology grade.

Human cervical cancer cell line (HeLa) were grown as monolayer in RPMI-1640 medium, supplemented with 10% (v/v) FBS, antibiotics

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(penicillin 100 U/mL, streptomycin 10 μg/mL) and 1 mmol/L sodium pyruvate under standard conditions, temperature (37 °C) in a controlled humidified atmosphere containing 5% CO_2.

2.2. Cell proliferation

For evaluation of cell proliferation MTT assay was used. In brief, HeLa cells (5000 cells/well) were seeded in a 96-well plate and kept overnight. The very next day fresh medium having different concentrations of galangin (25–100 μM) was added to the wells. The cells were allowed to grow for another 24 h. Four hours before completion of incubation, 20 μl of MTT (5 mg/ml) was added in each well. Media was removed after completion of incubation and 200 μl of DMSO was added to each well. The cells were again incubated for another 5–10 min at room temperature. The purple colour thus developed was measured at 595 nm using Bio-Rad micro plate reader. The % of viability was calculated using following formula:

$$\%\text{viability} = \frac{(\text{Mean OD of treated cells})}{\text{Mean OD of untreated cells (control)}} \times 100$$

2.3. Morphological changes

To study changes in morphology 5×10^5 cells/well were seeded in the 6 well plates and kept overnight. After attachment the cells were treated with different concentrations of galangin (25–100 μM). 24 hr post galangin treatment, the images of cells were captured under the microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.4. Clonogenic assay

Fixed numbers of cells (1000 cells/well) were seeded in six well plates having 2 ml of complete RPMI- 1640 media containing different concentrations of galangin. The plates were then incubated in CO_2 incubator at 37 °C for one week and media was changed twice a week. After one week the cells were washed twice with PBS and then fixed and stained with crystal violet solution. The excess stain was washed and colonies were observed with naked eye.

2.5. Wound healing assay

5×10^5 cells were seeded in 6 well plate and when cells became 80% confluent a scratch was made with 200 μl tip in each well of plate and media replaced with the galangin (25–100 μM) containing media. The changes in the scratched portion were noted at 0 h, 12 h and 24 h under microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.6. Micro drop migration assay

Cells were trypsinized, washed, counted and suspended in media (RPMI having 0.3% agrose). 40000 cells/2 μl were added to each well in duplicate in 96 well plate and then 100 μl of fresh media having different concentration of galangin was added. After 24 h of incubation the images were captured under microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.7. Total carbonyl content

Total carbonyl content was measured as per Dalle-Donne et al. [22], with some modification. In brief cells were trypsinized after 24 h of galangin treatment, washed and suspended in PBS (1×10^6 cells/100 μl in PBS). Suspended cells were sonicated (Barson) for 40 s at 25% amplitude. After sonication the proteins were precipitated with cold trichloroacetic acid (TCA, 15% final concentration). The precipitate was collected by centrifugation. A solution of 10 mM DNPH (Dinitrophenyl hydrazine) in 2 N HCl was added to the protein pellet of each sample. 2 N HCl was added to corresponding sample aliquot reagent blanks. Samples were allowed to stand in the dark at room temperature for 1 h with vortexing every 10 min; they were then precipitated with 15% TCA (final concentration) and centrifuged for 5 min. The supernatants were discarded; the protein pellets washed once more with 15% TCA, and further washed three times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Samples were then suspended in 6 M guanidine hydrochloride (dissolved in 2 N HCl) at 370 °C for 15 min with vortex mixing. Carbonyl contents was determined at 366 nm spectrophotometarically.

2.8. Detection of intracellular ROS levels

The intracellular ROS (Reactive oxygen species) generated by galangin in HeLa cells was measured using the 2′,7′-dichlorofluorescein-diacetate (DCFHDA) dye. For measuring the intracellular ROS in individual cells, 5×10^5 cells were seeded on cover-slips in a 6-well plate and incubated overnight for attachment. Next day cells were treated with fresh medium containing (25–100 μM) galangin. The cells were further incubated at 37 °C for 4 h. At the end of incubation, cover-slip was removed from the culture plate and stained with 40 μM 2′,7′-dichlorofluorescein-diacetate (DCFHDA) dye in PBS for 30 min at RT. Excess dye was removed by washing with 1× PBS. Cover-slip was mounted on glass slide and observed under a fluorescence microscope (Nikon ECLIPSE TiE, Tokyo, Japan) at 10 X magnification.

2.9. Apoptosis assay

Apoptosis in HeLa cells was detected by EtBr/AO staining solution. In brief cell suspension was mixed with EtBr/AO staining (1 mg/ml of AO in PBS + 1 mg/ml of EtBr in PBS) for 2 min. The cells were visualized under fluorescence microscope (Nikon ECLIPSE TiE, Tokyo, Japan) at 10 X magnification. The live, apoptotic and dead cells were differentiated on the basis of emitted fluorescence in merged image, that was green, orange and red respectively.

2.10. Glyoxalase-1 activity

Cytosolic fractions were prepared by lysing the cells in phoaphate buffer containing 1 mM EDTA by freez/thawing and sonication, This was followed by centrifugation at 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction. The glyoxalase-1 assay was performed using 0.2% MG and 2% glutathione in 0.1 M phosphate buffer (pH 6.8). Change in absorption was recorded at 240 nm for 2 min attributable to the formation of S-D-lactoylglutathione.

