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Antioxidant potential and radioprotective effect of soy isoflavone against gamma irradiation induced oxidative stress

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ABSTRACT

The in vitro antioxidant potential and in vivo radioprotective ability of soy isoflavones was studied. Male Wistar rats were orally administered with soybean isoflavones (60 mg/kg) for 21 days followed by gamma irradiation exposure. Survival studies in rats exposed at 10 Gy and endogenous spleen colony forming unit assay (CFU) at 6.0 Gy were performed in order to find radioprotective and immunomodulatory nature of the compound. The rat liver post mitochondrial supernatant and erythrocytes were used to measure lipid peroxidation (LPO) and glutathione (GSH) content along with various antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) after gamma irradiation exposure at 2.0 Gy. Pretreatment with soy isoflavone, prior to gamma irradiation resulted in the increased survival rate of the animals as compared to irradiated group. CFU counts in the isoflavone treated group followed by gamma irradiation at 6 Gy were significantly high as compared to control and the irradiated group, showing immunomodulatory nature of the isoflavones. Pretreatment with isoflavones also significantly reduced the LPO, enhanced the activity of antioxidant enzymes and improved haematological and histological parameters. The present study suggests that supplementation with isoflavone has potent antioxidant activity and act as probable radioprotector against gamma radiation induced oxidative damage.

1. Introduction

Ionising radiation causes damage to living tissues through a series of molecular events depending on the radiation energy. As human tissues contain 80% water, the major radiation damage is due to aqueous free radicals, generated by the action of radiation on water. The major free radicals formed upon aqueous radiolysis are hydroxyl radical ($^{\cdot}OH$), superoxide radical ($O_2^{\cdot-}$), hydroperoxy radical ($HO_2^{\cdot}$), among others (Mantena et al., 2008). The reactive oxygen species (ROS) cause several oxidative changes in various biomolecules such as lipids, proteins, carbohydrates as well as nucleic acids (Halliwell & Gutteridge, 1989). The radiation damage to the cell is potentiated or mitigated depending on several factors such as the presence of oxygen, sulphydryl compounds, vitamins and various other molecules in the cellular milieu. The scavengers of free radicals may work as effective radioprotector from radiation damage (Jagetia, Baliga, Malagi, & Kamath,

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2002). The search for radioprotective agents to counteract radiation damage is a necessity and will be of immense use in radiotherapy. Thus, radioprotector for use prior to exposure has been identified as one of the highest priority areas for research. The radioprotective agents minimise or prevent the damage from solar radiation exposure to the population as well as they can be utilised for protection from accidental radiation exposure caused by nuclear power facility, food radiation or other devices that releases radiation (Prasad, Srinivasan, Pugalendi, Venugopal, & Menon, 2006). Radioprotectors can be used to confer protection to the personnel involved in radiotherapy and in patients, who are exposed to radiation therapy. However, many of these radioprotector are either toxic or cannot be administered orally at doses that are effective (Lata et al., 2009).

Recent scientific investigations confirming the clinical significance of soybean neutraceuticals such as isoflavones, tocopherols, vitamin C, saponins, anthocyanin and phenols has helped in the acceptance of soy as food supplement due to their speculated role in the prevention of various diseases caused by oxidative stress (Dixit et al., 2010). Isoflavones are flavonoids compounds with two aromatic rings (C6) joined by a three-carbon chain. The content of total isoflavones ranges from 0.1% to 0.3% in soy flour. The rats were pretreated with isoflavone and exposed to gamma irradiation. The protection conferred by isoflavone was evaluated by survival curve studies, endogenous spleen colony forming unit assay, antioxidant enzymes and glutathione (GSH) in liver and erythrocytes. The histological evaluation of liver and haematology was also performed to observe the extent of cellular damage in various pretreated groups.

2. Materials and methods

2.1. Chemicals

All the chemicals and organic solvents used in this study were of analytical and HPLC grade. Acetonitrile (ACN), ethylenediaminetetraacetic acid (EDTA) and 1,1,3,3-tetraethoxypropane (TEP) were procured from Sigma–Aldrich, St. Louis, MO, USA. Soy isoflavone capsules were obtained from Raptakos Brett & Co. Ltd., Mumbai, India. Pyrogallol and H_2O_2 were procured from Merck India Ltd., Mumbai, India.

2.2. Animals

Healthy colony bred male albino rats of Wistar strain weighing 140 ± 10 g were used in the studies. The rats were housed in polypropylene cages (43 cm × 27 cm with floor area of 165.85 cm^2/animal under a temperature (23 ± 2 °C) and photo schedule. Animals had free access to rodent feed diet (Godrej Agrovet, Mumbai, India) and drinking water. The animal feed comprised of protein 20–22%, oil 3.5%, crude fibre 4%, ash 6%, calcium 1%, phosphorous 0.5%, lysine 1% and methionine 0.9%. All the experiments were conducted in accordance with the standard ethical guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Ministry of Environment, New Delhi, Government of India.

2.3. Determination of antioxidant properties

2.3.1. Preparation of isoflavone extract

Isoflavone (100 mg) was extracted in 100 ml of 50% ethanol at 25 °C in the dark overnight. The mixture was centrifuged at 3000*g* for 10 min. Extract was stored at 4 °C in the dark for further analysis.

2.3.2. Reducing power

The reducing power of the test samples was determined according to the method of John and Shahidi (2010).

2.3.3. Superoxide anion scavenging activity

The measurement of the superoxide anion scavenging activity (SASA) of the test sample was based on the method described by Liu, Ooi, and Chang (1997).

