

Evaluation of radioprotective efficacy of *Ficus racemosa* in Swiss albino mice exposed to electron beam radiation

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Abstract

The aim of this study was to evaluate the radioprotective potency of various doses (100, 200 and 400mg/kg b. wt) of ethanolic stem bark extract of *Ficus racemosa* (L.) on mice exposed to 6Gy electron beam radiation. Pre supplementation of *Ficus racemosa* for 15 days significantly reduced the malondialdehyde level in liver and was found to protect livers of irradiated mice from depletion of endogenous antioxidant enzymes like glutathione, GST, SOD, catalase and thiols. Pre supplementation also showed marked increase in WBC, spleen and thymus index. It also protected the liver, kidney and intestine from acute radiation effects, contributing to the over all radioprotective activity.

Keywords: Electron beam, Radioprotective activity, Antioxidant enzymes, *Ficus racemosa*

Introduction

Ionizing radiation induces the production of Reactive Oxygen Species (ROS), which includes superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). These free radicals can cause extensive cellular damage such as nucleic acid strand scission (Suganya et al. 2012) modification of polypeptides (Vaclav et al.

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2010) and lipid peroxidation (Fogarty et al. 2011). Majority of cancer patients will receive radiation therapy during the course of their treatment. Ionizing radiation employed in radiotherapy of various cancers, is nonselective in its action because it affects both tumor cells and normal cells. Most of its toxic effect is attributed to the ROS generated by the radiolysis of water. It is important to protect biological systems from radiation induced genotoxicity or lethality. Natural plant extracts have shown to protect cells and tissues against ionizing radiation without adverse effect such as feeling of tiredness, skin problems. Accordingly such plants can be used as an adjunct to conventional radiotherapy.

Ficus racemosa (FR) belongs to the family Moraceae is commonly known as Gular fig, Cluster fig in English, Gular in Hindi and Udumbara in Sanskrit. The stem bark of the plant exhibit wide spectrum of pharmacological activities such as anti-dysentery (Murthi et al. 2011), hypoglycemic, hypolipidemic, renal anti-carcinogenic, anti-diuretic, anti-tussive, hepatoprotective, radioprotective, anti-ulcer, antiinflammatory, antidiarrhoeal, wound healing, larvicidal, antifungal (Sumit et al. 2012) and cardioprotective activity (Faiyaz et al. 2012). Even though *in vitro* radioprotective potential of *Ficus racemosa* was studied by Veerapur et al. (2009) on V79 cell lines, there are no reports available for *in vivo* study. Hence in the present investigation, evaluation of radioprotective property of the *Ficus racemosa* in mice exposed to electron beam radiation was carried out since the plant requires intensive study with different parameters before drug development.

Materials and Methods

Preparation of the extract

Extract of *Ficus racemosa* stem bark was prepared by extracting 250g of shade dried stem bark powder in ethanol at 50 °C to 60 °C in a Soxhlet extractor for 72 hours. The cooled liquid extract was concentrated by evaporating its liquid contents in rotary evaporator, concentrated and dried in desiccators (yield: 28g). The dried ethanol extract was suspended in distilled water.

Animals

All the animal experiments were conducted on Swiss albino mice (10-12 weeks old weight 30-35g) chosen from animal house of Justice K.S. Hegde Medical College, Deralakatte, Mangalore. The animals were maintained under controlled conditions of temperature ($23\pm 2^{\circ}\text{C}$) and humidity (60-70%) and 12h light-dark cycle. They were kept in standard polypropylene cages with husk shavings as bedding and were allowed food and water ad libitum. Animals were randomly assigned to cages for grouping (n= 6 in each group). The studies were performed in accordance with the animal ethical committee of Justice K.S. Hegde Medical Academy.

Irradiation

The irradiation work was carried out at Microtron centre, Mangalore University, Mangalore, Karnataka, India. The unanaesthetised animals were restrained in well-ventilated perspex boxes and exposed to whole-body electron beam radiation (EBR) at a distance of 30 cm from the beam exit point of the microtron accelerator at a dose rate of ~ 100 Gy/min.