2.11. Immunofluorescence

For immunofluorescence studies the HeLa cells were grown on coverslips in 6 well plate with galangin for 24 h. Cells were then fixed with 4% formaldehyde in PBS for 15 min. After being washed with PBS and blocked with 1% BSA in 0.1% PBST, the cells were incubated with antibodies at 4 °C overnight. After being washed three times with PBST, the cells were incubated with Alexa Fluor 488–conjugated secondary antibodies for 1 h at room temperature and counterstained with DAPI. Antibody–antigen complexes were detected under a fluorescence microscope (Nikon ECLIPSE Ti, Tokyo, Japan), using a 60X objective lens.

2.12. Western blotting

Equal no of cells were seeded in 100 mm plates, when cells reached 80% confluence; they were treated with different concentrations of galangin. After 24 h of treatment, cells were trypsinized and washed with PBS and protein was extracted in RIPA (radioimmuno

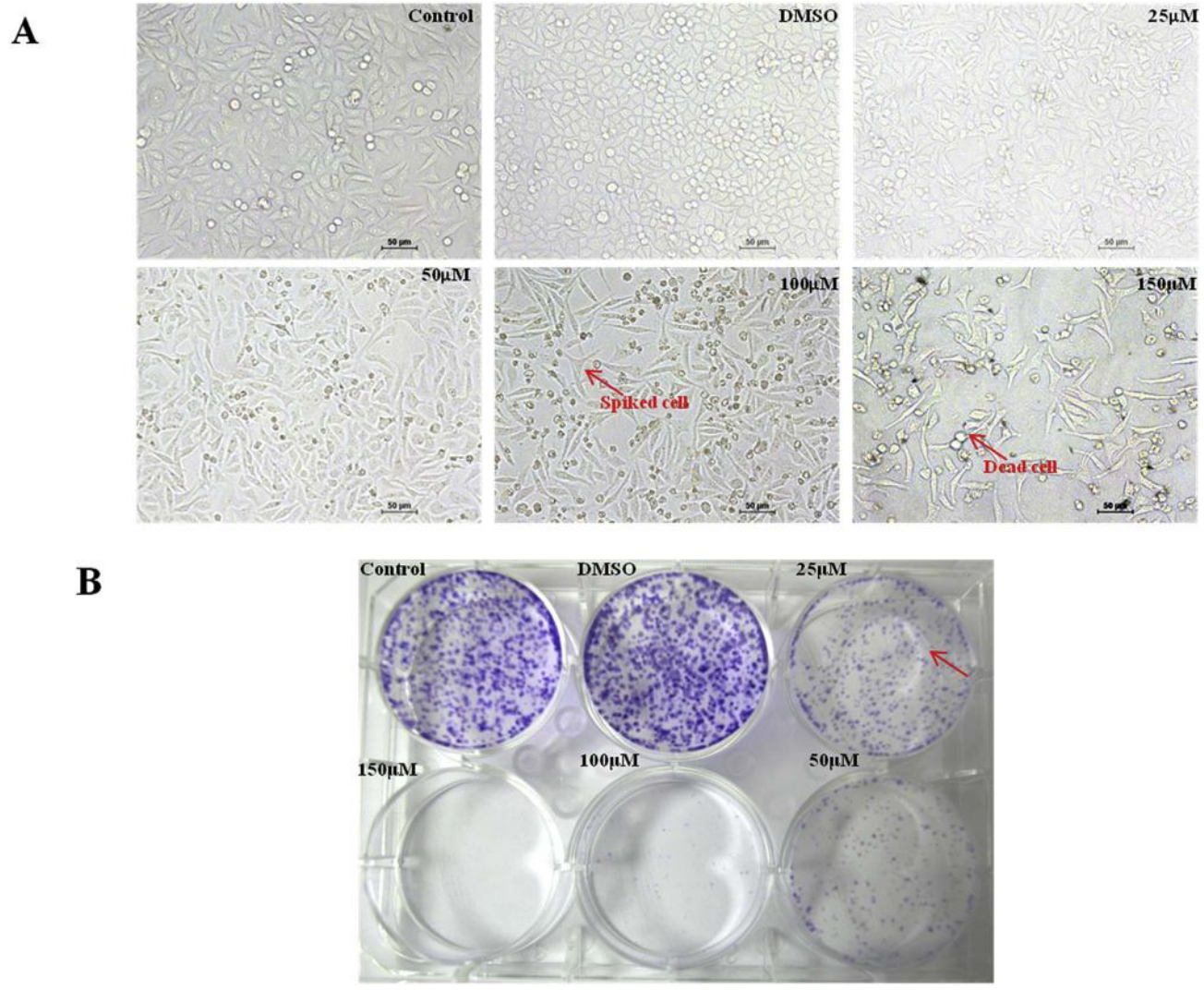

Fig. 1. Effect of different concentrations of Galangin on HeLa cells (A) Morphological changes like spikes formation, detachments, dead cells (Images were captured by Nikon inverted microscope at 10X magnification). (B) Colony formation. (Arrow shows decrease in size and number of colonies in treated groups).

