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Radioprotective Potential Of Punicagranatum Extract And Synthetic Ellagic Acid: A Biochemical And Hematological Study In Mice.

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Abstract

Radiation therapy has been used in cancer treatment for many decades; although effective in killing tumor cells, ROS produced in radiotherapy threaten the integrity and survival of surrounding normal cells. Recent studies have indicated that some commonly used medicinal plants may be good sources of potent but non-toxic radioprotectors. The pomegranate, Punicagranatum L., an ancient, mystical, and highly distinctive fruit, is the predominant member of the Punicaceae family. It is used in several systems of medicine for a variety of ailments. The objective of the present study was to investigate the protective effects of ethanolic extracts of pomegranate whole fruit (EPWF) and Synthetic Ellagic acid (EA) against Electron beam radiation (EBR) induced biochemical and hematological alterations in Swiss albino mice. The extract and synthetic compound were assessed for its radical scavenging property by DPPH and FRAP assays. The animals were treated with 200mg/kg body wt. of pomegranate extract and Ellagic acid for 15 days before exposure to 6Gy of EBR. The hematological parameters were determined in the blood and biochemical estimations were carried out in the serum of sacrificed animals. The plant extract and synthetic compound exhibited good radical scavenging and reducing properties. The pretreated animals exhibited protective effect on the hematological parameters. Radiation induced depletion in the level of reduced glutathione and total antioxidant capacity were prevented significantly by EPWF and EA administration. 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 extract and synthetic ellagic acid on radiation induced biochemical and hematological changes in mice may be due to its free radical scavenging and increased antioxidant levels.

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Keywords— PunicaGranatum, Ellagic Acid, Electron Beam Radiation, Biochemical Changes.

Introduction

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 (Borek et al., 1986).

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The major objectives in radiobiology have been the development of agents that can mitigate the damage produced by ionizing radiation to normal tissues and improvement of cancer radiotherapy. Radiation damages cells by direct ionization of DNA and other cellular targets and by indirect effect through ROS. The haematopoietic tissues due to their proliferate activity have received special attention (Anisworth and Leony, 1966).

After whole body exposure, manifestations of injury to mammalian tissue are well reflected in peripheral blood. Changes in blood cell counts are still considered the most sensitive biological evidences of excessive acute exposure to both external and internal irradiation. This is because of high sensitivity of blood and blood forming tissues to ionizing radiation. Radiation exposure alters the balance of endogenous antioxidant defense systems. Appropriate antioxidant intervention seems to inhibit or reduce free radicals toxicity and offer a protection against the radiation damage (Jacobson, 1954).

Ellagic acid has antiproliferative and antioxidant properties in a number of in vitro and small-animal models. These properties have generated interest in potential human health benefits. But there is only few very preliminary state of evidence supporting health benefits of ellagic acid in humans (Seeram et al., 2005; Madal and Stoner, 1998).

The pomegranate, *Punica granatum* L, an ancient, mystical, and highly distinctive fruit, is the predominant member of Punicaceae family. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments (Schubert, Lansky and Neeman, 1999).

Therefore the present study was carried out to investigate the comparative radio protective activity of extracts of *Punica granatum* (whole fruit and seeds) and synthetically available Ellagic Acid in Swiss Albino mice at hematological and biochemical levels.

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.

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 200 mg/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 :EA(200mg/kg b.w) + Radiation

Group IV: EPWF(200mg/kg b.w) Treatment for 15 days+Radiation

Group V: EPS (200mg/kg b.w) Treatment for 15 days+Radiation

Irradiation:

The irradiation work was carried out at Microtroncentre, Mangalore University, Mangalore, Karnataka, India. At the end of experimental period, the animals were restrained in well ventilated 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.

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

On day 15, following radiation, the animals were sacrificed and the blood was collected which was used for hematological and biochemical analysis.

Hematological Analysis:

The hematological parameters viz., White blood cells count, Red blood cells count, Hemoglobin concentration, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Number of Platelets (PLT) using Haematology Analyzer was measured in the blood using Hematology Analyzer (PCE 210VET).

