RESEARCH ARTICLE

Gossypetin ameliorates radiation-induced oxidative stress in the testis and lymphocytes

Amitava Khan[1,2], Krishnendu Manna[1,3], Dipesh Kumar Das[1,4], Chinchu Bose[5], Asoke Banerji[5], Sanjit Dey[1*]

ABSTRACT

Background: To explore the radioprotective action of gossypetin (GTIN) against gamma (γ) radiation-induced oxidative stress of the radiosensitive organ, testis, and lymphocytes. *Materials and Methods*: GTIN, a cost-effective and readily available phytochemical, was administered at 30 mg/kg body weight for three consecutive days before a 5 Gy γ radiation exposure. The radical-scavenging ability of the GTIN was considered in whole-body irradiated mouse models. The study included various physiological and cellular assessments, including biochemical analysis, the comet assay, the DNA fragmentation assay, and histopathology. *Results*: γ radiation-induced oxidative stress, evident through increased lipid peroxidation and reduced antioxidant markers in both the testis and lymphocytes. Histopathological analysis revealed radiation-induced structural changes in the testes, and the comet and DNA fragmentation assay confirmed a decrease in white blood cell (WBC) count due to radiation-induced DNA damage. However, pre-treatment with GTIN ameliorated these radiation-induced alterations in both lymphocytes and the testis. *Conclusion*: GTIN's radioprotective mechanism is attributed to the ability of the C ring within gossypetin, to quench the lone pair of electrons in free radicals generated by radiolysis. These chemical properties of this potent phytocompound safeguarded the cells from radiation-induced damage.

Keywords: Gamma Radiation, Gossypetin, Phytochemical, Testis, Lymphocytes.

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INTRODUCTION

Ionizing radiation exposure in humans may arise from various sources. Therefore, humans in particular have to be prepared for its exposure. The exposure could be from natural background, accidental release of radioisotopes, during planned radiotherapy, or nuclear accidents (e.g., Chernobyl), and air and space travel. The Fukushima nuclear power plant disaster, in particular, has prompted new questions regarding nuclear power's safety and utility. Radiation exposure has significant biological consequences, ranging from skin burns, hair loss, and birth defects to illness, cancer, and death, with continuous perturbation of physiological parameters [1,2]. The hazardous effects of radiation exposure on humans manifest primarily through two modes: direct interactions with target molecules or indirect damage mediated by free radical formation. Gamma radiation, for example, is capable of damaging the three functional components of the cell membrane - the lipid bilayer, a vast array of protein molecules, and the cytoskeleton, which alters its structure and impairs the protective barrier functions [3,4]. Critically, this activity leads to the oxidation of proteins and lipids and is also a major cause of DNA damage, operating either directly via strand breakage or indirectly through the generation of reactive oxygen species (ROS) [5,6].

Thus, the importance of developing radioprotectors, given their potential application during both planned (*e.g.*, radiotherapy) and unplanned radiation exposure (*e.g.*, in the nuclear industry, accidents, natural background radiation), is a valuable goal in the modern era. Several synthetic compounds like lipoic acid, deoxyspergualin, cysteine, cysteamine, 2-mercaptopropionyglycine, WR-2721, or amifostine (S-2-(3-amino-propilamino) ethylphosphorothioic acid) were tested and established as excellent radioprotectors [7, 8]. However, their applications are limited because of their adverse side effects. Therefore, the development of safe synthetic radioprotectors remains unanswered. The potential of natural radioprotectors has attracted considerable attention as possible alternatives to chemical radioprotectors [9]. Naturally occurring flavonoids with well-recognized pharmacological properties, including antioxidant activities,

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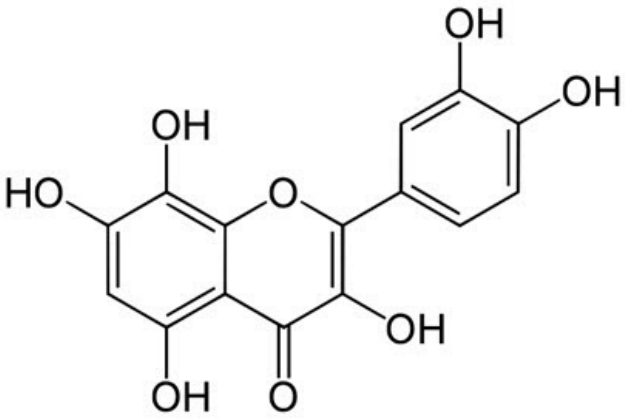

Figure 1: Structure of Gossypetin

are being explored as natural radioprotectors. The flavonoids are potent free radical scavengers, enhance the endogenous antioxidant status, and provide protection against whole-body irradiation [10,11].

