Study of Radioprotective Effects of Galangin and its Role in Modulation of Ionizing Radiation induced Signaling Events

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By

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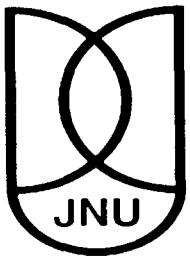

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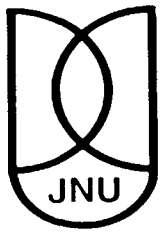

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CERTIFICATE

The research work embodied in this dissertation entitled "**Study of radioprotective effects of Galangin and its role in modulation of ionizing radiation induced signaling events**" has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India. The work is original and has not been submitted so far, in part or full for any degree or diploma of any university.

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ABBREVIATIONS AND SYMBOLS

UV	Ultraviolet
DNA	Deoxyribonucleic acid
DSBs	Double Strand Breaks
EtBr	Ethidium Bromide
ROS	Reactive Oxygen Species
IR	Ionizing Radiation
SSBs	Single Strand Breaks
Gy	Gray
H_2O_2	Hydrogen Peroxide
GSH	Glutathione
SOD	Superoxide Dismutase
CAT	Catalase
TCA	Trichloroacetic Acid
EDTA	Ethylene Diamine Trichloroacetic acid
DTNB	5, 5'-dithiobis-(2- nitro benzoic acid)
SDS	Sodium Dodecyl Sulfate
BSA	Bovine Serum Albumin
OD	Optical Density
DW	Distilled Water
µl	Microliter
EtOH	Ethanol
M	Molar
mM	Millimolar

TNB	5-thionitrobenzoic Acid
NADPH	Nicotinamide Adenine Nucleotide Phosphate
ml	Millilitre
mg	Milligram
µg	Microgram

CONTENTS

INTRODUCTION

1. INTRODUCTION

Ionizing radiations produce deleterious effects in the living organisms and the rapid technological advancement has further increased human exposure to ionizing radiations. Humans are exposed to ionizing radiations during diagnostic, therapeutic and industrial purposes. Apart from these humans also get exposed to radiations during air and space travel, nuclear accidents and due to extensive use of electronic devices like mobile phone.

The damaging effects of radiation exposure are attributed to radiation induced strand breaks as well as to the production of reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) [Halliwell & Gutteridge 2007]. Reactive species lead to damages such as DNA damage, chromosomal aberration, protein oxidation, DNA protein cross linking, membrane damage etc. (Yasuhiro *et al.,* 2002 and Genova *et al.,* 2004). However free radicals can be deleterious and cytotoxic at the pathogenic level and at the same time can serve as signalling and regulatory molecules at physiological levels. ROS also act as secondary messengers of signal transduction in several biological conditions (Lee, 2000).

Exposure of cells to ionizing radiations induces various signaling pathways that activate transcription factors like AP-1, NF-κ B, and p53. These transcription factors elicit various biological responses through induction/ repression of target genes. For instance, radiation induced DNA damage results in p53 activation leading to induction of p21, an inhibitor of cyclin-dependent kinases, resulting in arrest at the G1 phase of the cell cycle. This cell cycle arrest is thought to provide affected cells with ample time to repair their damaged DNA before entering S phase. On the other hand radiation induced double strand breaks, and indirect effects of radiation like ROS generation or sphingomyelin breakdown products are involved in the activation of NF-κ B,while as direct single strand breaks are considered to be

an unrelated event. In most of the cases expression of NF-κ B target genes promote cellular survival. ROS modulate NF-κ B response and NF-κ B target genes attenuate ROS to promote cellular survival.

One of the most important ways by which NF-κ B influence ROS level is via increased expression of antioxidant proteins such as Superoxide Dismutase (SOD), Catalase, Glutathione S-transferase pi(GST-pi), Glutathione peroxidase-1(GPx1) etc. However little is known regarding the signaling cascade by which radiation exposure results in NF-κ B induced activation and /or repression of target genes leading to cell death /survival.

NF-κ B is a dimeric transcription factor, composed of members of the Rel family that is kept in the cytoplasm of non stimulated cells through interaction with inhibitory proteins the IkBs. The IkBs, consist of three subunits IkBα, IkBβ and IkB which retain NF-κ B in the cytoplasm by masking the nuclear localization sequence embedded within the Rel homology domain. The most potent NF-κ B activators cause rapid phosphorylation of IkBs at two sites within their N-terminal regulatory domain. This phosphorylation event, which in the case of IkBα occurs on Ser-32 and Ser-36, results in polyubiquitination of the IkBs and their degradation by the 26S proteasome leads to liberation of NF-κ B. Sustained NF-κ B activation permits cells to escape apoptosis (Jung and Dritschilo, 2001). This high constitutive NF-κ B activity prevents cancerous cells from apoptosis leading to radioresistance (Smirnov *et al.*, 2001). Therefore NF-κ B is a crucial element of the cell's protective response to radiations and represents an attractive target for radiotherapy.

Attempts to protect normal cells against the deleterious effects of ionizing radiations by pharmacological intervention have been made in the past but the high toxicity of most of the chemical agents has necessitated search for alternative agents, which could be less toxic and highly effective at non-toxic dose levels.

In the last few decades there has been considerable interest in naturally occurring phytochemicals as potential radiotherapeutic agents due to their non-toxic nature and modulation of multiple targets. Plant extracts such as garlic (Gupta, 1988), ginseng (Pande *et al.*,1998a), Aloevera (Pande *et al.*, 1998b), Podophyllum (Goel *et al.*,1999), Amaranthus (Bhatia & Jain, 2003) and Mentha (Samarth and Kumar, 2003) have been found to have an advantage over the synthetic compounds in terms of low/no toxicity at the effective dose with minimum side effects.

Radioprotective activity of phytochemicals from plants like *Ginko biloba* (Hannequin, *et al.* 1986) *Centella Asiatica* (Goel et al 2001) *Hippohae rhannoides* (Mizina and Sitnikova *et al.*, 1999) and *Osimum sanctum*(Singh *et al.* 2003) have also been reported.

In the present study we purposed to evaluate Galangin (3,5,7 trihydroxyflavone) a bioflavonoid present in *Alpinia officinarum*. It is a Flavone and is also an important component of honey. It possesses various biological activities including antimicrobial, antimutagenic, Chemopreventive, anticlastogenic (Sohn *et al.*, 1998) and antioxidant properties (Russo and Longo, 2002; Tiong *et al.*, 2010). However much scientific literature is not available and its radiomodulatory potential has not been evaluated so far. With this background we evaluated the modulatory effect of Galangin and its mechanism of action against radiation induced damage using Swiss albino mice as model system.

AIMS & OBJECTIVES

2. AIMS AND OBJECTIVES

In the present study we purposed to evaluate Galangin (3,5,7 trihydroxyflavone) a bioflavonoid present in *Alpinia officinarum*. It is a lavone and is also an important component of honey. It possesses various biological activities including antimicrobial, antimutagenic, Chemopreventive, anticlastogenic (William *et al*, 1998) and antioxidant properties (Russo and Longo, 2002; Tiong *et al.*, 2010). The main aim of the present work is to evaluate Galangin for its radiomodulatory potential, using Swiss albino mice as a model organism. For this following objectives were set forth.

- To study the cytotoxicity of Galangin and its effect on radiation induced cell death.
- To study the effects of different doses of Galangin on radiation induced DNA damage.
- Study of the modulatory potential of Galangin on antioxidant enzymes in whole body irradiated mice.
- Study of the effects of Galangin on radiation induced signal transduction pathway.

REVIEW OF LITERATURE

3. REVIEW OF LITRATURE

3.1. Biological effects of Radiation

The biological effects of radiation are a result interactions of radiation energy with the atoms of the cells. Therefore radiation affects start from the lowest to the highest level or from atoms to whole body. There are mainly two mechanisms by which radiation interactions take place in the cells i.e. direct and indirect. Exposure of biological system to the ionizing radiation leads to the generation of species that are highly reactive and have the ability to alter all biological molecules such as DNA, lipids and protein (Kevin *et al.*, 2001, Sonnta, 1987). The biological effects of radiation range from damage to DNA, lipids, proteins and other macro molecules resulting into mutations, chromosomal aberration, cell death to death of the organism depending on the type of exposure.

3.1.1 Direct effects of radiation:

Ionizing radiation (IR) can act on biological molecules such as DNA, RNA, protein etc. and cause ionization and excitation. As a result, one or more chemical bonds break away resulting in atoms or molecules with unpaired electrons, which are very reactive and have a short span of life. The formation of these radicals takes place in the picoseconds time range. The bond may be repaired or cross-linking may take place due to radical-radical interactions. These free radicals may also react with lipids and may initiate chain reactions. This direct absorption of radiation energy by molecules is called direct effect of ionizing radiations.

$$IR + RH \longrightarrow R\bullet + \mathbf{H}\bullet$$

Both H· and R· radicals can react with another molecules.

$$R\bullet + R'H \longrightarrow R' + RH$$

Radicals can also produce cross linking reactions.

$$R\bullet + R\cdot \longrightarrow \mathbf{R\bullet - R\cdot}$$

These free radicals can react with other biological components of cells and cause further damage. The process of direct effects is more dominant in high LET radiation, such as neutrons or alpha particles.

3.1.2 Indirect effecst of radiation:

Water is the predominant molecule in living organism; therefore a major proportion of energy of radiation is absorbed by cellular water. The complex series of chemical changes that take place after exposure to radiation result in "radiolysis of water". Interaction of water with radiation leads to ionization and excitation process generating short lived free radicals (Daur *et al.*, 2010) which react with biological molecules and cause damage. Net result of the water radiolysis is the formation of hydrated electron (e_{aq}^-). Hydrogen atom (H•) and (OH•) free radicals.

$$IR + H_2O \longrightarrow H_2O+ + e\text{-}$$

$$H_2O + H_2O+ \longrightarrow H_3O+ + \mathbf{OH\bullet}$$

$$IR + H_2O \longrightarrow H_2O \longrightarrow H_2O + \text{photon emitted}$$

or

$$H_2O^* \longrightarrow \mathbf{OH\bullet + H\bullet}$$

and

$$H_2O + e^- \longrightarrow \mathbf{e_{aq}^-}$$

3.1.3 Bystander effects of radiation:

Until recently it was well known that genotoxic and carcinogenic consequence of ionizing radiation exposure are direct impact of energy absorption. However chromosomal damages have been reported in cells which were not directly exposed to ionizing radiation but existed

in the neighbourhood of irradiated cells (Prise *et al.*, 1998; Zhou *et al.*, 2000). Cytotoxic effects of ionizing radiation have be observed in the medium of irradiated cells when there was transfer of cell-free medium to non-irradiated cells (Mothersill and Seymour, 1997). These result shows that irradiate cells secrete some cytotoxic particles in the medium which cause killing of non irradiated cells. This is widely observed in fraction of the non irradiated cells which remain in direct contact with affected irradiated cells. Use of drugs such as lindane and octanol which inhibit gap-junction-mediated cell to cell communication, showed that there is suppression of bystander response (Azzam *et al.*, 2001; Zhou *et al.*, 2000) and thus gap junctions are important for bystander effects. The studies of direct damage of DNA by ionizing radiation are also challenged by showing that nuclear damage can be noticed after targeted cytoplasmic irradiation by using the newly developed microbeam irradiators (Prise *et al.*, 1998).

Reactive oxygen species also play an important role in various medium mediated bystander responses (Azzam *et al.*, 1998). These free radicals generate secondary long-lived organic radicals that cause bystander mutagenesis. The involvement of cell membrane in radiation induce bystander effect is also confirmed by suppression of SCEs and HPRT locus mutation in bystander cells with Filipin, a drug which is known to cause disruption of lipid raft (Nagasawa *et al.*, 2002).

Besides there are other pathways ways which have been shown to play a major role in the transduction of bystander effect such as COX-2 and NF-κ B (Zhou *et al* 2008). NF-κ B, COX-2, PGE_2, iNOS and NO play an important role in irradiated cells as well as in bystander cells.

NF-κ B is a transcription factor, activated by ionizing radiations and has a great role in cancer progression and regulation of immune system, regulation of expression of many genes

which have active role in cell proliferation, apoptosis, Invasion/metastasis angiogenesis etc (Huang *et al.,* 2001). NF-κ B is mainly regulated by Iκ B kinase (IKK), a multisubunit protein which has two catalytic subunits (IKKα ,IKKβ) and causes phosphorylation of Iκ Bα and a regulatory subunit IKKγ /NEMO (NF-κ B essential modulator) responsible for regulation of IKKα /IKKβ (Kim *et al.,* 2006).

3.1.4 Cellular and tissue response to radiations:

Cells have a good ability to repair damage, therefore radiation effects are some time reversible. In many cases the cells have the ability to repair completely and function normally (Bajinskis *et al.,* 2013). If the damage is severe, the affected cells die but in some instances cell are damaged but still have reproductive ability; however daughter cells lacking some life sustaining component finally die. The other possible result is that affected cells do not die but simply mutated, mutated cells reproduce and transmit the mutation, this could be beginning of tumor (Kamiya and Sasatani, 2012; Barcellos-Hoff, 2008).

Biological responses to radiation can be typically divided into two categories, depending on exposure. First category consists of exposure to high dose of radiation for short period of time. And the second category consists of exposure to low dose of radiation leading to chronic or long term effects (Joiner *et al.,* 2001). High dose of radiation normally kills the cells while low doses tend to damage or mutate them. Effects of low dose radiation occur at the molecular level of cells and may not be observed for many years.