2.3.4. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity (HRSA) was carried out as described by Halliwell, Gutteridge, and Aruoma (1987). The HRSA was measured as oxidation of deoxyribose inhibition.

2.3.5. Bleomycin dependent DNA damage

The bleomycin dependent DNA damage (BDDD) assay was done according to the method of Aeschlach et al. (1994).

2.4. In vivo radioprotective studies

2.4.1. Survival studies

Experiments were conducted with 10 animals per group which included control (Group 1), only irradiated (Group 2), only pretreated (Group 3) and pretreatment followed by radiation exposure (Group 4). The pretreatment was with isoflavones (60 mg/kg) for 3 weeks. In other studies, isoflavones at a dose of 100 mg/kg was fed to mice for 14 consecutive days (Song, Yan, & Cai, 2006). Isoflavone at a dose of 60 mg/kg was orally fed for 21 days to rats. The pretreated animals were exposed to a single whole body lethal dose of gamma irradiation at 10.0 Gy. The source of gamma irradiation was a ^{60}Cobalt unit (Theratron 780C, Ottawa, ON, Canada) used at the Department of Radiotherapy, Mahatma Gandhi Memorial Medical College, Cancer Hospital, Indore, India. The animals were kept in specially designed well-ventilated cages, their movements were restricted and exposed to whole body irradiation without anaesthesia at a rate of 1.22 Gy/min in a field size of 26 × 20 cm^2 at a source to animal distance of 65 cm. The effect of isoflavone pretreatment on gamma irradiation induced mortality and body weight changes of rats were investigated. Survival of rats was recorded up to 30 days post irradiation.

2.4.2. Endogenous spleen colony forming unit (CFU) assay

CFU assay was performed to determine the immunomodulatory nature of the soybean antioxidants. The groups of animals and pretreatment plan were as shown under survival studies, however, animals were exposed to a single whole body sublethal dose of gamma irradiation at 6.0 Gy. The conditions for gamma irradiation were similar as used in survival studies. The rats were sacrificed by mild anaesthesia on the

12th day post-irradiation. Spleens were removed, weighed and fixed in Bouin's solution for 24 h. CFU visible to naked eyes were scored from each spleen.

2.4.3. *Plan for assay of LPO and in vivo antioxidant enzymes*
The groups of animals and pretreatment plan were as shown under survival studies. Animals were exposed to a single dose of whole body gamma irradiation at 2.0 Gy. The animals were sacrificed by mild anaesthesia after 24 h post irradiation.

2.4.4. *Collection and treatment of the biological samples*
The rats were sacrificed under light anaesthesia and the blood was collected in the tubes containing 3.8% sodium citrate by the cardiac puncture. A part of blood was used for haematological examination. Liver was excised immediately and washed several times with ice cold 0.1 M phosphate buffered saline (PBS, 1:9), pH 7.4. The tissue was blotted dry, weighed and minced with stainless steel scissor to prepare tissue homogenate in PBS. A part of the liver was fixed in the Bouin's solution for the histological examination. The tissues were then embedded in the paraffin wax and sections were cut. The sections were stained with haematoxylin and eosin (H and E), mounted with Canada balsam and were examined microscopically.

2.4.5. *Preparation of tissue homogenate*
A 10% tissue homogenate was prepared in 0.1 M PBS (1:9), pH 7.4 using a Potter-Elvehjem type homogenizer for antioxidant enzymes assay. The homogenate was centrifuged at 16,000*g* for 25 min at 4 °C in Sorvall RC 5B plus centrifuge.

2.4.6. *Preparation of packed cell volume*
The blood samples collected in citrated vials was centrifuged at 1000*g* for 5 min to prepare packed cell volume (PCV). Plasma and the buffy coat were removed by gentle aspiration and the erythrocytes were washed with 0.1 M PBS, pH 7.4 thrice, to remove more than 99% of the white cells.

2.4.7. *Preparation of erythrocytes lysate*
The haemolysate was prepared by the method of McCord and Fridrovich (1969) by the precipitation of haemoglobin. PCV (0.5 ml of 5%) was added to 0.5 ml of distiled water. The solution was kept at 4 °C for 5 min followed by the addition of 0.4 ml chloroform–ethanol (3:5, v/v) mixture. The solution was shaken vigorously to precipitate haemoglobin. To the above solution, 0.15 ml of distiled water was added and the contents were shaken and centrifuged to obtain a clear lysate.

2.4.8. *Assay for lipid peroxidation in liver tissue*
Liver tissue was weighed and homogenate (20%) was prepared in 1.15% KCl using the Potter-Elvehjem homogenizer. The homogenate was centrifuged at 16,000*g* for 30 min. For alkaline hydrolysis of protein bound malondialdehyde (MDA), 0.4 ml of 6 M NaOH was added to the sample and it was incubated at 60 °C in a water bath for 45 min followed by centrifugation at 2000*g* for 5 min. The supernatant (1 ml) was diluted with an equal amount of acetonitrile (ACN) to precipitate proteins. The resulting suspension was then vortex for 30 s and centrifuged at 5000*g* for 10 min. The supernatant (0.5 ml) was transferred to 1.5 ml of eppendroff tube and mixed with 0.05 ml of dinitrophenylhydrazine (DNPH) solution (5 mM in 2 M HCl, pH 0.09) and incubated for 10 min at 37 °C (Tukozkan, Erdamar, & Seven, 2006).