Experimental design

Animals were divided into five groups of six animals each. Group I- whole body electron beam irradiated (6Gy), group II- untreated control (treated with vehicle only), group III- treated orally with 100mg/kg body weight, group IV- 200mg/kg body weight, group V- 400mg/kg body weight of stem bark extract, once daily for 15 consecutive days. 16th day the mice were exposed to 6Gy EBR (Madhu et al. 2011). Animals were observed for 15 days after irradiation and animals were sacrificed by cervical dislocation on 16th day.

Haematopoeitic protection

Blood was collected from the orbital artery and the white blood cells were counted using a hemocytometer. The spleen index and thymus index was determined by weighing method. $\text{Spleen/ thymus index} = \frac{\text{spleen/ thymus weight}}{\text{Body weight}} \times 100$ (Lin-Na Liu et al. 2012).

Micronucleus assay

Mouse bone marrow micronucleus test was carried out as described by Hosseinimehr, et al. 2003 and Schmid, 1975. The bone marrow cells from femur were flushed in the form of a suspension into a centrifuge tube containing 5% BSA. The cells were dispersed by gentle pipetting and collected by centrifuge at 2000 rpm for 5 min at 4°C . The cell pellet was resuspended in a drop of BSA and bone marrow smear were prepared. After air drying the smear were stained with May- Grunwald/Giemsa. Micronucleated polychromatic erythrocytes and Non chromatic erythrocytes were observed under Microscope. The percentage of micro nucleated polychromatic erythrocytes (MnPCEs), micronucleated non chromatic erythrocytes (MnNCEs) and ratio of PCE to (PCE + NCE) was calculated to assess the effects radiation on bone marrow proliferation.

Antioxidant enzymatic assays

The protein contents of 10% liver homogenates were determined by modified Lowry's method.

Reduced glutathione

Proteins were precipitated using 10% TCA, centrifuged and 0.5ml of supernatant was mixed with 0.3M phosphate buffer and 0.006mM DTNB. The mixture was incubated for 1min and the absorbance was measured at 412nm against appropriate blank. The glutathione content was calculated by using standard plot under the same experimental condition. (Sharma et al. 2009)

Glutathione -S-transferase

The activity of GST was determined by using CDNB substrate (Warholm et al. 1985). The reaction mixture contains 20mM CDNB, 20mM GSH, and 0.1M sodium phosphate buffer pH (6.5). The formation of the mixture GSH-CDNB conjugate was determined at 340nm and the activity was calculated by using $\epsilon = 9.6 \text{mM}^{-1} \text{cm}^{-1}$.

Catalase

The activity of catalase was determined by spectrophotometrically according to the standard protocol (Aebi 1984). Briefly, 30mM of H_2O_2 in 50mM phosphate buffer (pH 7.0), 0.02ml of the liver homogenate was added and read at 240 nm. Activity was calculated by using $\epsilon = 43.6 \times 10^{-3} \text{M}^{-1} \text{cm}^{-1}$.

Superoxide dismutase

The estimation of superoxide dismutase enzyme is carried out by Beauchamp and Fridovich method. The substrate used for the assay consists of nitro blue tetrazolium chloride which reacts with superoxide anions produced upon illumination of riboflavin in the presence of methionine as an electron donor, to produce formazan which is a blue colored complex. The SOD present in the sample will act on the superoxide anions produced by riboflavin and they reduce the net superoxide anions in the substrate leading to decreased production of formazan manifested by decreased intensity of the blue color formed. The decrease in the formation of formazan is directly proportional to the amount of SOD in the sample, 50% decrease in the formation of formazan is taken as one unit of SOD.

