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Research Article

Radiomodulatory Effects of different Phytochemicals on the Irradiated Mouse Model System

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Abstract

The present study was conducted to evaluate the radio-protective effect of three phytochemicals: emodin, acemannan and naringenin on the irradiated splenocytes, bone marrow and liver cells of mouse model system. γ- Radiation induces cellular and DNA damage which was studied by micronucleus assay and karyotypic study was done by metaphase arrest of chromosomes. The radio-protective efficacy of emodin was studied by cell viability assay, comet assay and plasmid relaxation assay. Acemannan showed to possess radio-protective potential when it elevated the levels of SOD and catalase anti-oxidant enzymes in the irradiated liver cells and improved cell proliferation in the irradiated splenocytes. The radio-protective effect of naringenin on the irradiated spleen cells and bone marrow cells was also studied via MTT assay and immunocytochemistry. All three studied phytochemicals were of natural origin and showed to exhibit good radio-protective properties, thereby providing an insight for improving defense mechanism of mouse model system against radiation exposure.

Keywords: *Splenocytes, bone marrow, liver cell homogenate, emodin, acemannan, naringenin*

1. Introduction

Radiotherapy is one of the most common alternatives for treating human cancers. It is needed by eighty percent of the cancer patients at some time or other, either for curative or palliative purpose. Normal tissues are transiently and / or permanently injured by ionizing radiation used to target tumor clonogens. Hence, there arises the need to protect the normal cells that surround the tumor from injury caused by radiation. The inadvertent radiation exposure during nuclear accidents, space travel, and the threats of nuclear terrorism has necessitated the development of potent radioprotectors to safeguard against human exposures. To develop treatment for immune depression after radiation exposure and radiotherapy of cancers, is a major challenge in medical science. Immune suppression is caused by damage to proliferating cells and depletion of peripheral blood lymphocytes and bone marrow cells, making organisms more susceptible to common infections and opportunistic pathogens that could be fatal (Goel, *et al*, 2007). Hence animal survival post irradiation is subject to presence of critical number of the hematopoietic stem cells and their proliferation level.

Identifying apoptosis as a means of eliminating cells treated with cytotoxic agents initiated novel studies deciphering the mechanism of apoptosis induced by phytochemicals. At present, there role in combination with chemotherapy with standard drugs to reduce toxicity and to enhance efficacy is being pursued vigorously. There supplementary inhibition effects on antigenic and metastasis regulatory actions had made these phytochemicals, sensible candidates as specific blocker of tumor associated events. All immunosuppressive drugs currently available have the potential to induce undesirable side-effects; however, the drug and dosage used determine the frequency/intensity of these effects. Plant derived natural products have the potential to be exploited in the novel drug development process (Pande, *et al*, 1998), and their numbers are constantly increasing in the treatment of some immune based diseases like cancers and CNS diseases.

Emodin (1,2,8-trihydroxy-6-methyl anthraquinone polyphenol), is known to be used as a preventive agent against autoimmune and other inflammatory diseases (Lin, *et al*, 2011). It is a component of the root and rhizome of *Rheum palmatum* (Brennan, *et al*, 2013). Emodin is also known to possess anti-bacterial, anti-oxidant, and anti-cancer effects in experimental model systems (Sharma, 2014). Other studies have reported immunosuppressive and anti-proliferative properties

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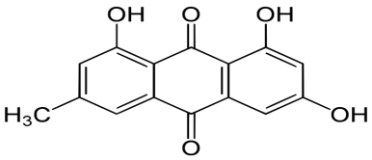
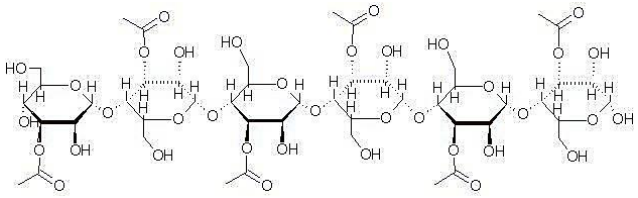
of emodin in *in vitro* and *in vivo* systems (Gao, *et al*, 2014, Liu, *et al*, 2009). However, till date, mechanisms of action are not completely interpreted.



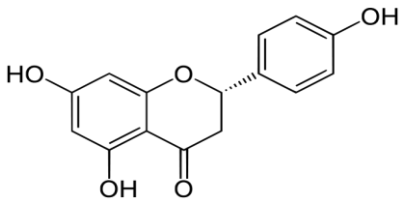
EMODIN

Acemannan is a major polysaccharide component of aloe vera gel. It has the ability to immunomodulate and provide protection against radiation induced mortality in mice. It possess various pharmacological properties like chemotherapeutic efficacy in mice (Harris, *et al*, 1991, Peng, *et al*, 1991). The anti-cancer activity of acemannan is mediated via immuno-modulation by secreting various cytokines and promoting the infiltration of immune cells at the cancer site (Harris, *et al*, 1991).



ACEMANNAN

Naringenin (*5,7-dihydroxy-2- (4-hydroxyphenyl) chroman-4-one*) belongs to the class of flavonoids called the flavanones which are abundant in citrus fruits such as grapefruit (*Citrus paradisi*) and the oranges (*Citrus sinensis*) (Ueng, *et al*, 1999). Naringenin has recently gained substantial interest for it's aforesaid role in the prevention and treatment of disease, with major interest in the use of these flavanones as anticancer and antiatherogenic compounds (Chetia, *et al*, 2012). Pre-treatment of cells with naringenin is reported to enhance the anti-oxidant levels in the irradiated mice (Kumar, et al, 2016).



NARINGENIN

The present study was initiated to determine the radio-modulatory effects of emodin, acemannan and naringenin. The advantage of this system as an experimental model is that it resembles a primary organ culture. Splenocytes consist of a mixture of about 80% B-lymphocytes and T-lymphocytes, the remaining mostly consisting of macrophages and granulocytes. Being highly radiosensitive, they serve as an ideal model for studies on radiation induced damage. Additionally, cell proliferation can be induced by external stimulation. The advantage of using bone marrow is that it provides pluripotent hematopoietic stem cells system having the ability to renew and differentiate into various types of blood cells (Wang, *et al*, 2013).

2. Materials and Methods

2.1 Model animal

Male Swiss Albino mice (7-8 weeks old) weighing 27-28g were random bred and maintained in the air-conditioned Central Animal House of the Jawaharlal Nehru University, New Delhi. A standard rat chow diet was fed to the swiss mice, along with access to water and were synchronized by maintenance of controlled environmental conditions (light, temperature, feeding time, etc.) for at least 2 weeks prior to and throughout the experiments. All the conducted experiments strictly adhered to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, constituted by the Animal Welfare Division, Government of India and Institutional Animal Ethics Committee (IAEC) on the use of animals in scientific research. The experimental protocols were approved by the IAEC-JNU (Jawaharlal Nehru University).