Condition for HPLC: Syringe filtered (Whatman, 0.2 μm) sample (20 μl) was injected into HPLC system (Young Lin, ACME 9000, Kyounggi-do, Korea), equipped with UV detector with C-18 silica column (Supleco, 5 μm with dimension of 150 × 4.6 mm). The mobile phase was ACN–distiled water (38:62, v/v) containing 0.2% (v/v) acetic acid. HPLC system was in isocratic condition at a flow rate of 1 ml/min and absorbance was set at 310 nm. The concentration of MDA equivalents in the sample was calculated by software Autochro 3000 after superimposing the chromatogram of the sample on the standard curve prepared from 1,1,3,3-tetraethoxypropane (TEP) and expressed as nmol/mg of protein.

2.4.9. *Assay for lipid peroxidation in erythrocytes*
One millilitre of 10% PCV was used for determination of LPO. Alkaline hydrolysis was performed as above. Sample preparation and HPLC conditions for determination of MDA equivalents in erythrocytes were same as given earlier.

2.4.10. *Assay for superoxide dismutase*
The ability of the enzyme to inhibit the autoxidation of pyrogallol in presence of EDTA was used as a measure of superoxide dismutase (SOD) activity (Marklund & Marklund, 1974). SOD activity was measured in the erythrocytes lysate and tissue homogenate. The assay was performed in a two folds concentration range.

2.4.11. *Assay for catalase*
The assay of catalase was performed according to the method of Aebi (1983). The decomposition of H_2O_2 in presence of CAT was followed by the decrease in absorbance at 240 nm.

2.4.12. *Assay for glutathione-S-transferase*
The method for the estimation of glutathione-S-transferase (GST) activity is based on the property of conjugation of –SH group with 1-chloro-2,4-dinitrobenzene (CDNB) mediated by GST (Habig, Pabst, & Jakoby, 1974).

2.4.13. *Determination of blood and tissue glutathione*
The method is based on the development of a relatively stable yellow colour with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with GSH (Beutler, Duron, & Kelly, 1963).

2.4.14. *Haematological analysis*
Haematological analysis was performed using automated haematology analyzer (Sysmex K-1000, Chicago, IL, USA).

2.5. *Statistical analysis*

The data obtained was analysed by one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparison procedure to calculate interrelation between the groups. *P* value was calculated by statistical software programme "SPSS evaluation version 14".

3. Results

3.1. Antioxidant properties

3.1.1. Reducing power

The reducing ability of the isoflavone extract was investigated by the reduction of ferric ions. The reducing capacity of the compound served as an indicator of its potential antioxidant activity. The reducing power of isoflavone increased with the increasing concentrations of isoflavone extract (Fig. 1a). The increase in reducing power was concentration dependent.

3.1.2. Superoxide anion scavenging activity

In phenazine methosulphate-NADH-nitroblue tetrazolium (PMS–NADH–NBT) system, superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction, reduces NBT. The decrease in absorbance at 560 nm with isoflavone indicates the consumption of superoxide anion in the oxidation mixture. The % inhibition of superoxide radical generation increased with increasing concentrations of isoflavone (Fig. 1b).

3.1.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined by the extent of inhibition of deoxyribose degradation. The oxidation of deoxyribose was inhibited by isoflavone extract, however, % inhibition of deoxyribose oxidation was not concentration dependent (Fig. 1c).

3.1.4. Bleomycin dependent DNA oxidation

The isoflavone due to higher reducing potential (in presence of bleomycin-$FeCl_3$) showed increased DNA oxidation (Fig. 1d). The increase in bleomycin dependent DNA damage was concentration dependent.

3.2. In vivo radioprotection study

3.2.1. Survival studies

Exposure of animals to 10.0 Gy gamma irradiation induced the symptoms of severe radiation sickness like irritability, lethargy, watering of eyes, ruffling of hairs, reduced food and water intake, diarrhoea, facial oedema and loss in body weight is shown in Fig. 2. The first mortality in the irradiation group was observed on day 6 and subsequent mortality at various post-irradiation days. All gamma irradiated animals without any pretreatment died within 13th day post irradiation. The pretreatment of rats with isoflavones (60 mg/kg) prior to gamma irradiation increased 30 days survival of the animals to 33.3% (Fig. 3). The pretreatment of animals with isoflavones delayed or reduced the severity of irradiation sickness and also delayed the onset of irradiation induced mortality when compared with the concurrent irradiation group.

3.2.2. Endogenous spleen colony forming unit (CFU) assay

Animals irradiated at a sub-lethal dose of 6.0 Gy (Group 2) or pretreated with isoflavone (Group 3) showed no CFU counts (Table 1). Pretreatment with isoflavones followed by 6.0 Gy of gamma irradiation (Group 4) led to the development of a significant number of colonies on rat spleen ($P < 0.001$). Spleen size and weight was also increased significantly in pretreated animals followed by gamma irradiation (Group 4) as compared to control group. The present data on spleen colonies demonstrate that pretreatment with isoflavone followed by