Biochemical Estimations:

After the treatment period, animals were euthanized and blood samples collected through cardiac puncture. Serum separation was done and used for estimation of Malondialdehyde (MDA) and Total antioxidant capacity (TAC). Reduced Glutathione level (GSH) was estimated in the whole blood.

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 (Buege and Aust, 1978). To the serum samples, 1mL of TCA-TBA-HCl reagent containing 15% Trichloroacetic acid (TCA) and 0.375% Thiobarbituric 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.

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 serum samples were 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. 100 μ L of the clear supernatant was transferred into a clean test tube and 1mL of Total Antioxidant Capacity (TAC) reagent containing 0.6M H₂SO₄, 28mM NaH₂PO₄ and 4mM ammonium heptamolybdate, was added to it and the mixture was then incubated in water bath at 90oC 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 (Prieto, Manuel and Miguel, 1999).

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 sulphhydryl 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. 500 μ L of the filtrate was taken and to this 2mL of phosphate solution (0.3M Na₂HPO₄) and 250 μ L of DTNB solution was added. Simultaneously a blank was maintained containing 200 μ L of distilled water, 300 μ L of precipitating solution, 2mL of phosphate solution and 250 μ L 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 (Sharma et al., 2009).

Statistical Analysis

The values were expressed as mean \pm 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

Table 1 represents the changes in hematological parameters in the blood samples of pretreated animals. Treatment with Ellagic Acid and P. granatum extracts exhibited protective effect on all the hematological parameters analysed compared to irradiated group.

Figure 1 represents the changes in GSH content, TAC levels and LPO levels in control, irradiated and pre treatment groups (EA, EPWF and EPS). Test groups exhibited

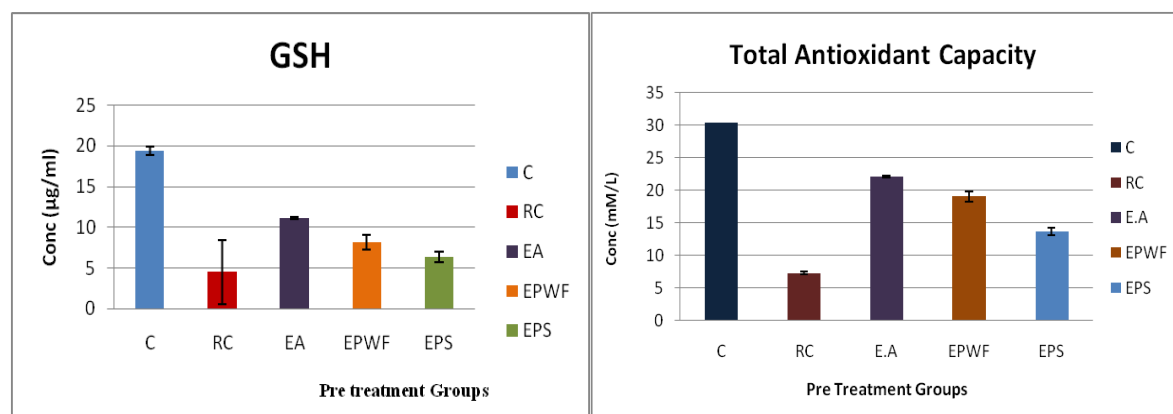
elevation in the levels of Total Antioxidant Capacity and reduced Glutathione compared to irradiated group. Changes in the TAC levels showed that radiation significantly decreased ($P<0.05$) the total antioxidant capacity in serum when compared to control group. Administration of EA, EPWF and EPS before irradiation caused significant elevation ($P<0.05$) in serum TAC levels when compared to irradiated group. The pre treatment groups (EA, EPWF and EPS) exhibited significant increase ($P<0.05$) in GSH 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 pre treated groups showed significant decrease ($P<0.05$) in serum MDA levels.

Table 1:

Comparison of the effect of Ellagic acid and P. granatum ethanolic extract administration on Hematological Parameters with Control and Whole Body Irradiated Swiss Albino Mice.