2.2 Isolation of splenocytes, bone marrow cells, liver cells

The mouse was euthanized by cervical dislocation. After dissection, the spleen was aseptically removed and washed with ice cold 0.9% saline and homogenized between the ends of frosted slides, which was passed through 100 µm cell strainer to obtain a single cell suspension. The suspended cells were centrifuged at 2000 rpm for 5 min. at 4° C. The supernatant was discarded. The pelleted cells were subjected to the hypotonic shock treatment with 800µl of ice cold distilled water, added immediately, to selectively lyse the RBCs. 5X PBS was added to make the cell suspension isotonic. The suspended cells were centrifuged at 2000 rpm for 5 min. at 4° C. The supernatant was discarded and the cells were washed by re-suspending them in 1X PBS. The slimy or fatty debris was carefully removed. The cell suspension was again centrifuged at 2000 rpm for 5 min. at 4° C to pellet the cells. The supernatant was discarded and the cells were resuspended in RPMI-1640 medium.

The mice was sacrificed by cervical dislocation and the femur was dissected. Bone marrow was flushed out with 1X PBS and the cells were isolated. The cell sample was diluted in the RPMI-1640 media in the ratio 1:5. 10 µl cell suspension was diluted in trypan blue and the cell count was performed using a haemocytometer under 40X magnification. The cell suspension was diluted further with RPMI to achieve the desired cell density as per the experimental requirement.

Preparation of the liver tissue homogenate was done by sacrificing the mice by cervical dislocation. The liver was perfused (0.9% saline ice cold) and dipped in liquid nitrogen. When need to be used, 1g of the tissue was weighed and homogenized in10 ml of 0.15 M tris KCl buffer.

2.3 Cell culture and treatment

The isolated splenocytes and bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% antibiotic (2 mM L-glutamine, 100 mg/ml penicillin and 100 mg/ml streptomycin) at 37° C in a humidified 5% CO_2 atmosphere. The cells were suspended in RPMI-1640 at a density of 1 x 10^6 cells/ml and treated with equal volumes of different concentrations of desired drugs. The drug treated cells were incubated at 37° C for 30 min. The cells were then exposed to γ- radiation at specific doses. All irradiation experiments were performed in air at room temperature in a γ- chamber (240 TBq ^{60}Co Model 4000 A) obtained from Isotope Division, Bhaba Atomic Research Centre (BARC), Mumbai, India. The samples were exposed to γ-radiation as per required doses of radiation. The dose rate used was 0.098 Gy/sec. Immediately after irradiation, the cells were kept for incubation at 37° C in the CO_2 incubator for specific time periods as per the experiment.

2.4 Micronucleus assay

Solution A was prepared by mixing 0.1 ml Triton X-100, 8 ml 1N HCl, 0.877 g NaCl and H_2O to make final volume up to 100 ml. Solution B contained 37 ml 0.1 M anhydrous citric acid, 63 ml 0.2 M $NaHPO_4$ having pH 6, 0.877 g NaCl, 34 mg EDTA disodium salt and 0.6 ml AO 1 mg/ml. Solution A and B were chilled on ice prior to use. The bone marrow cells were fixed in 1% glutaraldehyde in PBS for 5 min. and then rinsed twice with PBS. Solution A and solution B were mixed in 1:3 ratio. Contents were mixed by gentle inversion and incubated in dark on the ice for 30 min. followed by centrifugation at 3000 rpm for 5 min. The cellular pellet was retained and re-suspended in PBS**.** 5 µl of the cell suspension was smeared on the slide with the help of cover slip and visualized under fluorescence microscope.

2.5 Arrest of metaphase chromosomes

Colchicine stock solution (1 mg/ml) in PBS was prepared. Working solution was made in ratio 1:3 with 1X PBS. Hypotonic solution was prepared by dissolving 0.28 g KCl in 50 ml distilled water. Fixative solution was prepared by mixing methanol and glacial acetic acid in the ratio 4:1. The bone marrow cell culture sample was subjected to radiation (3 Gy) to study chromosomal aberration. 5 µl concanavalin A was added to promote cell proliferation followed by 20 hrs. incubation in CO_2 incubator. 20 µl colchicine was added to each sample for arrest of metaphase chromosomes followed by 2 hrs. incubation in CO_2 incubator. Centrifugation was done at 1500rpm for 7 min. and the supernatant was discarded. The hypotonic solution was warmed to 37° C and added to the pellet followed by incubation for 30 min. at 37° C. The pellet was retained after centrifugation. Fixative was added to the pellet and refrigerated. The cells were fixed on the slide and stained for visualization in the light microscope.

2.6 Assay for cell viability

The cell viability was assessed by trypan blue dye prior to all treatments. Trypan blue was dissolved in NaCl saline to obtain concentration 4 mg/ml i.e. 0.4%. Different concentrations of phytochemicals were tested for cytotoxicity against splenocytes and a representative γ- radiation dosage (3 Gy) was used for the study. Cell sample and trypan blue were mixed in the ratio 1:1. The cell count was performed with a hemocytometer under a light microscope at 40X magnification. The number of live and dead cells were counted and count was recorded. To calculate the viability, formula used was:

Viability = {no. of live cells/ (no. of live cells + no. of dead cells)} x 100%

2.7 Comet assay

2.7.1 Preparation of the reagents

Lysis Buffer

2.5 M NaCl, 100 mM EDTA, 10 mM Trizma or TRIS-base, 4 g NaOH. pH was adjusted to 10. Before use, add 1% Triton X-100 and 10% DMSO.

Electrophoresis Running Buffer

Solution A: 5 N NaOH
Solution B: 100 mM EDTA and pH 10
15 ml NaOH and 2.5 ml EDTA was mixed and final volume was made up to 500 ml.

Neutralization Buffer

24.25 g TRIS base was added to 400 ml of distilled water and pH was adjusted to 7.5. Final volume was made up to 500 ml.

Staining Solution (10X stock)

6 mg EtBr was dissolved in 30 ml distilled water. Working concentration used was 1X.

2.7.2 Preparation of the slides

The slides were sterilized. In 1X PBS, dissolve 1% NMA (normal melting-point agarose) by heating. When clear boiling solution was formed, ¾th of the slides were

coated and allowed to dry at room temperature. LMPA (low melting-point agarose) was melted in a water bath and maintained at 37° C. The spleen cell sample with LMPA was mixed in the ratio 1:4 and smeared over slide using cover slip and allowed to harden the gel at 4°C for 5-10 min. The coverslip was removed and third layer of LMPA was coated. Lysing solution was prepared by addition of 1% Triton-X 100 and 10% DMSO. The slides were immersed in the lysing solution and incubated for 2 hrs. at 4° C. Horizontal electrophoresis was performed at 4° C. The slides were neutralized in neutralization buffer 3 times at 5 min. intervals and were put in 100% chilled ethanol for 20 min., further dried at 50° C for 30 min. When required the slides were rehydrated in distilled water for 30 min. and stained with 1X staining solution and examined under fluorescence microscope. Images were scored using TriTek comet score freeware 1.6.1.13 software.