Total thiols

Briefly, 0.2 ml of liver homogenate was mixed with phosphate buffer (pH 8), 40 μl of 10 mM DTNB and 3.16 ml of methanol. The mixture was incubated for 10min and the absorbance was measured at 412nm against appropriate blanks. The total thiol content was calculated by using $\epsilon = 1.36 \times 10^{-4} \text{M}^{-1} \text{cm}^{-1}$ (Sedlak and Lindsay 1968).

Malondialdehyde

In brief, the liver homogenate was incubated with 15% TCA, 0.375%TBA and 5N HCL at 95°C for 15min, the mixer was cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm against appropriate blank. The amount of MDA was determined by using $\epsilon = 1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ (Braugher et al. 1987).

Total antioxidant capacity

Total antioxidant capacity was determined spectrophotometrically through the formation of phosphomolybdenum complex (Prieto et al. 1999). 100 μl of serum was mixed with 5% TCA, the mixtures was centrifuged, 1ml of TAC reagent was added and mixture was incubated at 90°C for 90min, and absorbance was measured at 695nm. The total antioxidant capacity content was calculated by

using standard plot under the same experimental condition.

Histopathological studies

Slice of liver, kidney and jejunum were fixed in 5% formaldehyde, embedded in paraffin wax, sectioned at 5µ thickness and stained with haematoxylin and eosin stain. Detailed microscopic examination these organs were carried out.

Statistical analysis

The data are expressed as Mean ± S.E.M. Statistical comparisons were performed by One-Way analysis of variance (ANOVA) followed by Dunnett post test using Graph pad Prism Version 5. 3.

Results

White blood cells

Compared with normal control mice, the number of the white blood cells in the four experimental groups was significantly reduced. However, the administration of FR attenuated the radiation induced reduction in white blood cells in a dose dependent manner, suggesting that FR confer radioprotection (Table 1).

Table 1: Effect of FR on WBC count in mice after whole body EBR.

Animals	WBC/ Cm ²
IR alone	3850±138.4
Control	6150±182.1
100mg/kg+IR	5083±132.7**
200mg/kg+IR	5300±332.7***
400mg/kg+IR	5683±331.1***

Values are means ±SE of 6 mice in each group, ***statistical significant at p<0.001 and **p<0.01 when compared to the radiation group.

Effect of FR on the spleen index and thymus index

EBR significantly reduced the spleen index and the thymus index (Table 2), suggesting that irradiation was destructive to spleen and thymus. Oral intake of 100, 200 and 400 mg/kg FR partially restored the spleen index and thymus index in a dose-dependent manner, suggesting that FR favorably modulate the immune system in mice.

Table 2: Effect of FR on spleen and thymus indices of the mice after whole body EBR.

Animals	Spleen index	Thymus index
IR alone	0.264±0.02	0.268±0.01
Control	0.519±0.01	0.501±0.03
100mg/kg+IR	0.376±0.03 *	0.431±0.02
200mg/kg+IR	0.463±0.03***	0.523±0.02 ***
400mg/kg+IR	0.518±0.01***	0.532±0.04 ***

Values are means ±SE of 6 mice in each group, ***statistical significant at p<0.001 and *p<0.05 when compared to the radiation group.

Estimation of the anti-mutation effects of FR by determining the micronucleus rates of polychromatic and non chromatic erythrocytes in bone marrow

EBR caused a marked increase in the micronucleus rate of polychromatic and non-chromatic erythrocytes (Table 3), compared with the control group. Oral intake of FR suppressed the increase in the micronucleus rate of polychromatic and non chromatic erythrocytes in a dose-dependent manner and also the ratio of PCE to (PCE + NCE) increased when compare to irradiated group.

Table 3. Effect of FR on micronucleus formation in bone marrow cells of Swiss albino mice exposed to EBR.

