

The Antioxidant Role of Mulberry (*Morus alba L.*) Fruits in Ameliorating the Oxidative Stress Induced in γ -Irradiated Male Rats

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Abstract

Based on the radioprotective effect of substance possessing antioxidant effect previously reported, it was hypothesized that mulberry fruit, a substance possessing antioxidant activity, might be able to protect against oxidative stress induced by γ -rays. Gamma-irradiation (2.5 Gy \times 3 delivered every other day) resulted in a significant decrease in hepatic Glutathione contents (GSH), Xanthine Dehydrogenase (XDH), Superoxides Dismutase (SOD) and Catalase (CAT) activity, the level of insulin and testosterone as well as the concentration of High Density Lipoprotein-Cholesterol (HDL-C). Moreover, a remarkable increase in the Malondialdehyde (MDA) concentration, xanthine oxidase activity, the activity of some liver enzymes, the level of glucose and the concentrations of Total Cholesterol (TC), Triglycerides (TG), low density- and very low density lipoprotein-cholesterol was observed in γ -irradiated rats. In contrast, administration of Mulberry Fruit Powder (MFP) to γ -irradiated rats was found to offer protection against γ -irradiation induced oxidative stress, by elevating the activity of antioxidant enzymes, enhancing liver function, in addition to improving the lipid metabolism. All results in this study suggested that mulberry fruit had high potential to be developed as radio protective agent.

Keywords: Gamma-irradiation; Mulberry fruits; Antioxidants; Insulin; Testosterone

Introduction

All living organisms are exposed to some amount of radiation coming from outer space or emitted from the radioisotopes present in the environment [1]. Radiations are commonly used in a number of medical and industrial situations; however, their prooxidative effects limit their applications [2]. The deleterious effects of ionizing radiation in biological systems are commonly mediated through the generation of Reactive Oxygen Species (ROS) [3], causing oxidative damage in several organs, including testes [4].

The scavenging of free radicals and inhibition of lipid peroxidation has been suggested to be the key target activities for developing successful radioprotection strategies [5,6]. Natural antioxidants play a major role by continuously inactivating ROS, to keep only a small amount necessary to maintain normal cell function [7]. Considerable epidemiological evidence has been gathered to suggest an association between consumption of fruits containing antioxidants, and a reduced risk of certain chronic diseases [8,9].

Mulberry (*Morus alba L.*) belongs to the family *Moraceae*. Mulberry fruit is widely regarded as a nutritious food, and it can be eaten freshly or widely used in the production of wine, fruit juice, jam and canned food [10,11]. Mulberry fruit is not only used as fruit, but also it has been used effectively in natural medicine for the treatment of sore throat, fever, hypertension and anemia [12,13]. Moreover, mulberry fruit is used to protect against liver and kidney damage, strengthen the joints, improves eyesight, and have anti-aging effects [11,14]. Anthocyanins and water extracts from mulberry fruit can scavenge free radicals, inhibit Low-Density Lipoprotein (LDL) oxidation, and have beneficial effects on blood lipid and atherosclerosis [15,16].

Therefore, the present study was undertaken to investigate the possible ameliorative effects of mulberry fruits on oxidative damage, resulting from exposure of normal male rats to γ -radiation.

Materials and Methods

Materials and plant preparation

Standard commercial rodent diet and fresh purple-colored

mulberry fruits were purchased from the local market (Cairo, Egypt). All berries were dried at 70°C for 4 days and grounded to powder [17].

Radiation facility

Whole body gamma irradiation of rats was performed using a Canadian gamma cell-40, (¹³⁷Cs) housed at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. The dose rate was 0.43 Gy/min at the time of the experiment. Rats were exposed to fractionated dose of 7.5 Gy γ -irradiation administered as 2.5 Gy, every other day.

