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Modulation of Haematopoetic System and Antioxidant Enzymes by *Emblica Officinalis* Gaertn and its Protective Role Against γ-radiation Induced Damages in Mice

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Radioprotection/Emblica/Radiation/Oxidative stress/Antioxidants.

The radio protective effect of the fruit pulp of *Emblica officinalis* Gaertn (Emblica) was studied in adult Swiss albino mice. Mice were treated with 2.5g/kg b.wt of Emblica for 10 consecutive days before irradiation and exposed to a single dose of 700 rads (7Gy) of radiation after the last dose. One group was given Emblica continuously for another 15 days after irradiation. Changes in the total leukocyte count, bone marrow viability and hemoglobin were studied after whole body irradiation. Administration of Emblica significantly increased these levels, which were lowered by irradiation. Animals were sacrificed at various time points after irradiation and the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST), and levels of glutathione were assayed in the blood. The damage to the cell membrane after whole body irradiation was studied by measuring the tissue lipid peroxides levels. Administration of Emblica significantly enhanced the activity of the various antioxidant enzymes and GST as well as glutathione system in the blood. Treatment with Emblica also lowered the elevated levels of lipid peroxides in the serum. The data clearly indicated that the extract significantly reduced the bioeffects of radiation. Emblica extract may be useful in reducing the side effects produced during therapeutic radiation.

INTRODUCTION

The interactions of radiation with the components of living system results in the generation of several oxygen free radicals (OFRs). OFRs so formed are responsible for many of the detrimental effects of radiation on living system. They can attack virtually all components including DNA, protein and causes membrane lipid peroxidation. They also impair the indigenous antioxidant defense mechanism.[1)]

Since radiation causes enormous damage to normal cells several strategies are designed in order to minimize the lethal consequences of radiation exposure to normal cells. Use of radioprotectors is one among such strategy.[2)] Several compounds have been tried as radioprotective agents in experimental animals and as well as in human volunteers. This includes sulfhydryl compounds like cysteine, WR2721, antioxidants like vitamin C & E, cytoprotective agents like MESNA and several biological modifiers like γ-interferon.[2)] However due to toxicity produced, these radioprotective agents had only limited use in clinical medicine. Recently there have been renewed interests in the search of potential drugs of plant origin for their potential use as radioprotectors.

Emblica officinalis Gaertn (syn. *Phyllanthus Emblica* Linn), family-Euphorbiaceae, is being extensively used in traditional Indian system of medicine and is a constituent of several poly herbal preparations. Brahma Rasayana, which contains Emblica, is reported to have an excellent radio protective activity in animal models as well as in human volunteers undergoing radiotherapy.[3,4)] Emblica is an excellent antioxidant and a free radical scavenger. It helps in protecting the skin from damaging effect of UV radiation.[5)] Emblica was found to inhibit nitrosation reaction in stomach, which will be highly helpful in reducing stomach cancers.[6)] It was found to be antimutagenic and reduced the clastogenicity induced by various metals.[8)] Emblica was found to be hepatoprotective,[9)] anti-diabetic[10)] and reduced the ulcer of the stomach[11)]. Emblica inhibited N-Nitroso diethyl amine-induced hepatocarcinogenesis and 20-methyl chloranthrene-induced sarcoma in experimental animals.[12)] Emblica was found to be non-toxic to human and experimental animals. Many polyphenols such as ellagitannins, phyllemblin, ellagic acid, trigalloylglucose, phyllantidin, mucic acid, emblicannin, and furosin[13)] are reported to be present in Emblica.

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We have earlier identified epigallocatechin gallate in the extract of Emblica as one of the major polyphenol, which is also a major constituent of green tea.[14] Antioxidant activities of many of these polyphenols are reported.[14] There are no reports available on the radio protective activity of Emblica. In the present study we have evaluated the possible role of Emblica as a radioprotective agent against sub-lethal dose of γ-radiation in mice.