2.8 Plasmid relaxation assay

To the plasmid DNA (300 bp), 1X PBS was added. 100 µM emodin was added to plasmid DNA and incubated for 2 hrs. Sample was exposed to 3 Gy and 12 Gy γ-radiation. Incubation at RT for 1 hr. was done. Gel was run at 60 V for 30 min. followed by visualization of plasmid DNA band under gel documentation system.

2.9 SOD assay

Swiss albino mice was exposed to whole body γ-irradiation (3 Gy) and pretreated with acemannan for 7 days at 25, 50 and 100 mg/kg body weight. 0.1M sodium phosphate buffer was prepared (pH 8), along with 3 mM EDTA mono sodium salt and 25% Triton X-100. Pyrogallol was prepared by dissolving 10.2 mg in 10 ml distilled water. Add 8.6 µl of conc. HCl. Liver cytosolic sample was mixed with distilled water in the ratio of 1:10 to prepare cytosolic mixture. 5 µl Triton X-100 was added followed by 30 min. incubation on ice. Reaction mixture was prepared: 500 µl Na_2P buffer + 367 µl H_2O + 33 µl EDTA + 60 µl pyrogallol + 10 µl detergent treated cytosolic sample, followed by incubation for 2 min. OD was recorded at 420 nm for 2 min.

Reaction:

pyrogallol + O_2 $\xrightarrow{\text{auto oxidation}}$ O^{-2} $\xrightarrow{\text{oxidized}}$ H_2O_2

SOD activity = { (OD of reference – OD of sample) x V x D}/(OD of reference x v x protein conc.)

where, V= total reaction volume, D= dilution factor, v= sample volume

2.10 Catalase assay

Swiss albino mice was exposed to whole body γ-irradiation (3 Gy) and pretreated with acemannan for 7 days at 25, 50 and 100 mg/kg body weight. 5 ml Triton X-100 (25%), 0.1 M Na_2P buffer (pH 7) and 1X of H_2O_2 were prepared. Ethanol was diluted in ratio 1:100. The liver cytosolic sample was prepared in the ratio of 1:10 and 5 µl Triton X-100 was added, followed by incubation on ice for 30 min. 5 µl ethanol was added followed by incubation on ice for 30 min. In the reaction mixture: 380 µl H2O, 500 µl Na_2P buffer, 100 µl H_2O_2, 20 µl sample followed by 1 min. incubation on ice. OD was recorded at 240 nm at 15 sec. intervals for 2 min.

Catalase activity = (change in OD x V x D) / (v x E x protein concentration)

where, V= total reaction volume, D= dilution factor, v= sample volume, E= extinction coefficient

2.11 MTT assay

Preliminary experiments were conducted to standardize the number of cells to be loaded onto the 96-well plates. The treated splenocytes were loaded to flat bottomed 96-well plates at a density of 5 x 10^4 cells per well in RPMI-1640 medium. In γ-radiation dose response studies, the cell samples were left untreated and exposed to γ- radiation at dose of 5 Gy followed by acemannan treatment at dose of 1, 10, 50, 250, 500 µg/mL and naringenin treatment at dose of 0.01, 0.1, 1, 10, 250, 1000, 2000, 3000 µg/mL. The plates were incubated at 37° C for 48 hrs. under a humidified and sterile atmosphere containing 5% CO_2. After 48 hrs., MTT solution (5 mg/ml) was added to wells. After 4 hrs. of incubation at 37° C in a 5% CO_2 incubator, 0.02% acidified DMSO was added to each well. The plates were incubated at 37° C in dark for 15 min. The optical density of each well was measured using an automatic microplate reader with a test wavelength of 570 nm and reference wavelength of 690 nm. The cell viability % was calculated as follows:

$$\% \text{ Cell Viability} = \frac{\text{Average O.D of test wells X 100\%}}{\text{Average O.D of control wells}}$$

(O.D. = Optical Density; Test wells = treated wells)

2.12 Immunocytochemistry

Sterilized coverslips were coated with 0.1% gelatine-coating solution, and incubated for 10 min. at RT. The coverslips were air dried for 15 min. 1X PBS (0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4) was prepared. 4% formaldehyde solution was prepared in 1X PBS (pH 6.9). Wash buffer: 0.1% BSA in 1X PBS. The splenocytes and bone marrow cells were cultured in the media, exposed to γ-radiation (3 Gy) and pre-treated with naringenin at dose of 10, 50, 100 µM followed by addition to the wells of a cell culture plate containing gelatine-coated coverslips. When cells have reached the desired density, culture media from each well was removed and washed twice with PBS. 2-4%

formaldehyde fixative solution was added to each well, and incubated for 20 min. at RT. The wells were washed twice with PBS and covered with 400 µL of wash buffer. The fixed cells were washed two times with wash buffer. Non-specific staining was blocked by adding blocking buffer, and incubated for 45 min. at RT. Blocking buffer was removed. The unconjugated primary antibody was diluted in dilution buffer. For fluorescent ICC staining of cell smears, it was incubated at RT for 1 hour. Washing was done two times with wash buffer. The secondary antibody was diluted in dilution buffer and added to the wells followed by incubation at RT for 1 hour in the dark. Rinsing was done two times in 400 µL of wash buffer. 300 µL of the diluted DAPI solution was added to each well, and incubated for 5 min. at RT. Wells were rinsed once with PBS and once with water. 1 drop of anti-fade mounting medium was dispensed onto the microscope slide per coverslip followed by visualization under fluorescence microscope.

3. Results

3.1 Micronucleus assay

Potential genotoxic carcinogens that act by initiating genetic damage are toxicologically screened by micronucleus assay. The *in vivo* test normally uses mouse bone marrow or mouse peripheral blood. When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is expelled. If any micronucleus formation takes place, it remains in the anucleated cytoplasm. Due to lack of a main nucleus, micronuclei visualization is facilitated in these damaged cells. Induced chromosome damage is indicated by increased frequency of micronucleated polychromatic erythrocytes in the treated animals. A micronucleus is the erratic (third) nucleus which is created during the anaphase of mitotic or meiotic cell division cycle. Micronuclei are cytoplasmic bodies having a portion of acentric chromosome or whole chromosome which was not carried to the opposite poles during the anaphase. Daughter cell lacks a part or all of a chromosome, when micronuclei formation takes place. The whole chromosome or its fragments usually develop nuclear membranes and form as micronuclei as a third nucleus. Post cytokinesis, one daughter cell remains with one nucleus and the other having one large and one small nucleus, i.e., micronucleus. In case of major genetic damage, there is a possibility of presence of more than one micronucleus in a cell. Genotoxic assessment of varied chemicals is analyzed by micronucleus test.

Radiation is known to induce DNA damage. Micronucleus assay was performed in the erythroblast to check the presence of micronucleus formed as a result of radiation induced chromosome damage. Small dot shaped structures in the cells indicate micronucleus which is a separated part from the nucleus, generally located around the edges of the cells. Fig.1 shows the cells unexposed to γ- radiation whereas Fig. 2 shows the cells exposed to γ- radiation (3 Gy) showing presence of large no. of micronuclei formation.