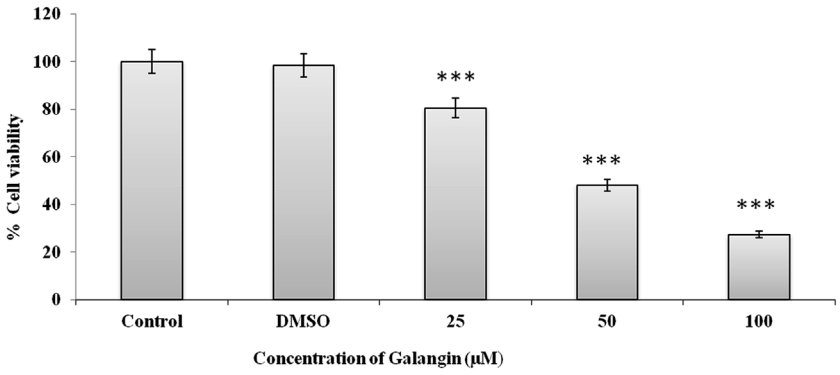

Fig. 2. Effect of galangin on cell viability by MTT assay. Data are presented as mean ± S.D. of three separate experiment ***P < 0.001 with respect to control.

precipitation assay) buffer and protein concentration was estimated using the bradford dye. Extracted protein was separated in 8% SDS-PAGE gel and transferred to nitrocellulose membrane under semidry condition. The membrane was blocked using 5% BSA in PBS and probed with primary antibody (Nrf-2, glyoxalase-1 and β actin) followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The bands were detected using ECL kit and images were permanently captured in X-ray films and results were analyzed with respect to loading control (β-actin).

2.13. Data analysis

Mean and standard deviation was calculated using Graph Pad Prism 5.03. A statistical test was done using one-way ANOVA and p values less than 0.05 were considered significant.

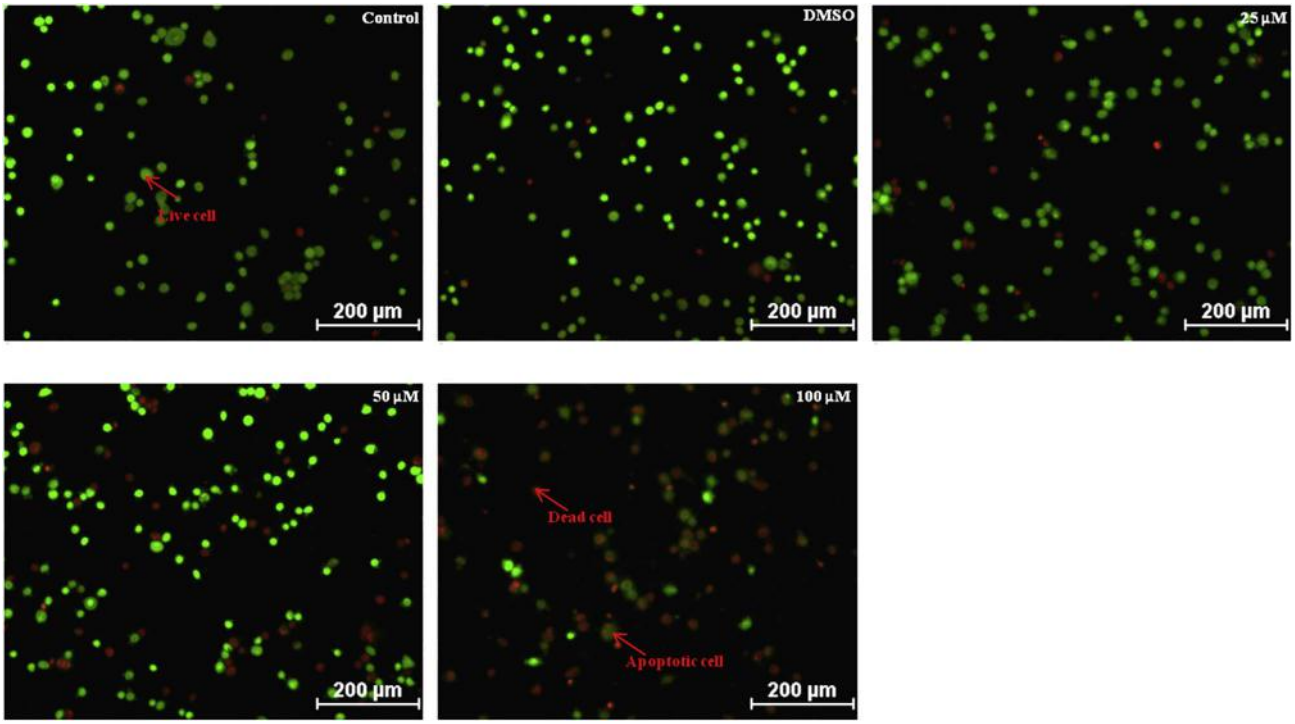

Fig. 3. Galangin induced apoptosis in HeLa cells as detected by EtBr/AO staining. Green, orange and red fluorescence represents live, apoptotic and dead cells respectively. Images were analyzed by NIS elements Nikon analysis software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