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium (NBT), riboflavin, reduced glutathione (GSH), 5-5' dithiobis (2-nitrobenzoic acid) (DTNB), and 1- chloro-2, 4-dinitrobenzene (CDNB) were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Thiobarbituric acid was purchased from Hi-media laboratories, Mumbai, India, 1,1,3,3-tetramethoxy propane was procured from Sigma-Aldrich chemicals, USA. All other chemicals and reagents used in this study were of analytical grade.

Preparation of extract

Fresh Emblica fruits were purchased from local market and washed thoroughly in water. A voucher specimen (Voucher No. Eup-10) of the plant and fruit is kept in the herbarium of Amala Ayurvedic Hospital and Research Centre. Emblica weighed 1Kg was cut into small pieces, grinded and extracted twice with 75% methanol (1000 ml each time) at room temperature for 24hr. Extract was filtered through Whatmann filter paper and supernatant was evaporated with the help of rotary evaporator at 40°C under vaccum. The final liquid suspension was lyophilized. The yield of the preparation was 0.8%. The lyophilized powder was resuspended in water at desired concentrations in 0.2ml and administered orally by gavage once daily to animals.

Animals

Inbred 4–6 weeks old female Swiss albino mice (20–25g) were obtained from Small animal breeding station, Kerala Agricultural University, Mannuthy, Thrissur. They were kept in well-ventilated cages under standard conditions of temperature, pressure and humidity. The animals were provided with normal mouse chow (Sai Durga feeds and foods, Bangalore) and water *ad libitum*. All animal experiments conducted during the present study got prior permission and followed the guidelines of Institutional Animal Ethics Committee (IAEC).

Irradiation

Animals were treated with a single dose of radiation of 700 rads (7Gy). The source of radiation was a ^{60}Co Theratron-Phoenix teletherapy unit (Atomic Energy Ltd, Canada). Animals were restrained in specially designed, well-ventilated cages without anesthesia and exposed to whole body radiation at a rate of 1.33 Gy/min in a field size of 25 × 25 cm^2 and at a distance of 80 cm from the source.

Determination of effect of Emblica on haematological parameters of irradiated animals

Twenty-one mice were randomly divided into three groups of 7 animals each. Group I was treated as irradiated control served with vehicle. Group II was treated with Emblica (2.5g/Kg b.wt) ten days prior to irradiation. Group III was administered Emblica (2.5g/Kg b.wt) ten days prior to irradiation and continued for another fifteen days after irradiation. All the three groups were irradiated with a single dose of 700 rads.

Body weights of all the animals were determined one day prior to irradiation and every third day thereafter. Blood was collected from tail vain into heparinised tubes and the following parameters were analyzed one day before radiation and every third day thereafter. The parameters analyzed were total WBC count (haemocytometer method), differential count (Leishman's staining method) and haemoglobin by Drabkin's method.[15]

Determination of effect of Emblica on bone marrow viability and antioxidant parameters of irradiated animals

Thirty-six animals were divided in to four groups of nine animals each. For group I–III treatment protocol were similar as described above. Group IV was treated as normal animals without any treatment. On days 5, 10 and 15 after irradiation (700rad) three animals from each group was sacrificed. Blood was collected to heparinised tubes, and plasma was removed and following parameters were assayed in the blood. Activity of the enzyme SOD was measured by NBT reduction method of McCord and Fridovich.[16] CAT activity was estimated by the method of Aebi[17] by measuring the rate of decomposition of hydrogen peroxide (H_2O_2) at 240 nm. Level of GSH was assayed by the method of Moron *et al*[18] based on the reaction with DTNB. Assay of GPX followed the method of Hafeman *et al*[19] based on the degradation of H_2O_2 in the presence of GSH. The method of Habig *et al*[20] was followed to assay the activity of GST based on the rate of increase in the conjugate formation between GSH and CDNB.