We selected lymphocytes because they are blood-forming elements, and the testis was preferred because it is a radiosensitive organ. Thus, the effects of radiation should be visible to cells immediately after any stress in these body parts.

The current work aims to evaluate and establish a novel compound regarded as safe for the organism yet effective against radiation-induced stress. Gossypetin (GTIN) is 3, 5, 7, 8, 3', 4' hexahydroxy flavones. Hibiscus species provide a very economical source of GTIN and related compounds. It was isolated from *Hibiscus vitifolius* and *H furcatus* [12]. The extracts of these plants are traditionally used for treating diabetes and inflammation [13]. GTIN has two pairs of ortho hydroxyls (positions 7, 8, and 3', 4') and a pair of para hydroxyls at positions 5 and 8 (Figure 1). Therefore, it is oxidizable due to the possible formation of two o-quinone and one p-quinone intermediates, making it a strong free radical scavenger [14]. Previous reports have shown that GTIN exhibits strong bactericidal activity, which can protect alveolar bone and limit inflammation in ligature-induced periodontitis in mice [15-17]. We previously examined strong antioxidant activity *in-vitro* and good antioxidant protection against radiation-induced DNA damage in *ex-vivo* settings. It can also ameliorate the radiation-induced oxidative stress in the liver. Therefore, our choice of GTIN was based on its demonstrated safety and compatibility with normal mammalian organs, tissues, and cells, as reported in our previous research [7,18]. In the present article, we examined whether the radioprotective principle of the phytochemical GTIN confers protection against biological perturbations in the testis and lymphocytes following radiation exposure. This article provides significant insights into how the GTIN mitigates radiation effects in rapidly dividing systems, preventing significant physiological and DNA damage. The effects will be positive, as these cost-effective approaches will advance future space research and address extreme physiological conditions.

MATERIALS AND METHODS

Chemicals

High melting point agarose (HMA), Low melting point agarose (LMA), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 2,4,6-tripyridyl-s-triazine (TPTZ), Ethylenediaminetetraacetic acid (EDTA), tris-base, and ethanol were purchased from Merck. All other chemicals used were of the highest analytical quality. The GTIN was obtained by extraction from the dried flowers of *Hibiscus vitifolius,* as described previously [6].

Animals

Swiss albino male mice (*Mus musculus*), 6–7 weeks old (weighing 25 ± 2 g), were randomly chosen. They were kept under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%), and a 12-hour light-dark cycle. Animals were given standard mouse feed and water ad libitum. All animal experiments were under the project, which was certified by CCSEA (then CPCSEA). The care and use of the animals reported in this study were approved by the Institutional Animal Ethics Committee (IAEC/proposal/SD-4/2011 dated 04.04.2011) of the University of Calcutta, Kolkata, India.

Irradiation

Animals were preserved in well-ventilated perplex boxes and exposed to γ radiation (5 Gy) throughout their bodies at a dose rate of 1 Gy/min and a source-to-surface distance of 77.5 cm.

They were irradiated with a ^{60}Co source of γ radiation.

Experimental Design

Swiss albino male mice were chosen from a congenital colony and divided into four groups of eight animals each. The optimum dose of GTIN (30mg/kg body weight) was selected based on survival, as previously reported [18]. After the survival study, the dose (30 mg/kg body weight) was selected by administering different GTIN doses and determining the optimal protective dose.