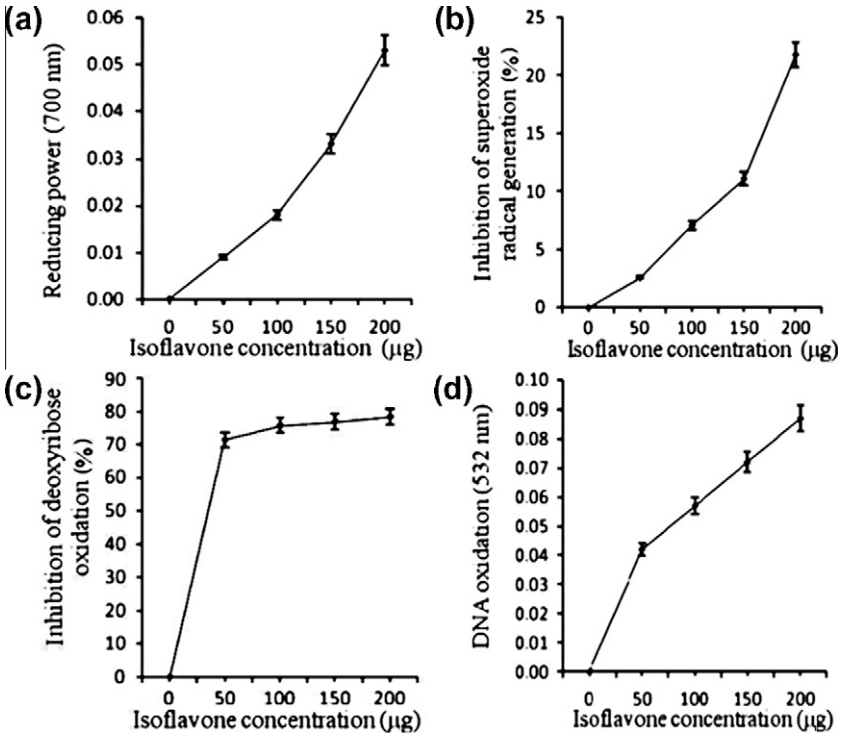

Fig. 1 – Reducing power (a), superoxide anion scavenging activity (b), hydroxyl radical scavenging activity (c) and bleomycin dependent DNA damage (d) of the isoflavone extract. Values are mean ± SE of four experiments. All values are significant ($P < 0.05$) as compared to control.

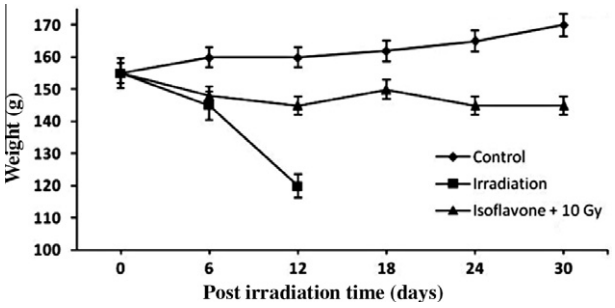

Fig. 2 – Effect of gamma irradiation on the body weight (g) of rats pretreated with isoflavone (60 mg/kg) prior to 10 Gy of gamma irradiation. Values are mean ± SE of 10 animals remained after mortality. Body weight was observed for 30 days after gamma irradiation exposure. Experiments were repeated four times.

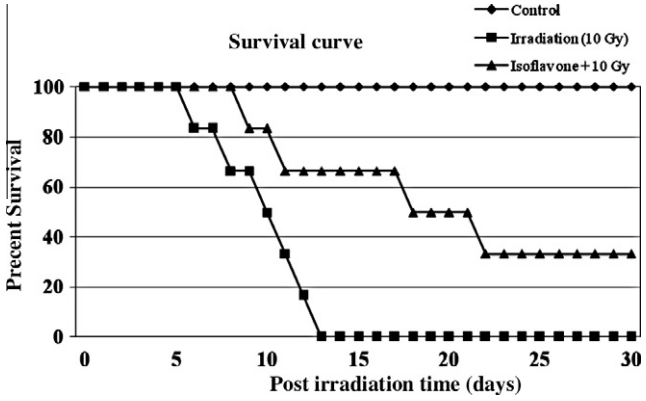

Fig. 3 – Survival curve of rats pretreated with isoflavone before exposure to 10 Gy of gamma irradiation. Survival was observed for 30 days after exposure. Experiments were repeated four times.

gamma irradiation showed an immunomodulatory nature of the soybean antioxidants and gave a significant protection to the haemopoietic system of the animals.

3.2.3. Effect of isoflavone pretreatment on LPO and antioxidant enzymes

The effect of isoflavone pretreatment on LPO and antioxidant enzymes in gamma irradiated rats is presented in Table 2. The MDA equivalent in liver of gamma irradiated rats (Group 2) was found to be nearly three folds higher than the control (Group 1). Rats pretreated with isoflavone for 3 weeks (Group 3) showed no significant increase in liver and erythrocytes LPO as compared to control (Group 1). The treatment with isoflavone for 3 weeks followed by gamma irradiation at 2.0 Gy (Group 4) showed significant increase in LPO of liver and erythrocytes as compared to control (Group 1). However, a significant decrease was observed in both liver and erythrocytes LPO of the rats pretreated with isoflavone without or with gamma irradiation exposure (Groups 3 and 4) as compared to gamma irradiated rats (Group 2).

Rats exposed with gamma irradiation (Group 2) showed a significant decrease in liver and erythrocytes SOD activity as compared to control (Group 1). Isoflavone pretreatment (Group 3) showed increase in liver SOD as compared to control and gamma irradiated rats (Groups 1 and 2). Isoflavone pretreatment prior to gamma irradiation of 2 Gy (Group 4) showed increase in liver SOD as compared to gamma irradiated rats (Group 2) and was similar to control (Group 1). Erythrocytes SOD decreased in isoflavone pretreated rats without or with gamma irradiation (Groups 3 and 4) as compared to control (Group 1). However, significant increase in erythrocytes SOD was found in rats pretreated with isoflavone treatment without or with gamma irradiation exposure (Groups 3 and 4) as compared to gamma irradiated rats (Group 2).

