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Ameliorative Activity of *Punica granatum* Extracts and Ellagic acid against Radiation Induced Biochemical Changes in Swiss Albino Mice.

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ABSTRACT

Radiation therapy has been used in cancer treatment for many decades; although effective in killing tumor cells, Reactive Oxygen Species (ROS) produced in radiotherapy threaten the integrity and survival of surrounding normal cells. ROS are scavenged by radioprotectors before they can interact with biochemical molecules, thus reducing harmful effects of radiation. The objective of the present study was to investigate the protective efficacy of ethanolic extracts of pomegranate whole fruit (EPWF) and seeds (EPS) and Synthetic Ellagic acid (EA) against Electron beam radiation (EBR) induced biochemical alterations in Swiss Albino mice. The animals were exposed to sub-lethal dose (6Gy) of Electron Beam Radiation and then treated with100, 200 and 400mg/kg body wt. of pomegranate extracts and synthetic ellagic acid for 15 consecutive days. The biochemical estimations were carried out in the liver homogenate of the sacrificed animals. Test groups exhibited elevation in the levels of antioxidants and antioxidative enzymes estimated compared to irradiated group. Also there was significant reduction in the levels of membrane lipid peroxidation in the treated groups compared to irradiated control. The findings of our study indicate the protective efficacy of pomegranate extracts and synthetic ellagic acid on radiation induced biochemical changes in mice may be due to its free radical scavenging and increased antioxidant levels.

Keywords: *Punica granatum* extract, Ellagic acid, Radiation, Biochemical changes.

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INTRODUCTION

Radiation occurs in nature, but it can also be produced artificially, as in medical x-rays and microwaves for cooking. Radiation can be either beneficial or harmful, depending on its use and control. Exposure to ionizing radiation negatively impacts human health, increasing the risk of incidence of serious pathologies such as cancers and cardiovascular diseases [1–6]. Humans are often exposed to ionizing radiation from various terrestrial sources. Ionizing radiation causes harmful effects through the generation of free radicals. Absorption of ionizing radiation by living cells can produce chemical and biological modifications directly. It can also act indirectly through radiolysis of water, thereby generating reactive chemical species that may damage DNA, proteins and lipids [7]. It is now fairly well established that radiation produces deleterious effects on the organisms and widespread use of radiation in diagnosis, therapy, industry, energy sector and in adverent exposure during air and space travel, nuclear accidents and nuclear terror attacks requires safeguard against human exposures. Radiation protection is an area of immense significance due to its wide applications in planned radiotherapy as well as unplanned radiation exposure [8-11].

Ionizing radiations inflict damage to biological systems essentially through direct deposition of energy into crucial bio-macromolecules or by radiolysis of milieu water and generation of reactive free radicals [9-10]. These reactive oxygen species in turn react with different bio-molecules viz., lipid, DNA, proteins and inflict oxidative damage in them [12].

Since the dawn of civilization, herbal drugs have been used in the ancient civilizations and their use in treatment of various diseases is on a rise especially in the developing and underdeveloped countries primarily due to its easy affordability, non toxic nature, easy acceptability, less toxic or no toxic effects and easy availability. A number of drugs of both synthetic and biological origin have been tested for radioprotection in the last few decades in various parts of the world. The synthetic radioprotectors and radiosensitizers currently available are toxic at their effective doses and consequently natural products, particularly herbals, are receiving attention. Medicinal plants with radioprotective properties contain a plethora of bioactive compounds that exhibit diverse properties eg: antioxidative, immunostimulative, cell proliferative, antiinflammatory and antimicrobial etc. and usually act in a dose dependent manner [13-14].

The pomegranate, *Punica granatum* L., an ancient, mystical, and highly distinctive fruit, is the predominant member of the Punicaceae family. The pomegranate tree, which is said to have flourished in Eden, has been used extensively in the folk medicine for a number of therapeutic purposes [15]. In addition, it has recently been reported that pomegranate contains some species of flavonoids and anthocyanidins in their seed oil and juice, and shows an antioxidant activity three times more potently than red wine and green tea extract [16-22].