Determination of total phenolic compounds

The concentration of total phenolic compound was measured by a modified Folin-Ciocalteu colorimetric method [18]. Briefly, a sample diluted was added to a test tube containing 1.58 mL of distilled water. Folin-Ciocalteu reagent of 100 μ L was added, and the tube was stirred and allowed to stand at room temperature for 8 min. 300 μ L of Na₂CO₃ (7%, w/v) was added to the mixture and the absorbance was measured at 765 nm, after 120 min at room temperature, using a spectrophotometer. The results were expressed as milligram of Gallic Acid Equivalents (GAE) per gram fresh matter of fruit (mg GAE/ g fruit).

Determination of antioxidant activity by the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

DPPH assay was determined by previously method described

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[19]. Each sample (0.5 mL) was added to 0.5 mL of 0.4 mm DPPH (2,2-Diphenyl-1-Picrylhydrazyl) in methanol. The mixture was shaken vigorously and allowed to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. Percent inhibition of DPPH radical was calculated for each dilution of berry extract, according to formula: %inhibition=[(ADPPH-Aplant)/ADPPH]×100, where ADPPH is the absorbance value of the DPPH versus blank solution, and Aplant is absorbance value of the sample solution. A lower level of absorbance indicated a stronger radical scavenging activity.

Experimental animals

Adult male albino rats reared in NCRRT animal house were used in the present experiments. Matched weight animals (150 ± 10 g) were selected and housed in plastic cages, under controlled condition, and fed on standard commercial rodent diet.

Experimental design

Male albino rats were exposed. 24 animals were randomly divided into four groups as follows: Group (C): (control group) rats fed on balanced diet for 6 weeks, Group (MFP): rats fed on balanced diet contained 1% Mulberry Fruits Powder (MFP), Group (Irr.): (irradiated group) rats were exposed at the 1st week of the experiment to fractionated γ -irradiation dose of 7.5 Gy administered as 2.5 Gy, every other day and fed on balanced diet for 6 weeks, and Group (Irr.+MFP): irradiated rats fed on balanced diet contained 1% mulberry fruits powder.

At the end of the experiment, animals from each group were sacrificed, 24 hrs post the last dose of treatment. Blood samples were collected through heart puncture, after light anesthesia and allowed to coagulate, and centrifuged to obtain serum for biochemical analysis. Also, liver tissue was removed for biochemical investigation.

Biochemical analysis

The lipid peroxidation was determined colorimetrically as Malondialdehyde (MDA) [20]. Hepatic Xanthine Oxidase (XO) and Xanthine Dehydrogenase (XDH) (Table 1) were determined according to Kamiaski and Jewezska [21]. Whereas, the value of hepatic Glutathione content (GSH) and the activity of Superoxides Dismutase (SOD) and Catalase (CAT) were measured by the method of Gross et al. [22], Minami and Yoshikawa [23] and Allain et al. [24], respectively. In addition, Total Cholesterol (TC), Triglycerides (TG) and High-Density Lipoprotein-Cholesterol (HDL-C) were determined, according to procedure described by Allain et al. [24], Fossati and Prencipe [25] and Demacker et al. [26], respectively, while low-density lipoprotein cholesterol and very Low-density lipoprotein-cholesterol were evaluated according to Friedwald et al. [27] and Norbert [28] formulas, respectively, by the following equations: LDL-C (mg/dl)= TC-(TG/5+HDL-C), vLDL (mg/dl)=TG/5. The activity of serum Aspartate Transaminase (AST) and Alanine Transaminase (ALT) was estimated according to Reitman and Frankel [29], serum Gamma Glutamyl Transferase (GGT) was assessed according to Rosalk [30], as well as serum Alkaline Phosphatase Activity (ALP) was assessed, according to Kind and King [31]. Total bilirubin was analyzed using the method reported by Malloy and Evelyn [32]. Serum glucose was evaluated by the method of Trinder [33]. Finally, the serum testosterone concentration was measured by the Enzyme Linked Immunosorbent Assay (ELISA), according to the method of Engrall and Pelmann [34], and also insulin hormone level was determined by radioimmunoassay kit supplied by Diasari, Italy.