Catalase activity was significantly decreased in liver and erythrocytes in gamma irradiated (Group 2) rats as compared to control (Group 1). Liver CAT activity in rats pretreated with isoflavone (Group 3) was similar to control (Group 1) but significantly ($P < 0.001$) higher than gamma irradiated group (Group 2). However, no significant change was observed in the erythrocytes CAT activity after isoflavone pretreatment (Group 3) as compared to gamma irradiated rats (Group 2). Similarly, isoflavone pretreatment prior to gamma irradiation (Group 4) showed no significant change in erythrocytes CAT activity as compared to gamma irradiated rats (Group 2).

3.2.4. Effect of isoflavone pretreatment on GSH and GST activity

The effect of isoflavone pretreatment on GSH content and GST activity in gamma irradiated rats is presented in Table 3. The liver GSH content showed no significant change in gamma irradiated group (Group 2) as compared to control (Group 1). However, erythrocytes showed significant increase in GSH content of gamma irradiated rats (Group 2) as compared to control (Group 1). Isoflavone pretreatment without or with gamma irradiation exposure (Groups 3 and 4) showed no

Table 1 – Effect of isoflavone pretreatment on endogenous spleen colony forming units (CFU assay).

Treatment	Spleen wt. (g)	Colony count/spleen
Control	0.32 ± 0.02	0.0 ± 0.0
Irradiation (6 Gy)	0.31 ± 0.03	0.0 ± 0.0
Isoflavone	0.34 ± 0.01	0.0 ± 0.0
Isoflavone + irradiation	0.38 ± 0.01^{by}	6.5 ± 1.2^{cz}

Animals were pretreated with isoflavone (60 mg/kg) prior to 6.0 Gy of gamma irradiation. Animals were sacrificed on 12 post irradiation day. Values are initially mean ± SE of 10 animals.
^{b} $P < 0.01$; $^{c}P < 0.001$ as compared to control group.
$^{y}P < 0.01$; $^{z}P < 0.001$ as compared to irradiation group.

Table 2 – Effect of isoflavone pretreatment on LPO and antioxidant enzymes in liver and erythrocytes of rats.

Group	LPO[1]		SOD[2]		CAT[3]	
	Liver	Erythrocytes	Liver	Erythrocytes	Liver	Erythrocytes
1	0.74 ± 0.05	ND	06.64 ± 0.34	20.13 ± 0.68	207.52 ± 13.74	74.51 ± 8.61
2	2.23 ± 0.32^{c}	384.68 ± 9.37^{c}	04.75 ± 0.37^{a}	07.98 ± 0.73^{c}	137.85 ± 14.85^{a}	32.82 ± 6.60^{b}
3	0.79 ± 0.06^{z}	ND^{z}	09.13 ± 0.45^{bz}	11.18 ± 0.90^{cx}	241.50 ± 16.26^{z}	47.22 ± 8.61^{NS}
4	1.24 ± 0.07^{ay}	148.91 ± 8.75^{cy}	06.82 ± 0.45^{x}	11.87 ± 0.80^{cx}	179.73 ± 13.18^{NS}	46.06 ± 5.64^{NS}

Rats were pretreated orally with isoflavone (60 mg/kg.), for 3 weeks prior to whole body gamma irradiation exposure at a dose of 2.0 Gy. Animals were sacrificed after 24 h of irradiation.
Values are mean ± SE of six animals.
Groups 2, 3 and 4 as compared to Group 1. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.
Groups 3 and 4 as compared to Group 2. $^{x}P < 0.05$; $^{y}P < 0.01$; $^{z}P < 0.001$. NS Not significant.
[1] nmoles MDA equivalents formed/mg protein for liver and nmoles MDA equivalents formed/g Hb for erythrocytes.
[2] units/mg protein.
[3] μmoles H_2O_2 decomposed/min/mg protein.

Table 3 – Effect of isoflavone pretreatment on GSH content and GST activity in liver and erythrocytes of rats.

Group	GSH[1]		GST[2]	
	Liver	Erythrocytes	Liver	Erythrocytes
1	0.04 ± 0.01	27.34 ± 1.90	1.41 ± 0.11	0.46 ± 0.04
2	0.05 ± 0.01^{NS}	48.93 ± 1.69^{c}	0.39 ± 0.11^{c}	0.04 ± 0.05^{c}
3	0.04 ± 0.01^{NS}	23.85 ± 1.89^{z}	1.73 ± 0.14^{z}	0.10 ± 0.05^{c}
4	0.06 ± 0.01^{NS}	37.86 ± 1.90^{ay}	0.83 ± 0.14^{NS}	0.10 ± 0.05^{c}

Experimental details were as shown in Table 2.
Values are mean ± SE of 6 animals.
Groups 2, 3 and 4 as compared to Group 1. $^{a}P < 0.05$; $^{c}P < 0.001$.
Groups 3 and 4 as compared to Group 2. $^{y}P < 0.01$; $^{z}P < 0.001$. NS Not significant.
[1] μmoles DTNB conjugated/mg protein for liver and μmoles DTNB conjugated/g Hb for erythrocytes.
[2] μmoles GSH conjugated/min/mg protein.

significant change in GSH content of liver as compared to control and gamma irradiated rats (Groups 1 and 2). However, isoflavone pretreatment without or with gamma irradiation exposure (Groups 3 and 4) showed significantly lowered GSH content in erythrocytes as compared to gamma irradiated rats (Group 2).