Not all the cells give equal response to radiation or are equally sensitive to radiation (West *et al.,* 1993; Krueger *et al.,* 2007). Those cells which are actively reproducing have more sensitivity as compare to those which are not. A direct interaction of the radiation to the dividing cells could result in death or mutation of the cells, whereas interaction with dormant cells, lead to less effect. As a result lymphocytes and the cells which produce blood are

constantly dividing and are therefore most sensitive as compare to reproductive and gastrointestinal cells which have less regenerative capacity. The muscle and nerve cells are slowest in regeneration and are least sensitive to radiation (Koukourakis 2012).

During radiotherapy radiation treatment can also cause loss of function in normal tissues. In renewal tissue like bone marrow or GI tract loss of function may be connected with loss of proliferative activity of stem cells while in other tissues loss of function occurs through damage to mature cells / damage to supporting vasculature and stroma (Kim and Tannock 2005).

The effect of radiation treatment on normal tissues has been divided into early (or acute) responses and late responses, based on functional and histopathological endpoints. In early responses clinical symptoms manifest within a few weeks of radiation treatment whereas in late response clinical symptoms may take many months to years to develop (Soren, 2006).

Acute responses: Primarily occur in tissues with rapid cell renewal where cell division is required to maintain the function and integrity of the organ such as stem cells in the crypts of the small intestine or basal layer of the skin etc. Acute responses occur within three months of start of radiotherapy but are not usually limiting in fractionated radiotherapy due to ability of tissues to undergo rapid repopulation. Radiation-induced apoptosis has also been detected in many cells and tissues, such as hematopoietic cells, intestinal crypts and spermatogonia (Allan and Travis, 2005). In lymphoid and myeloid tissue a fraction of the functional cells die by apoptosis and, thus this mode of death plays an important role in the temporal response of these tissues to irradiation (Clarke *et al.,* 2005). Following irradiation of skin there is early erythema after irradiation (Collen and Mayer 2005), and this is believed to be related to release of 5-hydroxytryptamine by mast cells causing increase in vascular permeability (Pamela E *et al.,* 2001). Similar mechanisms may also lead to the early nausea and vomiting

observed following irradiation of the intestine. The severity of these reactions and time of recovery depend on the dose received, volume of skin irradiated and number of surviving basal cells which repopulate the tissue (Collen and Mayer, 2005).

Late tissue responses: Late tissue responses occur in organs whose parenchymal cells do not divide frequently and hence do not show mitosis linked death until called upon to divide. They also occur in tissues which show early reactions, such as skin/subcutaneous tissue and intestine. Late responses (generally regarded as those which occur more than 3 months after treatment) limit the dose of radiation during radiotherapy. The nature and timing of late reactions mainly depends on the tissue involved and can be expressed as loss of organ function, for example radiation-induced nephropathy (Moulder and Cohen, 2005). In general response of the tissue or organs to the radiation mainly depends on three factors (Hall and Giaccia, 2012).

- Inherent sensitivity of individual cells of the tissue.
- Kinetics of the tissue as a whole of which the cells are part.
- Way of organisations of the cells in a tissue.

The epithelial and haemopoietic bone marrow cell components are continuously regenerating compartments, the doubling time of clonogenic cells in crypts of small intestine is less than one day and whole GI tract renew within a few days (Barker *et al.,* 2008). The endothelial cells turn over in conduit blood vessel *in vivo* is one to several years, while only 0.1% cells actively proliferate in quiescent vessels (Cines *et al.,* 1998). Similarly only 0.3% submucosal fibrobast cells of intestine are found in proliferative phase (Koukourakis *et al.,* 2005). The proliferative rate in these cells increases drastically under stressful conditions. However neurons never proliferate. On the basis of distinct cellular compartmentalisation Koukourakis

(2012) has purposed that exposure of tissues to ionizing radiation can have five main types of effects.

Type I

Early radiation effects or cellular depletion involve cellular death and cellular depletion of a tissue which is followed by a proliferative response of stem cells.

Type II

Early radiation effects or reactive gene activation, it involves cellular and tissue dysfunction which is followed by, tissue oedema, increased vascular permeability, production of growth factor and cytokines by fibroblasts and endothelial cells and attraction of macrophages and other white blood cells, resulting into radiation induced inflammation. It is the result of activation of genes leading to inflammation, vascular permeability, angiogenesis, apoptosis inhibition etc. (Boerma *et al.,* 2006).

Type III

Late radiation effects or tissue disorganisation: If radiation damage is high, type II early effects can change into permanent tissue disorganisation and dysfunction, and proliferation of fibroblasts and vessels, leading to the known clinical aspects of oedema, fibrosis can result. Severe damage to the vascular component can also lead to necrosis. This is a result of a complicated cycle of growth factor and cytokine production, leading to organ dysfunction (Tsoutsou and Koukourakis, 2006).

Type IV

Radiation effects or stochastic effects, resulting in mutations in the exposed somatic cell genome and are passed to their progeny. This can occur in blood cells, leading to haematological malignancies, or connective tissue cells, that lead to solid tumours (Travis *et al.,* 2006).

Type V

Bystander effects, this effect was known for decades (Tsoutsou and Koukourakis, 2006), but now it has become the matter of intense research due to its mechanism which is not well known (Baskar, 2010 , Prise and O'Sullivan, 2009).In this effect there is damage in cells or tissues which are not directly exposed to radiation. This is a result of molecular signalling through gap junctions between cells, or via secreted molecules by the radiated cells. Both targeted and non targeted cells shows same type of DNA damage such as single and double strand break (Ilnytsky *et al.,* 2009 and Hei, 2003).

3.1.5 Radiation induced generation of ROS:

Ionizing radiation cause radiolysis of water and ionized water (H_2O^+), hydroxyl radical (. OH), hydroperoxyl radical (HO_2.), hydrogen radical (H.), and hydrogen peroxide (H_2O_2) are generated in a very short period of time ($\sim 10^{-8}$ s) [Riley, 1994]. Except H_2O_2, all are unstable and disappear in very short time. The ROS that have prominent biological effects are superoxide radical ($O_2.^-$), H_2O_2, alkyl hydroperoxide (ROOH), .OH, peroxyl radical (ROO.), and singlet oxygen. Most ROS are labile and disappear fast except H_2O_2 and ROOH, which are more stable. Ionizing radiation not only cause generation of ROS derived from water radiolysis; but also increase intracellular level of ROS (Zheng *et al.,* 2008). Radiolysis of water plays a major role in DNA damage (Wallace 1998 and Ward, 1988). It is becoming clear that reactive oxygen species produced secondarily have some role to play in other biological processes such as, genomic instability apoptotic signalling after IR, and radiation-induced bystander effects (Daur *et al.,* 2010). All these effect finally affects the cell integrity and survival.

There are several reports that NADPH oxidase is involved in ionizing radiation induced ROS production (Zheng *et al.,* 2008). Several studies also suggest that mitochondria are responsible for generation of reactive oxygen species (Mikkelsen, *et al.,* 2001). However, it is not fully known that how ionizing radiation induces ROS generation from mitochondria. It is

reported that ETC complex inhibitors such as rotenone and antimycin enhance electron leakage, indicating that the electron flow through the ETC greatly influences mitochondrial ROS production (Brand, 2010; Murphy, M. P 2009). Although it has been suggested that IR promotes mitochondrial ROS production, it remains to be elucidated how IR triggers it.

In response to ROS, cells have developed defence mechanism such as antioxidant enzymes that dismutate superoxide (O_2^-) into H_2O_2 (SOD) or degrade H_2O_2 (catalase, Peroxiredoxins and glutathione peroxidases) [Engelhardt, 1999; Rhee *et al.*, 2003]. When cellular generation of ROS crosses its threshold antioxidant capacity, a state of oxidative stress is reached that leads to serious cellular injuries and contributes to the pathogenesis of many diseases.

In response to radiation induced ROS different redox-sensitive transcription factors are also activated which depend upon level of ROS and coordinate distinct biological responses. At low oxidative stress Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes is activated (Halliwel & Gutteridge 1999). Now it is well established that H_2O_2 is the main signalling molecule in ROS induced signalling events which have the potential to inhibit tyrosine phosphatases through oxidation of cysteine residues in their catalytic domain, which activate tyrosine kinases and downstream signalling (Tonks, 2005). An intermediate amount of ROS causes activation of NF-κ B and AP-1 and triggers an inflammatory response, and a high level of oxidative stress induces alteration of the mitochondrial PT pore and disruption of the electron transfer, and results in apoptosis or necrosis (Halliwel & Gutteridge, 1999).

3.1.6 Radiation induced DNA damage and genomic instability:

Radiation causes various lesions in DNA, like rupture of the strands, alteration to the bases, destruction of sugars, cross linking and formation of dimers etc, leading to genomic instability, which is transmitted through many generations via surviving cells. Double strand

breaks are the most common DNA damage event caused by ionizing radiations, and are directly proportional to radiation dose (Uder, *et al.* 2005). Double strand DNA breaks are recognised by ATM and activated ATM mediates phosphorylation of different nuclear proteins such as p53 CHK2/Cds1, BRCA1, NBS1, and histone H2AX. (Jackson, 2002; Zhou and Elledge, 2000; Khanna *et al.,* 2001; Shiloh and Kastan, 2001; Shiloh, 2003). p53, a tumor protein is accumulated and activated through phosphorylation. Depending upon the cell type, the level of p53 rises rapidly which then cause accumulation of other genes products such as p21. GADD45, BAX, and p53AIP1 (Vogelstein *et al.,* 2000; Wahl and Carr, 2001; Oren *et al.,* 2002; Vousden and Lu, 2002). All of this leads to, cell cycle arrest at G_1/S, apoptosis, and growth arrest (senescence). One single DNA double-strand break is enough to activate p53 (Huang *et al.,* 1996). It should be noted that ionizing radiation can also induce genomic instability in cell lines which have no functional p53 (Kadhim *et al.,* 1996). For example, induced delayed DNA damage could cause dicentric chromosome formation, leading to inhibition of cell division by forming chromosomal bridges, and this process does not require p53 function.

Recent studies have also shown that bystander effects (Price *et al.,* 1998; Nagasawa and Little, 1999; Zhou *et al.,* 2000) can also lead to radiation-induced genomic instability (Barcellos-Hoff and Brooks, 2001; Mothersill and Seymour, 2001; Morgan *et al.,* 2002; Lorimore and Wright, 2003).Although the mechanism is not fully known, it is also hypothesized that it stimulates the production of reactive oxygen species after irradiation (Clutton *et al.,*1996; Narayanan *et al.,* 1997; Limoli *et al.,* 1998; Roy *et al.,* 2000; Morgan *et al.,* 2002). These results show that not only intracellular factors but also extracellular factors are involved in genomic instability. Now there is considerable evidence that non irradiated cells also express delayed gene mutation and different types of chromosomal aberrations (Wright, 1992, 1997). The delayed mutations are mostly point mutations, similar to

spontaneous mutations, while early effects of ionizing radiation are usually deletion mutation (Vetrovs, *et al.,* 1997). Although the mechanism for these delayed effects of ionizing radiation is not well known, excessive production of reactive oxygen species and nitrogen species have been implicated (Wright, 1998; Little, 2000; Iyer and Lehnert, 2000; Yamamori *et al.,* 2012)

3.1.7 Radiation induced Apoptosis:

DNA damage leads to a network of signal transduction pathways involved in cell cycle arrest, apoptosis and the activation of DNA repair processes. Ionizing radiation has the capacity to interact directly with the target and cause ionisation, thus initiating the signalling events which finally lead to biological changes (Valentin, 2006; Lehnert, 2007).

Not all signalling pathway are initiated at the site of DNA damage but also through activation of receptor and sphingomyelinases (SMase). In some cell types, the breakdown product of sphingomyelin such as ceramide (Haimovitz *et al.,* 2008) acts as a secondary messenger mediating apoptosis induction after IR. Ionizing radiation also induces apoptosis through generation of free radical scavenging (ROS).

P53-mediated apoptosis is an important function of its tumour suppressor activity and also an important mechanism by which many anti-tumour agents kill cells; however, the mechanisms by which p53 causes apoptosis are not completely known. One pathway is via the transcription of the apoptosis - inducing protein, Bax, but transcription-independent activation of caspase-3 also occurs after ionizing radiation. p53 can mediate the apoptotic signals through a Bax/cytochrome c-independent pathway, which involves protein kinase A. Some p53-induced genes (PIG) expressed before the apoptosis were studied and many were noticed to encode proteins that could respond to oxidative stress conditions (Polyak *et al.,* 1997).

3.1.8 Radiation induced signalling pathways:

Ionizing radiations can induce multiple signalling pathways in cells. Radiation can activate pathways dependent on the expression of growth factor receptors; however it is not necessary that the one pathway that gets activated in one cell type also gets activated in other cell types. In some cell types, enhanced basal signalling by onco-genes such as Ras may provide a radioprotective signal. In other cell types, this may be done by PI3K, NF-kB or MAPK etc. Radiation-induced signalling also takes place through growth factor receptors like EGF receptor channel by different downstream signalling pathways. Receptor signalling mainly depends on auto-crine factors.