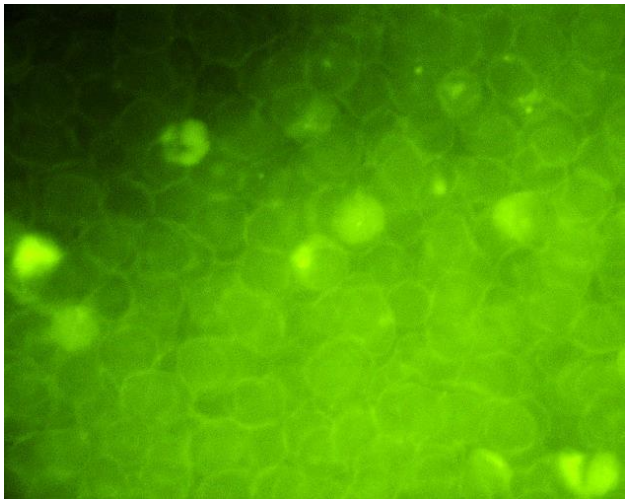


Fig. 1 Cells without micronucleus

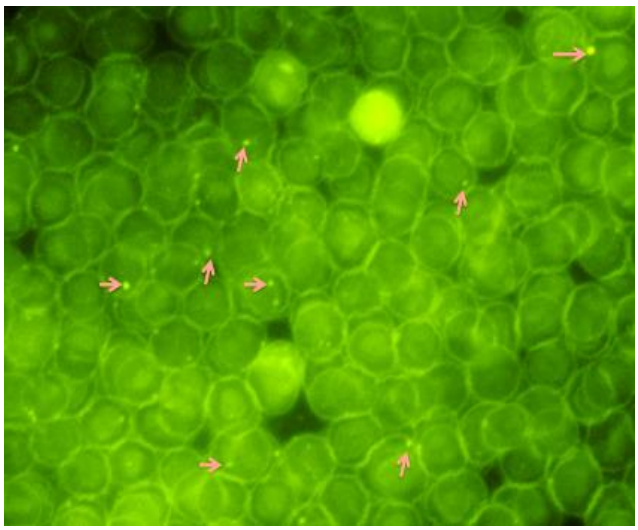


Fig. 2 Cells having micronucleus

3.2 Arrest of metaphase chromosomes

Colchicine is a secondary metabolite and a product possessing natural toxicity, originally derived from the extract of the plants of genus *Colchicum*. Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Colchicine actively functions as a mitotic spindle poison, provided tubulin availability that is essential for mitotic cell division. Thereby, inhibiting mitosis which could be used for the study of cellular genetics. To see the chromosomes of a cell under a light microscope, it is important that they must be viewed near the point in the cell cycle in which they are most dense. This occurs near the middle of mitosis, so

mitosis must be stopped before it completes. Karyotypic studies are performed by adding colchicine to a culture during mitosis as part of the standard protocol. To visualize clastogenic effect due to radiation at cellular level, colchicine is injected into Swiss albino mice. Chromosomal aberration was analyzed by colchicine arrest of metaphase chromosomes of bone marrow in mice. Cell sample not exposed to radiation had intact 40 Chromosomes as shown in Fig. 3. Whereas cells exposed to radiation of (3 Gy) showed aberration caused by induced DNA damage that resulted in breakage of chromosomes as shown in Fig. 4.

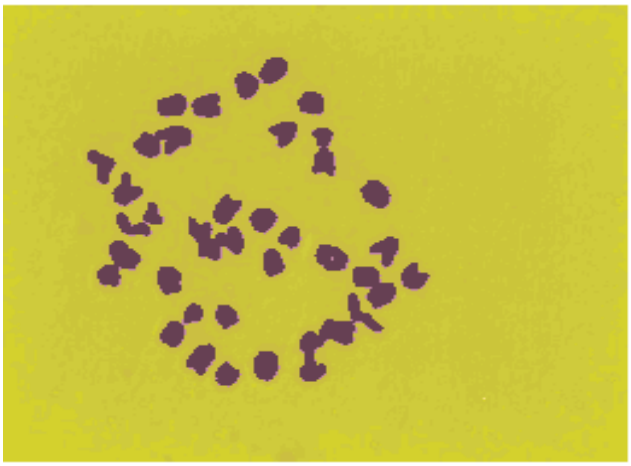


Fig. 3 Chromosomes without damage

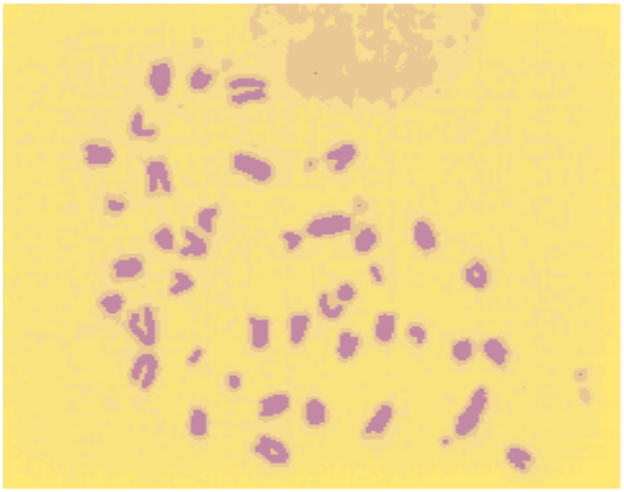


Fig. 4 Chromosomal aberration

3.3 Effect of emodin in radioprotection:

3.3.1 Cell viability assay

Radiation is a potent inducer of apoptosis via DNA damage that leads to cell death. To measure the radioprotective potential of emodin, the cell viability assay was performed. To measure the cell death, trypan blue was used which stains the dead cells leaving the live cells unstained hence giving the proportion of live cells and dead cells. In case of radiation treatment (3 Gy) there was a decrease observed in the cell viability which was restored by 100 µM emodin by 16% ± 6% enhancement in comparison to radiation control as shown in Fig. 5. There was no significant toxic effect observed in the spleen cells when emodin was given alone.

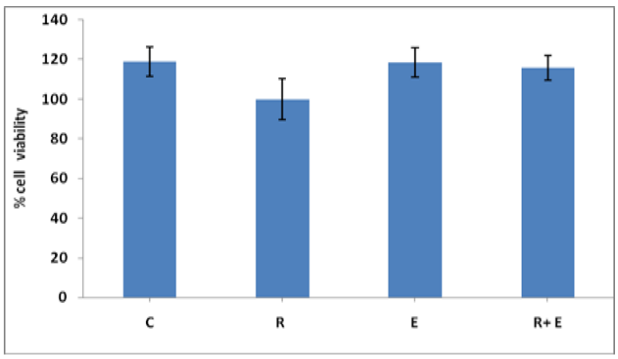


Fig. 5 Effect of emodin in enhancement of cell viability. (C= control cell population with no radiation or dose treatment, R= radiation dose, E= emodin dose)

3.3.2 Comet assay

DNA is primary target of the radiation, which induces apoptosis in the cell. For this purpose, comet assay was performed to check the radioprotective effect of emodin. Greater is the DNA damage, more is the amount of migrated DNA from the nucleoid, hence greater is the tail length. In the case of spleen cells exposed to radiation (3 Gy) alone, there was a significant increase of 77% ± 3% in the tail length of the comet as compared to the control. When 100 µM emodin was given after radiation treatment to the splenocytes, there was a drop in the tail length by 69% ± 3% as compared to radiation control as shown in Fig. 6 and Fig. 7.