A highly significant ($P < 0.001$) decrease was observed in GST activity of both liver and erythrocytes in gamma irradiated rats (Group 2) as compared to control (Group 1). Isoflavone pretreatment without or with gamma irradiation exposure (Groups 3 and 4) showed no significant change in the liver GST activity as compared to control rats (Group 1). Isoflavone pretreatment (Group 3) showed significantly higher GST activity in liver as compared to gamma irradiated rats (Group 2). Isoflavone pretreatment prior to gamma irradiation (Group 4) showed no significant change in liver GST activity as compared to control and gamma irradiated rats (Groups 1 and 2). However, erythrocytes GST activity significantly decreased in rats pretreated with isoflavone without or with gamma irradiation exposure (Groups 3 and 4) as compared to control (Group 1) and was statistically similar to gamma irradiated animals (Group 2).

3.2.5. *Effect of isoflavones pretreatment on haematological parameters*

The haematological parameters of rats against gamma irradiation damage are depicted in Table 4. The WBC, erythrocytes, platelet count and haemoglobin content decreased significantly ($P < 0.001$) in gamma irradiated rats (Group 2) as compared to the control (Group 1). There was no significant change observed in WBC count of isoflavone pretreated animals (Group 3) as compared to control (Group 1). However, isoflavone pretreatment prior to gamma irradiation (Group 4) showed significant decrease in total leucocyte count (TLC) as compared to control (Group 1) but was significantly higher than gamma irradiated rats (Group 2). RBC, haemoglobin content and platelet counts of rats pretreated with isoflavone without and with gamma irradiation (Groups 3 and 4) were normal as compared to the control (Group 1).

3.2.6. *Effect of isoflavone extract on histology of rat liver*

The control animals (Group 1) showed normal cellular characteristics of liver, i.e., normal parenchyma, unremarkable hepatocytes, canaliculi, canals of Hering, sinusoids, sinusoidal lining cells, endothelial cells, Kupffer cells, pit cells, space of Disse, stellate cells and reticulin fibrils (Fig. 4A). The liver histology of the rats exposed with gamma irradiation at a dose of 2.0 Gy (Group 2), revealed subintimal oedema, haemorrhage involving small hepatic veins, patchy sinusoidal congestion and focal areas of necrosis (Fig. 4B). The histology of liver of the rats treated with isoflavone for 3 weeks without or with gamma irradiation exposure showed normal cellular architecture (Fig. 4C and D).

Table 4 – Effect of isoflavone pretreatment on haematological parameters of rats.

Parameter	Group I	Group 2	Group 3	Group 4
WBC ($\times10^3/\mu l$)	8.1 ± 1.1	1.3 ± 0.8^{c}	7.6 ± 0.8^{z}	3.6 ± 0.5^{bx}
Lymphocytes ($\times10^3/\mu l$)	1.8 ± 0.3	1.0 ± 0.4	6.6 ± 0.5^{cz}	2.8 ± 0.4^{az}
Monocytes ($\times10^3/\mu l$)	1.2 ± 0.2	0.2 ± 0.1^{c}	0.6 ± 0.2^{x}	0.6 ± 0.2^{xb}
Granulocytes ($\times10^3/\mu l$))	5.1 ± 0.8	0.1 ± 0.1^{c}	0.4 ± 0.1^{c}	0.2 ± 0.1^{c}
RBC ($\times10^6/\mu l$)	8.7 ± 0.8	3.2 ± 1.2^{c}	8.0 ± 0.8^{z}	5.0 ± 0.5^{x}
Hgb (g/dl)	13.7 ± 2.4	7.82 ± 1.6^{c}	11.5 ± 1.2^{z}	11.0 ± 1.7^{y}
HCT (%)	43.0 ± 3.6	18.2 ± 2.4^{c}	35.0 ± 2.6^{az}	28 ± 1.8^{cy}
MCV (fl)	91.6 ± 8.2	30.78 ± 2.6^{c}	58.2 ± 2.4^{cz}	51.3 ± 3.4^{cz}
MCH (pg)	29.2 ± 2.6	9.2 ± 0.8^{c}	19.1 ± 1.6^{cy}	14.6 ± 1.3^{cx}
MCHC (g/dl)	31.8 ± 3.2	24.6 ± 2.1^{b}	32.8 ± 2.1^{z}	26.6 ± 1.6^{a}
RDW (%)	12.6 ± 2.1	8.0 ± 1.0^{a}	12.8 ± 0.9^{y}	10.8 ± 0.6
Platelet ($\times10^3/\mu l$)	272 ± 23	150 ± 21^{c}	361 ± 45^{bz}	302 ± 39^{z}
MPV (fl)	8.8 ± 0.7	5.5 ± 0.4^{a}	7.4 ± 2.1^{x}	6.9 ± 0.4
PDW (fl)	9.7 ± 0.9	5.2 ± 0.6^{b}	6.8 ± 0.5^{a}	6.7 ± 0.7^{a}

White blood cell count (WBC), red blood cell count (RBC), haemoglobin content (Hgb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), RBC distribution width (RDW), platelet count (PLT), mean platelet volume (MPV) and platelet distribution width (PDW).
Rats were pretreated orally with isoflavone (60 mg/kg), for 3 weeks prior to whole body gamma irradiation exposure at a dose of 2.0 Gy. Animals were sacrificed after 24 h of irradiation.
Values are mean ± SE of six animals.
Groups 2, 3 and 4 as compared to Group 1. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.
Groups 3 and 4 as compared to Group 2. $^{x}P < 0.05$; $^{y}P < 0.01$; $^{z}P < 0.001$.