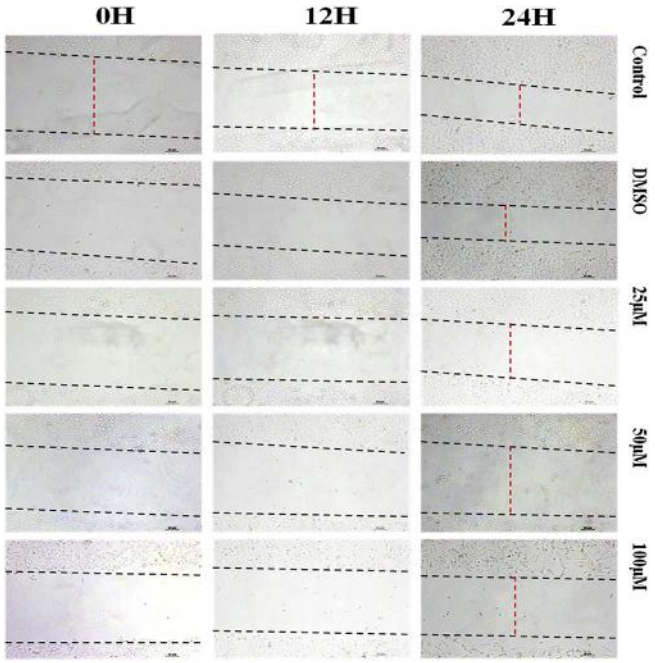

Fig. 4. Effect of galangin on HeLa cells migration by monolayer scratch assay at different time points. Mark narrowing gap with time in control at 24 h but very little migration in galangin treated samples.

3. Results

3.1. Effect of galangin on morphology and cell survival

The galngin induced changes in morphology of HeLa cells after 24 h (Fig. 1, A). The treated cells showed cell shrinkage, spindle shape, detachment, and spike formation. The extent of change in morphology as well as number of cells showing changes was concentration dependent.

Besides the effect of galangin on cell survival was evaluated by colonogenic assay. A colony was defined as to consist of at least 50 cells. The colonogenic assay showed that galangin significantly decreased the number and size of colonies in a concentration dependent manner (Fig. 1B).

3.2. Effect of galangin on cells proliferation

Galangin inhibited *in vitro* proliferation of HeLa cells up to 50% at 50 µM (Fig. 2). The EtBr/AO dye staining showed that galangin treatment for 24 h induced apoptosis in dose dependent manner (Fig. 3). The number of apoptotic and dead cells in galangin treated samples increased significantly.

3.3. Effect on the metastatic potential

Galangin at concentration of 25–100 µM demonstrated a clear dose dependent antimigratory effect in wound healing assays of human cervical cancer cell line. In untreated controls 50% migration took place while as cells treated with 25 µM of galangin only 20% cells migrated, after 24 h (Fig. 4). This was further confirmed by microdrop migration/invasion assay. Galangin inhibited the migration of HeLa cells in concentration dependent manner (IC50 12.5 µM) & at 75 µM cell invasion in to agerose gel was completely inhibited (Fig. 5).

3.4. Galangin & reactive oxygen species

Galangin treatment was able to increase the intracellular ROS level in treated HeLa cells as compared to untreated control cells as measured by fluorescence microscopy (Fig. 6). From the fluorescence images of cells it is clear that the intracellular fluorescence intensity of individual cells markedly increased and positively correlated with increased galangin concentration.

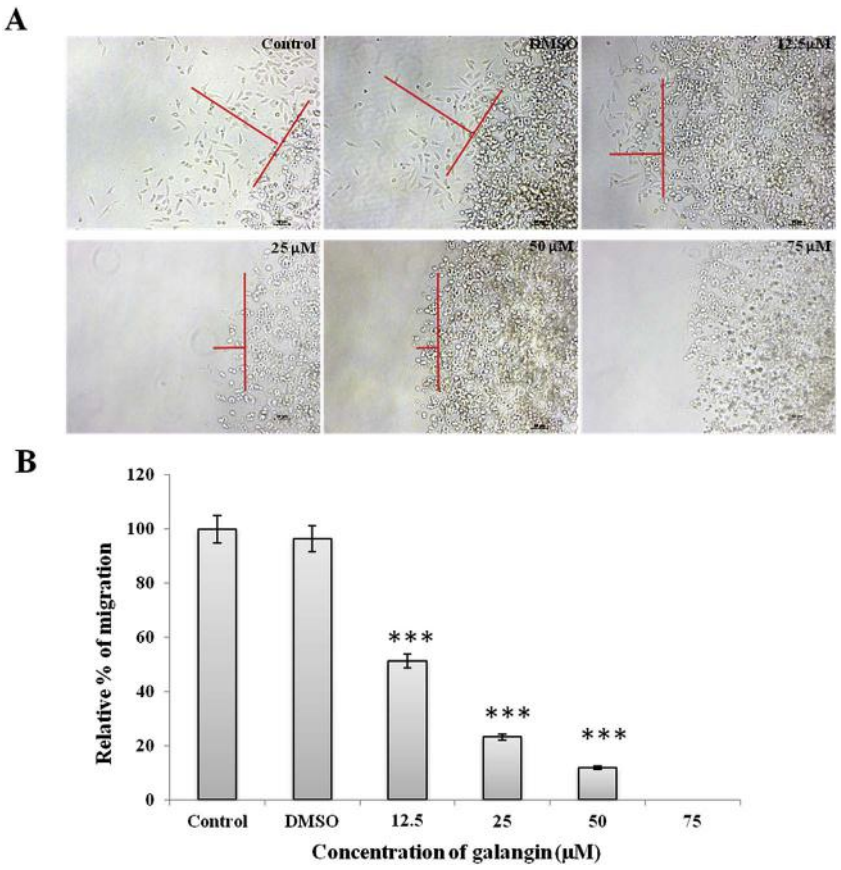

Fig. 5. Effect of Galangin (12.5 µM - 75 µM) on (A) migration of HeLa cells out of agerose microdroplet (B) % migration of HeLa cells (***P < 0.001 with respect to control).