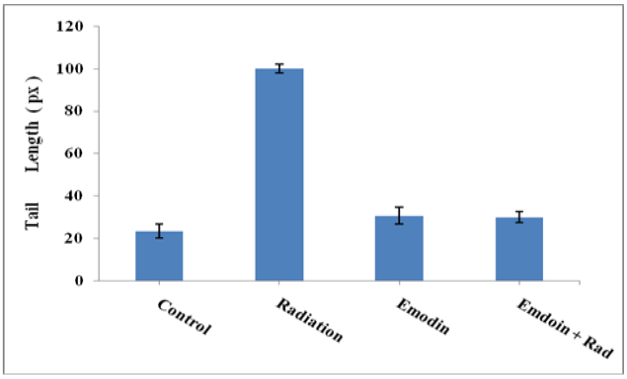


Fig. 6 Effect of emodin on radiation induced apoptosis of splenocytes

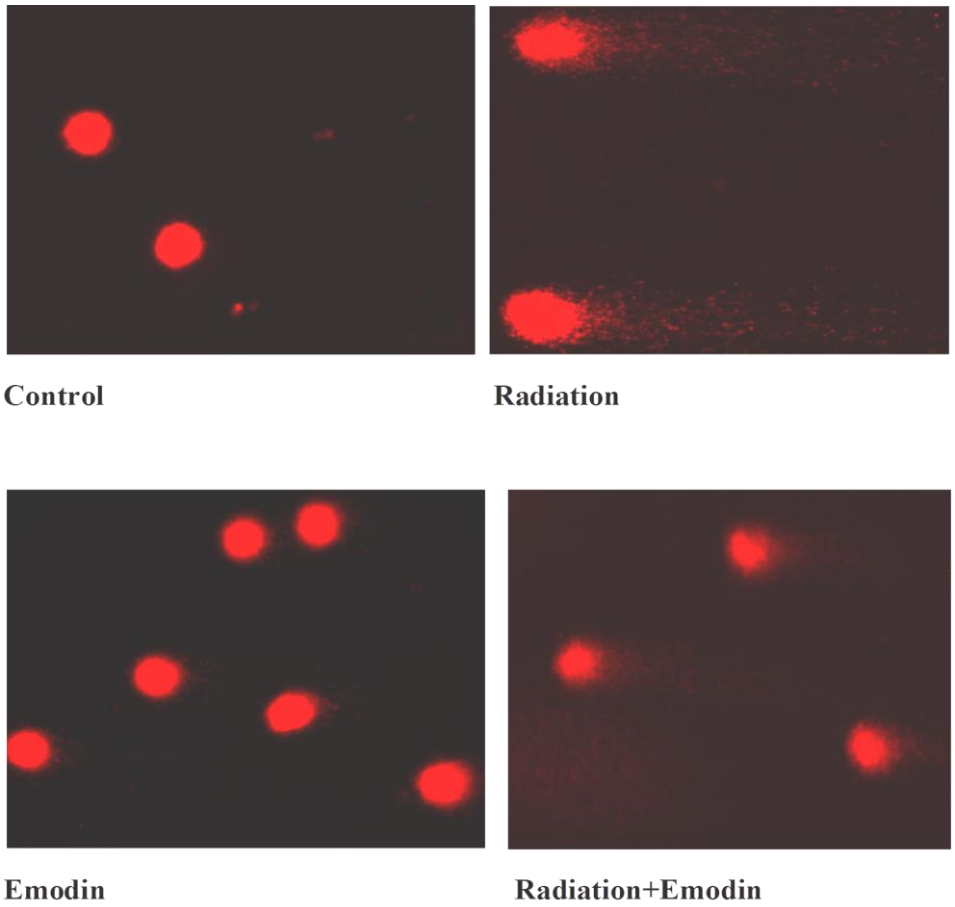


Fig. 7 (i) **Control** is showing the intact nucleoid (DNA head) with no tail formation. (ii) **Radiation** dose (3 Gray) is showing DNA damage depicted by the formation of long tail as compared to other experimental groups. (iii) **Emodin** alone has no effect on the spleen cell population. (iv) **Radiation + Emodin** treatment had tend to repair the DNA damage caused by radiation by showing shorter tail length

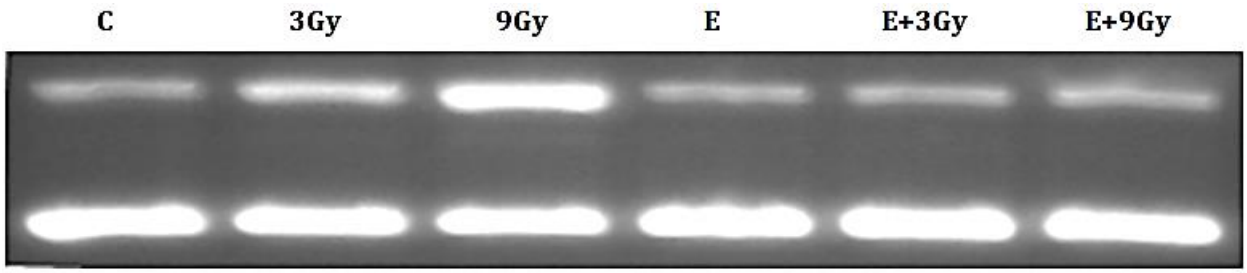


Fig. 8 Visualization of plasmid DNA under gel documentation system (Gy = Gray ; C = control ; E = emodin)

3.3.3 Plasmid relaxation assay

Plasmid relaxation assay was performed to estimate the pBR322 plasmid DNA damage induced by exposure to different doses of ionizing radiation (3 Gy and 9 Gy). Exposure of plasmid DNA to γ- radiation resulted in production of strand breaks as a result of which the super-coiled form of plasmid DNA gets converted to open circular form. Pre-treatment with emodin resulted in recovery of super-coiled form of plasmid DNA. The addition of emodin prior to radiation treatment reduced the induced strand breaks in plasmid DNA in a dose dependent manner at high as well as low doses of radiation. Pre-treatment resulted in inhibition of the conversion of super-coiled DNA to open circular form against low as well as high doses of γ- radiation as illustrated in Fig. 8. Band width was found maximum in case of plasmid exposed to 9 Gy radiation dose while it decreased in case of pre-treatment with emodin.

3.4 Effect of acemannan in radioprotection:

Cells have various anti-oxidant enzymes like super oxide dismutase, catalase to scavenge free radicals that are generated due to radiation treatment.