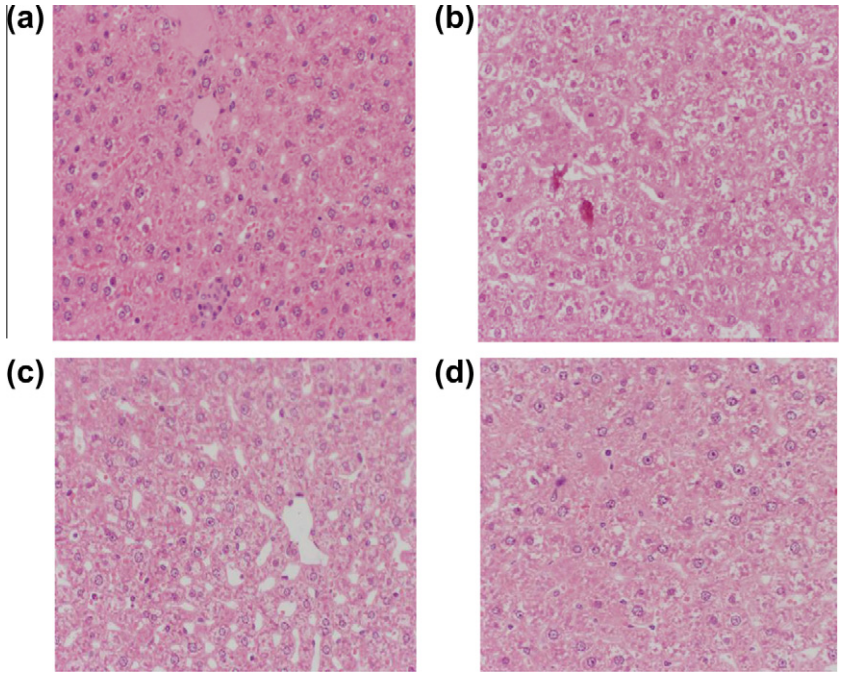

Fig. 4 – H and E stained section of liver from control rats (a). Section of liver showing subintimal oedema, haemorrhage involving small hepatic veins, patchy sinusoidal congestion and focal areas of necrosis in rats exposed to 2.0 Gy of whole body gamma irradiation (b). Section of liver from isoflavone pretreated rats showing normal cellular structure (c). Section of liver from isoflavone pretreated rats exposed with gamma irradiation at a dose of 2.0 Gy showing normal cellular structure (d) under 20×.

4. Discussion

The present study was to evaluate the effects of gamma irradiation on the antioxidant defence system and the radioprotection afforded by isoflavone. Isoflavones are considered as one of the major antioxidant components found in soybean and has been reported to break the chain reaction of lipid by scavenging several ROS and inhibiting chemiluminesence reactions (Marquele et al., 2005). Among the principal properties in foods that may account for the potential health

benefits of flavonoids and phenolics is their antioxidant activity (Chandrasekara & Shahidi, 2011; Shahidi, Alasalvar, & Liyana-Pathirana, 2007). Several in vitro studies have demonstrated that flavonoids can scavenge superoxide (Afanas'ev et al., 1989), hydroxyl (Husain, Cillard, & Cillard, 1987) and peroxyl radicals (Lotito & Fraga, 1998) and inhibit LPO in various systems. Several mechanisms may account for the antioxidant activity of flavonoids, in addition to free radical scavenging viz., chelation of transition metal ions (Morel, Lescoat, Cillard, & Cillard, 1994), inhibition of oxidant enzymes (Ueno, Kohno, Yoshihira, & Hirono, 1984) or by regeneration of α-tocopherol from α-tocopheroxyl radical (Salah et al., 1995). Isoflavone due to its higher reduction potential in presence of bleomycin $FeCl_3$ showed increased DNA oxidation. The results indicate that the isoflavone extract has high SASA, HRSA, BDDD and reducing ability. The results indicate a concentration dependent response of antioxidants properties in isoflavone extract. Exposure to lethal dose of ionising radiation severely increases the oxidative burden on the body and the endogenous antioxidant defence mechanism cannot cope with this increase stress (Lata et al., 2009). It is a well-established fact that ionising radiation at cellular level can induce damage in the biologically important macromolecules such as DNA, proteins, lipids and carbohydrates in the various organs. In some organs, damage is expressed early while in others, it may be expressed over a period of time depending upon the cell kinetics and the radiation tolerance of the tissues (Jagetia & Baliga, 2003).

The induction of symptoms of radiation sickness like reduction in food and water intake, irritability, epilation, weight loss, emaciation, lethargy and ruffling of hairs within 3–5 days by 10.0 Gy of gamma irradiation is in agreement with the earlier studies (Jagetia & Baliga, 2003; Mantena et al., 2008). The death due to irradiation from 8 to 13 days is due to the haemopoietic damage inflicted by irradiation. The survival after exposure to high doses of irradiation, i.e., 10.0 Gy depends on the survival of a critical number of haemopoietic stem cells (HSC) and the ability of these cells to generate an effective level of mature cells of multiple lineages to repopulate the depleted haematopoietic compartment (MacVittie, Monroy, Patchen, & Souza, 1990). The main cause of bone marrow syndrome is the severe depletion of the HSCs, since these being more sensitive to radiation than the committed and mature peripheral blood cells. The 30-day time period after lethal whole body irradiation for survival studies indicates the capacity of the isoflavone to modulate the recovery and regeneration of the gastrointestinal (GI) epithelium and the haemopoietic progenitor cells in the bone marrow, the two most radiosensitive organs that are essential for sustaining the life. The pretreatment of rats with isoflavone resulted in reduction of radiation-induced mortality as compared to irradiated animals. It was reported that the gastrointestinal syndrome occurs between doses 5.0 and 12.0 (primarily >10.0) Gy of irradiation exposure and death occurs within 3–10 days, while bone marrow syndrome occurs between dose 2.5 and 8.0 Gy and death occurs within 1–2 months (Weiss & Kumar, 1998). The 30 days survival of the rats following whole body lethal irradiation can be correlated with haemopoietic recovery and regeneration.