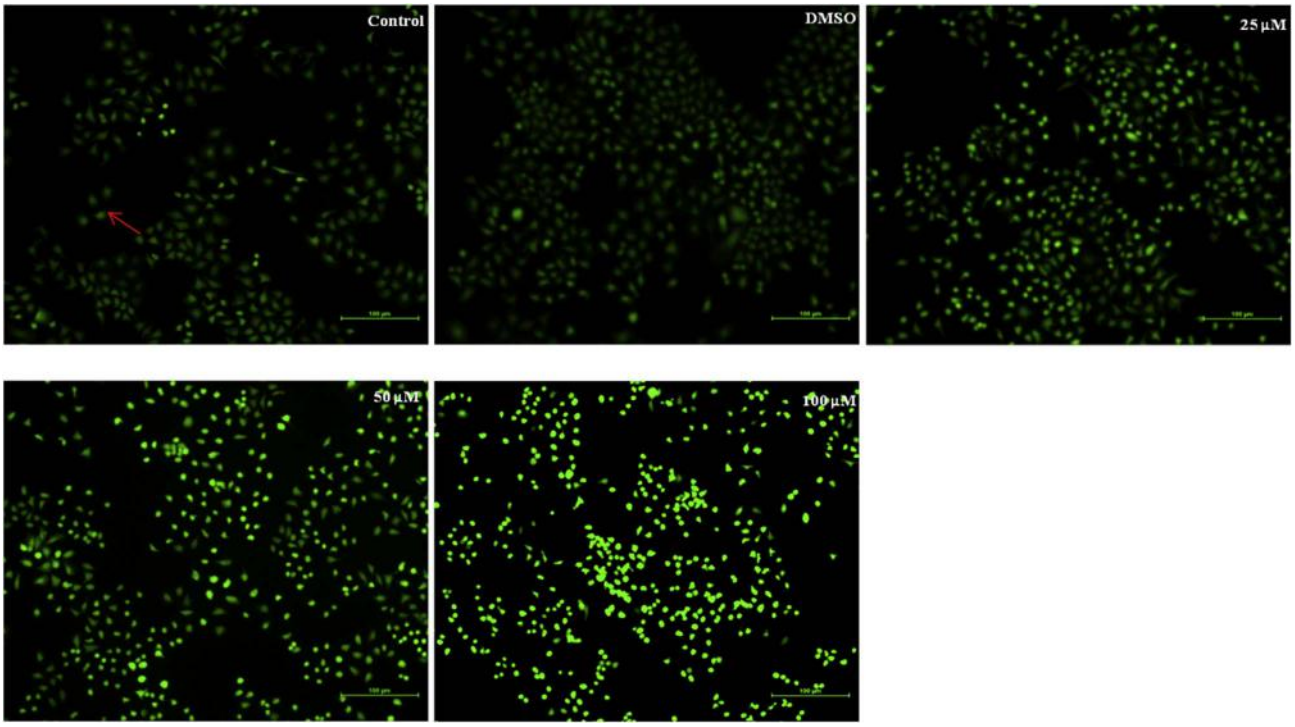

Fig. 6. Effect of different concentrations of Galangin on ROS level in HeLa cells after 4 h of treatment. Mark increased fluorescence with increasing concentration of Galangin.

3.5. Total carbonyl content after galangin treatment

The total carbonyl content (an indicator of oxidation damage) in galangin treated and control cells was measured using DNPH spectrophotometeric analysis. Galangin treatment increased the total carbonyl content as a function of dose. Treatment of cells with 50 µM of galangin increased the carbonyl content by about 1.5 fold in comparison to untreated controls cells (Fig. 7).

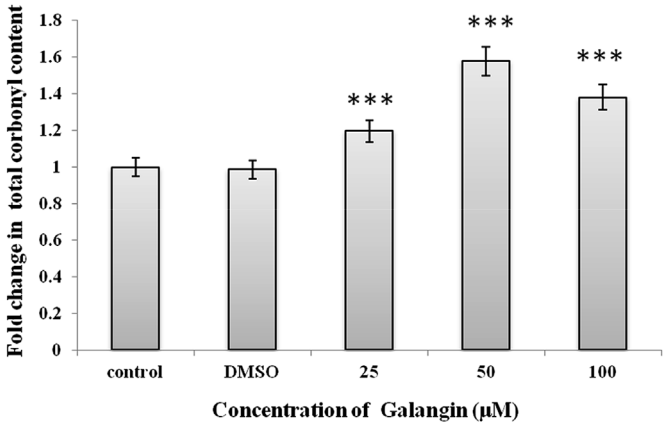

Fig. 7. Effect of Galangin on total carbonyl content in HeLa cells 24 h post treatment. (Data are presented as mean ± S.D. of three separate experiment ***P < 0.001).

3.6. Molecular mechanism of galangin induced cell death

To understand the molecular mechanism of cell death associated with galangin the level of glyoxalase-1 protein was studied. As can be seen in Fig. 8 the galangin treatment decreased the glyoxalase-1enzyme activity in a concentration dependent manner which could be correlated with a significant increase in carbonyl content.