Parameters	C	MFP	Irr.	Irr.+MFP
MDA (n mol/ml)	193.27 ± 3.24 ^a	181.76 ± 2.83 ^a	388.51 ± 4.72 ^c	242.53 ± 4.61 ^b
XO (mU/mg protein)	2.44 ± 0.07 ^a	2.30 ± 0.06 ^a	3.72 ± 0.07 ^c	2.56 ± 0.05 ^b
XDH (mU/mg protein)	3.15 ± 0.16 ^a	3.19 ± 0.14 ^a	1.56 ± 0.11 ^c	2.83 ± 0.13 ^b
GSH (mg/g tissue)	27.31 ± 0.92 ^a	27.73 ± 0.86 ^a	15.68 ± 0.64 ^b	25.86 ± 0.75 ^a
SOD (U/mg protein)	46.08 ± 1.06 ^a	47.10 ± 0.88 ^a	30.11 ± 0.81 ^c	41.63 ± 0.74 ^b
CAT (U/g protein)	3.21 ± 0.02 ^a	3.34 ± 0.02 ^a	1.79 ± 0.02 ^c	2.83 ± 0.03 ^b

Table 1: Effect of MFP supplementation on MDA, Xanthine Oxidoreductase system (XO and XDH), GSH, SOD and CAT of γ -irradiated rats.

Parameters	Fresh matter of mulberry fruits
Total phenolics (mg GAE/g)	519.35
Total antioxidant activity (μ g/mL)	232.25

Table 2: The phenolic compounds and total antioxidant activity contents of MF.

Statistical analysis

Statistical analyses were performed using computer program, Statistical Packages for Social Science (SPSS) [35], and values were compared to each other, using one-way analysis of variance (ANOVA).

Results

The amount of total phenolic compounds and the total antioxidant activity of Mulberry Fruit (MF) was shown in table 2; the results obtained were that the total phenolic contents was 519.35 mg GAE/g, while the total antioxidant activity of MF was 232.25 μ g/mL fresh matter of fruit.

The data presented in table 1 revealed a significant decrease in the value of hepatic GSH contents and the activity of XDH, SOD, and CAT activity, associated with a significant increase in MDA level and XO activity of rats exposed to γ -radiation, as compared to the corresponding values of control and all treated groups; while rats receiving MFP after γ -irradiation exposure had a lower concentration of MDA and XO activity, and higher level of GSH, as well as SOD and CAT activity, than the γ -radiated group.

As a result of γ -irradiation exposure, levels of TC, TG, LDL-C and vLDL-C were highly increased, with a significant decrease in HDL-C concentration, as compared with control and all treated groups, while treatment with MFP after γ -irradiation exposure, minimizing the hyperlipidemic effects of γ -irradiation by reducing the concentration of TC, TG, LDL-C and vLDL-C, and elevating the level of HDL-C, as compared with irradiated group only.

Also, the results presented in table 3 revealed a significant elevation in the concentration of total bilirubin and the activity of AST, ALT, ALP and GGT in γ -irradiated group, compared to control; whereas, the level of total bilirubin in addition to the activity of liver enzymes, were decreased in the group of γ -irradiated rats supplemented with MFP.

Finally, the data summarized in table 4 indicated that exposure of rats to γ -irradiation resulted in an obvious rising in the glucose concentration, associated with reduction in the level of insulin and testosterone. In contrast, it was noticed that MFP administration after γ -irradiation exposure reduced the level of glucose, as well as enhanced the level of insulin and testosterone.

Parameters	C	MFP	Irr.	Irr.+MFP
AST (U/ml)	30.15 ± 0.45 ^a	29.71 ± 0.48 ^a	53.27 ± 0.73 ^c	36.57 ± 0.83 ^b
ALT (U/ml)	23.54 ± 0.71 ^a	22.92 ± 0.59 ^a	41.32 ± 0.62 ^c	28.41 ± 0.83 ^b
ALP(U/100ml)	8.92 ± 0.31 ^a	8.83 ± 0.45 ^a	15.87 ± 0.61 ^c	11.06 ± 0.52 ^b
γ GT (U/ml)	3.96 ± 0.29 ^a	3.88 ± 0.36 ^a	6.48 ± 0.47 ^c	4.91 ± 0.52 ^b
Bilirubin(mg/dl)	0.58 ± 0.02 ^a	0.57 ± 0.02 ^a	1.12 ± 0.03 ^c	0.70 ± 0.02 ^b

Table 3: Effect of MFP supplementation on the level of total bilirubin and the activity of some liver enzymes of γ -irradiated rats.