Different studies have proved that the epidermal growth factor receptor (EGFR) is activated in response to irradiation of various carcinoma cell types. The threshold dose at which radiation could induce ErbB1 phosphorylation in carcinoma cells appeared to be ≈ 0.5 Gy (Glazer *et al.,* 2001). Ionizing radiation can activate the MAPK pathway via the activation of EGFR to same level that is observed by physiological, growth-stimulatory, EGF concentrations (0.1 nM). It is also shown that, Irradiation of tumor cells can increase transcription of TGFα and activate the EGFR; this is one of the mechanism by which ionizing radiation can increase proliferation rate of surviving cells.

Autocrine ligands also play an important role in the activation of receptors after radiation exposure. TGFα has been shown to cause secondary activation of ErbB1 and the downstream MAPK and JNK pathways after irradiation in many carcinoma cell lines (Hagan and Dent, 2000). Ionizing radiation caused cleavage of pro- TGFα in the plasma membrane, which leads to its release and initiates downstream signalling. Increasing the radiation dose enhance both the secondary activation of ErbB1 as well as secondary activation of the MAPK and JNK pathways, suggesting that radiation can promote a dose- dependent increase in the cleavage of pro-TGFα. Radiation-induced MAPK activation has also been shown to

increased expression of the DNA repair proteins ERCC1 and XRCC1. More recent findings have shown that radiation also activates other ErbB family members such as ErbB2, ErbB3 and ErbB4 (Rupert Schmi *et al.,* 2003). Since ErbB2 activation does not depend on ErbB1, it can be concluded that radiation may causes activation of multiple plasma membrane receptor tyrosine kinases.

In addition to ErbB family there are also other growth factors and cytokine receptor which play an important role in radiation response of cell. Cytokines such as TNFα , IL6, urokinase-type plasminogen activator (uPA), and TGFI3 have all played an important role in cell survival after radiation exposure (Wirth and Sagan, 2002). Radiation causes rapid activation of TNFα receptor and, in addition, radiation induced MAPK and p38 pathways are known to enhance the synthesis of TNFα ligand (Wirth and Sagan, 2002). TNFα signaling after irradiation may lead to the activation of both pro-caspase enzymes as well as the transcription factor, nuclear factor kappa B (Price *et al.,* 1998).

NK-kB also acts as a radioprotector.Interleukin 6 (IL-6) acts both as pro-inflammatory and anti-inflammatory cytokines, and regulates immune cell function. Several studies have shown that IL6 can induce anti-apoptosis signals in cells that are protective against the damaging effects of ionizing radiation (Friedhelm Herrmann *et al.,* 1993). The radiation induces expression of IL6 mediated by PI3K pathway and activation of the NF-κ B depending upon the cell type.

3.2. Radiation protection

Accidental exposures to high doses of ionizing radiation and radiotherapy leads to increased rate of DNA damage and cell death of normal cells and tissue disorganization. To protect cells and tissues from this damaging effect of radiation, several radio-protective agents have been identified which can effectively reduce the formation of free radicals produced in cells as a result of exposure to radiation. Radio-protectants also help to minimize accumulation of genetic mutations in radiotherapy patients and people going on to space missions (Testard *et al.,* 1995). Radiation protection is an area of great importance due to its increased application in planned radiotherapy as well as unplanned radiation exposure in our daily life.

The use of radiation therapy to treat cancer also involves exposure of normal tissues. Radiation therapy also injures/destroys cells in the exposed area by damaging their genetic material. As a result, patients may experience symptoms during the therapy and for a few weeks after therapy or months to years later. Research has been progressing in whole world for development of different chemical and biological compounds to protect against radiation induced unwanted damage. Compounds are being developed which protect the normal tissue from radiation and heal injury caused by radiation. Various natural and synthetic compounds such as antioxidants, vitamins, DNA binding molecules, cytoprotective agents, immunomodulators, have been evaluated extensively for their radioprotective properties in both *in vitro* and *in vivo* models (Paul *et al.,* 2011).

An ideal radio-protective agent should have following properties (Milosevic *et al.,* 1990)

1- It should have easy route of administration and should be stable even after liver detoxification.
2- It should have fast reabsorbing properties and easy bio-availability to all tissues and organs in the body.

3- It should have minimal side effects even on repeated administration.

4- It should be effective against damaging effects of different types of radiations. (X, gamma, neutrons)

5- It should have a property of differential protection to normal tissue over cancerous tissue.

At the molecular level, exposures to high doses of radiation induce free radical formation that damages DNA and cellular organelles, which activate repair pathways. Damage if completely repairable lead to cell survival, otherwise process of apoptosis is triggered leading to cell death. Improper damage repair with mutations in surviving cells sometimes cause carcinogenesis. Radio-protective agents can act at each of these steps to reverse the destructive effect of radiation.

3.2.1 Mechanistic classification of radio-protectants:

Blockers of oxygen consumption:

Free radicals formed as a consequence of exposure of water molecules to ionizing radiations, further react with intracellular oxygen to form oxygen-free radicals, which damage DNA directly. Mitochondrial and other cellular functions also g*et al*tered by these free radicals leading to mutagenesis and death of the cell. Agents that can reduce oxygen consumption of the cell thus reducing the formation of oxygen free radicals can act as radioprotectants. Amifostine is the only well known radioprotectant that has been approved for clinical use under this category (Glover *et al.*, 1984). It scavenges free radicals and reduces oxygen tension in normal tissue shifting the metabolism to anaerobic pathways. (Koukourakis *et al.*, 2004). Another method involves the stabilization of HIFs in the normal cells, which directly acts on LDHA (Lactate Dehydrogenase-A) leading to its over-expression, which suppresses oxidative phosphorylation thus oxygen consumption of the cell (Semenza *et al.*, 1996). This

reduces radiation damage by reactive oxygen species. Radioprotective agents that utilize this pathway include Cobalt chloride (CoCl2); the iron chelator, desferriox-amine; and the organomercurial compound, mersalyl, which induce HIFs and mimic hypoxia (Piret *et al.,* 2002; Jiang *et al.,* 2005). Another radioprotectant Clioquinol, a copper (Cu) (II)/ zinc (Zn) (II) chelator, inhibits the degradation of HIF-1α .

Free radical scavengers:

Free radical scavengers are endogenously present in all cells, which are normally expressed under stressful conditions. Zn, Cu or the mitochondrial manganese (Mn) superoxide dismutase (SOD) are some enzymes that neutralize free oxygen radical by converting it into a less toxic hydrogen peroxide (Gutteridge and Halliwell, 2000). Glutathione, an endogenous thiol group containing molecule also acts as free radical scavenger (Bump and Brown, 1990). Compounds that can induce the expression of these scavengers act as radio-protectants like Amifostine and Cerium oxide (CeO2) nanoparticles (Liu SC *et al.* 2004). Amifostine is a potent inducer of MnSOD and its dephosphorylated thiolic form (WR-1065) exogenously scavenges free radicals. Cerium oxide (CeO2) nanoparticles also act as free radical scavengers and can induce endogenous SOD production (Colon *et al.,* 2010) Nitroxide compounds like tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) also have radio-protective properties (Hahn *et al.,* 1992).

DNA repair boosters:

DNA damage caused by interaction with free radicals immediately activates DNA repair pathways that involve action of complex enzymatic machineries to accurately repair the damage and allow the cells to survive. WR-33278 the disulfidic form of amifostine has structural similarities to polyamines putrescine, spermidine and spermine (Mitchell *et al.,* 1995) which are involved in DNA repair. WR-33278 can scavenge hydroxyl-radicals and

induce packaging of DNA into liquid-crystalline condensates (Savoye *et al.,* 1997) making it inaccessible to radiolytic attack. It can also remove Platinum adducts from DNA like WR-1065 (Treskes *et al.,* 1992). Resveratrol (3,4-trihydroxy-trans-stilbene), a polyethanol extract from red grapes has an ability to stimulate sirtuins expression in cells, which is involved in DNA repair, based on their deacetylases and mono-ribosyltransferase activity (Howitz *et al.,* 2003). Butin, a 7,3,4-trihydroxydihydroflavone enhances the expression of Oxoguanine DNA glycosylase (OGG1), an enzyme that initiates base excision repair pathway of the oxidised purine bases and also protects mitochondrial DNA from oxidative damage (Dobson *et al.,* 2002; Zhang *et al.*, 2011).

Inhibitors of death signalling pathways:

Free radical induced endoplasmic reticulum and mitochondria damage immediately triggers the pro-apoptotic cell response involving p53 expression as the first step of apoptosis. The cell cycle gets arrested at G1 phase and all the repair pathways are triggered. If the damage is repairable the cell cycle arrest is released if not, then apoptosis is induced whereby mitochondrial caspases are released finally leading to death. Compounds that can block this pathway can act as potent radioprotectants like Sodium orthovanadate (NaV04) and Pifithrin-a (Imino-tetrahydrobenzothiazol-tolylethanonehydrobromide) which inhibit p53 (Morita *et al.,* 2010). This method of radioprotection may prevent Type I toxicities but Type IV stochastic effects would increase leading to the enhancement of radiation carcinogenesis.

Growth factors:

15 - 20 days post exposure to radiation type I early toxicity symptoms appears where tissues tend to repopulate cells by synthesizing various growth factors that induce stem cell proliferation and toxicity healing. Hemopoietic growth factors like erythropoietin (EPO) and G (or GM)-CSF help treat neutropenia and anaemia post radiotherapy (Hensley *et al.,* 2009).

Applications of recombinant forms of these growth factors can radio protect tissues by mimicking their action and repopulating the tissues post damage. Becaplermin, (a recombinant platelet-derived growth factor B-chain homodimer), telbermin [recombinant human (rh) vascular endothelial growth factor (VEGF)] are few examples of radio-protectants under this category (Moore *et al.,* 2009; Henry *et al.,* 2003).

Blockers of radiation inflammation and chemotaxis:

Immediately after exposure to radiation the cells undergo type II radiation toxicity showing inflammatory response caused by cytokine release, leukocytes infiltration leading to vascular dysfunction and tissue oedema, followed by fibrosis in most of the cases. Radioprotectants target these steps of radiation inflammatory response to subside the harmful effects. IL-10 is an anti-inflammatory interleukin that can suppress leukocyte extra vasation and reduce Angiotensin-II mediated oxidative stress and vascular dysfunction. (Didion *et al.,* 2009) Recombinant IL-10 is under clinical trials (Colombel *et al.,* 2001). IL-10 inducers like Noradrenaline, Y-40128 (Tsujimoto *et al.,* 2009), the peroxisome proliferator-activated receptors-a ligand PPAR-alpha WY14643 (Yanagisawa J *et al.,* 2009) and the anti-tumour alkyllysophospholipid analog edelfosine (Mollinedo *et al.,* 2009) are potent radio protectants.

Blockers of autocrine/paracrine pathways:

Radiation toxicity can lead to deregulation of stromal cells resulting in fibroblast and endothelial proliferation and thus tissue disorganization and failure. This autocrine/paracrine loop mediated pathway can be modulated by specific inhibitors which come under this category of radio protectants. Accumulation of HIF1, 2 directly increases the expression of VEGF which is involved in fibrosis via VEGF receptors on fibroblasts, myofibroblasts and endothelial cells. (Yoshiji *et al.,*2003; Chintalgattu *et al.,* 2003). Inhibitors of this pathway like vatalanib, pazopanib and cediranib (the VEGF tyrosine kinase inhibitors) can block these

loops and prevent fibrosis (Hamada *et al.,* 2005). Similarly Retinoic acid; TNP- 470 (anti-angiogenic compound); PX-478 and YC-1 (HIF inhibitors) [Williams *et al.,* 2009; Park *et al.,* 2008]; naringenin, halofuginone and relaxin (Inhibitors of TGFb signalling) [Liu *et al.,* 2006; Xavier *et al.,* 2004; Heeg *et al.,* 2005] and COX inhibitors; are few compounds that belong to this class of radioprotectants.

Antimutagenic keepers of genomic integrity:

Exposure to radiation during radiotherapy and occupational exposure may cause mutagenesis and increased chances of radiation carcinogenesis in normal tissues. Free radical mediated DNA double strand breaks are the hallmark of radiation induced damage that lead to mutagenesis. Radioprotectants like Amifostine along with free radical scavenging can also reduce the mutation load by reverting back specific p53 mutations (Maurici *et al.,* 2001; Grochova *et al.,* 2008) as well as suppressing the activation of c-myc gene (Liu *et al.,* 1997) by interfering at the genetic level. Non-homologous end joining (NHEJ) is an error prone repair pathway for double strand DNA breaks where as homologous recombination pathway is error free (Mahaney *et al.,* 2009). Vanillin derivatives have antimutagenic properties where they block NHEJ pathway and shift the balance towards the error free homologous recombination (Durant and Karran, 2003). TGFb is a growth factor that can clean cells that have centrosome aberrations and genomic instability mediated by p53 activation (Maxwell *et al.,* 2008). NF-κ B also has some role in protecting cells from genomic instability (Natarajan *et al.,* 2008).

Protectctors against bystander cells:

Non-targeted effects of radiation are mainly mediated by gap junctions that allow the flow of small molecules between cells and account for bystander effects. Agents that can target gap junctions and their constituent proteins can act as radio protectants for example Gamma

hexachlorocyclohexane (lindane) can induce gap junction endocytosis (Segretain *et al.* 2004). TGFβ-3 can down-regulate connexion 43 (Jin *et al.,* 2008). Phorbol ester 12-O-tetradecanoylphorbol-13-acetate and chlorohydroxyfuranones, also inhibit gap junction (Hakulinen *et al.,* 2004). Long range bystander effect is mediated via cytokine signalling by macrophages (Lorimore *et al.* 2008). Agents like (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (Al-Abed *et al.,* 2005) and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride can inhibit macrophage activation (Radzioch, and Varesio 1988) thus acting as radio protectants.