Owing to these significant biological activities, pomegranate juice is being increasingly popularized. Ellagic acid has antiproliferative and antioxidant properties in a number of in vitro and small-animal models [23].

These properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption [24]. As with other polyphenolic antioxidants, ellagic acid has a chemo protective effect in cellular models by reducing oxidative stress [25-26].

No published comparative studies are available till date describing the protective role of pomegranate whole fruit and seeds and synthetic ellagic acid against the damaging effects of electron beam radiation on antioxidants and antioxidative enzymes.

With this background, the present investigation was carried out to evaluate the protective effect of pomegranate whole fruit and seeds and synthetic ellagic acid against radiation induced biochemical alterations in swiss albino mice.

MATERIALS AND METHODS

Collection of Plant Material and Preparation of Extract

The fruits of *Punica granatum* were collected from the local market, Mangalore and the specimens were identified.

The whole fruit (peel+ seeds) and seeds of *P.granatum* were dried in hot air oven at 40°-50°C for a period of one week. The dried plant material was powdered using mixer grinder, and subjected to soxhlet extraction with 99% ethanol for 24 hours. The mixture was evaporated to dryness in a rotary flash evaporator and stored in refrigerator.

Synthetic compound, Ellagic acid was purchased from Sigma Aldrich.

Experimental Animals

Adult male Swiss Albino mice (6-8 weeks old/20-25g) were procured from the Institutional Animal House, K.S Hegde Medical Academy, Nitte University, Mangalore. Animal care and handling was carried out according to the guidelines set by WHO (World Health Organization, Geneva, Switzerland). They were housed under standard animal house conditions and fed with standard laboratory pellets and water *ad libitum.* Experimental protocol was approved by the Institutional animal ethical committee.

Irradiation

The irradiation work was carried out at Microtron centre, Mangalore University, Mangalore, Karnataka, India. The animals were restrained in well ventillated perspex boxes and exposed to sublethal dose (6Gy) of whole body electron beam radiation at a distance of 30 cm from the beam exit point of the Microtron accelerator at a dose rate of approximately 72Gy/min.

Preparation of drug and mode of administration

The required amount of ellagic acid and ethanolic extracts of *P.granatum* (EPWF and EPS) was dissolved in double distilled water (DDW) and administered orally once daily for 15 consecutive days at three different concentrations i,e 100, 200 and 400mg/kg body weight. The animals were divided into the following groups:

- Group I: Control - Animals in this group were administered with distilled water.
- Group II: Radiation Control – distilled water + Irradiation
- Group III, IV, V- were administered with standard ellagic acid (EA) at three different doses i,e: 100,200 and 400 mg/kg body wt. respectively via oral gavage for 15 consecutive days after irradiation.
- Group VI, VII, VIII - were administered with ethanolic extracts of pomegranate whole fruit (EPWF) at three different doses i,e: 100,200 and 400 mg/kg body wt. respectively via oral gavage for 15 consecutive days after irradiation.
- Group IX, X, XI - were administered with ethanolic extracts of pomegranate seeds (EPS) at three different doses i,e: 100,200 and 400 mg/kg body wt. respectively via oral gavage for 15 consecutive days after irradiation.

Food, water intake and body weight changes were recorded throughout the study period.

Biochemical Estimations

After the treatment period, animals were euthanized on day 16 and liver tissue was excised. 10% liver homogenate (1g liver in 10mL of 0.4M phosphate buffer, pH 7.0) was prepared using a glass homogenizer. It was centrifuged at 10,000rpm for 15 minutes at 4°C. The supernatant was used for biochemical estimations of antioxidants (Reduced Glutathione, Total Antioxidant Capacity), lipid peroxidation and antioxidative enzymes (Superoxide Dismutase, Catalase, Glutathione S Transferase) according to standard protocols using Double Beam Spectrophotometer.