3.4.1 SOD assay

Destruction of superoxide free radical (O^{2-}) into hydrogen peroxide (H_2O_2) is catalyzed by SOD metalloenzyme and molecular oxygen and consequently provide a defense mechanism against superoxide radical toxicity. It protects oxygen-metabolizing cells against harmful effects of superoxide free radicals. Dismutation is a redox reaction in which species is simultaneously oxidized and reduced to form two different species. Pyrogallol (1,2,3-Tri Hydro Benzene) autoxidation method is used for assay of superoxide dismutase activity in different tissues of the mouse. SOD is essential for biological defense against superoxide anion. SOD activity increased markedly with elevation of the oxygen concentration. Method is used for crude tissue homogenates. It does not interfere with concentration of ascorbic acid and glutathione present in the tissue homogenates. SOD activity was determined by measuring the inhibition of the photochemical reduction of pyrogallol by the enzyme in the reaction mixture. When in alkaline solution, pyrogallol absorbs oxygen from the air, turning brown from a colorless solution. It can be used in this way to calculate the amount of oxygen in air. SOD prevents reduction of pyrogallol by oxygen.

$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$

To check the anti-oxidant activity of superoxide dismutase in providing a defense mechanism against superoxide radical toxicity, the level of SOD enzyme in the liver tissue was measured. To check the radioprotection when mice was exposed to whole body irradiation (3 Gy), there was a drop in the level of SOD by 18% ± 5% in comparison to control. But when cells were pretreated with acemannan for 7 days, there was an enhancement in the level of SOD by 29% ± 13% in comparison to radiation control. It was also observed that acemannan alone also improves the level of SOD in the liver tissue and radio-protection was found maximum at the dose of 50 mg/kg body weight as depicted in Fig. 9.

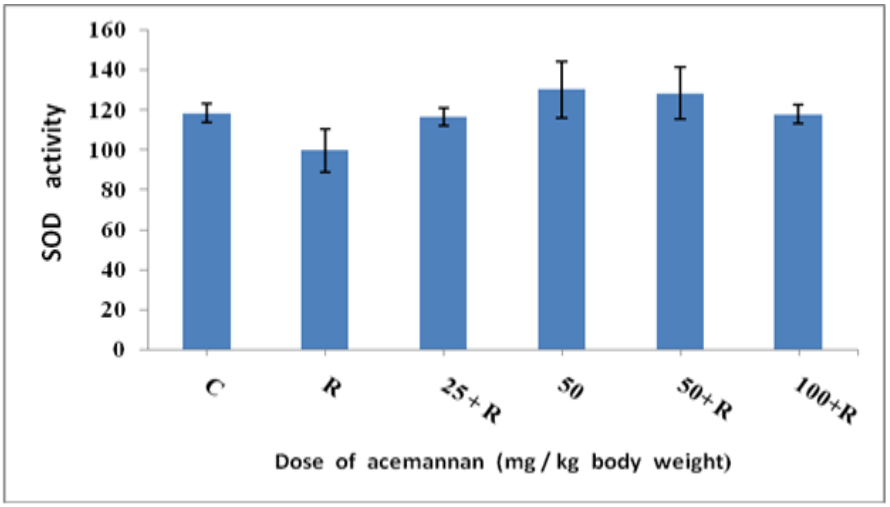


Fig. 9 Effect of acemannan against cytotoxic effects induced by radiation on SOD activity as measured by SOD assay. (C= control cell population, R= radiation dose)

3.4.2 Catalase assay

Hydrogen peroxide (H_2O_2) is a strong and substantially harmful oxidizing entity, and it's conversion to water (H_2O) and molecular oxygen (O_2) is catalyzed by catalase. H_2O_2 is also used by catalase for oxidizing potential toxins that include alcohols, formaldehyde and formic acid. The absorbance of H_2O_2 is recorded at 240 nm for calculating the rate of the reaction as water and oxygen cease to absorb energy at this wavelength window. The rate of the reaction is proportionally elevated in the catalase presence. Absorbance decline at 240nm was measured to study the CAT activity due to H_2O_2 degradation, in the reaction mixture having 50 mM sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2 and expressed in nmol of H_2O_2 utilized mg^{-1} protein min^{-1}.

To check the anti-oxidant activity of the catalase enzyme, the level of catalase enzyme in the liver tissue was measured. To check the radioprotection when mice was exposed to whole body irradiation (3 Gy), there was a drop in the level of catalase by 10% ± 4% in comparison to control. But when cells were pretreated with acemannan for 7 days, there was an enhancement in the level of catalase by 33% ± 6% in comparison to radiation control. It was also observed that acemannan alone also improves the level of catalase in the liver tissue and radio-protection was found maximum at the dose of 100 mg/kg body weight as shown in Fig. 10.

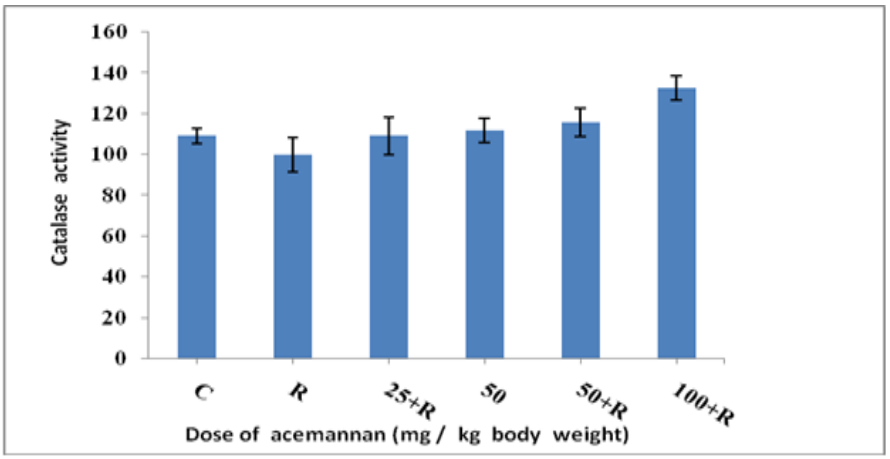


Fig. 10 Effect of acemannan against cytotoxic effects induced by radiation on catalase activity as measured by catalase assay. (C= control cell population, R= radiation dose)

3.4.3 MTT assay

Radiation is known to affect cell proliferation and cause cytotoxic effect. To observe the radioprotective effect of acemannan, the assay for cell proliferation was performed with the use of (0.5 mg/ml) MTT. The MTT assay relies on the mitochondrial dehydrogenase enzyme's ability from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes. Addition of dimethyl sulphonate (DMSO) leads to solubilization of crystals giving rise to a purple color. The number of surviving cells is directly proportional to the amount of formazan product formed which in turn is directly proportional to the intensity of the color. When the spleen cells were exposed to the radiation treatment (5 Gy), there was a drop observed in the cell proliferation by 15% ± 6% as compared to the control as illustrated in Fig. 11. Whereas, when the irradiated cells were treated with *Aloe vera* extract (acemannan), the cell proliferation was enhanced by 31% ± 8% which was maximum at the concentration of 50 µg/ml acemannan in comparison to the treatment group given only radiation.