3.2.2 Phytochemicals as radioprotectors:

Prevention of adverse effects of radiation in clinical trials, by antioxidant vitamins and other biological molecules suggested that, high doses of antioxidants can be used as adjuvant for increasing radiation treatment efficiency (Jena *et al.,* 2010). The fact that there is no synthetic radioprotector developed which has all the quality such that it does not produce toxicity, provide effective long time protection and persist for number of years and can be easily administered, plant based phytochemicals are being explored. They are non-toxic with proven great therapeutic benefits and have been used since ancient times for curing various diseases and disorders. Today more than 70% people depend upon plant based remedies for their health care needs (Benkovic *et al.,* 2008). Various plants have been proved to be beneficial for free radical-mediated conditions in humans like arthritis, cancer, Parkinson's disease, Alzheimer's and inflammatory disorders (Paul *et al.,* 2011). Plants are rich sources of phytochemicals which include anthocyanins, flavonoids etc. Among all of these, several flavonoids (quercetin, myricetin-flavonol, luteolin-flavone) have been reported to have potent antioxidants properties with radioprotective abilities *in vitro* and *in vivo.* (Piya *et al.,* 2011)

3.3. Review of literature for Galangin:

Galangin (GA, 3,5,7 -trihydroxyflavone), is an active constituent of the rhizome of *Alpinia galanga*, a plant of the ginger family Zingiberaceae. It is also found in root of *Alpinia officinarum*, which is a common spice and constituent of herbal medicine in Asia. Besides plants it is also present in propolis, a resinous material produce by activities of honey bees from gum of various plants. Propolis contains up to about 9% galangin (Park *et al.* 1995).

Galangin is a member of the flavonol class of flavonoids. It is a flavonol derivative that does not have a hydroxyl group in the B-ring, but has 2,3-double bond with 3-hydroxyl group in the C-ring and 5,7-dihydroxyl groups in the A-ring (fig. 1). Therefore galangin is the most lipophilic compound among similar flavonols such as quercetin, kaempferol, morin and myricetin, etc. It has been shown to possess a variety of biological activities at non-toxic concentrations in organisms.

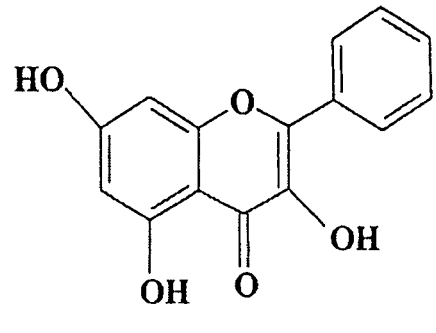


Fig 1: The chemical structure of Galangin

3.3.1 Anti-genotoxic activity

Galangin has been reported to have multiple bioactivities and affects in many cell systems. Results from many *in vitro* and *in vivo* studies have shown that galangin is capable of suppressing the mutagenicity (Sohn *et al.*, 1998) and clastogenicity (Heo *et al.*, 1992) of

some alkylating agents. A study of the antigenotoxicity of galangin against n-methyl-n-nitrosourea (MNU) showed it to be anti mutagenic toward MNU (Heo et., al 1998). Further anti-genotoxic activity against polycyclic aromatic hydrocarbons (PAHs), different DNA cross-linking agents and radiomimetic chemicals has also been reported (Huang *et al.,* 1983). Galangin exerts anti-genotoxicity by inhibiting the formation of DNA adducts by alkylating agents or PAH. It causes a potent, dose-dependent inhibition of CYP1A1 and CYP1A2 activities (Zhai *et al.,* 1998) and is a strong inducer of the phase II enzyme. Repressor and inhibitor of phase I or inducers /activators of phase II enzymes are known to have a good chemopreventive potential.

3.3.2 Anti-microbial activity:

Antibacterial activity of galangin was investigated against 17 strains of 4-quinolone resistant *Staphylococcus aureus* using an agar dilution assay. The strain which possessed an amino acid alteration in the GrlB subunit of topoisomerase IV had increased susceptibility to galangin. The topoisomerase IV enzyme may therefore be implicated in the antibacterial mechanism of action of galangin (Cushine *et al.,* 2006). The bioactivity of the flavonoids galangin 3-methyl ether was investigated *in vitro* against amastigote stages of *Leishmania amazonensis* and found to have significant activity (Campana *et al.,* 2009). Aggregatory effect of galangin on bacterial cell was also investigated. In preparatory time-kill assays, galangin was found to reduce colony counts of *Staphylococcus aureus.*

3.3.3 Enzyme-modulating activity:

Galangin, a dietary flavonoid, inhibited cytochrome P450 1A1 (CYP1A1) expression induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This inhibitory activity remained after permeating human intestinal epithelial Caco-2 cell monolayers, but was reduced when galangin permeated TCDD-pretreated Caco-2 cells (Hamada *et al.,* 2010). In a study several

flavonoids were tested for their potential function in inhibiting acetylcholinesterase (AChE) activity from the brain *in vitro*. Among all the tested flavonoids, galangin showed an inhibitory effect on AChE activity with the highest inhibition (Guo *et al.*, 2010). Investigation of dietary benefits of bioflavonoids to the inhibition of ATP synthase found that galangin has significant inhibitiory potential (Chinnam *et al.*, 2010). Galangin reversibly inhibited human butyrylcholinesterase (BChE, EC 3.1.1.8) and was found to be the most potent BChE inhibitor among the tested flavonoids, which showed 12 times higher preference for binding to BChE than to the related enzyme human acetylcholinesterase (AChE, EC 3.1.1.7) (Katalinic *et al.*, 2010).

3.3.4 Anti-oxidant activity:

Antioxidant activity of galangin was measured and found to show significant DPPH radical scavenging activity (Lascar *et al.*, 2010). Honey has been used since long time both in medical and domestic needs. Antioxidant property of honey was investigated and found to have significant antioxidant activity (Jaganathan *et al.*, 2009). Antioxidant activity of galangin on Fe2+/citrate-mediated membrane lipid peroxidation in isolated rat liver mitochondria was investigated and results suggest that 2, 3-double bond in conjugation with the 4-oxo function in the flavonoid structure are major determinants of the antioxidant activity of flavonoids in mitochondria (Dorta *et al.*, 2008). Galangin has anti-oxidative activity and free radical scavenging effect without significant pro-oxidant activity (Heo and Yang, 2000; Silva *et al.* 2000). Galangin helps to preserve other protective antioxidants such as vitamin E, vitamin C, and other flavonoids, and can also prevent lipid peroxidation (Sulaiman and Boukraa, 2010).

3.3.5 Anti-inflammatory activity:

Topical anti-inflammatory activity of selected flavonoids commonly found in propolis was investigated and it has found that after topical application of galangin for 3 h, there was more

than 50%, reduction in croton oil-induced oedema in a mouse model (Toit *et al.*, 2009). Inhibition of human neutrophil degranulation by galangin was also evaluated using released elastase as a biomarker and galangin was found to have significant Inhibitory potency (Kanashiro *et al.*, 2007).

Galangin has been found to simulate calcium deposition and alkaline phosphatase, indicating that it is capable of stimulating extracellular matrix formation and calcification (e.g. stimulating bone formation) and is capable of increasing osteoblast activity. It is a strong activator of the bone morphogenetic protein receptor (BMP-receptor). Hence, galangin can be advantageously used in a method for maintenance of bone health or the treatment, alleviation and/or prevention of bone disorder (Huh *et al.*, 2013).

3.3.6 Anti-cancer activity:

Galangin suppresses beta-catenin induced transcription, which is aberrantly up-regulated in colorectal and liver cancers, by promoting the degradation of intracellular beta-catenin. Galangin down-regulated the intracellular beta-catenin levels in cancer cells with inactivating mutations of adenomatous polyposis coli or Axin (Junsung *et al.*, 2011). The *in vitro* preferential cytotoxicity of galangin evaluated against a PANC-1 human pancreatic cell line displayed the most potent preferential cytotoxicity in the nutrient-deprived medium and triggered apoptosis-like morphological changes (Li *et al.*, 2010). Galangin was found to have inhibitory effects against various cancer cells (Tauil *et al.*, 2009). It has been found to be an interesting candidate for combination therapy in the treatment of imatinib-resistant leukemias. It caused a decrease in Bcl-2 levels and markedly increased the apoptotic activity of imatinib both in sensitive or imatinib-resistant Bcr-Abl+ cell lines. (Tolomeo *et al.*, 2008). Galangin inhibits cyclo-oxygenase (COX) enzymes, suppresses the aggregation of platelets, and thus provides anti-inflammatory effects. It is considered as most potent COX-2 inhibitor

among natural products (Gabor and Razga, 1991). A study on breast tumor cells found galangin blocked the "transition of cells from the G0/G1 to the S phases of cell growth through the elimination of cyclin D3. A study on leukemia cells showed that galangin blocks aryl hydrocarbon receptor activation and PAH immunotoxicity (Murray *et al.,* 2000).Anti-proliferative activity on the growth of an estrogen receptor-positive human breast cancer cell line was reported by So *et al.,* (1997). Activity of chemically induced aryl hydrocarbon receptors (AhR), as well as cell proliferation has also been reported to be suppressed by galangin. (Murray *et al.* 2006). Therefore galangin may be a useful chemopreventive agent against potential long-term health effects from genotoxic environmental agents.

3.3.7 Effect on metabolic process:

The Investigations on the effects of galangin on oxidative stress, inflammatory cytokine levels and NF-kappa B activation in fructose-fed rat liver showed that galangin prevented the rise in plasma glucose, insulin and triglycerides and improved insulin sensitivity, however, treatment with galangin downregulated the expression of the cytokines. Translocation of NF-kappa B into the nucleus was also increased in fructose diet-fed animals, which was prevented by galangin (Sivakumar *et al.,*, 2011). Effect of the flavonoid galangin in isolated rat thoracic aortic rings was investigated. Galangin relaxed aortic rings with or without endothelium and caused nitric oxide release from aortic rings (Morello *et al.,* 2006).

The exceptional diverse biological characterstics of galangin prompted us to examine its radiomodulatory activity. This study is an attempt to study the effect of galangin, a molecule offering broad therapeutic window, on radiation induced damage, which in future may become a member of anticancer armamanteriun.

MATERIALS & METHODS

4. MATERIALS AND METHODS

4.1 Chemicals:

Galangin, RPMI medium 1640, FBS, antibiotic solution (Himedia), primary monoclonal antibody NF-κ B, Bcl-2 and Bax, agarose, Ethidium Bromide (EtBr), reduced glutathione (GSH) bovine serum albumin (BSA), 1,6 diphenyl-1,3,5-hexatriene (DPH), thiobarbituric acid (TBA), 5,5-dithio-bis(2-nitrobenzoic acid (DTNB), 1 chloro 2-4, dinitrobenzene (CDNB), agarose, Triton X-100 and MTT [3(4,5-dimethyl thiozol-2-yl] were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Secondary anti-mouse HRP-conjugated antibody was purchased from Bangalore Genei. Spectra multicolour broad range protein ladder was purchased from fermentas, Fisher scientific India. All other chemicals used were of analytical grade.

4.2 Animals:

Male Swiss albino mice (6-8 weeks old) weighing 28-30g were used for the present experiments. They were maintained in the animal house of the university. Standard feed (Hindustan Lever Ltd, Mumbai, India) and water were provided ad libitum. All the experiments were conducted adhering to the ethical guidelines of the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and Jawaharlal Nehru University Institutional Animal Ethics Committee, on the use of animals for scientific research.

4.3 Gamma-irradiation:

All experiments were performed at room temperature in gamma chamber (240 TBq ^{60}Co Model 4000 A) obtained from the Isotope Division of Bhabha Atomic Research Centre (BARC), Mumbai, India. The dose rate used was 0.041 Gy s^{-1}. Mice were irradiated

***In vitro* studies:**

4.4 Preparation of primary splenocytes:

The animals were sacrificed by cervical dislocation. For *in vitro* studies, spleens were dissected out and washed in 0.9% saline. Spleens were crushed in between two frosted slides in 1ml PBS and single cell suspension was made after bursting RBCs by hypotonic shock. The remaining splenocytes were washed twice in PBS (pH 7.4). Cells were counted and suspended in RPMI 1640 supplemented with 10% FBS and 1% anti-biotic-antimycotic solution. Typically each culture consisted of an initial density of 1.5 X 10^6 cells/ml of medium.

4.5 pBR322 plasmid relaxation assay:

Plasmid relaxation assay was performed to estimate the pBR322 plasmid DNA damage induced by exposure to various doses of ionizing radiation. Plasmid DNA (250-300 ng) in 10 µl of 1X PBS (pH 7.4) was exposed to 3 Gy gamma radiation. We choose 3 Gy as dose response studies were already done in lab (Richi et al., 2012).

To study the effect of different doses of Galangin (10, 25, 50 and 100 µM), plasmid DNA was pre-treated with Galangin for 2 hr prior to exposure to radiation. The DNA was allowed to stabilize for 30 min on ice before subjecting it to electrophoresis on 1% agarose gel in 100 mM Tris acetate, EDTA (2 mM buffer) (pH 8.3), to study DNA strand breaks. The gel was run at 5V/cm for 1 hr at room temperature. The gel image was taken by gel doc system (Alpha Innotech Corp) and the bands were quantified using spot denso band analysis software provided along with the system.