The femurs of the above animals were dissected out and bone marrow cells were flushed into phosphate buffered saline (pH 7.4) containing 2% fetal calf serum. The cells were washed and bone marrow viability was determined by the method of Sredni *et al*[21]. The results were expressed as number of live bone marrow cells × 10^6/ femur.

The liver of the sacrificed animals were excised quickly, washed in ice-cold saline and kept at –70°C till the day of analysis. On the day of analysis 25% homogenate was prepared in ice-cold tris–HCl buffer (0.1M, pH7.4). The homogenate was centrifuged at 12000 rpm for 30 minutes and

supernatant was used to determine the tissue lipid peroxide levels (LPO) using the TBA method of Okhawa *et al*[22].

Statistical analysis

Data was expressed as mean ± standard deviation (SD). Significance levels for comparison of differences were determined using Student's t test. The mean of Emblica treated group was compared with that of radiation alone treated group. The radiation alone treated group was then further compared with untreated group. The differences between means were considered to be statistically significant if $p \leq 0.001$.

RESULTS

Radiation treatment at the dose level used here did not produce a statistically significant reduction in the body-weight of the exposed animals. Initial body weight of animals were 24.87 ± 3.77, 24.32 ± 4.89 and 25.21 ± 3.37 respectively for group I, II and III. On day 6 body weight was reduced to 22.02 ± 3.47, 21.11 ± 4.30 and 20.80 ± 2.42 respectively ($p > 0.05$).

Radiation significantly lowered the total leukocyte count in irradiated animals (Fig. 1 A). Administration of Emblica was