Cells	IR alone	Control	100mg/kg+IR	200mg/kg+IR	400mg/kg+IR
MnPCE/PCE (%)	27.30 ±1.18	0.30 ±0.06	11.40±0.7***	7.49±0.72***	2.68±0.28***
MnNCE/NCE (%)	12.67 ±7.30	0.27±0.02	4.5±0.35***	3.77±0.40***	2.16±0.18***
PCE/PCE+NCE(%)	25.91 ±1.43	63.58±4.26	40.53±2.3**	41.34±2.67**	46.03±2.93***

Values are means ±SE of 6 mice in each group, ***statistical significant at p<0.001, ** p<0.01 when compared to radiation group. PCE: Polychromatic erythrocytes, NCE: Non chromatic erythrocytes, MnPCE: Micronucleated Polychromatic erythrocytes, MnNCE: Micronucleated Non chromatic erythrocytes.

Antioxidant enzymatic assays.

Glutathione

EBR decreased the GSH level in the liver (Fig 1), pretreated with FR significantly restored the GSH level.

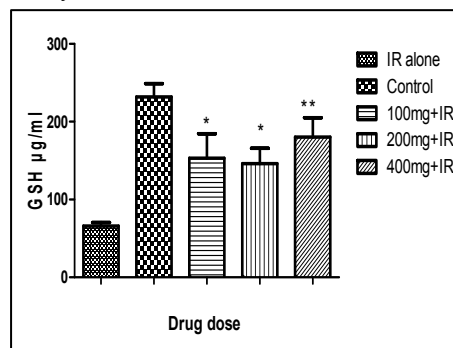


Figure 1: Effects of FR on GSH in liver after whole body electron beam irradiation. Values are means±SE of 6 mice in each group, **statistical significant at p<0.01 and *p<0.05 when compared to the radiation group.

Glutathione-S-transferase

EBR decreased the GST (moles/min/mg of protein) activity in the liver (Fig 2), FR treated mice significantly restored the GST activity in a dose-dependent manner

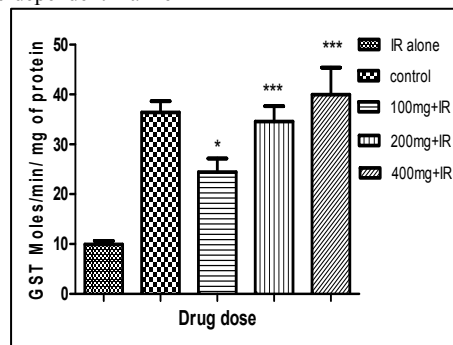


Figure 2: Effects of FR on GST activity in liver after whole body electron beam irradiation. Values are means ±SE of 6 mice in each group, ***statistical significant at p<0.001, *p<0.05 when compared to radiation group.

Catalase

EBR decreased the catalase activity (moles/min/mg of protein) in the liver (Fig3), pre treatment with FR in all the three concentrations restored the catalase activity.

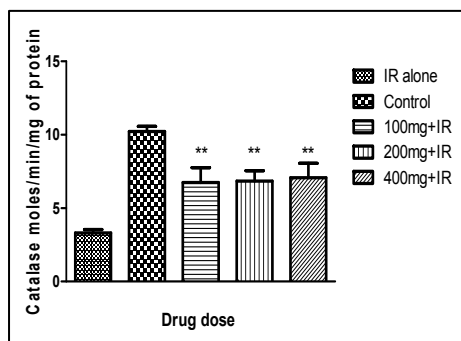


Figure 3: Effects of FR on catalase activity in liver after whole body electron beam irradiation. Values are means \pm SE of 6 mice in each group, **statistical significant at $p < 0.01$ when compared to the radiation group.

Superoxide dismutase

The whole body EBR decreased the SOD activity (U/mg of rotein) in the liver (Fig4), FR dose-dependently restored the SOD activity.