4.6 Treatment of the cells:

Preliminary studies were carried out to study the effect of different doses of radiation on splenocytes. After checking the toxicity of galangin on splenocytes, four doses of Galangin were selected (10, 25, 50 and 100 μM) for further experiments. The splenocytes were incubated with different concentration of Galangin for 2 hr, at 37 ^{0}C in a humidified atmosphere of 5% CO_2 prior to radiation exposure (3 Gy). Samples were incubated for another 24 hr post irradiation at 37 ^{0}C in a humidified atmosphere of 5% CO_2 for cell viability assays.

4.7 Cell proliferation / viability assay:

The proliferation of the cells was determined by the MTT assay. After various treatments 4 x 10^4 cells /well were seeded in 96-well microtiter plates. After 48 hr, 5 mg/ml MTT in PBS was added to each well. Mitochondrial dehydrogenase metabolized MTT to purple formazan crystals in living cells, which were dissolved in 100 μl of DMSO after 4 hr and absorbance of all the samples was read at 570 nm with 690 nm as a reference wavelength using microplate reader (Molecular Devices, Inc. US). Cell viability was proportional to the absorbance measured.

In Vivo Studies:

To investigate the effect of Galangin (G A) *in vivo* mice were divided into different groups.

Group 1: control, animals (No treatment)

Group 2: Vehicle control, received corn oil.

Group 3: Radiation control (1.5Gy)

Group 4: Galangin (10 mg/kg)

Group 5: Galangin (50 mg/kg)

Group 6: Galangin (100mg/kg)

Group 7: Galangin (10 mg/kg) +Radiation

Group 8: Galangin (50 mg/kg) +Radiation

Group 9: Galangin (100mg/kg) +Radiation

For present studies Galangin was given orally, once a day for three consecutive days. Corn oil was used as vehicle. Food and water were provided *ad libitum* to the animals. The dosage of Galangin is based on earlier report (Sohn et al., 1998). At the end of 4^{th} day, whole body irradiation was given and then after 24hr mice were sacrificed by cervical dislocation. The liver, kidney and spleen tissue was dissected out and washed in ice-cold saline and snap frozen in liquid nitrogen and finally stored at -80°C for further use.

4.8 Preparation of tissue homogenates:

0.5 g of tissue was weighed for each sample and homogenized in 0.5 ml of Tris;KCL Buffer (0.15 M, PH 7.4), from this 500 µl of homogenized sample of corresponding tissue were taken for GSH assay. Homogenized samples were centrifuged at 15000 g for 15 m at 4^0C. Further supernatant transferred to ultra centrifuge and centrifuged at 40,000 rpm for 1 hr at 4^0C followed by separation of cytosolic and microsomal fraction in separate eppendorf. Cytosolic fractions were used to assess the specific activity assay of Catalase, SOD, GST and GSH.

4.9 Protein quantification:

Cytosolic protein was determined by Bradford Assay. 250µl of 1x Bradford reagent was mixed with each sample and incubated at room temperature for at least 5 min. OD was taken

at 595 nm in the spectrophotometer. The protein was estimated from the standard curve using BSA.

4.10 Biochemical assays:

GSH content

Glutathione content was determined by method described by Moron et al.(1979).

Principal: The reduced glutathione was estimated by reaction with DTNB generating a yellow coloured complex (5-Thiol-2-nitrobenzoate). OD is absorbed at 412nm.

Reduced Glutathione + DTNB $\longrightarrow$ 5-Thio-2-nitrobenzoate

Assay: Immediately after homogenizing, 0.5ml crude tissue homogenate was added to 0.1ml of 25% TCA, which caused precipitation of the protein. It was centrifuged at 5000rpm for 5min at 4^0C. 0.1ml supernatant was mixed with 0.9ml of 0.1M phosphate buffer (PH 8.0) and 2ml of DTNB. The absorbance was read at 412nm.

Specific activity of Catalase

Principal: it catalyses the breakdown of hydrogen peroxide (H_2O_2) into H_2O and O_2.

$$H_2O_2 \longrightarrow 2H_2O + O_2$$

Assay: The supernatant was incubated with ethanol (10 µl/ml) for 30 m on ice. Then triton X-100 (1%) was added and further kept for 30 m on ice. The treated supernatant was added to assay mixture containing 0.05 M sodium phosphate buffer (PH 7.0), 10 mM H_2O_2 decrease in absorbance was measured at 240 nm. The activity was calculated with extinction coefficient 0.04 $nmole^{-1}cm^{-1}$. One unit of catalase activity was defined as amount of enzyme needed to decompose one mole of H_2O_2 per minute.

Specific activity of superoxide dismutase

Superoxide dismutase was assayed by the method of Marklund and Marklund., (1974) by measuring the inhibition of autoxidation of pyrogallol at wavelength of 420 nm. SOD Assay mixtures (1 ml) contained 0.05 M sodium phosphate buffer (pH 8.0), 0.1 mM EDTA and 0.27 mM pyrogallol. The reaction was initiated by the addition of enzyme sample, pretreated with Triton X-100 on ice for 30 min. One unit of enzyme was defined as the amount of SOD required to produce half-maximal inhibition of auto-oxidation.

Specific activity of glutathione S-transferase

Glutathione S-transferase (GST) was assayed in accordance with the method of Habig et al. (1974), by measuring the formation of GSH-CDNB (1-chloro-2,4-dinitrobenzene) conjugate at 340 nm. The reaction volume (1 ml) contained final concentrations of 0.1 M sodium phosphate buffer (pH 6.5), 1 mM CDNB in ethanol (95%) and 1 mM GSH. The reaction was initiated by the addition of the enzyme sample.

The specific activity was calculated using an extinction coefficient 9.6 mM^{-1} cm^{-1} and expressed in terms of milimole CDNB-GSH conjugate formed min^{-1} mg^{-1} protein.

4.11 Western blot analysis:

Nuclear protein extracts were prepared using methods described previously (Zhou et al., 1999). Tissue samples were immediately immersed in 1ml ice-cold lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of pepstatinA , leupeptin and L-leucinethiol and homogenized. They were kept on ice for 15 min, and then 15µl of 10% Igeapal CA 630 was added to the homogenate. After a brief vortexing, they were further centrifuged at 12,500 rpm for 5 min. The pelleted nuclei were resuspended in 50-200 µl of nuclear extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of pepstatinA,

leupeptin, and L-leucinethiol and kept on ice for 30 min. The nuclear suspension was centrifuged at 12,500 rpm for 15 min at 4^0C to collect the supernatants containing the nuclear protein extracts. Nuclear extract was store at -80^0C till further use.

The protein content was quantified by Bradford assay as described earlier. About 20μg of protein was resolved over 12% SDS-polyacrylamide gel at 80 V for 3 hr and transferred to a nitrocellulose membrane. Membranes were blocked in PBST buffer containing 5% skimmed milk overnight. The membranes were then incubated with monoclonal primary antibody NF-κB in PBST overnight at 4^0C. The membranes were washed four times in PBST and incubated for 1 hr at room temperature with anti-mouse secondary antibody horseradish peroxidise, diluted in PBST. Membranes were washed five times and detected by chemiluminescence reagents (ECL plus kit, GE Healthcare) and autoradiographed using X-ray film (Kodak Co. USA).

4.12 Statistical Analysis:

Mean and standard deviation was calculated by Sigma 3.5sofware. Statistical analysis was done using normality test then Mann-whitney rank sum test. The values are mean ±SD. P values <0.05 was considered as significant.

RESULTS

5. RESULTS

5.1 Effect of different doses of radiation on cell death in splenocytes:

Effect of different dose of radiation (1-7 Gy) was studied by MTT assay (Fig 2). Radiation decreased the cell viability in a dose dependent manner. At low dose of 1 Gy no significant change in viability was observed against radiation. Cell viability was inhibited by 42.58, 46 and 53% at 3, 5, and 7 Gy dose of radiation.

5.2 Cytotoxic effects of galangin on splenocytes:

To check the toxic effect of galangin on splenocytes, a range of drug doses (25-200 μM) were tested. Doses up to 75 μM did not show toxic effect but at high dose of 200 μM cell viability decreased by 19 % (Fig 3).

5.3 Effect of galangin on radiation induced cell death:

MTT assay was used to check effect of galangin on irradiated cells. On irradiation with 3 Gy, there is was a significant ($p<.05$) decrease in cell viability as compare to unirradiated cells. The cells pre-treated with different doses of galangin and then exposed to radiation showed a better survival potential. Radiation induced death in cells treated with a drug dose of 75 μM showed the significant protection ($p<.05$) (fig.4).

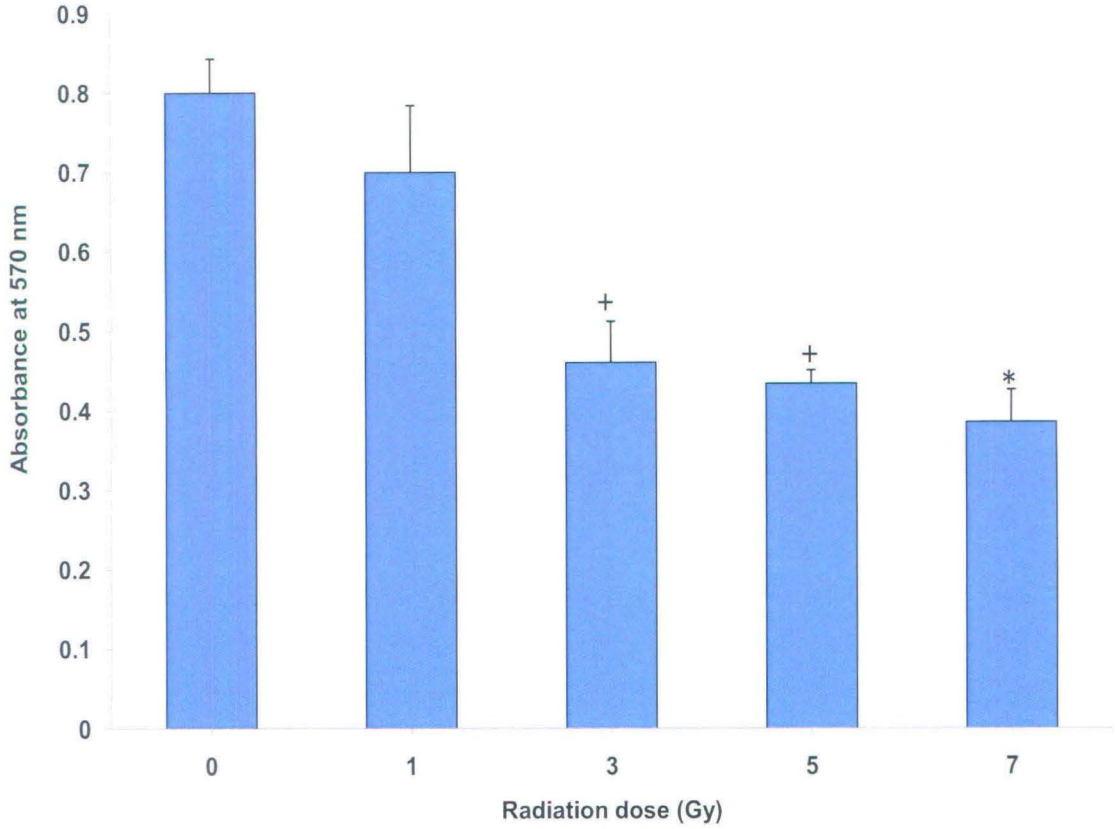


Fig: 2. Cell viability measured by MTT reduction assay 48 hr post irradiation (0-7 Gy). Data is expressed as mean ±SD from 6 wells per sample. Three experiments were performed. *Significant at $p<0.01$, +Significant at $p<0.001$ compared to unirradiated control .

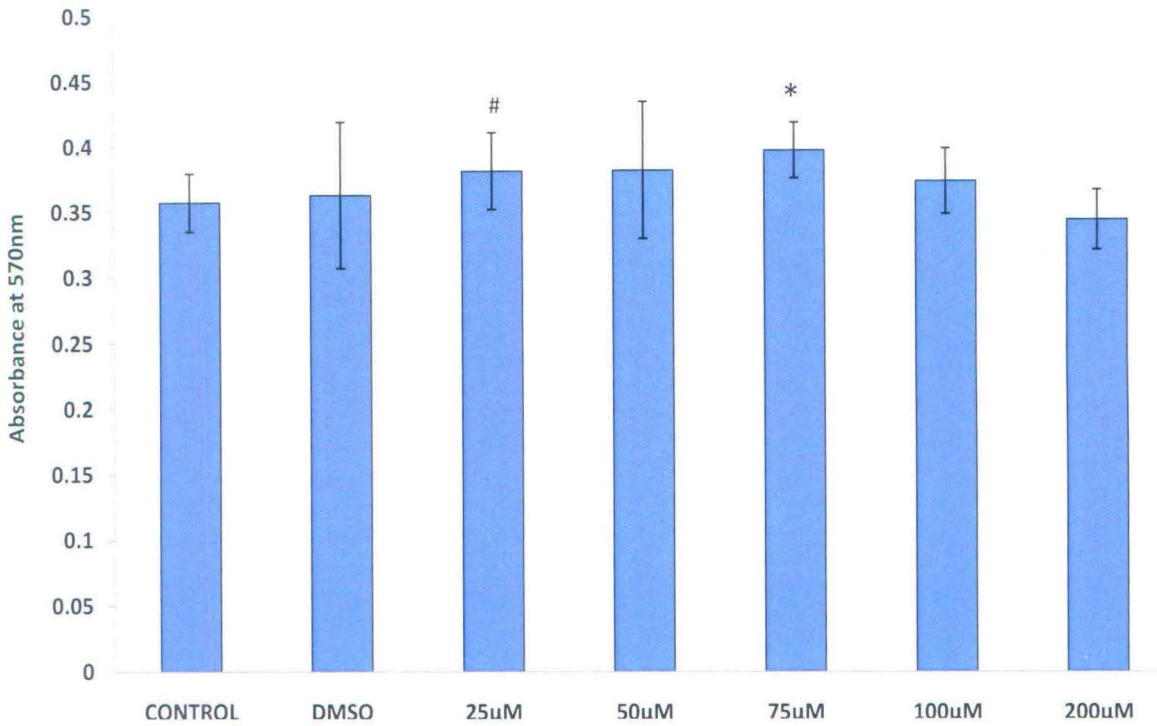


Fig: 3. Galangin cytotoxicity in murine normal splenocytes was measured by MTT reduction assay 48 hr post galangin treatment. Data is expressed as mean ±SD from 6 wells per sample. *Significant at $p<0.01$, #Significant at $p<0.05$ compared to untreated control.