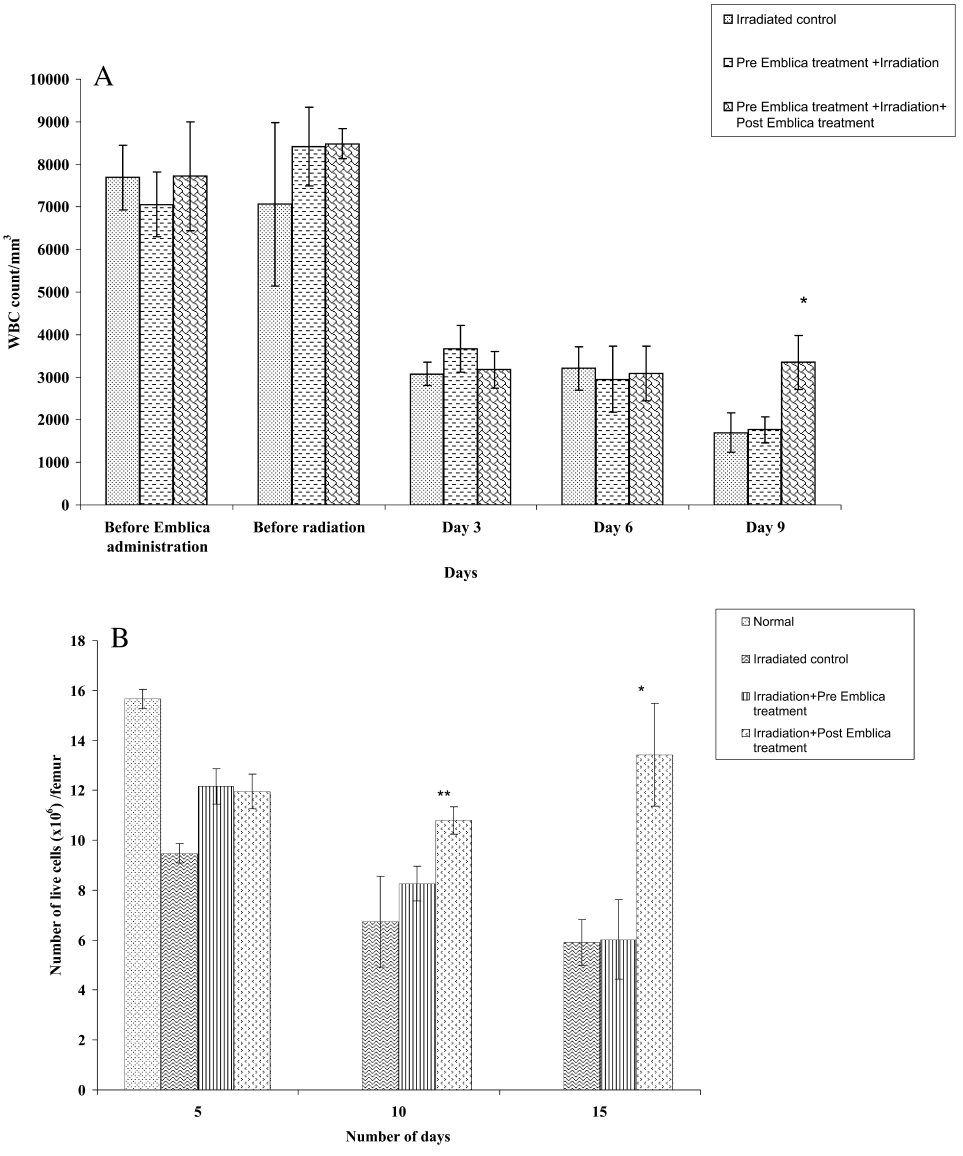

Fig. 1. Effect of administration of Emblica fruit pulp extract on the total WBC count of irradiated animals. Irradiation lowered the total WBC count and continuous administration of Emblica elevated the levels (1 A). Effect of administration of Emblica fruit pulp extract on the bone marrow cellularity of radiation treated animals. Emblica treatment elevated the levels of bone marrow cellularity (1 B). Data is expressed as mean ± S.D, * denotes $p \leq 0.001$, ** denotes $p \leq 0.005$.

found to increase the count. In the initial days after irradiation both group II (Emblica pre treated group) and group III (Emblica continuously administered group) showed almost similar number of WBC. But at later days after irradiation, group III showed a significantly elevated WBC as compared with group I (radiation alone treated group) and group II. This indicated that continuous Emblica administration stimulated the hemopoetic system in a concentration dependent manner. This observation is further supported by the increased bone marrow viability found in-group III (Fig. 1 B). Bone marrow viability in normal animals was (Group IV) was $15.66 \pm 0.50 \times 10^6$ cells/femur. Bone marrow viability was significantly decreased after irradiation. After 15th day of post- irradiation group II possessed a value of 5.9×10^6 cells/ femur where as group II and III showed 6.03×10^6 and 13.43×10^6 cells/ femur respectively. The hemoglobin levels were significantly reduced after irradiation (data not shown). On day 6 radiation alone treated group had a hemoglobin level of 10.51 ± 3.19 where as Emblica continuously administered group had a value of 11.89 ± 2.89. The differential count did not show any significant variation (data not shown)

The activity of both SOD and CAT, two of the major enzymes involved in the antioxidant defense mechanism were found to be decreased after irradiation (Fig. 2 A and 2