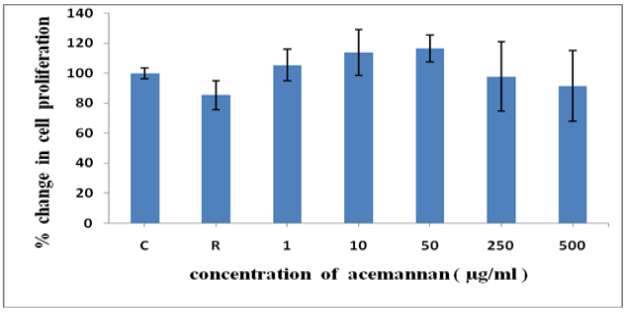


Fig. 11 Effect of acemannan against cytotoxic effects induced by radiation on cell proliferation after 48 hrs. of radiation treatment (5 Gy). (C= control cell population , R = radiation dose)

3.5 Effect of naringenin in radioprotection:

3.5.1 MTT assay

When the spleen cells were exposed to the radiation treatment (5 Gy), there was a drop observed in the cell proliferation by 14% ± 5% as compared to the control. Whereas, when the irradiated cells were treated with naringenin, the cell proliferation was enhanced by 16% ± 4% which was maximum at the concentration of 10 µg/ml naringenin in comparison to the treatment group given only radiation as illustrated in Fig. 12.

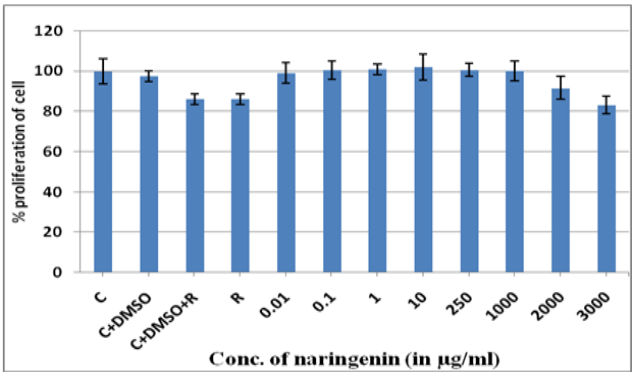


Fig. 12 Effect of naringenin against cytotoxic effects induced by radiation on cell proliferation after 48 hrs. of radiation treatment (5 Gy). (C= control cell population, DMSO = dimethyl sulphoxide, R= radiation dose)

3.5.2 Immunocytochemistry

Immunocytochemistry (ICC) utilizes antibodies targeting specific peptides or protein antigens via specific epitopes in the cell. Location of sub-cellular compartments of the cell that are expressing the antigen are visualized by an immuno-positive signal.

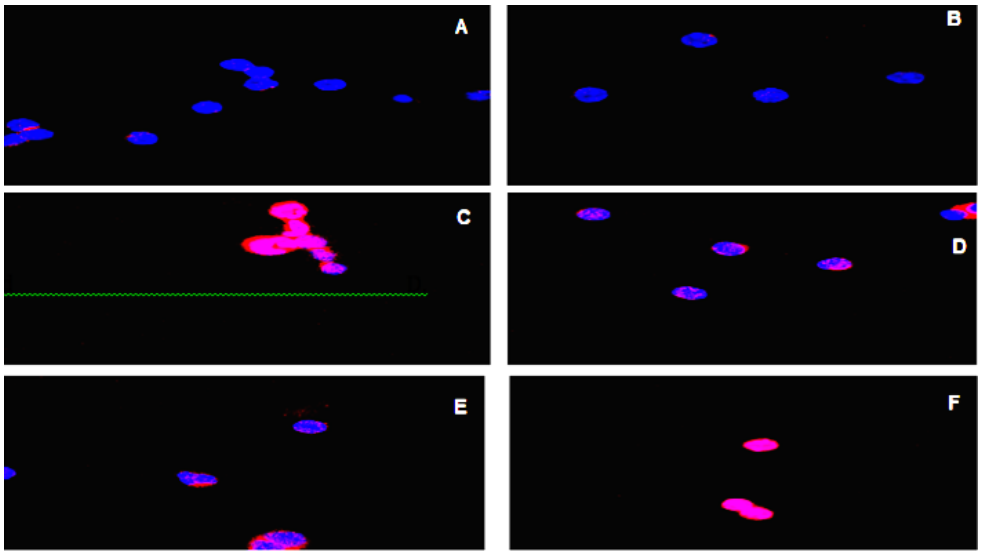


Fig. 13 NF-κB expression in the spleen cells (A) Control, (B) DMSO Control, (C) Radiation control, (D) 10 μM Naringenin + Radiation, (E) 50 μM Naringenin + Radiation, (F) 100 μM Naringenin + Radiation

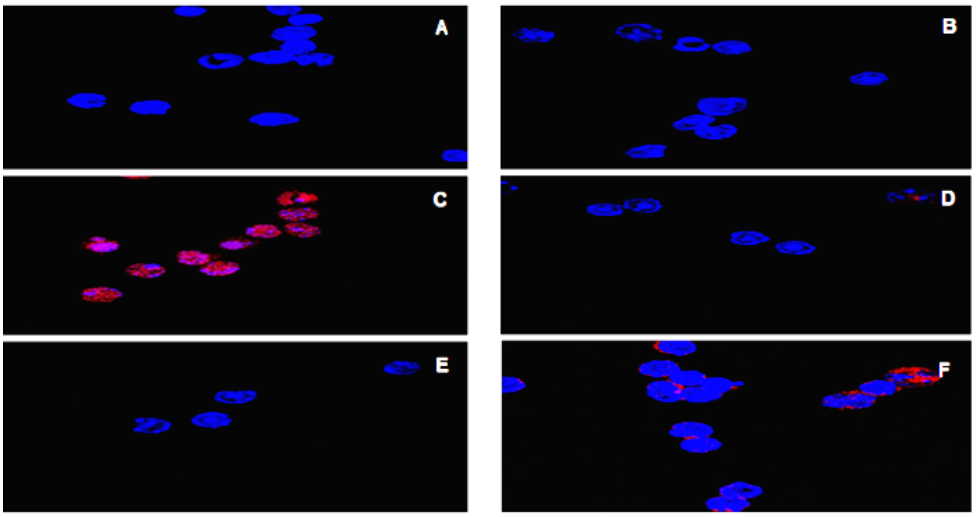


Fig. 14 NF-κB expression in the bone marrow cells (A) Control (B) DMSO Control (C) Radiation control (D) 10 μM Naringenin + Radiation (E) 50 μM Naringenin + Radiation (F) 100 μM Naringenin + Radiation

ICC is an efficient laboratory technique, used to identify specific protein or antigen in cells (cultured cells, cell suspensions) by use of a specific antibody, which binds to it, thereby allowing visualization and examination under a microscope. Alternatively, the secondary antibody may be covalently linked to a fluorophore (FITC) which is detected in a fluorescence or confocal microscope. The fluorescence location depends on the target molecule, which is internal for cytoplasmic proteins and external for membrane proteins. Immunofluorescence when used in conjunction with confocal microscopy, is an efficient technique for locating the proteins and studying dynamic processes like exocytosis, endocytosis, etc.