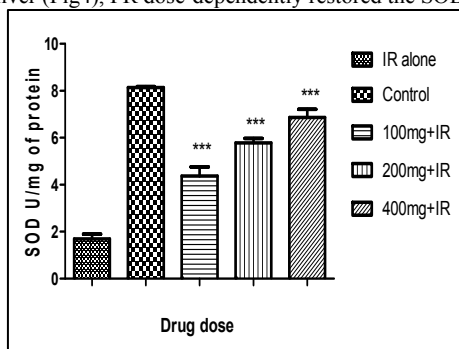


Figure 4: Effects of FR on SOD activity in liver after whole body electron beam irradiation. Values are means \pm SE of 6 mice in each group, ***statistical significant at $p < 0.001$ when compared to the radiation group.

Total thiols

EBR decreased the total thiols (μ moles/mg of protein) level in the liver (Fig 5), FR dose-dependently restored the total thiols level.

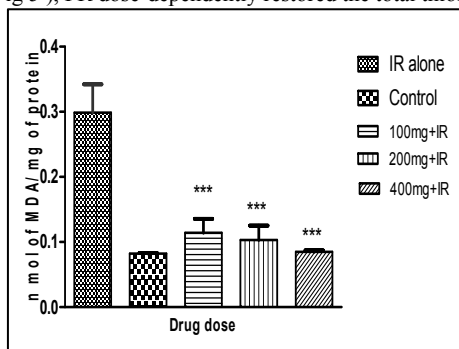


Figure 5: Effects of FR on total thiols in liver after whole body electron beam irradiation. Values are means \pm SE of 6 mice in each group, ***statistical significant at $p < 0.001$ when compared to the radiation group.

Malondialdehyde

EBR increased the malondialdehyde (n mole of MDA/mg of protein) level in the liver (Fig 6), FR treated mice decreased the malondialdehyde level in a dose-dependent manner.

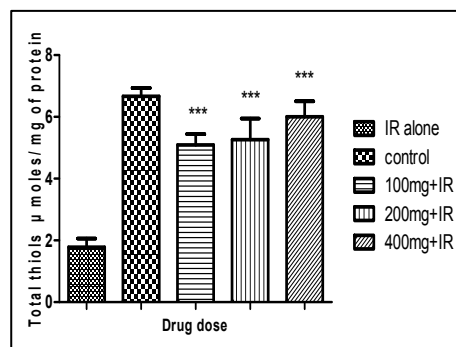


Figure 6: Effects of FR on MDA in liver after whole body electron beam irradiation. Values are means \pm SE of 6 mice in each group, ***statistical significant at $p < 0.001$ when compared to the radiation group.

Total antioxidant capacity

EBR decreased the level of total antioxidant capacity in serum (Fig 7). FR treated mice restores the total antioxidant capacity in a dose-dependent manner.

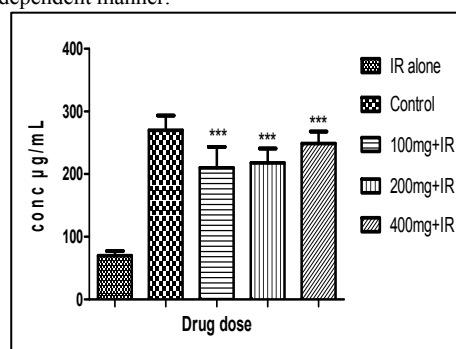


Figure 7: Effects of FR on total antioxidant capacity in serum after whole body electron beam irradiation. Values are means \pm SE of 6 mice in each group, ***statistical significant at $p < 0.001$ when compared to the radiation group.

Histopathology of jejunum

Histopathology of jejunum (Fig 10), showed significant decrease in the number of villi, villus height, crypts and goblet cells in irradiated mice (B), when compared to normal mice (A). Also increase in the number of dead cells, inflammatory cells was observed in irradiated mice compared to normal. Pretreatment with FR protected the villi, maintained the villus height and decreased number of dead and inflammatory cells was observed (C).