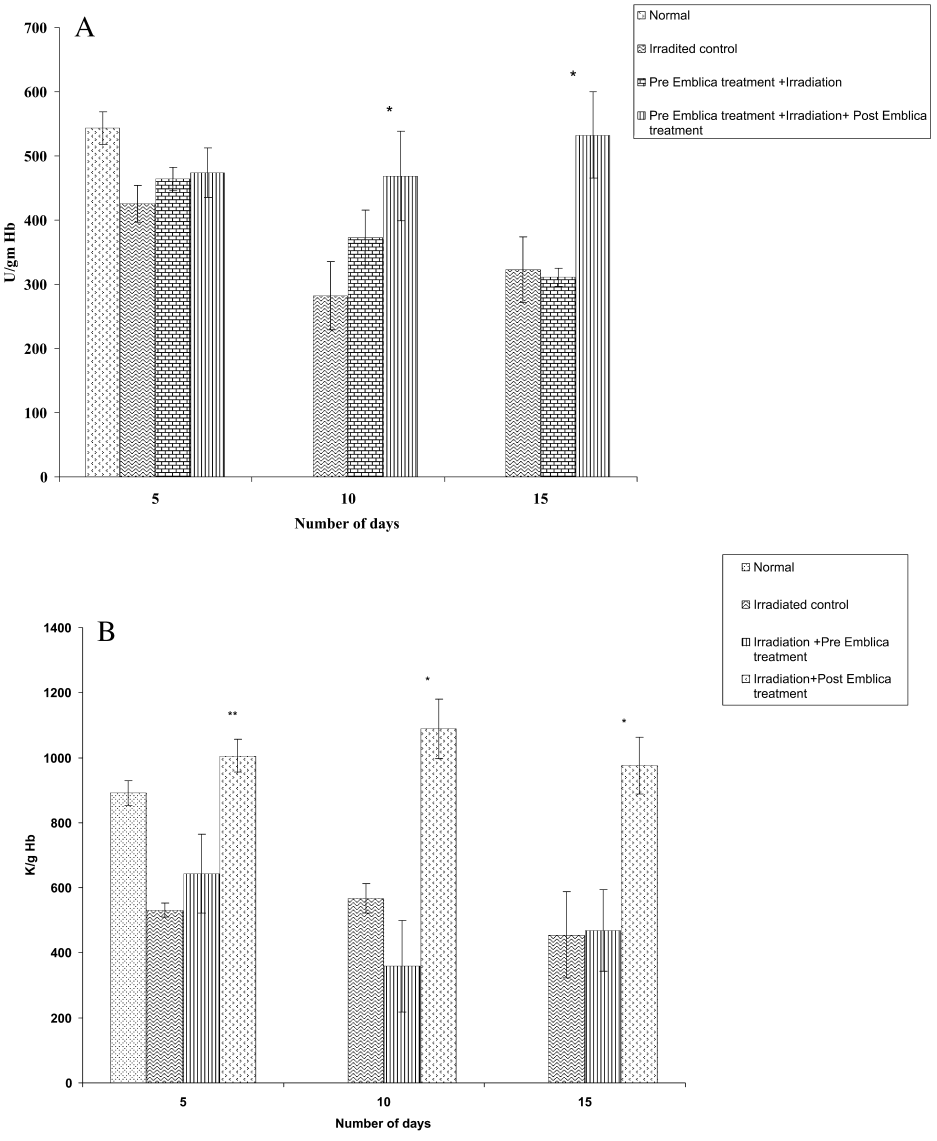

Fig. 2. Effect of administration of Emblica fruit pulp extract on the activity of the enzyme superoxide dismutase (SOD) in blood. Radiation treatment lowered the activity of SOD and continuous administration of Emblica elevated these levels (1 A). Effect of administration of Emblica fruit pulp extract on the activity of the enzyme catalase (CAT) in blood. Emblica administration significantly elevated the lowered levels of catalase after irradiation (2 B). Data is expressed as mean ± S.D, * denotes $p \leq 0.001$.

B). The continuous administration of Emblica enhanced SOD activity, which showed the maximum value on 15th day after irradiation and CAT on 10th day after irradiation.

Activity of GPX was also found to be decreased after whole body irradiation (Table 1). Continuous administration of Emblica elevated the activity of GPX. On day 15 after irradiation group I had an activity of 1211.18 ± 113.23 U/L of haemolysate whereas group III showed an activity of 1967.64 ± 131.77 demonstrating that Emblica administration stimulated GPX activity ($p \leq 0.001$).