Reduced Glutathione (GSH)

This method is based upon the development of a relatively stable yellow color, when 5, 5'-dithiobis 2-nitro benzoic acid (DTNB) is added to sulphydryl compounds including glutathione. The diluted samples were treated with 1.5mL of precipitating solution (glacial m-phosphoric acid, EDTA and NaCl per 100mL of distilled water), and kept for 10 minutes for the precipitation to complete. The solutions were then filtered through a whatmann No.1 filter paper.500µL of the filtrate was taken and to this 2mL of phosphate solution (0.3M Na_2HPO_4) and 250µL of DTNB solution was added. Simultaneously a blank was maintained containing 200µL of distilled water, 300µL of precipitating solution, 2mL of phosphate solution and 250µL of DTNB. The intensity of the yellow color formed was spectrophotometrically read immediately (within ten minutes) at 412nm against the blank in an UV-Visible Double Beam Spectrophotometer and the GSH concentration was calculated from the standard graph [27].

Total Antioxidant Capacity (TAC)

This quantitative assay is based on the conversion of Molybdenum(Mo VI) by reducing agents like antioxidants to molybdenum (Mo V), which further reacts with phosphate under acidic pH resulting in the formation of a green coloured complex. The diluted tissue homogenate was pipetted out into a clean test tube and 5%TCA was added to it to precipitate out the proteins in the sample, the mixture was then allowed to stand for about five minutes and centrifuged. 100µL of the clear supernatant was transferred into a clean test tube and 1mL of Total Antioxidant Capacity (TAC) reagent containing 0.6M H_2SO_4, 28mM NaH_2PO_4 and 4mM ammonium heptamolybdate, was added to it and the mixture was then incubated in water bath at 90^oC for 90 minutes. A blank was also maintained simultaneously by substituting distilled water instead of sample in the reaction mixture. Following the incubation, the reaction mixture was cooled and the optical density of the greenish to bluish colour formed was read at 695nm against blank in a Spectrophotometer and TAC was calculated from the standard graph [28].

Lipid Peroxidation

Malondialdehyde (MDA) formed by the breakdown of poly unsaturated fatty acids (PUFA) serves as a convenient index to determine the extent of lipid peroxidation. This was assessed according to the method of Buege and Aust 1978 [29]. To the liver homogenate samples 1mL of TCA-TBA-HCl reagent containing 15% Trichloroacetic acid (TCA) and 0.375% Thibarbutric acid (TBA) 0.25N Hydrochloric acid was added. The samples were kept in boiling water bath for 15 minutes.

The reaction mixture was cooled and centrifuged. The supernatant was taken and the optical density of the pink colour formed was read at 535nm in a spectrophotometer. The concentration of malondialdehyde in the sample was obtained by plotting the obtained absorbance against the standard graph. The optical density of the pink colour formed is directly proportional to the concentration of malondialdehyde in the given sample.

SOD Activity

The estimation of superoxide dismutase enzyme was carried out by Beauchamp and Fridovich method. The substrate used for the assay consists of nitro blue tetrazolium chloride (NBT) 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 coloured complex. The SOD present in the sample acts on the superoxide anions produced by riboflavin and thereby 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.

The supernatant of liver homogenate was used for the assay. For each sample analyzed a corresponding control was maintained. A common standard and blank was kept for each set of illumination.

Test: 2.5ml Methionine, 0.3 ml Riboflavin, 0.1mL NBT, 100µl Tissue homogenate.

Control: 2.5ml Methionine, 0.3ml Riboflavin, 0.1ml phosphate buffer 0.05 M (pH 7.8), 100 µl Tissue homogenate. Standard: 2.5ml Methionine, 0.3 ml Riboflavin, 0.1ml NBT, 0.1 ml phosphate buffer 0.05 M (pH 7.8) Blank: 2.5ml Methionine, 0.3ml Riboflavin, 0.2ml phosphate buffer 0.05 M (pH 7.8)

The test tubes labeled as Test, Standard and Control were subjected to illumination for 10 minutes in an illumination chamber lined with aluminium foil, and fitted with a 15W fluorescent lamp. Following illumination, immediately the optical density of all the reaction mixtures was read at 560nm. Units of enzyme present in the sample were calculated and expressed as U/mg protein.