Parameters	C	MFP	Irr.	Irr.+MFP
Glucose (mg/dl)	111.32 ± 3.25 ^a	106.81 ± 3.48 ^a	149.27 ± 5.73 ^b	119 ± 0.83 ^{a,b}
Insulin (μ U/ml)	34.85.54 ± 2.71 ^a	35.27 ± 2.59 ^a	20.32 ± 2.62 ^c	28.95 ± 2.83 ^b
Testosterone (ng/dl)	202.53 ± 3.12 ^a	219.57 ± 3.12 ^a	141.63 ± 2.75 ^c	191.27 ± 3.03 ^b

Table 4: Effect of MFP supplementation on glucose, insulin and testosterone of γ -irradiated rats.

Parameters	C	MFP	Irr.	Irr.+MFP
TC (mg/dl)	191.17 ± 3.11 ^a	184.31 ± 2.60 ^a	260.12 ± 3.01 ^c	214.26 ± 2.57 ^b
TG (mg/dl)	114.14 ± 2.14 ^a	112.54 ± 1.97 ^a	184.71 ± 2.37 ^c	134.82 ± 3.11 ^b
HDL-C (mg/dl)	51.12 ± 0.65 ^a	52.97 ± 0.91 ^a	33.36 ± 0.78 ^c	45.47 ± 0.81 ^b
LDL-C (mg/dl)	117.22 ± 1.25 ^a	108.83 ± 2.11 ^a	189.82 ± 2.05	141.83 ± 2.17 ^b
vLDL-C (mg/dl)	22.83 ± 0.11 ^a	22.51 ± 0.09 ^a	36.94 ± 0.15 ^c	26.96 ± 0.12 ^b

Table 5: Effect of MFP supplementation on lipid profile of γ -irradiated rats.

Discussion

It is well documented that dietary antioxidants play an important role in mitigating the damaging effects of oxidative stress on cells. Yang et al. [36] indicated that mulberry fruit is a natural health food with antioxidant effects, and these beneficial effects may be because of phytochemical constituents, which might include fiber, fatty acids, phenolics, flavonoids, anthocyanins, vitamins and trace elements.

Many reports have revealed that the physiological function of natural foods can be attributed to the antioxidative capacity of their phenolic components. The results in table 2 demonstrated that the Total Phenolic Content (TPC) of mulberry fruit was 519.35 mg GAE/g, whereas the total antioxidant activity was 232.25 μ g/mL, and these results were in agreement with Kaewkaen et al. [17].

According to the data obtained, it appears that the detrimental damage of radiation is associated with the alteration of XOR system, and conversion of XDH into XO activity. The significant increase in XO activity might be attributed to radiation-induced hypoxia, where insufficient oxygen availability elevates calcium concentration, which activates a protease capable of converting the dehydrogenase to oxidase form [37,38]. Also, the level of MDA in the present study was elevated in the serum of untreated irradiated animals. The observed increased Thiobarbituric Acid Reactive Substances (TBARS) level in irradiated rats could be attributed to the peroxidation of membranes lipid, resulted in cellular structure modifications and oxygen radicals-mediated tissue damage [39,40].

In the present study, the activities of Superoxide Dismutase (SOD) and Catalase (CAT) were significantly decreased in irradiated rats. The existence of a mutually supportive relationship between enzymatic antioxidants; SOD and CAT against accumulation of ROS inactivates the superoxide anion and peroxide radicals, by converting them into water and oxygen. In this study, the observed decrease in SOD activity suggests inactivation of the enzyme, possibly due to increased superoxide radical production or an inhibition by the H_2O_2 ,

as a result of corresponding decrease in the activity of CAT, which selectively degrades H_2O_2 . In previous studies, activities of SOD, CAT and GPx have been reported to decrease in the liver of irradiated rats [41,42]. The significant decrease in GSH levels observed in untreated irradiated animals may lead to decreased protection against oxidants. This decrease could be due to an enhanced utilization in large amount, to combat the radiation-induced free radical damage, as glutathione is a major non-enzymatic antioxidant [43]. Similar decrease in hepatic GSH [40], and testicular GSH has been reported, following gamma irradiation in rats [44].