Animal survival in the presence of high dose of ionising radiation suggests the occurrence of physiological adaptive mechanisms, supported by pretreatment with isoflavone, which protect against excessive radiation damage. The percent of survival after isoflavone pretreatment indicates the effectiveness of the antioxidant in arresting gastrointestinal death. This reduction in gastrointestinal death may be due to the protection of intestinal epithelium, which would allow proper absorption of the antioxidants. There are reports that oral administration of isoflavone genistein significantly enhanced protection against radiation-induced lethality (Landauer, Srinivasan, & Seed, 2003). Davis, Mungunsukh, Zins, Day, and Landauer (2008) also reported that isoflavone, i.e., genistein induces radioprotection by HSCs quiescence.

The haemopoietic protective ability of the isoflavone extracts used was further confirmed by the spleen weight and endogenous CFUs on 12th day post irradiation after 6.0 Gy of whole body gamma irradiation exposure. The incidence of colony formation as well as the spleen size was significantly increased in pretreated animals prior to gamma irradiation exposure as compared to control, irradiated and only pretreated group. These results suggest of an immunostimulatory and haemopoietic ability of isoflavone in gamma irradiated rats. The result suggested that the isoflavone was very effective in stimulating regeneration of HSCs as evidenced by the increase in endogenous spleen colonies. The mechanisms by which extracts stimulate the regeneration of haematopoietic cells are not known. One possible mechanism may operate through the isoflavone induced suppression of prostaglandin production as prostaglandins are reported to block proliferation of haematopoietic cell (Gentile, Byer, & Pelus, 1983).

LPO in vivo can affect the structural and functional integrity of cell membranes and it can impair cell function by reacting with various macromolecules including proteins and nucleic acids (Rice-Evans & Burdon, 1993). Gamma irradiation exposure to rats resulted in a marked increase in LPO in liver and erythrocytes, which indicates increased oxidative stress. However, a decrease in LPO was observed more commonly in animals pretreated with isoflavone prior to gamma irradiation. It is suggested that pretreatment with isoflavone prior to gamma irradiation prepares the animals for oxidative assault and the animal is better prepared to sustain oxidative stress. SASA as well as reducing power may be important factors in the inhibition of LPO as already reported (Yadav & Bhatnagar, 2007). Isoflavone extract may scavenge superoxide anion and chelate metal ions to make them unavailable for iron induced LPO.

Antioxidant enzymes, i.e., SOD, CAT and GST activity in liver and erythrocytes showed significant decrease with gamma irradiation exposure, probably due to increased oxidative stress. The superoxide anions are generally dismutated by SOD to form H_2O_2, which is decomposed by CAT (Fridovich, 1978). It is suggested that decrease in liver and erythrocytes antioxidant enzymes activity of gamma irradiated animals may be due to the denaturation of enzymes structure by gamma irradiation exposure. Moreover, decrease in CAT activity in animals exposed with gamma irradiation was probably due to the inactivation of CAT, as a flux

of superoxide anions have been shown to reduce CAT activity (Kono & Fridovich, 1982). The earlier studies in which the animals were exposed to gamma irradiation also showed marked increase in LPO and inhibition of SOD, CAT and GST activity (Devipriya, Sudheer, Srinivasan, Venugopal, & Menon, 2008; Prasad et al., 2006).

Gamma irradiation also resulted in a significant increase in GSH content in erythrocytes. The efflux of GSH from liver is the source of GSH to other organs (Yadav & Bhatnagar, 2007). GST detoxifies a variety of electrophiles generated during oxy-radical detoxication by conjugating them with GSH (Hayes & Pulford, 1995). The result showed that gamma irradiation decreased the GST activity in liver and erythrocytes of rats. The liver GST activity was significantly increased in pretreated animals as compared to gamma irradiated rats, suggesting that pretreatment with isoflavone lowers oxidative stress and facilitates the removal of electrophiles.

The results showed that gamma irradiation exposure resulted in a significant decrease in the number of WBC, RBC, haemoglobin content and platelet count, which may be due to alteration in bone marrow as well as haemopoietic system of the animals. Pretreatment with isoflavone prior to gamma irradiation exposure showed no significant haematological changes as compared to control. The rapidly dividing cells of the blood system, especially leucocytes and erythrocytes are highly prone to gamma irradiation-induced damage as reactive oxygen species (ROS) decreases blood cellular components, including reticulocytes. The decline in reticulocytes following gamma irradiation reflects the early damage of the bone marrow haematopoietic function. The results showed that pretreatment with isoflavone prior to gamma irradiation resulted in the recovery of haematological parameters and attenuated the gamma irradiation induced damages to the blood system. The histology of liver revealed normal liver architecture in animals treated with isoflavone as compared to gamma irradiation. The results indicate that there was recovery from liver damage caused by gamma irradiation in isoflavone pretreated groups. The isoflavone has been shown to counteract gamma irradiation induced oxidative stress due to their antioxidant properties.

5. Conclusions

It is concluded that soy isoflavone scavenge free radicals produced by radiation exposure and thus inhibit radiation-induced cellular damages. It is therefore suggested that the inclusion of soybean in daily diet at a low non-toxic dose may find better prospects as antioxidant and radioprotector.

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