Catalase Activity

Catalase activity was measured in terms of decomposition of Hydrogen peroxide (H_2O_2) into water molecule, which was measured as decreased absorbance at 240nm.

The diluted tissue homogenate was added to 3ml of 30mM hydrogen peroxide. 3ml of distilled water was maintained as blank and 3 ml of hydrogen peroxide served as reagent blank. The decrease in absorbance was followed for 2 minutes at 15 seconds interval at 240nm. The catalase activity was expressed as U/mg protein i.e., 1µmole of H_2O_2 converted into H_2O in 1 minute [31].

GST Activity

Glutathione S-transferase activity was assayed by the method of Warholm et al. 0.01M (pH 6.5) phosphate buffer containing 1 mm EDTA, 20 mm GSH and 20 mm CDNB were added to the supernatant in a cuvette and the increase in absorbance was noted at 340 nm. The activity of GST was expressed as U/mg protein i.e., µmole of GSH-CDNB conjugate formed/min /mg of protein [32].

Tissue Protein Content

All the samples were analysed for protein content by Biuret method using commercially available kits [33].

Statistical Analysis

The values were expressed as mean ± SD. The data were statistically analysed by one way ANOVA using Prism Software. It was then followed by Tukey's test for multiple comparison between groups. The $P< 0.05$ was considered statistically significant.

RESULTS

Figure 1 represents the changes in tissue GSH content, TAC levels and LPO levels in control, irradiated and post treatment groups (EPWF, EPS and EA). The study revealed that

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GSH content decreased significantly in the irradiated group (P<0.05) compared to control. The post treatment groups (EA, EPWF and EPS) at three different concentrations (100mg, 200mg and 400mg/kg body wt) exhibited significant increase(P<0.05) in GSH levels when compared to irradiated group. However Ellagic acid at the concentration of 200mg/kg body weight was more effective than 100 and 400mg/kg body weight.