In this study, irradiated rats treated with Mulberry Fruits Powder (MFP) showed a significant decrease in the level of MDA content and XO activity, with concomitant significant increase in the activity of XDH, SOD and CAT, and in the content of GSH. Thus, MFP has potential as an anti-peroxidative agent, and as an antioxidant. Yang et al. [36] reported that MFP contained vitamin C and low levels of vitamin E, in addition to anthocyanins and flavonoids; all of which are powerful natural antioxidants that increase SOD and GSH-Px activities, and decrease TBARS concentration and improve lipid profiles in rats [45,46]. In addition, mulberry fruit contains many trace elements including Cu, Mn, Zn and Fe, which are necessary components of SOD [37]. Among these, Cu, Mn and Fe are prosthetic groups of SOD and play a decisive role in its enzyme activity. Zn stabilizes the structure of SOD [47]. Se is an important element of GSH-Px, which regulates lipid metabolism, prevents fatty liver formation, and improves antioxidant ability in rats [48].

The present results revealed that the levels of TC, TG, LDL and vLDL-C in serum were significantly higher in irradiated rats, than those of the control group. On the other hand, radiation exposure resulted in a significant decrease in HDL-C level in serum of the irradiated rats. Significant increase in the levels of serum lipid profile and LDL are demonstrated post radiation exposure of rats (Table 5), possibly as a result of liver injury. This indicates that ionizing-radiation-induced oxidative stress, which might alter hepatic lipid metabolism and serum lipoproteins. It seems that there is an association between radiation-induced oxidative stress and elevated levels of lipid fractions and LDL [49]. This association is similarly observed in other conditions, characterized by increased oxidative stress [50,51]. Therefore, it is suggested that oxidative stress might be an important determinant of altered lipid metabolism, due to radiation exposure [52].

Administration of MFP to irradiated rats resulted in significant declines in serum lipid profile, LDL-C and vLDL-C, associated with remarkable elevation in HDL-C, as compared to γ -irradiated group. The physiological effects of mulberry as antioxidant take place via its content like flavonoid, therefore suggesting their role in prevention of coronary heart disease [53], including atherosclerosis. Wan et al. [53] and Jenkins et al. [54] reported that flavonoids may decrease the risk of cardiovascular disease by lowering LDL: HDL ratio and reducing oxidized LDL in human, and make LDL less susceptible to oxidative stress. Flavonoids may work by making liver cells more efficient, to remove LDL-C from blood by increasing the LDL receptor densities in liver, and by binding to apolipoprotein B [55,56]. Also, the increase in HDL-C concentration could protect the LDL against oxidation in vivo because lipids in HDL are preferentially oxidized, before those in LDL [57].

The activities of ALT, AST, ALP and GGT, as well as the level of total bilirubin in serum, showed a significant rise, following γ -irradiation exposure. The increase in aminotransferase activities by radiation may be due to the damage of cellular membranes of hepatocytes, which in

turn, leads to an increase in the permeability of cell membranes, and facilitates the passage of cytoplasmic enzymes outside the cells, leading to the increase in the aminotransferase activities in liver and blood serum [58,59]. Also, it is proposed that oxidative stress is linked to the organ damage, following exposure to ionizing radiation [52,60].

However, the activity of liver enzymes was decreased, as a result of MFP administration to γ -irradiated rats. Several studies revealed that mulberry fruit, leaves, bark and branches have been used in Chinese medicine to treat fever, facilitate discharge of liver, protect the liver damage and lower blood pressure [61,62]. Hsu et al. [63] investigated the protective mechanisms of Mulberry Water Extracts (MWEs) in carbon tetrachloride (CCl₄)-induced hepatic injury, and observed that the levels of serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP) were reduced via cotreatment with MWEs, compared with CCl₄ treatment alone. Also, the authors concluded that MWEs exhibit protective and curative effects against CCl₄-induced liver damage and fibrosis, via decreased lipid peroxidation and inhibited proinflammatory gene expression.