Further to understand, glyoxalase-1 regulation, the level of Nrf-2 transcription factor was also checked. The result showed that the galangin decreased the Nrf-2 protein in a dose dependent manner after 24 h of galngin treatment both in western blotting & immunoflurescence studies (Fig. 9).

4. Discussion

Present studies investigated the mechanism/s associated with cell death induced by galangin in cervical cancer cell line (HeLa). The morphological changes in cells clearly indicate that galangin is cytotoxic and induces cell death in a concentration and time dependent manner. Within 24 h of treatment, cells undergo apoptosis and show decreased proliferation. This was further confirmed by colony formation assay that showed decrease in size and number of colonies as compared to control (Fig. 1). Cytotoxicity of Galangin has been reported in a number of cancer cell lines like HCT-15, HT-29, (human colon cancer cells), B16F10 (melanoma cells) and Caki-1 and 786–0 (renal carcinoma cells) [15–17]. The IC-50 value for different cancer cells has been reported between 25 μM and 150 μM [15–20]. In the present case also the IC 50 value for HeLa cells was approximately 50 μM (Fig. 2).

Galangin has been reported to decrease the antioxidant defence *in vitro* and *in vivo* in murine models by modifying the xenobiotic and anti oxidant enzymes and decreasing the total antioxidant levels resulting in accumulation of intracellular ROS [[15–17,21]. Our results also

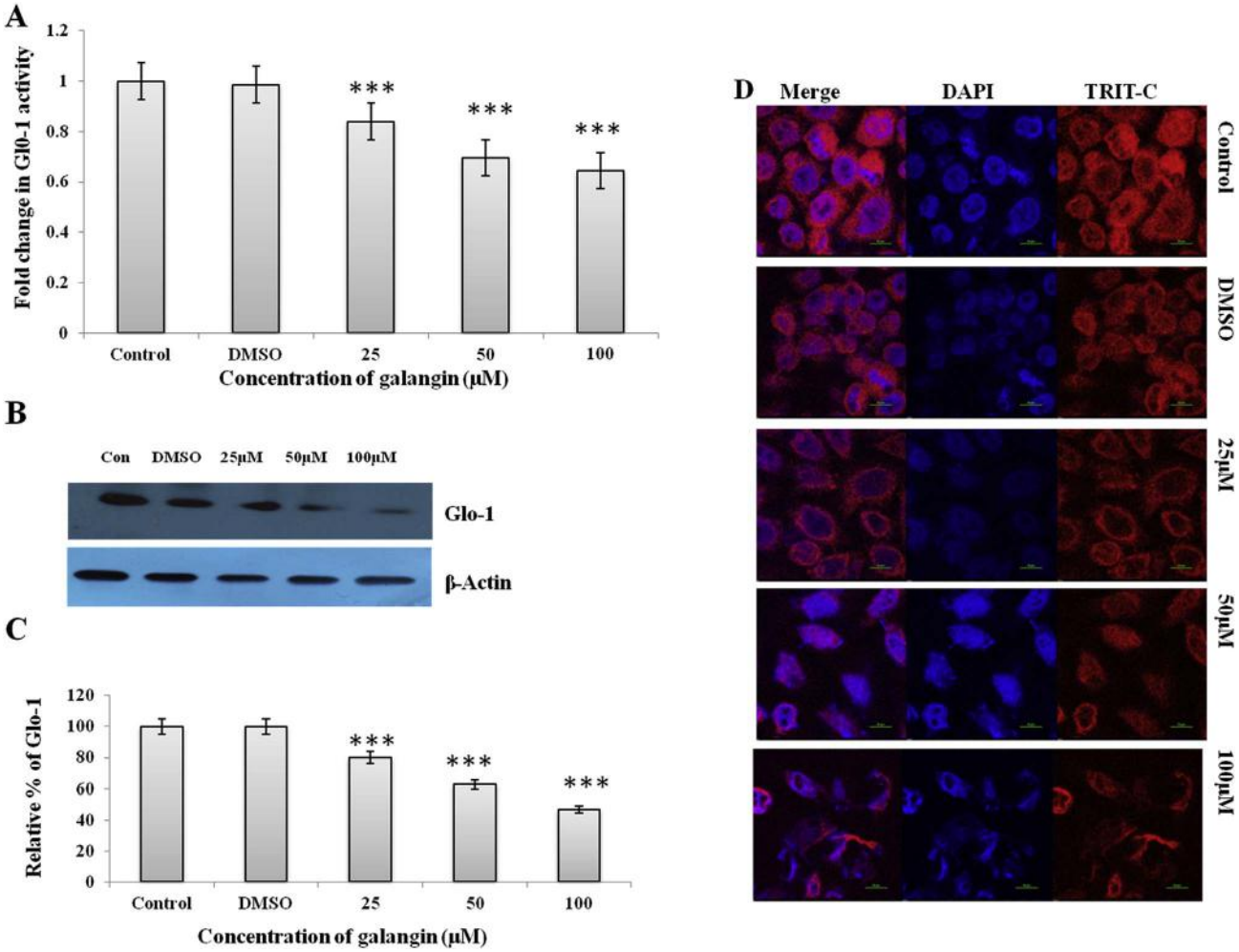

Fig. 8. Effect of 24 h treatment of galangin (25 μM–100 μM) on Glyoxalase-1 (A) Enzyme activity (B) protein expression using western blotting (C) % Relative expression level of glyoxalase-1 corresponding to loading control β-Actin (D) immunoflurescence images (Nikon ECLIPSE Ti) at 60X magnification (Data expressed as mean ± SEM of triplicates.***P < 0.001 significant vs. control).