The levels of the major cellular antioxidant GSH increased after Emblica administration (Table 2). The levels of GSH were brought down after irradiation. On day 15 the levels of GSH increased almost three times in Emblica continuously administered group as compared with radiation alone treated group indicating that Emblica administration elevated the GSH levels ($p \leq 0.001$). It could be presumed that an increased level of anti-oxidant enzymes and GSH is a direct consequence of Emblica administration and could be seen in unirradiated animals as well (data not shown). Emblica administration also elevated the activity of GST (Table 3) an enzyme involved in the glutathione mediated detoxification system. On day 15 group III showed an activity of 2.28 ± 0.15 (nanomoles of CDNB-GSH conjugate formed) ($p \leq 0.01$) where as group I had an activity of 1.45 ± 0.38 only.

Radiation increased the levels of lipid peroxidation in all the radiation treated animals (Table 4). On day 15, it was 3.50 ± 0.11(nanomoles of MDA formed/mg of protein) for

Table 1. Effect of administration of Emblica on blood GPX levels of irradiated animals (values are expressed as U/L of haemolysate)

Group	Day 5	Day 10	Day 15
Normal	2174.40 ± 82.18	ND	ND
Irradiated Control	1611.53 ± 147.52**	1311.33 ± 151.72*	1211.18 ± 113.23*
Emblica Pre-treatment + Irradiation	1759.90 ± 111.23	1623.66 ± 186.93	1296.24 ± 155.61
Emblica Pre-treatment + Irradiation + Emblica Post- treatment	1951.74 ± 69.83	1725.76 ± 185.82**	1967.64 ± 131.77*

*$p \leq 0.001$, **$p \leq 0.01$., ND-Not Determined

Table 2. Effect of administration of Emblica on blood GSH levels of irradiated animals (values are expressed as nmol/ml)

Group	Day 5	Day 10	Day 15
Normal	26.08 ± 3.64	ND	ND
Irradiated Control	17.65 ± 3.12	17.59 ± 1.24	16.80 ± 1.31**
Emblica Pre-treatment +Irradiation	22.82 ± 2.54	17.70 ± 3.50	16.20 ± 1.32
Emblica Pre-treatment+Irradiation + Emblica Post -treatment	23.99 ± 5.36	37.68 ± 2.35*	44.37 ± 5.17*

*$p \leq 0.001$, **$p \leq 0.01$. , ND-Not Determined

Table 3. Effect of administration of Emblica on blood GST levels of irradiated animals (values are expressed as nano-moles of CDNB-GSH conjugate formed/min/gm Hb)

Group	Day 5	Day 10	Day 15
Normal	1.27 ± 0.20	ND	ND
Irradiated Control	1.43 ± 0.39	1.62 ± 0.22	1.45 ± 0.38
Emblica Pre-Treatment + Irradiation	1.30 ± 0.21	1.23 ± 0.27	1.25 ± 0.17
Emblica Pre-treatment +Irradiation + Emblica Post- treatment	2.19 ± 0.09*	2.17 ± 0.12*	2.28 ± 0.15*

*$p \leq 0.001$, ND-Not Determined

Table 4. Effect of administration of Emblica on tissue lipid peroxide levels of irradiated animals (values are expressed as nano moles of MDA formed/mg of protein)

Group	Day 5	Day 10	Day 15
Normal	0.86 ± 0.04	ND	ND
Irradiated Control	3.11 ± 0.14*	3.44 ± 0.21*	3.50 ± 0.11*
Emblica Pre-treatment+Irradiation	2.20 ± 0.12	2.18 ± 0.16	2.39 ± 0.38
Emblica Pre-treatment + Irradiation + Emblica Post- treatment	1.88 ± 0.22*	1.95 ± 0.13*	2.01 ± 0.17*

*$p \leq 0.001$, ND-Not Determined

group I, whereas in-group III it was significantly reduced to a level of 2.01 ± 0.17 ($p \leq 0.001$).