These groups were – (i) Control group: The control groups were given distilled water through oral gavages once a day for three consecutive days, (ii) IR group: Mice were given distilled water for three consecutive days before exposing them to a single dose of 5-Gy ^{60}Co γ -irradiation, (iii) GTIN group: Mice were administered with GTIN (30 mg/kg body weight) orally for three consecutive days, and (iv) GTIN+IR group: Mice were administered with GTIN (30 mg/kg body weight) orally for three consecutive days. One hour after administering the last dose, the animals were exposed to a single 5-Gy γ-irradiation dose. All the animals were necropsied by cervical dislocation at 6 hours post-irradiation. The testis and blood cells were collected for further experiments.

Measurement of alkaline phosphatase (ALP) and acid phosphatase (ACP) activities from the testis

Testis ALP and testis ACP levels were measured using spectrophotometric assay kits of Randox Laboratories Ltd (Antrim, United Kingdom) according to the manufacturer's instructions [19].

Hematological Study

Blood was collected from the retro-orbital plexus in a vial containing 0.5 M EDTA. RBC and WBC counts were conducted using standard laboratory procedures with hemocytometers. For hemoglobin estimation, 4 µL of blood was added to 1-mL of Drabkin's solution and incubated for 3 minutes. The absorbance was taken at 550 nm. 15 gm/dL of hemoglobin is considered the standard.

Isolation of Lymphocytes from Mice Blood

Lymphocytes were isolated using the procedure described earlier [20,21], with slight modifications. Briefly, 3 mL of whole blood was diluted 1:1 with RPMI 1640 and overlaid onto the lymphocyte separation medium in a centrifugation tube. After centrifugation for 15 min at 400 g, the lymphocyte layer at the interface between blood plasma and the medium was carefully transferred using a Pasteur pipette into a tube containing 5 mL of RPMI 1640 culture medium. The lymphocytes were washed twice with RPMI 1640 and centrifuged at 250 g for 10 min. The cell pellet was resuspended in 6 mL of RPMI, and 1 mL of the suspension was used for the comet assay, DNA fragmentation assay, and antioxidant parameters.

Alkaline single-cell gel electrophoresis (comet assay)

Radiation-induced DNA double-strand breaks in lymphocytes were estimated using single-cell gel electrophoresis (comet assay) as previously described [22,23]. In brief, frosted slides were incubated in 1% NMA in PBS until solidification; a second layer of LMA containing approximately 2×10^5 cells was then added at 37°C, and coverslips were immediately placed. After solidification of the LMA, the cover slips were removed, and chilled lysing solution containing 2.5 M sodium chloride (NaCl), 100 mM disodium EDTA (Na2-EDTA), 10 mM Tris–HCl at pH 10, and 1% DMSO, 1% Triton X-100 was applied overnight at 4°C. After removal of the slides from the lysing solution, they were placed in a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na_2-EDTA, and 0.2% DMSO, pH ≥ 13.0). The slides were equilibrated in the same buffer for 20 minutes, and electrophoresis was carried out at 25V, 180 mA for 20 minutes. After electrophoresis, the slides were washed with 0.4M Tris–HCl buffer, pH 7.4, to remove the alkali. Subsequently, these were stained with 50 µL of EtBr (20 µg/mL) and observed under a microscope equipped with bright-field phase-contrast and epi-fluorescence capabilities (Leica DC 300 FX, Wetzler, Germany) at 400X magnification. The DNA strand breaks were quantified using the Comet Score software, by which % DNA in tail, tail length, tail moment, and Olive tail moment were obtained directly.

DNA Fragmentation Assay

The DNA Fragmentation assay was performed as described in a previous report[24]. About 2×10^6 of isolated lymphocytes were transferred to a microcentrifuge tube. The cells were lysed with 0.5 mL ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100). Fragmented DNA was divided from intact chromatin by centrifugation for 10 min at 13000 g, 4°C (preparation B). The supernatant was carefully moved to a tube (preparation A). 0.5 mL of lysing buffer was added to the pellet-containing preparation B. 0.5 mL of 25% TCA was added to the A and B preparations, and the preparations were vortexed vigorously. The tubes were placed at 4°C, and the precipitate was left overnight. The precipitates were centrifuged for 10 min at 13000 g. The supernatants were aspirated and discarded. After complete removal of the supernatant, 80 µl of 5% TCA was added to each pellet, and the DNA was hydrolyzed by heating at 83°C for 20 min in a water bath. 160 µL of DPA solution was added to the test tubes, and a blank containing 80 µL 5% TCA. All tubes were vortexed and then left overnight at room temperature. To measure optical density (OD), the collected supernatants were transferred to 96-well plates, and OD was measured at 620 nm using an ELISA reader.