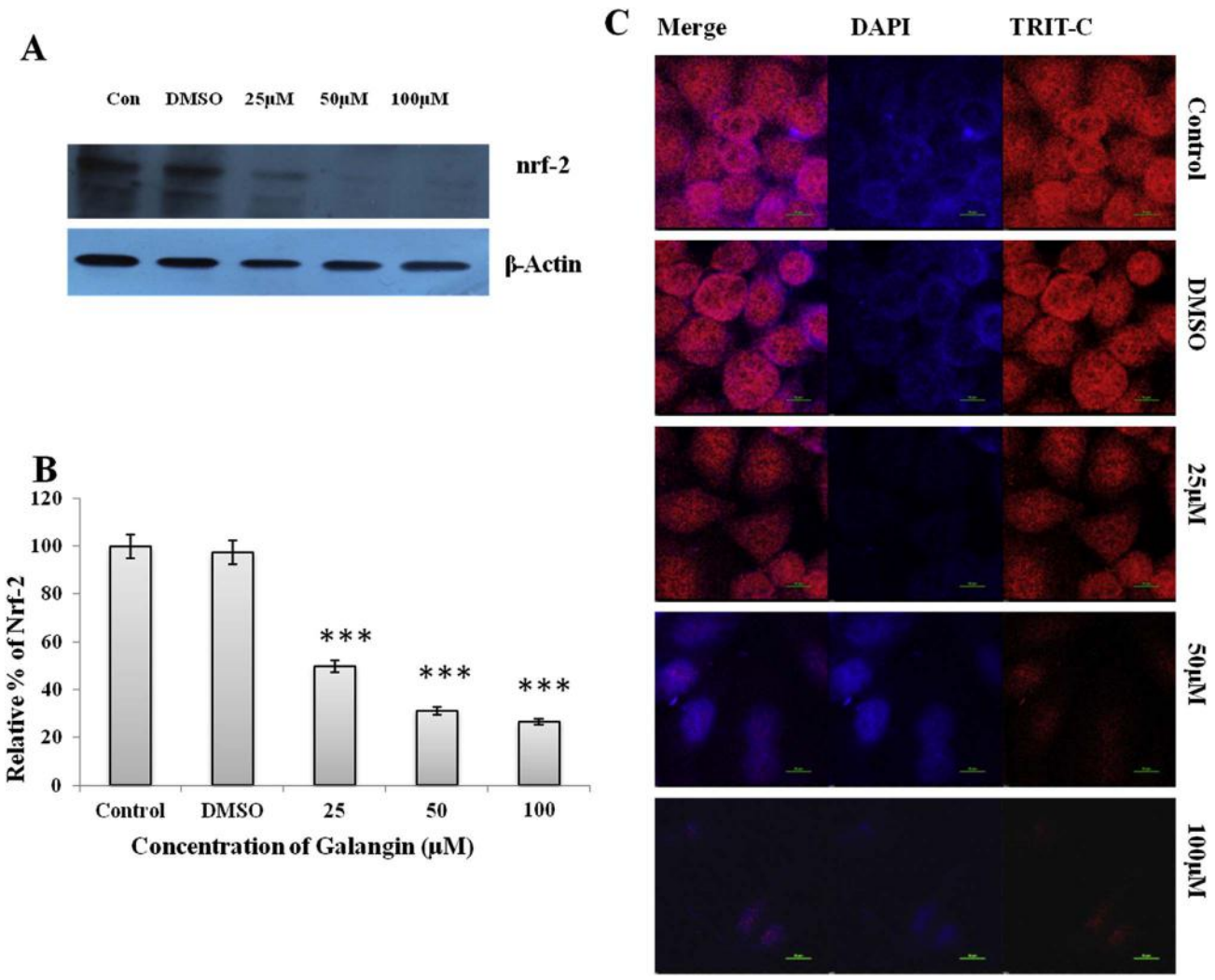

Fig. 9. Effect of 24 h treatment of galangin (25 μM–100 μM) on Nrf-2 (A) protein expression using western blotting (B) % Relative expression level of Nrf-2 corresponding to loading control β-Actin (C) immunoflurescence images. (Nikon ECLIPSE Ti) at 60X magnification (Data expressed as mean ± SEM of triplicates.***P < 0.001 significant vs. control).

indicate that the intracellular ROS level is significantly increased in galangin treated HeLa cells (Fig. 6). Increase of ROS beyond a threshold results in DNA damage that could result in the activation of cell death via various mechanisms including apoptosis. Galngin could induce apoptosis in Bcr-Abl expressing blood cancer cells (both imatinib mesylate sensitive as well as resistant cells K562 and KCL22) by arresting cell cycle and decreasing pRb, cdk-4, cdk-1 and cycline B levels [14]. Galangin has also been reported to induce apoptosis and cell death in human colon cancer cells and B16F10 melanoma cells by intrinsic mitochondrial pathway through altering mitochondriyal membrane potential and dysfunction by activating the caspase-3 and -9 in a dose dependent manner [16].