DISCUSSION

Radiation is used therapeutically for the treatment of various types of malignancies. The severe side effects of radiotherapy resulted from the damage of normal cells. Rapidly dividing cells of gastrointestinal tract, heamatopoetic systems are more prone to radiation-induced damages[23]. In the present study the myelosuppression produced as a result of whole body irradiation is significantly lowered by continuous administration of Emblica extract. Moreover the bone marrow viability was also elevated by Emblica treatment indicating that Emblica stimulated the haematopoetic system.

The interaction of ionizing radiation with biological system results in the generation of many highly reactive short lived reactive oxygen species (ROS) mainly due to the hydrolysis of water. The major ROS resulting from aqueous radiolysis include H, OH, RO_2, H_3O^+, etc. These ROS the attack cellular macromolecules like DNA, RNA, proteins, membranes, etc and cause its dysfunction and damage. ROS increased the membrane lipid peroxidation, which in turn can alter the integrity of membrane structure leading to inactivation of membrane bound enzymes, loss of permeability of the membrane and decrease in membrane fluidity.[1,24] Whole body irradiation increased the levels of tissue lipid peroxidation. Treatment of Emblica was found to be effective in reducing the lipid peroxidation.

Radiation produced a change in the antioxidant enzymes levels in the body. The values of SOD were significantly higher in Emblica treated animals. Radiation caused an increase in the levels of superoxide radicals and the increased SOD activity seen after Emblica administration was therefore to eliminate superoxide radicals. As a result of dismutation, highly reactive H_2O_2 is formed which was degraded by CAT and GPX. While CAT converts H_2O_2 to H_2O and O_2, GPX removes H_2O_2 by coupling its reduction to H_2O with oxidation of GSH to GSSG. The values of GSH were decreased in radiation treated animals when compared to the normal ones. Administration of Emblica extract increased the GSH levels Emblica showed excellent antioxidant activity *in vitro*[25] and present study also revealed its *in vivo* antioxidant potential.

The possible mechanism of action Emblica is shown in Fig. 3. Radiation has been shown to induce DNA strand breaks and mutation and induced peroxidative changes to lipids and proteins. Emblica extract has been shown to have significant antioxidant activity, which reduces the oxidative changes induced by radiation. Emblica extract was also found to inhibit mutagenisis by direct binding to certain mutagens as well as by inhibiting carcinogen activation. It stimulates hemopoiesis thus reducing the myelosuppression induced by radiation. Moreover it produces a protective layer in stomach thus reduces the mucosal damage of gastrointestinal linings during irradiation. Presences of a variety of polyphenols are reported in Emblica. These polyphenols are excellent scavengers of oxygen radicals produced in the body by radiation, thus affording protection to the body. It can be hypothesized that antioxidant activity, potent stimulation of hemopoetic system, non-toxicity as well as the easy availability of Emblica make it as an excellent choice for further development as a natural radioprotector.

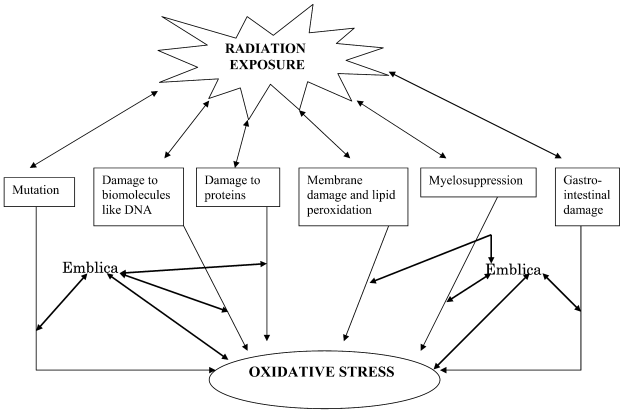

Fig. 3. The schematic diagram represents various consequences of radiation exposure to cells resulting in oxidative stress. The bold double-headed arrows (⟷) represents the possible site of action of Emblica resulting in oxidative stress induced by irradiation.

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