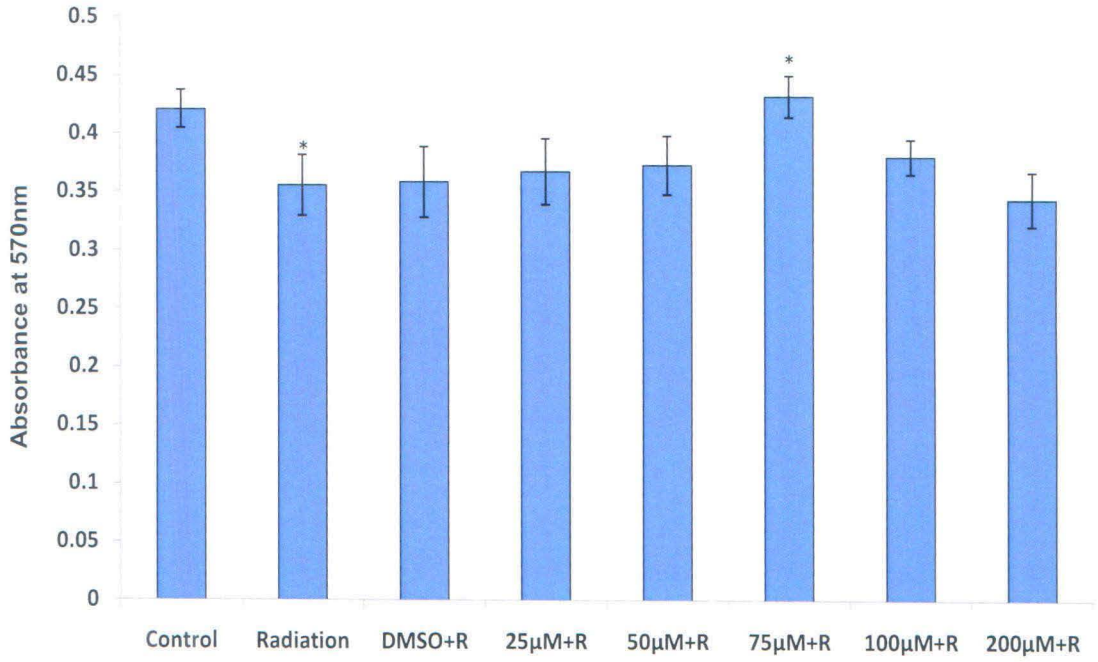


Fig: 4. Cell viability measured by MTT reduction assay. Samples were incubated with Galangin for 2 hr before irradiation (3Gy). Data is expressed as mean ±SD from 6 wells per sample. *Significant at $p<0.01$ compared to radiation control.
R- radiation control.

5.4 Radiomodulatory effect of galangin on DNA damage

DNA damage was assessed by plasmid pBR322 relaxation assay.Exposure of plasmid DNA to gamma radiation resulted in production of strand breaks, as a result supercoiled form of DNA was converted to relaxed form. The reduction in supercoiled form of plasmid DNA was found to be directly related to radiation dose and showed radiation dose dependent pattern (Fig.5). Exposure to radiation even at one gray also result in decrease in supercoiled upto 10%, as compared to unirradiated control. At dose of 10Gy supercoiled form was reduce by 55.42% and at high dose (50Gy) all of the supercoiled form was converted to relaxed form.

The treatment of different doses of galangin (10 μM, 25 μM, 50 μM and 100 μM) prior to 3 Gy of radiation resulted in recovery of supercoiled form of DNA. When DNA was exposed to radiation there was 18% inhibition in supercoiled form (Fig. 6A, Lane 3). At 25 μM and 50 μM of drug concentration galangin reduced the strand breaks in plasmid and supercoiled form was restored.

Thus the above data show that pre-treatment of galangin results in inhibition of radiation induced DNA strand breaks and as a result of which into supercoiled form is not converted into open circular.

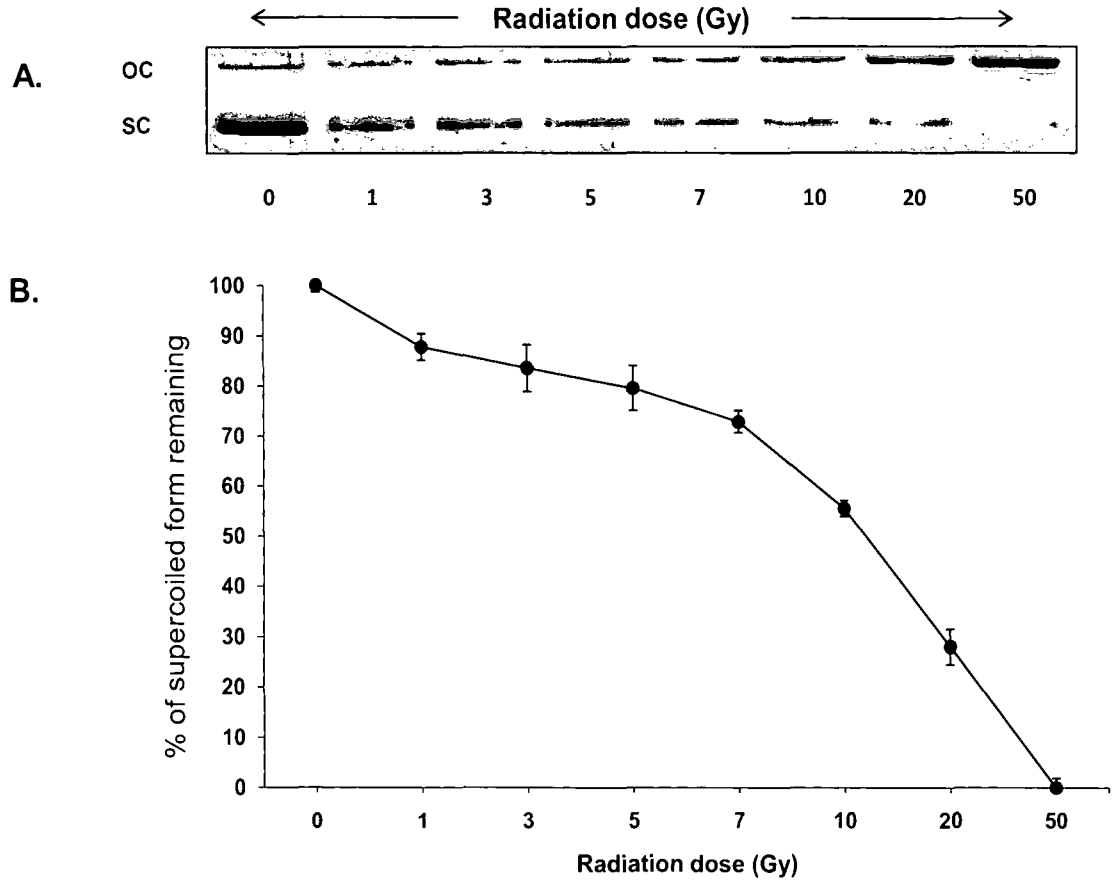
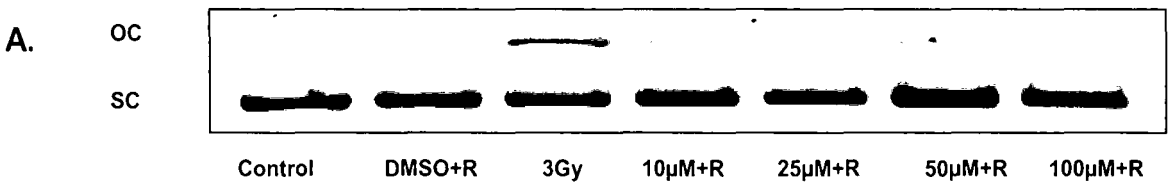


Fig: 5. pBR322 plasmid DNA exposed to different doses of radiation (1-7 Gy) .Figure (A). Figure (B) is representative graph of experiments in figure (A). Each point represents the mean ± S.E.M. from three individual experiments. OC - open circular, SC - supercoiled form of plasmid DNA.



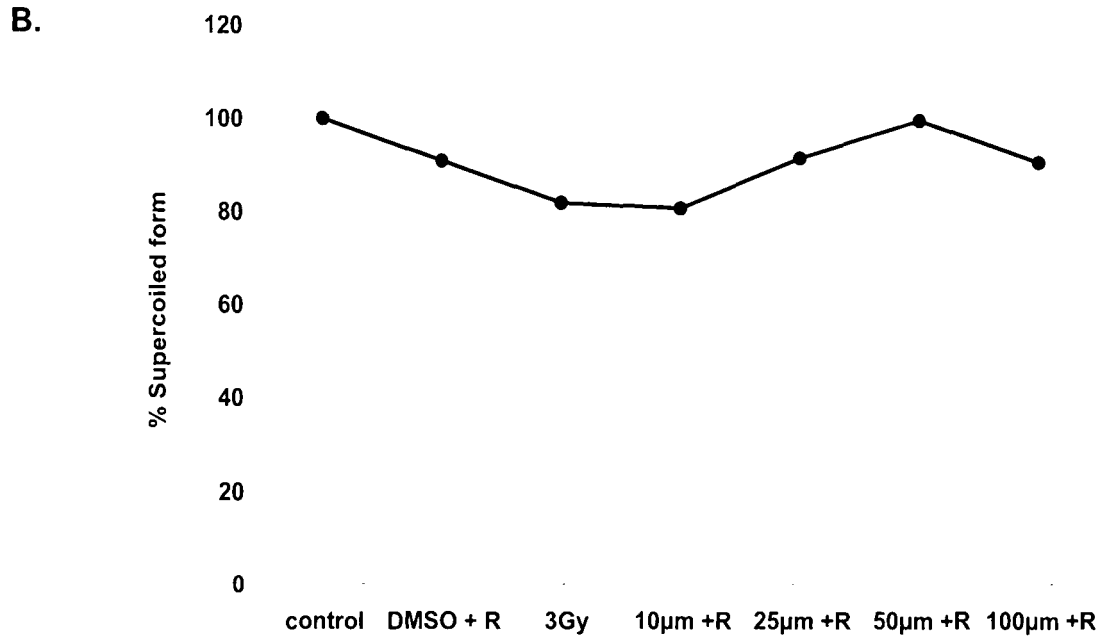


Fig: 6. Protection of pBR322 plasmid DNA by different doses of Galangin against gamma-radiation(3Gy), 25μM AND 50μM show the maximum protection.

5.5 Radiomodulatory effect of Galangin on antioxidant enzyme profile :

Antioxidant status is one of the most important determinants of radiation response of cells/tissues. This system is composed of glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), catalase and glutathione-s-transferase (GST).

To understand the radiation response of various enzymes involved in antioxidant functions in mice, mice were pretreared with different dose of galangin (10, 50 and 100mg/kg body weight of mice) and mice were sacrificed 24hr post irradiation. Various organs were dissected out to study activity of antioxidant enzymes. Corn oil was used as a vehicle control.

Galangin pretreatment at 10, 50,100 mg / Kg body wt showed an increase the GSH content. (Fig.7)The change was significant at 50 and 100 mg / kg body wt. However mice treated with radiation alone did not show a significant change in GSH content. (Fig. 8) In mice pretreated with galangin and then exposed to radiation a significant increase in GSH level was observed at 100mg / kg body wt.

Specific activity of superoxide dismutase (SOD), glutathione-s-transferase (GST) and catalase (CAT) was checked in mice kidney. In mice treated with different doses of only galangin no significant change in ezymee activity was observed at any of the doses tested. (Fig 9, 10, 11). The mice pretreated with galangin and exposed to radiation showed an increase in the specificity activity of GST and CAT. This increase was significant at higher doses (50 and 100 mg per kg body wt) only (Fig 13, 14).However SOD does not show any significant change (Fig. 12).