In this study, rats exposed to gamma radiation had a significant elevation in serum glucose level and noticeable reduction in insulin concentration, compared to the control group. Ellefson and Caraway [64] stated that hyperglycemia may be caused by metabolic disorder, as a result of endocrine dysfunction and increased level of glucose. The recorded hyperglycemia in the present results could be attributed to endocrine abnormalities induced by irradiation, that promote the secretion of peptide which has relation to carbohydrate metabolism, by increasing glyconeogenesis in liver [65,66]. Lee et al. [67] and Hamza and Osman [68] attributed the lowering effect of γ -irradiation exposure on insulin level to the production of free radicals that induced oxidative stress, resulted in reduction in insulin secretion and DNA damage.

Results of γ -irradiated rats receiving MFP revealed an obvious reduction in glucose level and elevation in insulin concentration, in comparison to γ -irradiated group. The antidiabetic activities of mulberry leaf extract in experimental animals have also been reported by many researchers [69,70], with the studies supporting the usage of mulberry reduced blood glucose in rats with diabetes, induced by streptozotocin or alloxan [71-73]. In addition, mulberry has long been used in Chinese medicine for the prevention and treatment of diabetes, because as we now know, they contain chemical compounds that suppress high blood sugar levels (hyperglycemia), following a carbohydrate-rich meal. Mudra et al. [74] concluded that the co-ingestion of mulberry extract with 75 g sucrose significantly reduced the increase in the blood glucose level. Moreover, Liu et al. [75] observed a significant decline in blood glucose, accompanied with evident increase in plasma insulin level in diabetic mice treated with the hybrid of 1-deoxyynojirimycin and polysaccharide (HDP) from mulberry. The authors reported that HDP could protect pancreatic β -cells from damage induced by alloxan, due to the ability to scavenge the free radical and repair the destroyed pancreatic β -cells, and restored the serum insulin in HDP treated mice to normal.

In this study, a significant reduction in the concentration of testosterone was observed, as a result of γ -irradiation exposure. In contrast, the value of this hormone was obviously increased, post treatment of γ -irradiated rats with MFP. Liu et al. [75] and Michael and Amer [76] found that the level of testosterone was decreased after whole-body irradiation dose of 4 and 5 Gy due to alterations in DNA-single strand break, cell apoptosis and oxidative stress. Popoff and Kapich [77] observed a positive correlation between a decline in

testosterone affinity and exposure to gamma irradiation. Also, Oi-Kano et al. [78] reported that γ -irradiation exposure (6 Gy) resulted in a significant decline in testosterone concentration due to generation of free radicals.

However, the effect of MFP on testosterone level might be attributed to its phenolic contents that have antioxidant capacity, and prevent oxidative damage induced by γ -irradiation. Jeong et al. [79] proposed the mechanism of phenolic compounds, whose supplementation enhances lipid and protein metabolism, owing to hormonal regulation by the stimulation of noradrenalin secretion, thereby affecting the levels of steroid hormones, including testosterone and corticosterone, and other hormones in rats. Also, the effect of MFP could be linked to the abundance of flavonoids (which is an effective aromatase inhibitor) [79]. The cytochrome P-450 aromatase is required for the conversion of androgens to estrogens, and hence, aromatase inhibitors would decrease the concentration of estrogens and maintain a higher level of testosterone.

Conclusion

In conclusion, the present study revealed that the mulberry fruit is the potential functional food that can protect against oxidative damage induced by gamma-irradiation in male rats, through its positive effects on the activity of some antioxidant enzymes, liver enzymes, elevation of the level of insulin and testosterone, inhibition of lipid peroxidation, as well as improvement of lipid profile. Moreover, the ameliorating effects of MF attributed to its phenolic and flavonoid contents that possess antioxidant activity.

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