The percentage of fragmented DNA was calculated according to the following formula:

% fragmented DNA= {OD 620 nm tube A/(OD 620 nm tube A+ OD tube 620 nm tube B)}× 100

Biochemical Estimation

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) in lymphocyte and testis tissue homogenates were estimated according to the previously described protocol [25,26]. TBARS in the testis and lymphocytes were assessed by using the standard protocol. Briefly, the homogenate was incubated with 15% TCA, 0.375% TBA, and 5N HCl at 95°C for 15 min; the mixture was cooled, and the absorbance of the supernatant was measured at 535 nm against a suitable blank. The amount of lipid peroxidation was determined using $\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}.

Superoxide dismutase activity

The superoxide dismutase activity of lymphocytes and testis tissue was determined using the modified protocol [27]. 3 mL of Tris buffer (50 mM) was mixed with 50 µL of the sample. The mixture was incubated at 25°C for 10 minutes. 100 µLof pyrogallol was added, and the absorbance was measured at 420 nm using a spectrophotometer.

Catalase activity

Catalase activity in lymphocytes and testis tissue was evaluated by measuring the decrease in absorbance resulting from catalase-catalyzed decomposition of H_2O_2 [28]. The

standard reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 30 mM hydrogen peroxide, and 3 µL of tissue homogenate, for a total volume of 1.0 mL. The reaction was run at 20°C, and only the initial linear catalytic phase for one minute was used to determine catalase activity. Enzyme activity was determined by measuring the rate of H2O2 decomposition (1 µmol/min) and expressed as 1 U, using εH_2O_2 at 240 nm (43.6 M^{-1} and cm^{-1}).

Reduced glutathione

Reduced glutathione levels in lymphocytes and testis tissue were determined using the method described earlier [29,30]. Lymphocytes and testis tissue homogenate were treated with 0.1 mL of 25% TCA, and the resulting precipitate was pelleted by centrifugation at 3900 g for 10 min. Free endogenous sulfhydryl was assayed in a total volume of 3 mL by adding 2 mL of 0.5 mM 5,5′-DTNB prepared in 0.2 M phosphate buffer (pH 8) to 1 mL of the supernatant. The GSH reacts with DTNB, forming a yellow complex. The absorbance was read at 412 nm.

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) levels of lymphocytes and testis tissue homogenate were evaluated using the previously described method [31]. 1 mL FRAP reagent and 10 µL sample (Tissue homogenate) were mixed and kept in the water bath at 37°C for 4 minutes. The optical density was measured at 593 nm. Concentration was calculated against a $FeSO_4$ standard curve. The FRAP unit is equal to 100 µmol/dm^3 Fe^{2+} [32].

Histological Analysis of Testis Tissue

A small portion of the testis was cleaned and fixed in 10% buffered formaldehyde for histological analysis. Testes were then processed and embedded in paraffin wax. Paraffin blocks were prepared, and sections of 5 µm thickness were processed and stained with hematoxylin (H) and eosin (E) for histopathological evaluation [33]. The stained slides for each group were examined using a light microscope (Olympus 207444, Tokyo, Japan) at 200× and 400× magnification. The photomicrographs were taken using a Canon PowerShot S70 digital camera.

The gray value was calculated for the selected area using ImageJ version 1.46. The photomicrographic images were further analyzed quantitatively. The reduction of the gray value indicates the loss of sperm cells in the central lumen of seminiferous tubules, and the enhancement of the gray value indicates the intactness of the seminiferous tubule lumen, which was chiefly occupied by sperm cells. 3D analysis of histological slides from the selected area was performed using ImageJ version 1.46.