In most cancer cells Glyoxalase-1 is highly expressed and inhibition of glyoxalase-1 can selectively induce apoptosis [3]. Galangin treated samples also showed a decrease in Glyoxalase-1 activity. In the present study we further observed that galangin treatment led to an increase in the total carbonyl content indicating an increase in oxidative damage (Fig. 7). Total carbonyl content is a general indicator for protein oxidation and is used as a measure of oxidative injury [22]. The decreased glyoxalase-1 activity can result in accumulation of dicarbonyles like methylglyoxal and other derivatives including AGEs, DNA and protein derivatives resulting in cell death [23–26]. Since Nrf-2 is known to transcriptionally regulate glyoxalse-1, to provide defence against dicarbonyl stress, we also checked the level of the transcription factor Nrf-2. A decrease in the levels of Nrf-2 was observed in protein expression studies that was further confirmed by immunofluorecence studies (Fig. 8). Previous studies have also shown that modulators of Nrf-2 modulate Glyoxalase-1 expression [25]. Nrf-2 binds at ARE site of GLO-1 gene to regulate the expression of glyoxalase-1 enzyme in response to stress. Besides glyoxalase-1, Nrf-2 also regulates expression of antioxidant enzymes resulting in reduced antioxidant defence. In the present study also we observed a decrease in Nrf-2 as well as Glo-1 levels in galangin treated samples.

From the above discussion it can be speculated that the galangin modulates Nrf-2 levels resulting in decreased expression of glyoxalase-1 leading to decreased detoxification of MG. Increased MG levels can lead to increased levels of oxidative stress resulting in cell death. MG, Glyoxalase-1 & dicarbonyl stress have emerged as the targets of Nrf-2/Keap system [25]. Thus Galangin can induce cell death in HeLa cells by multiple mechanisms. Inhibition of Nrf-2 can be linked to decrease in glyoxalase-1 expression & increased accumulation of MG and oxidative stress in combination with carbonyl stress produced by MG, thus could induce cell death in the HeLa cells (Fig. 10).

Besides cell death galangin was able to decrease the metastatic potential of the Hela cells *in vitro*. Both migratory and invasive potential were found to decrease in a dose and time dependent manner. A significant inhibition of Hela cell migration was observed *in vitro* at concentration well below the ant proliferative IC-50 values. Galangin has been reported to efficiently inhibit TPA-induced invasion and migration of HepG2 cells through a protein kinase C/extracellular signal-

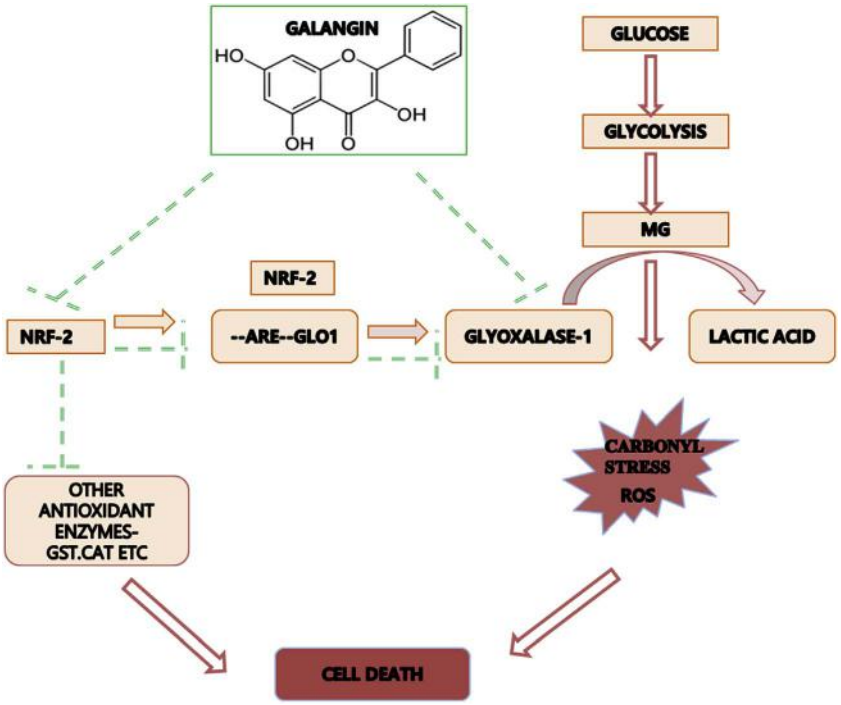

Fig. 10. Proposed mechanism for the cell death induced by Galangin in HeLa cells.
MG–Methylglyoxal, ARE–Antioxidant response elements, Nrf-2–Nuclear factor erythroid 2–related factor 2.

regulated kinase (PKC/ERK) pathway [18]. Galangin and kaempferol have also been reported to play a role in decreasing invasive potential by decreasing MMP-9 expression by blocking activation of NF-κB and AP-1 [19]. Knockdown of GLO1 is associated with reduced tumor-associated properties such as migration and proliferation in cancer cells [27]. On the basis of the present studies, it can be inferred that galangin induced inhibition of metastatic potential was due to the decreased glyoxalase-1 enzyme.

In conclusion it can be postulated that the galangin modulates Nrf-2 level resulting in decreased expression of glyoxalase-1 leading to decreased detoxification of MG. Increased MG levels lead to increased levels of oxidative stress resulting in cell death. Thus inhibitors of Nrf-2 like galangin can be linked to induced cell death and decrease in metastatic potential of cancer cells via decrease in glyoxalase-1 expression & increase in damage caused by MG and ROS.

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