To measure the expression of γ- radiation induced NF-κB level in the splenocytes and bone marrow cells, it was checked via ICC (Immunocytochemistry). Effect of naringenin on cellular level of NF-κB after γ-radiation treatment: NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB present in majority of animal cell types, is actively engaged in cellular reciprocation to stimuli such as free radicals, stress, ultraviolet irradiation, cytokines, and bacterial or viral antigens. NF-κB is a protein responsible for cytokine production and cell survival. γ - Radiation is known to induce the NF-κB levels. Since NF-κB regulates a large number of transcription factors required in cell cycle, the level of NF-κB was measured. It was found that the level of NF-κB was increased in case of spleen cells and bone marrow cells exposed to radiation (3 Gy) as depicted in Fig. 13 and Fig. 14 respectively. Whereas, when the cells were pretreated with naringenin, the level of NF-κB was decreased which was minimum at 50 μM naringenin dose after γ-radiation treatment.

Conclusion

Radiation exposure causes damage to living tissues through a series of molecular events depending on the radiation energy. The resulting damage to the target components may be induced either directly or indirectly. As human tissues comprises of 80% water content, the aqueous free radicals are formed as a result of major radiation damage, generated by the action of radiation on water. Early research on radiation protection has unravelled the basic mechanisms and yielded a large number of radio-protecting compounds. However, most of the compounds failed in their transition from laboratory to clinic. Acute toxicity and their inability to differentiate between tumor and normal cells are the main reasons for their failure in clinical applications. On treatment with emodin, we found an increase in the cell viability of the spleen cells after radiation exposure. Similarly, there was a decrease in the DNA damage in case of irradiated cells pre-treated with emodin as observed by comet assay. When plasmid DNA was exposed to the γ- radiation in the presence of emodin, underwent reduced DNA damage as compared to the control. Thus, it shows that pre-treatment with emodin reduced the formation of DNA strand breaks as a result of which there was an increase in super-coiled form of DNA against different doses of gamma radiation. It exhibits DNA damage repairing mechanism. Hence it could be an effective drug in radioprotection. On treatment with *Aloe vera* extract acemannan, we found an increase in the cell proliferation in the spleen cells after radiation exposure. An increase in the SOD activity as well as catalase activity was also found in case of treatment of irradiated mice with acemannan. Naringenin treatment of the spleen cells also resulted in an increase in the cell proliferation after radiation exposure. Spleen cells and bone marrow cells showed reduced level of NF-κB upon naringenin treatment after radiation treatment. The present study depicts that naringenin inhibits the NF-kB pathway by down-regulating radiation-induced apoptotic proteins, thereby, radio-protecting at cellular, tissue and organism level. It has the ability to protect normal cells against radiation induced apoptosis. There is a continued interest in the identification and the development of effective, innocuous, and low-cost radio-protective agents as well as compounds that could potentially shield the detrimental effects of radiation exposure as well as increase the therapeutic index of radiation therapy for cancer treatment. Further research employing these phytochemicals is required to explore their radioprotective properties that can be useful for mankind.

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Conflict of Interest

No potential conflict of interest was declared by the authors.

References

Goel H C, Prakash H, Ali A, and Bala M, (2007), Podophyllum hexandrum modulates gamma radiation-induced immunosuppression in Balb/c mice: Implications in radioprotection, *Mol Cell Biochem*, 295, 93-103. doi:10.1007/s11010-006-9277-5.

Pande S, Kumar M and Kumar A, (1998), Evaluation of radiomodifying effects of root extract of Panax ginseng, *Phytother Res*, 12, 13-17.

Lin S, Chen H, Wei W, Ye S, Liao W, et al, (2011), Antiproliferative and Antimetastatic Effects of Emodin on Human Pancreatic Cancer, *Oncology Reports,* 119, 623-626.

Brennan J C, Denison M S, Holstege D M, Prokopios M, Jerry

L D, et al, (2013), 2,3-cis-2R, 3R-(-)-epiafzelechin-3-O-p-coumarate, a novel flavan-3-ol isolated from Fallopia convolvulus seed, is an estrogen receptor agonist in human cell lines, *BMC Complement Altern. Med*, 13:133.

Sharma R and Tiku A B, (2014), Emodin, an anthraquinone derivative, protects against gamma radiation-induced toxicity by inhibiting DNA damage and oxidative stress, *Int J Radiat Biol,* 90:275-283.

Gao J, Wang F, Wang W, Su Z, Guo C, et al, (2014), Emodin suppresses hyperglycemia- induced proliferation and fibronectin expression in mesangial cells via inhibiting cFLIP, *PLoS One,* 9:e93588.

Liu Y X, Shen N Y, Liu C and Lv Y, (2009), Immunosuppressive effects of emodin: An in vivo and in vitro study, *Transplant. Proc*, 41: 1837-1839.

Harris C, Pierce K, King G, Yates K M, Hall J, et al, (1991), Efficacy of acemannan in treatment of canine and feline spontaneous neoplasms, *Mol Biother*, 3: 207-213. http://www.ncbi.nlm.nih.gov/pubmed/1768373

Peng S Y, Norman J, Curtin G, Corrier D, McDaniel H R, et al, (1991), Decreased mortality of Norman murine sarcoma in mice treated with the immunomodulator, Acemannan, *Mol Biother*, 3: 79-87. http://www.ncbi.nlm.nih.gov /pubmed/1910624

Ueng YF, Chang YL, Oda Y, Park SS, Liao JF, et al, (1999), In vitro and in vivo effects of naringenin on cytochrome P450-dependent monooxygenase in mouse liver, *Life Sci,* 119: 623-626.

Chetia P, Bala A, Khandelwal B and Haldar P K, (2012), Comparative in vitro free radical scavenging property of â-carotene and naringenin with respect to vitamin C and Nacetyl cysteine, *Pharmacologia*, 3 (12), 724-728.

Kumar S, Tiku A B, (2016), Biochemical and Molecular Mechanisms of Radioprotective Effects of Naringenin, a Phytochemical from Citrus Fruits**,** *J Agric Food Chem***,** 64 (8**)**, 1676-1685**.** DOI: 10.1021/acs.jafc.5b05067

Wang J, Yu L, Jiang C, Chen M, Ou C, et al, (2013), Bone marrow mononuclear cells exert long-term neuroprotection in a rat model of ischemic stroke by promoting arteriogenesis and angiogenesis, *Brain Behav Immun*, 34: 56-66. doi*:*10. 1016/ j.bbi.2013.07.010*.* PMC 3795857 PMID 23891963*.*