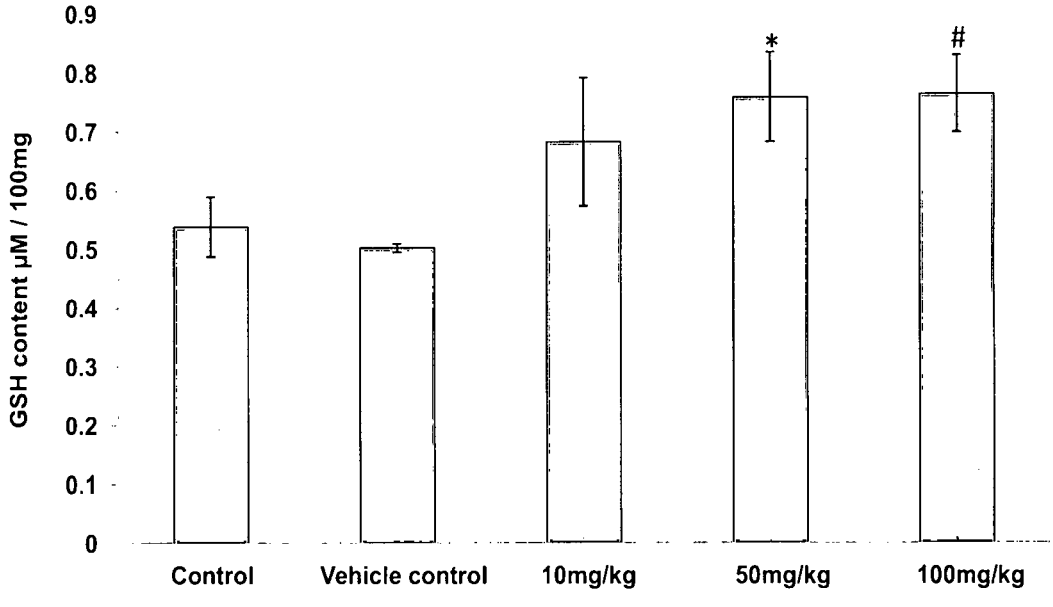


Fig: 7. Effect of different doses of Galangin (10, 50 and 100mg/kg body weight of mice) on GSH (Glutathion) content in kidney of mice. *Significant at $p<0.01$, #Significant at $p<0.05$ compared to untreated control.

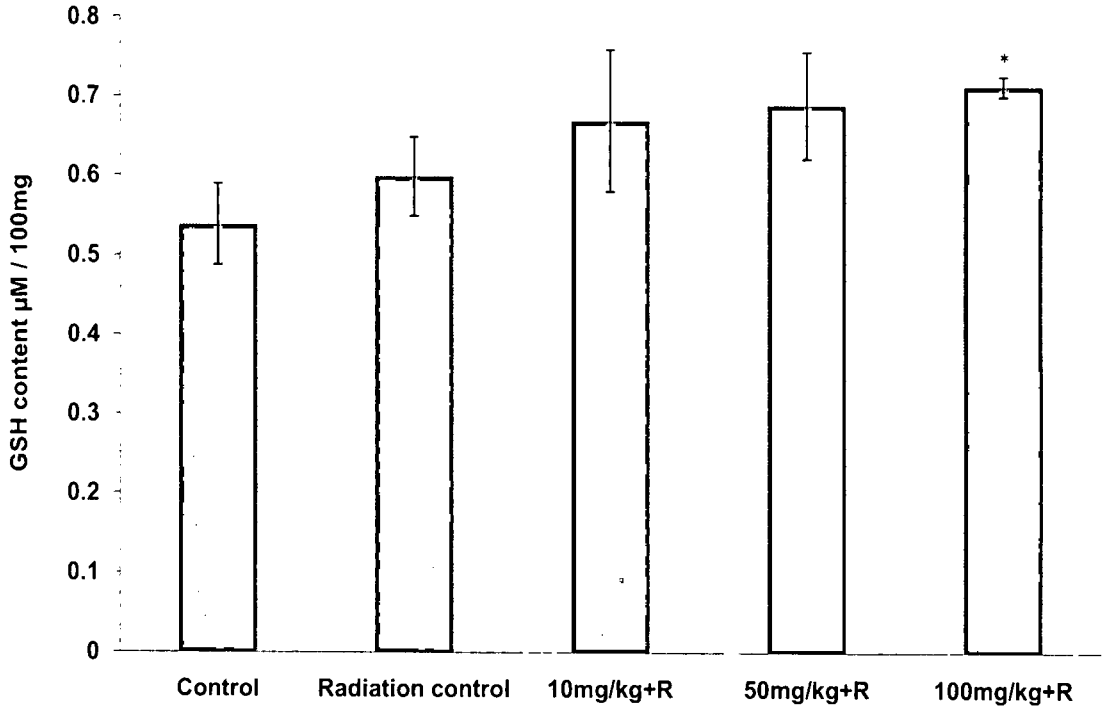


Fig: 8. Effect of different doses of Galangin (10, 50 and 100mg/kg body weight of mice) on GSH content, 24hr post irradiation (1.5 Gy) in kidney of mice. *Significant at $p<0.01$ compared to irradiated control.

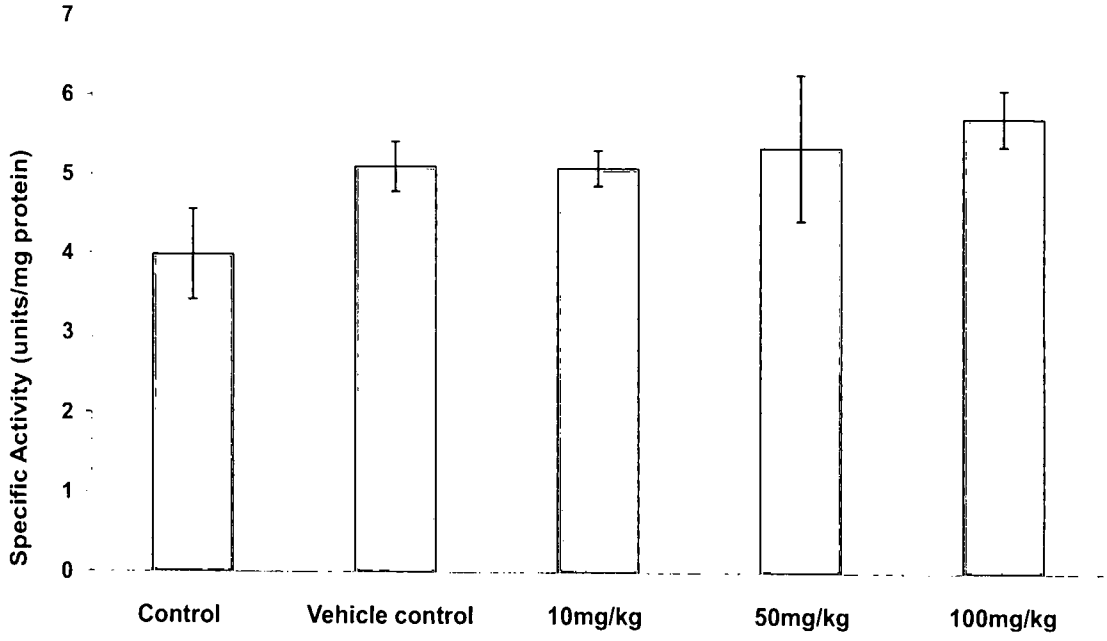


Fig: 9. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *SOD* (*Superoxide Dismutase*) in kidney of mice.

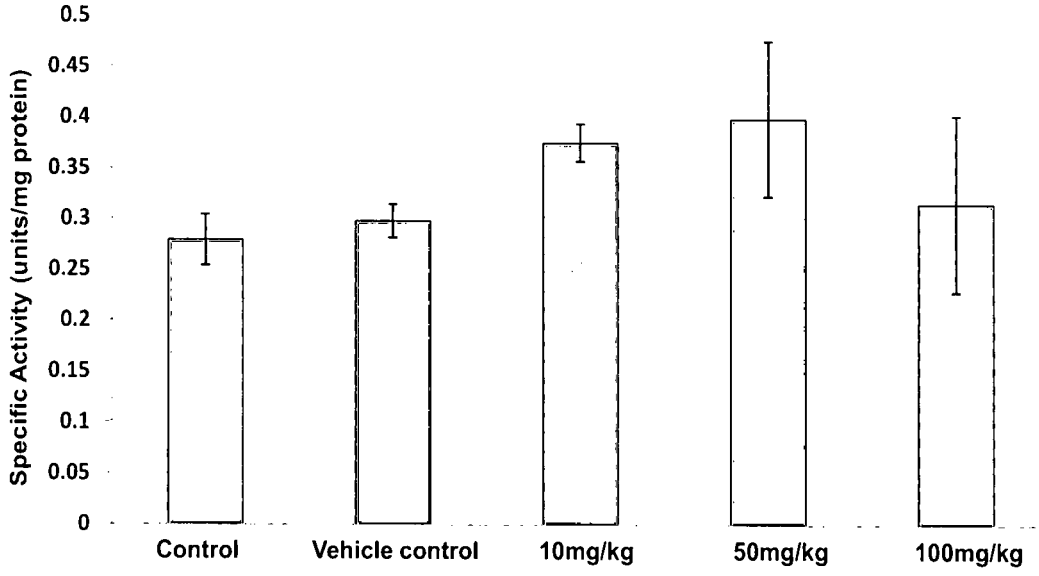


Fig: 10. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *GST* in kidney of mice.

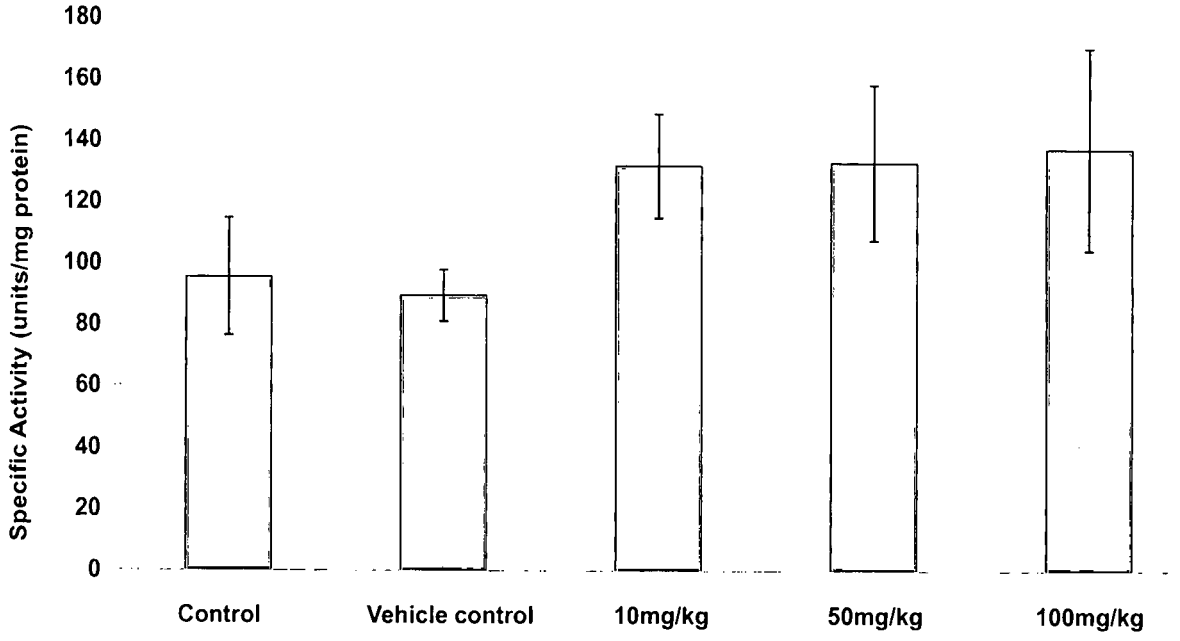


Fig: 11. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *Catalase* in kidney of mice.

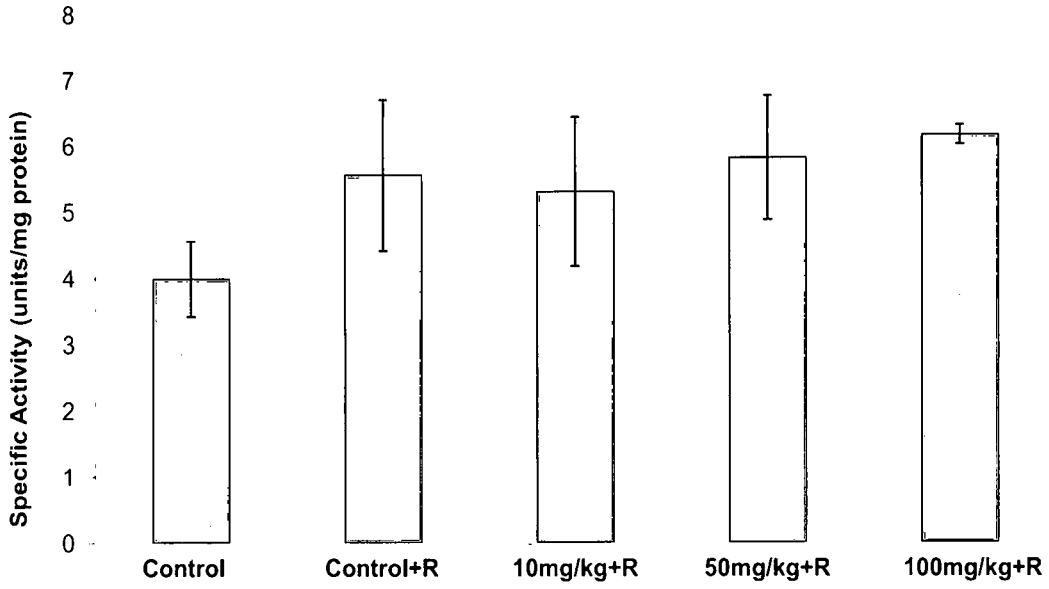


Fig: 12. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *SOD* 24 hr post irradiation (1.5 Gy) in kidney of mice.