Statistical Analysis

The values were given as mean ± standard deviation (SD). Analysis of variance (ANOVA) with Tukey's post hoc test was performed to evaluate the data and determine the significance of differences among groups. $p<0.05$ was considered the level of significance.

RESULTS

Gossypetin ameliorated gamma radiation-induced RBC, WBC count, and Hemoglobin concentration

The total WBC count in the control group decreased significantly ($p < 0.05$) after 6 hrs of radiation exposure in the irradiated group. In the GTIN+IR group, the fall in WBC count was significantly ameliorated compared to the IR group alone. A similar trend was observed in RBC count. After irradiation, the RBC count decreased significantly compared to the control. Treatment with GTIN before irradiation significantly increased cell counts compared with the IR group ($p < 0.05$). The hemoglobin concentration in the irradiated group was significantly lower than that in the control group. However, pre-treatment with GTIN in irradiated animals significantly increased hemoglobin concentration (Table 1).

Study of radiation-mediated DNA damage in lymphocytes by alkaline comet assay

The alkaline comet assay showed that γ radiation induced DNA damage. The comet parameters substantiated DNA damage. γ-radiation exposure (5 Gy) significantly increased all comet parameters, i.e., tail length, % DNA in tail, tail moment, and olive tail moment, compared with the control (Figure 2). Tail length was significantly increased in IR compared to control, but significantly reduced in GTIN+IR than in the IR groups. In IR groups, DNA content increased significantly compared with the control, whereas it decreased significantly in the GTIN+IR groups. Similar results were observed in the case of the tail moment and the olive tail moment. Tail moment was significantly higher in IR in contrast to control, whereas it was significantly decreased in GTIN+IR groups. The

Table 1: Table showing statistical comparison of WBC, RBC count and Hemoglobin concentration in between four different groups i.e., Control, IR, GTIN, GTIN+IR

Parameter	*Control*	*IR*	*GTIN*	*GTIN+IR*
RBC (millions/mm3)	5.02 ± 0.76	3.22 ± 0.62*	5.12 ± 0.39	4.3 ± 0.39^
Hemoglobin (g/dL)	11.85 ± 0.51	7.48 ± 0.48*	11.65 ± 0.39	8.79 ± 0.42^
W.B.C. (Cells/mm3)	2981.4 ± 83.68	792.2 ± 54.36*	2662 ± 164.71	1892.4 ± 153.95^

*Control vs IR,^IR vs GTIN+IR, Data are Mean ± SD (n=8). $p<0.05$ was considered significant

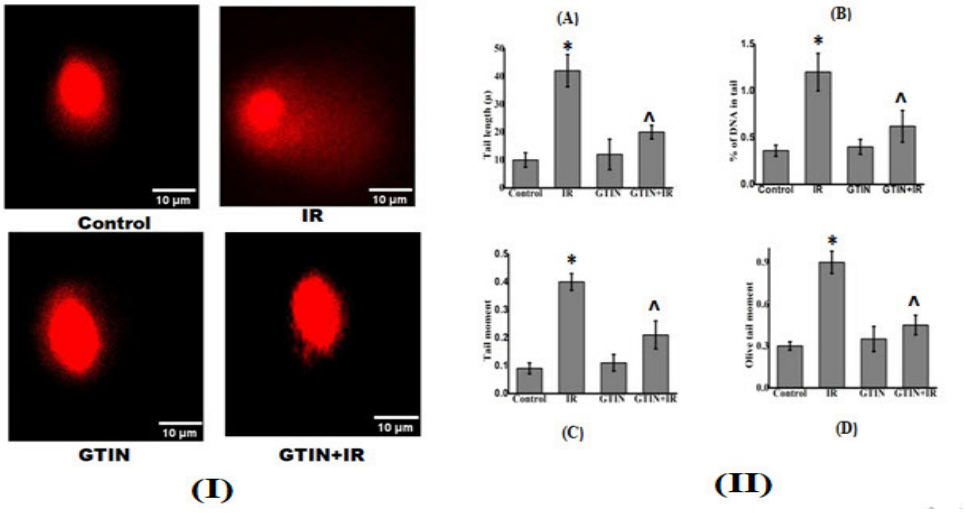

Figure 2: (I) DNA damage study using lymphocytes determined by alkaline comet assay (single cell gel electrophoresis). **(II):** Different comet parameters of figure 2.1. (Two cells were selected for quantification from each group.) (A) Tail length (B) % of DNA in tail (C) Tail moment (D) Olive tail moments. The bars were given as mean ± standard deviation (SD). (n=8). p<0.05 was considered as level of significance.* Control vs IR, ^IR vs GTIN + IR

olive tail moment was significantly elevated in the IR group compared with the control, whereas GTIN pre-treatment markedly reduced it relative to the IR group.