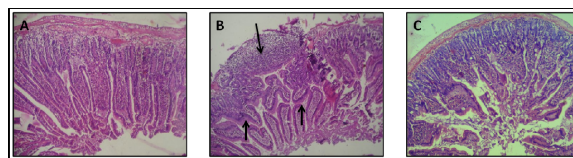


Figure 10: Histopathology of Jejunum. A: Normal control. B: Irradiated. C: Pretreated with 200mg/kg FR

Discussion

Plants are one of the most important sources of medicines. Today the large numbers of drugs in use are derived from plants (Sumit et al. 2012). The present results demonstrate that administration of ethanolic stem bark extracts of FR for 15 consecutive days before

exposure to EBR was radioprotective in mice. Whole body electron beam irradiation induced a marked reduction in white blood cell count, spleen index and thymus index, and a marked increase in the micronucleus rates of polychromatic erythrocytes in bone marrow in these mice. As expected the mice exposed to whole body EBR had significantly reduced GST, catalase and SOD activity in liver, and also caused lipid peroxidation in the liver. Irradiation also caused decreased level of GSH, total thiols and total antioxidant capacity, and it also causes damage to liver, kidney and jejunum.

The thymus and spleen are very sensitive to radiation and are important components of the immune system. Radiation remarkably reduced the spleen and thymus index, indicating impaired immune function in mice. The present results showed that FR prevented the decline in the spleen index and thymus index in mice radiated by EBR, suggesting the extract could at least partially restore immunological function, and improve the pathological status of mice. This result is similar to that reported by Lin-Na Liu et al. (2012), who showed that exposure to γ irradiation resulted in a significant decrease in body weight, liver weight and spleen index of rats in a dose-dependent manner compared with control mice.

Bone marrow cells are very sensitive to radiation, which causes chromosomal aberrations and increases the micronucleus rates of polychromatic erythrocytes. The irradiation-induced changes in micronuclei and chromosomal aberrations are dose dependent. The administration of FR in the present study partially prevented the increase in the micronucleus rate of polychromatic erythrocytes and non chromatic erythrocytes in the bone marrow of these mice, indicating that FR is anti-mutagenic. This finding is supported by the observation that pre - and post- treatments with LC & LA to γ -irradiation was found to decrease the Mn-PCEs compared with irradiated group (Sally et al. 2010).

Irradiated mice showed depletion in the GSH level when compared to the normal mice. In the normal conditions, cells are intact healthy and GSH is restored by synthesis. FR treatment in irradiated mice protected GSH and the levels are close to normal mice. Similar results were observed by (Raafat et al. 2011). GST is an enzyme involved in the detoxification process. FR was found to increase GST activity compared to irradiated mice. The liver has the high-est contents of antioxidants and antioxidant enzymes indicating that it plays an important role in pro-oxidants detoxification (Jalal et al. 2010). In this study, SOD activity was decreased by EBR radiation, but it was significantly restored when mice were pretreated with extract, the results are similar to (Raafat et al. 2011). Administration of FR significantly restored the EBR radiation induced suppression of catalase activity. Total thiols are important biological markers and a source of -SH- groups in enzyme formation and DNA synthesis. Pretreatment with extract increased total thiol content in irradiated mice, and maintains the desired level of SH groups. MDA is generated by free radical attack on cell membrane phospholipids and circulating lipids, and acts as a sensitive biomarker for oxidative stress that occurs as part of the pathogenesis of various diseases. The present study found that stem bark extract of FR significantly reduced the MDA level. Constituents of *Ficus racemosa* bark such as flavonoids, tannins and glutathione have antioxidant activity. So stem bark extract of plant may inhibit lipid peroxidation by scavenging free radicals (Shivalinge et al. 2011). Pretreatment with FR also restored the total antioxidant capacity.

Conclusion

In conclusion, electron beam irradiation of pretreated mice for fifteen consecutive days with FR showed significant declines in

MDA levels and micronucleus rate which in turn showed increase in survivability of animals. FR offer as a promising radioprotective agent since all the studied assays attributed to several possible mechanisms, such as normalization of intracellular antioxidant levels and free radical scavenging activity.

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