Group s	RBC($\times 10^6/\mu\text{l}$)	WBC($\times 10^3/\mu\text{l}$)	Hb(g/dl)	LY (%)	MO (%)	GR (%)	PLT($\times 10^3/\mu\text{l}$)
Control	8.06 \pm 0.96	6.73 \pm 1.06	11.5 \pm 0.56	74.08 \pm 0.09	7.68 \pm 0.01	25.66 \pm 2.11	369.33 \pm 18.5
RC	6.73 \pm 0.183	4.7 \pm 0.264	7.45 \pm 1.06	66.53 \pm 3.40	6.63 \pm 0.28	35.3 \pm 1.11	244.33 \pm 10.4
EA (I)	7 \pm 0.19	4.733 \pm 0.20	10.35 \pm 0.2	66.7 \pm 3.40	6.8 \pm 0.1	28.55 \pm 0.05	265.7 \pm 5.13
EPWF (I)	6.85 \pm 1.88	4.4 \pm 0.14	10.25 \pm 0.3	64.63 \pm 3.9	6.7 \pm 0.14	31.73 \pm 2.36	245 \pm 8.38
EPS I	6.93 \pm 0.84	4.35 \pm 0.35	9.4 \pm 0.3	61.7 \pm 0.42	6.55 \pm 0.07	33.66 \pm 1.72	240.5 \pm 6.36



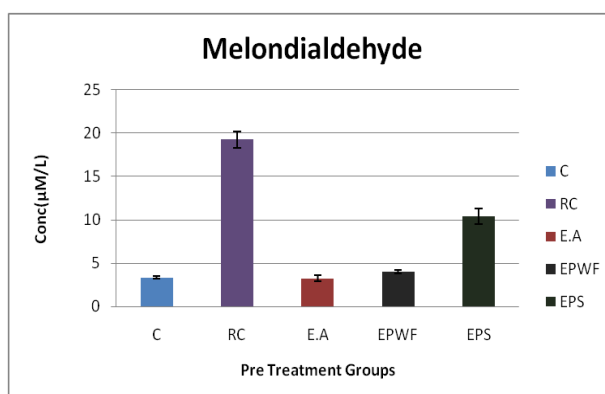


Fig 1 : Graph showing variations in antioxidants (A: Reduced Glutathione(GSH)in RBC lysate, B: Total Antioxidant Capacity(TAC),membrane lipid peroxidation (MDA) in in serum of control, radiation control(6Gy) and Pre treatment groups EA, EPWF, EPS. Data are expressed as Mean±SD. (n=6), P<0.05, statistically significant

Discussion

Through acute and subacute toxicity studies, the LD50 of EA, EPWF and EPS was found to be greater than 2000 mg/kg b.wt.

In the present study, a significant decrease in the hematological constituents of peripheral blood in animals of the irradiation alone group was observed. The decline in hematological constituents may be attributed to direct destruction of mature circulating cells, loss of cells from the circulation by haemorrhage, or leakage through capillary walls and loss of production of cells by radiation (Casarett, 1968).

In the present investigation, Ellagic acid and pomegranate whole fruit extract pre-treatment showed a gradual recovery of hematological constituents in the peripheral blood of Swiss albino mice.

Also it was observed that Ellagic acid and pomegranate whole fruit extract treatments significantly elevated GSH and TAC level and decreased MDA formation in the serum of Swiss albino mice.

However the pomegranate seeds extract did not produce significant results.

The GSH and Total Antioxidants represent an important defense against oxygen derived free radicals and cellular lethality from exposure to anticancer drugs or ionizing radiation (Orrhinius and Moldeus, 1984; Biaglow et al., 1989).

Thus, the results of the present study suggest that Ellagic acid and pomegranate whole fruit extract modulates the radiation induced hematological and biochemical alterations in Swiss albino mice.

Conclusion

The findings of our study indicate the radioprotective efficacy of pomegranate whole fruit extract and synthetic ellagic acid in mice may be due to its phytochemical constituents, free radical scavenging potential and increased antioxidant levels.

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