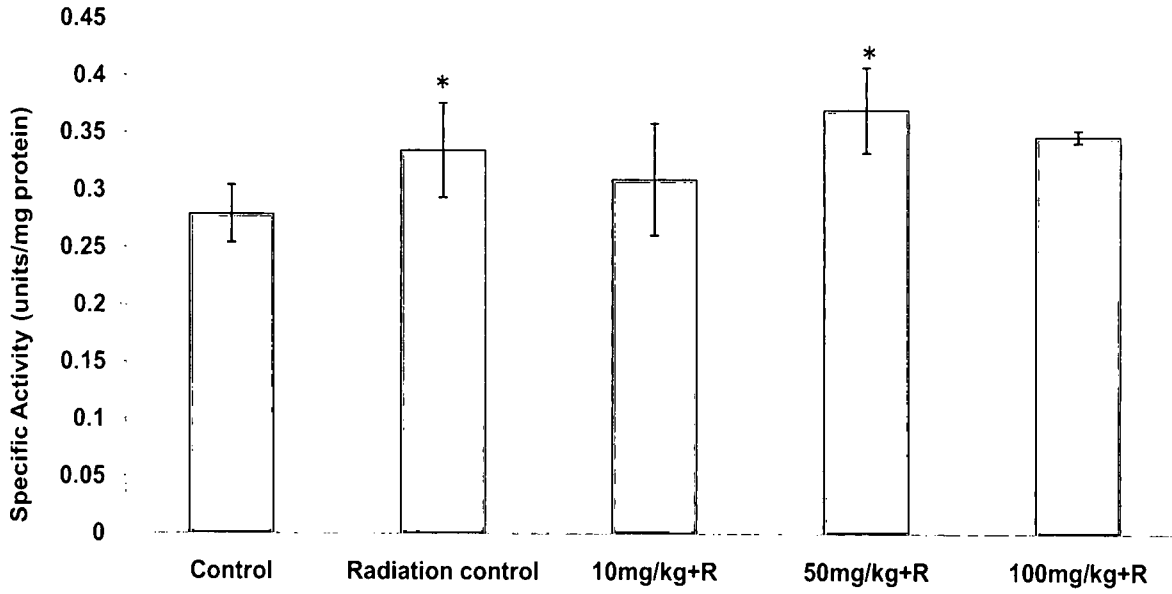


Fig: 13. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *GST* 24 hr post irradiation (1.5 Gy) in kidney of mice. *Significant at $p<0.01$ compared to irradiated control.

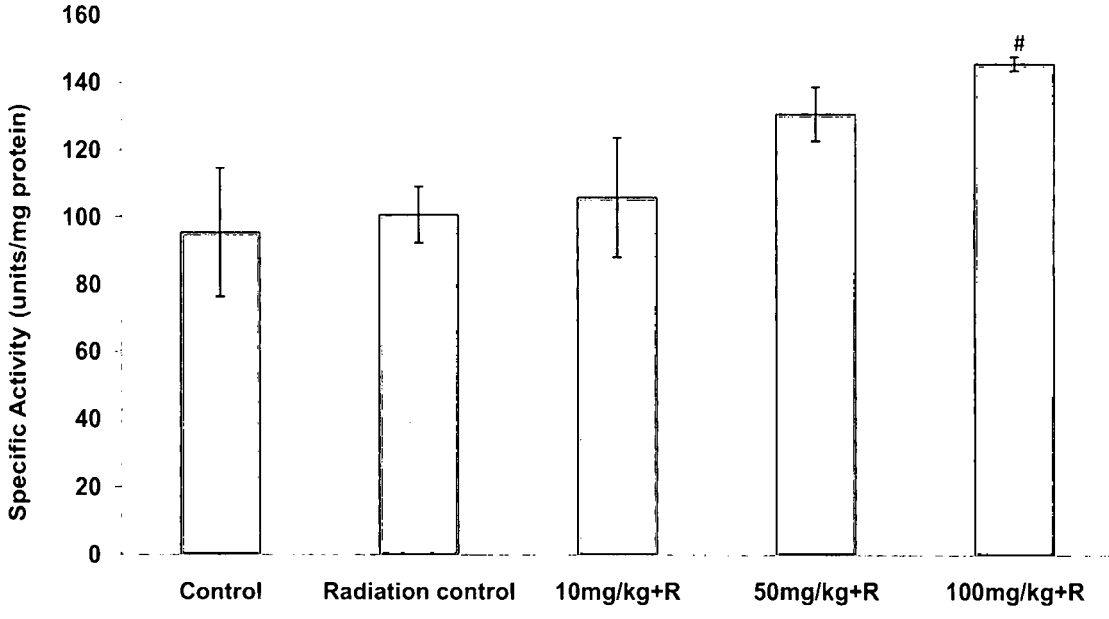


Fig: 14. Effect of different doses of Galangin (10, 50 and 100 mg/kg body weight of mice) on specific activity of *Catalase*, 24hr post irradiation(1.5 Gy) in kidney of mice. #Significant at $p<0.05$ compared to irradiated control.

5.6 Effects of galangin on expression of NF-kB:

NF-kB is a transcription factor which increases in response to radiation and further regulates different downstream genes such as COX-2 etc.

Effect of different doses of radiation on NF-kB expression

Mice were irradiated with different radiation dose (0.1, 1 3 and 6 Gy) and sacrificed after 24hr and western blot analysis was done. There was a linear increase in expression of NF-kB with radiation. Radiation dose of 3 Gy dose was chosen for further experimentation

Radiomodulatory effects of galangin on NF-kB expression

To study the radiomodulatory effect of galangin on NF-kB expression, groups of mice given prior treated with different doses of galangin (10, 50, 100mg/kg) were irradiated with 3Gy of radiation dose. Data was analysed with western blot analysis.

Western blot shows that there is decrease in NF-kB expression in irradiated mice pretreated with galangin when compared to radiation control. The expression of NF –kB decreased with with increase in galangin dose. 100mg/kg shows the least expression (fig. 15 Lane 6).

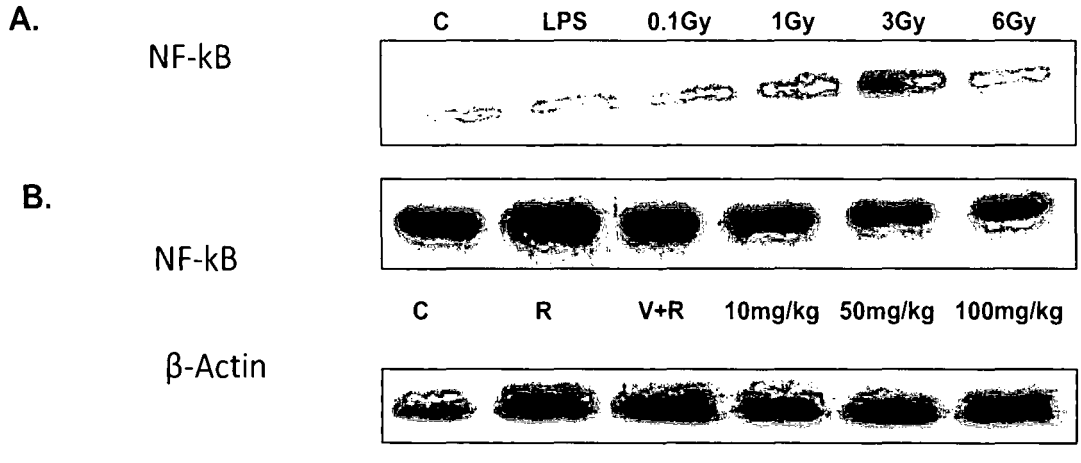


Fig: 15. NF-kB expression in spleen of mice (24hr post irradiation). A. Effect of different doses of radiation. B. Effect of different concentration of galangin against radiation (3 Gy). Unirradiated control (C), R- 3 Gy radiaion control, V+R- vehicle+Radiation, lane 4-6 (different doses of Galangin+Radiation) LPS-lipopolysaccharide.

DISCUSSION

&

SUMMARY

6. DISSUSSION

In the present study it was shown that exposure of plasmid pBR322 to gamma radiation results in strand break leading to conversion of supercoiled of plasmid DNA into the relaxed form. Free radicals and reactive oxygen/nitrogen species produced as a result of radiarion exposure cause DNA single strand and double strand breaks. Reactive oxygen species are considered to be the primary reactive species responsible for radiation induce damage to biomolecules including DNA. In our studies galangin conserved the supercoiled form of DNA by preventing the formation of radiation induced strands breaks. Galangin has been reported to have free radical scavenging activity due to presence of hydroxyl group on its carbon ring thus protecting DNA from free radical attack (Heo *et al.,* 1996). Thus, the radioprotective effect of galangin can be explained by its capacity to trap free radicals before they cause harm.

Galangin is a flavonol found in honey, *Alpinia officinarum*, It has been reported to show nti-mutagenic, anti-clastogenic, anti-oxidative, radical scavenging, metabolic enzyme modulating activity. It is also effective against certain types of DNA damages. In our experiments splenocytes treated with different doses of galangin prior to irradiation were found to have increased viability when compare to untreated cells suggesting that galangin reduced the damaging effect of radiation and protects them from death. However the mechanism of protection still needs to be evaluated.

To prevent the damaging effect of free radicals living beings have antioxidant system, represented by a complex system of enzymes. Main function of antioxidant enzymes is to control and inhibit the free radical processes beyond a threshold level. The most important components of the antioxidant system enzymes superoxide dismutase (SOD), glutathione-s-transferase and Catalase which neutralize reactive oxygen species (ROS).

In our study, group of mice pre-treated with different doses of galangin, alone did not show significant change in antioxidant enzymes but when mice were treated with low dose of radiation (1.5Gy) with prior treatment with galangin there was a significant change in level of antioxidant enzyme activity when compared to the irrradiated groups. GST and catalase showed significant increase in specific activity at the drug dose of 50mg/kg and 100mg/kg body weight of mice. GSH content in galangin treated micewas also enhanced significantly.

The normal functioning/ survival of irradiated cells is also dependent on enzymes involved in the metabolism of reactive oxygen species. The increased level of cellular defence is suggestive of an attempt made by the cells to minimize the radiation damage. Elevation of GSH content can help to detoxify various electrophiles and free radicals responsible for oxidative damage as well as help in the maintenance of the redox state of irradiated cells.

Apart from its antioxidant activity, galangin has been reported to have anti-inflammatory effects by affecting the expression of many other genes. This could be attributed to the fact that flavanoids can inhibit the phosphorylation of signal transduction proteins including regulation of transcription factors like NF-kB (Baeuerle and Henkel, 2006) NF-kB is a heterodimeric protein composed of two subunits. It remains in the cytoplasm as an inactive complex with its inhibitory protein of IkB family. IkB protein masks the nuclear localization signal sequences within the NF-kB. Upon stimulation by radiation, the inhibitory subunit IkB is phosphorylated by a specic kinase (IKK) and cause the proteasomal degradation (kim *et al.*, 2006). The active NF-kB is then translocated to the nucleus from the cytoplasm. Since active NF-kB can initiate cascade of inflammatory reactions, inhibition of NF-kB translocation can relive inflammation and IR induced by inflammatory factors. Many natural compounds are shown to have anti-inflammatory properties by inhibiting NF-kB at concentrations comparable to those of classical anti-inflammatory drugs. Compounds such as resveratrol, green tea polyphenols, curcumin and capsaicin are potent inhibitors of IKK

activity. It is possible that galangin might block the activity of IKK and suppress the degradation of inhibitory subunit thus prevent the activation of NF-kB there by suppress the production of IL-6 and TNF-α production (kim *et al.,* 2006). In our study we found that galangin decreased the expression of NF-kB in a dose dependent manner, the groups of mice treated with 100mg/kg body wt. of galangin show maximum decrease in NF-kB activity. Therefore galangin may have its protective ability by suppression of genes activated by NF kB. Galangin being one of the main components of propolis has been reported to be responsible for the anti-inflammatory activity of propolis (Blonska *et al.,* 2006) Radiation also plays an important role in the development of oxidative stress and production of pro-inflammatory cytokines that activate many signaling factors including NF-kB. Thus radioprotective effects of galangin could be attributed to diverse mechanisms from enhancement of antioxidant function, to anti-inflammatory role via NFkB. However this is only a preliminary study further experiments are under way.

7. SUMMARY

Our results show that galangin has cyto-protective effects and increases the viability of irradiated splenocytes. It also improved the proliferation of cells exposed to gamma radiation. Galangin protected DNA (pBR322 plasmid) against radiation induced damage. Galangin reduced the damaging effect of radiations by reducing the activity of nuclear factor NF-kB and thus suppressing further inflammatory reactions. Galangin increased antioxidant activites which in turn protected from free radicals resulting from ionizing radiation. Present study showed that galangin can protect mice from radiation induce DNA damage, oxidative stress and can modulate signalling pathyways involving NFkB. However this is only a preliminary study further experiments need to be done to confirm its role as radioprotective agent and decipher the exact mechanism of action.

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