Gossypetin treatment before whole-body irradiation ameliorated radiation-induced DNA damage in lymphocytes.

This method is based on the principle that extensively fragmented double-stranded DNA can be separated from chromosomal DNA through centrifugal sedimentation. In our results, whole body γ radiation exposure induced a significant percentage (%) of DNA fragmentation compared to the group (Figure 3). Only the GTIN treatment did not affect the percentage of DNA fragmentation when compared with the control. GTIN pre-treatment, combined with 5 Gy γ radiation (GTIN+IR), demonstrated a significant reduction in DNA fragmentation compared to IR alone.

Gossypetin ameliorated gamma radiation-mediated lipid peroxidation (LPO) of murine lymphocytes

TBARS, such as malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serve as an index of the extent of peroxidation. γ-irradiation induced a significant ($p < 0.05$) increase in LPO levels compared with the control group. In contrast, GTIN treatment before irradiation ameliorated the effects of radiation exposure, as TBARS values decreased significantly ($p<0.05$) compared with the irradiated group (Figure 4A).

Gossypetin preserved gamma radiation-mediated antioxidant perturbations in murine lymphocytes.

After radiation exposure, the endogenous antioxidants, namely Catalase activity, GSH level, SOD activity, and FRAP level, were reduced. Our results showed a significantly greater enhancement of antioxidant parameters in the GTIN+IR groups compared to the IR groups (Figure 4B-4E).

Gossypetin pre-treatment ameliorated gamma radiation-induced lipid peroxidation in the testis.

Thiobarbituric acid reactive substances (TBARS), such as malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, served as an index of the extent of peroxidation. In the IR group, TBARS values increased significantly ($p<0.05$) compared with the control group. On the other hand, in GTIN+IR, significant inhibition of LPO ($p < 0.05$) was observed compared with the IR group (Table 2).

Gossypetin pre-treatment upheld the radiation

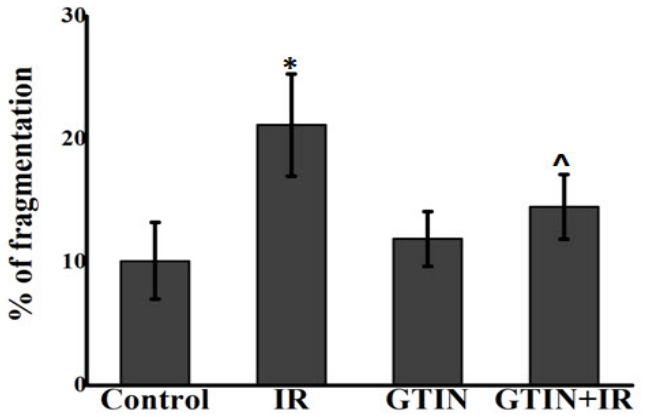

Figure 3: Gamma radiation induced lymphocytes DNA fragmentation and its prevention by GTIN pre-treatment were measured by the Diphenyl Amine (DPA) method. Bars are mean ± standard deviation (SD) for n=8. p<0.05 was considered a level of significance. *Control vs IR and ^IR vs GTIN+IR