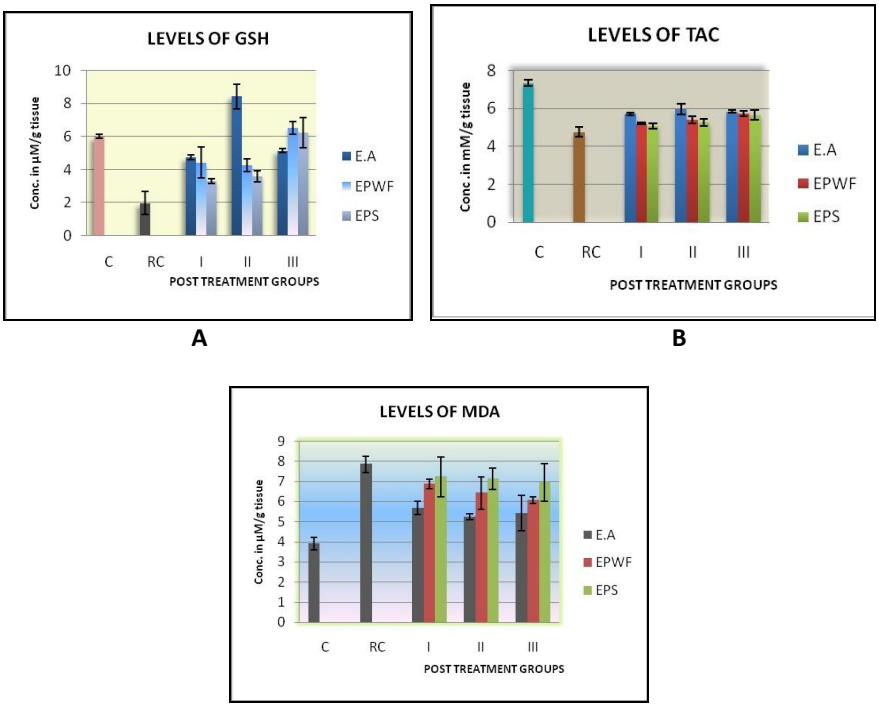

Fig 1: Graph showing variations in antioxidants A)Reduced Glutathione, B)Total Antioxidant Capacity) and C)membrane lipid peroxidation (MDA) in liver homogenates of control, radiation control(6Gy) and Post treatment groups(EA,EPWF,EPS) at three different concentrations : I-100mg/kg body wt,II-200mg/kg body wt and III-400 mg/kg body wt. respectively. Data are expressed as Mean±SD. (n=6), P<0.05, statistically significant.

Changes in the TAC levels showed that radiation significantly decreased (P<0.05) the total antioxidant capacity in liver tissue when compared to control group. Administration of EA, EPWF and EPS at three different dosages to the animals after irradiation caused significant elevation (P<0.05) in liver TAC levels when compared to irradiated group.

The results of lipid peroxidation demonstrated that the changes in MDA levels in irradiated animals were appreciably counteracted by EA, EPWF and EPS administration. Irradiated animals exhibited significant increase (P<0.05) in MDA levels compared to control, whereas the post treated groups showed significant decrease (P<0.05) in the liver MDA levels. However treatment with ellagic acid exhibited much pronounced effect than EPWF and EPS administration.

Figure 2 demonstrates the effect of ellagic acid and EPWF, EPS on radiation induced changes in Superoxide Dismutase (SOD), Catalase and Glutathione S Transferase (GST) activities.

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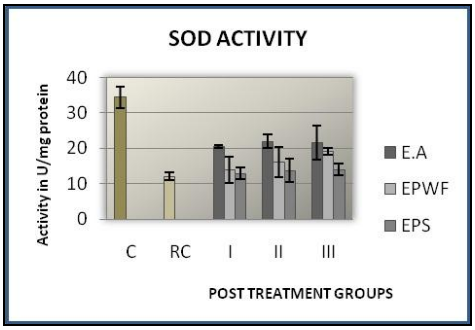
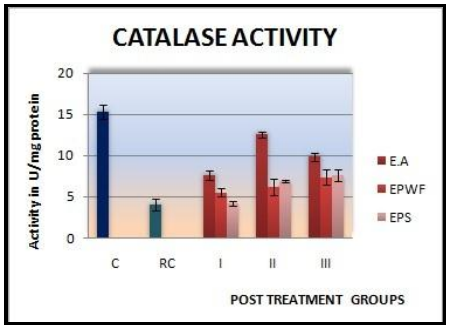
SOD, Catalase and GST activities decreased significantly (P<0.05) in irradiated group compared to non-irradiated control animals. The post treated groups exhibited significant increase (P<0.05) in all the antioxidative enzyme activities determined. However treatment with ellagic acid showed much pronounced effect on the activity of antioxidative enzymes compared to EPWF and EPS administration.





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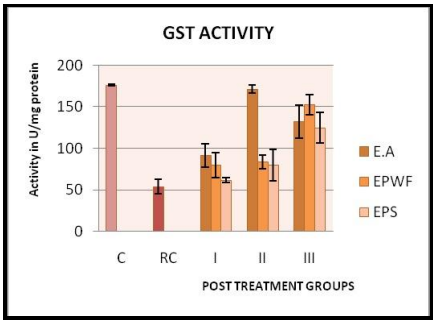

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Fig 2: Graph showing variations in Antioxidative enzymes D)Superoxide Dismutase, E)Catalase and F)Glutathione S Transferase in liver homogenates of control, radiation control(6Gy) and Post treatment groups(EA,EPWF, EPS) at three different concentrations : I-100mg/kg body wt,II-200mg/kg body wt and III-400 mg/kg body wt. respectively. Data are expressed as Mean±SD. (n=6), P<0.05, statistically significant.

DISCUSSION

Several plant and herbal products form the supplements of daily human diet. The potential of dietary ingredients for radiation protection has remained unexplored area till now. The dietary supplements if found radioprotective, may be of crucial importance, as they are in daily human use, non-toxic and have wide acceptability [34].

The pomegranate tree, which is said to have flourished in the Garden of Eden, has also been extensively used as a folk medicine in many cultures, documented at least as far back as the Egyptian Papyrus of Ebers,1550 B.C. [35]. The most famous usage worldwide has been as a vermifugal or taenicidal agent [36-37] i.e., a killer and expeller of intestinal worms. According to one account, the alkaloids contained in the root, tree bark, and to a lesser extent, fruit rind, cause the "tapeworm to relax its grip on the wall of the intestine" thus allowing the weakened parasites to be easily expelled by a second herbal drug, one which is cathartic [35].

The second major property of pomegranate hulls exploited in folk medicine is their strong astringency, making them a popular remedy throughout the world, in the form of an aqueous decoction (i.e., boiling the hulls in water for 10-40 minutes), for dysentary and diarrhea, and also for stomatitis [38-40].

Other ethnomedical explorations have documented pomegranate hull and/or root extract usage both orally and intravaginally to prevent fertility [41-42] and abortion [43] and to ameliorate assorted gynecological problems [44-45]. Other traditional uses of these materials have included treatments for snakebite, [46] diabetes, [47] burns and leprosy [48] The fresh fruit itself has been used as a refrigerant to lower fever [49].

In the present study, the biochemical analysis carried out in the liver homogenate of Swiss albino mice exposed to sublethal dose of Electron beam radiation ascertained its radioprotective potential.

The protective effect of ethanolic extracts of pomegranate (EPWF and EPS) and synthetic Ellagic acid (EA) on antioxidants and antioxidative enzymes was investigated in the liver homogenate of Swiss albino mice exposed to sublethal dose (6Gy) of Electron Beam radiation.

The effect of EPWF, EPS and EA administration at three different dosages on the hepatic antioxidant level namely, reduced Glutathione, Levels of Total Antioxidant Capacity and Melondialdehyde level in post irradiated animals is shown in figure 1. The post treatment groups (EA, EPWF and EPS) at three different concentrations (100mg, 200mg and 400mg/kg body wt) exhibited significant increase($P<0.05$) in GSH levels when compared to irradiated group. Administration of EA, EPWF and EPS at three different dosages to the animals after irradiation caused significant elevation ($P<0.05$) in liver TAC levels when compared to irradiated group.

The results of lipid peroxidation demonstrated that the changes in MDA levels in irradiated animals were appreciably counteracted by EA, EPWF and EPS administration. The antioxidant effects of *P.granatum* ethanolic extracts and synthetic Ellagic acid were probably responsible for this alleviation.

The post treatment of irradiated group with the ethanol extracts of *P.granatum* (EPWF and EPS) and synthetic Ellagic acid (EA) increased the activity of antioxidative enzymes in the liver. The post treated groups exhibited significant increase ($P<0.05$) in the activity of antioxidative enzymes – Superoxide dismutase, Catalase and Glutathione S Transferase determined. The ability of the extracts and the compound to increase the expression of the enzymes probably contributed for this elevated enzyme activity.

CONCLUSION

The findings of the present investigation suggest that *P.granatum* ethanolic extracts and synthetic Ellagic acid has a worthy protective efficacy against the damaging effects of electron beam radiation on hepatic antioxidants and antioxidative enzymes. The ameliorative activity of the extracts and synthetic compound against the radiation induced

biochemical alterations in mice may be due to their free radical scavenging properties and their ability to induce antioxidant enzymes.

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