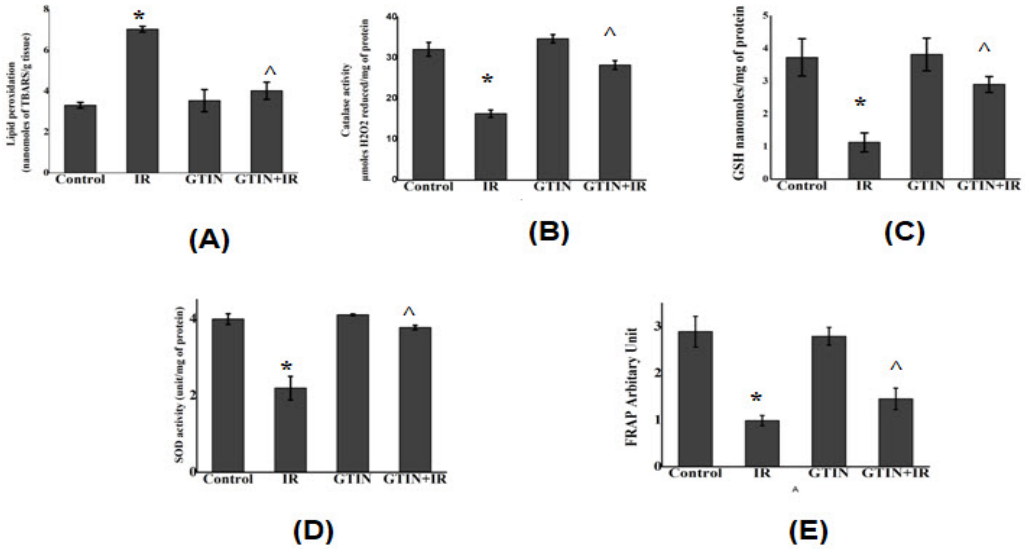

Figure 4: Lipid peroxidation and antioxidant parametetrs of mice lymphocytes. In these four panels Bar 1: control group, Bar 2: mice irradiated with 5 Gy gamma radiation (IR), Bar 3: mice pretreated with GTIN (30 mg/kg body wt) for 3 days (GTIN), Bar 4: GTIN treated plus irradiated (GTIN+IR). . Bars are mean ± standard deviation (SD) for n=8. p<0.05 was considered a level of significance. In all these cases statistical comparison: *Control vs IR, ^ IR vs GTIN+IR(A) Effect of GTIN on radiation (5 Gy) induced LPO of mice lymphocytes in terms of TBARS (nanomoles of TBARS/mg of protein). (B) Effect of GTIN on radiation (5 Gy) induced alteration of catalase activity (μmol H_2O_2 reduced/mg protein) in murine lymphocytes. (C) Effect of GTIN on radiation (5 Gy) induced alteration of reduced glutathione (nmol/mg protein) in mice lymphocytes. (D) Effect of GTIN on radiation (5 Gy) induced alteration of superoxide dismutase activity (unit/mg protein) in murine lymphocytes. (E) Effect of GTIN on radiation (5 Gy) induced alteration of Ferric Reducing Antioxidant Power (FRAP Unit) in murine lymphocytes.

Table 2: GTIN pre-treatment ameliorated γ-radiation-mediated testicular alterations

	LPO (nanomoles of TBARS/g tissue)	*ACP(IU/L)*	*ALP (IU/L)*
Control	1.72 ± 0.25	34.10 ± 3.70	37.72 ± 1.27
IR	5.14 ± 0.37#	45.58 ± 2.60#	31.98 ± 1.53#
GTIN	1.64 ± 0.17*	32.24 ± 2.77*	38.64 ± 1.67*
GTIN+IR	3.04 ± 0.51**	34.26 ± 1.87**	36.72 ± 1.39**

#Control vs IR, * IR vs GTIN, ** IR vs GTIN+IR, Data are Mean ± SD (n=8). p<0.05 was considered significant.

induced testicular ACP and ALP activity

ACP usually remains in the acrosome of spermatozoa and also in the lysosome of Sertoli cells, spermatocytes, and spermatids. In the irradiated group, the ACP activity in the testis showed a significant (p<0.05) increase when compared with the control group. In GTIN-pretreated irradiated (GTIN+IR) mice, a significant recovery in ACP activity was observed when compared to IR. ALP activity in the testes showed a significant decline (p<0.05) in the irradiated group compared with the control group. In the GTIN+IR group, the ALP activity was significantly (p<0.05) increased compared to irradiated mice (Table 2).

Histological study, quantification, and 3D analysis

In the control testis, a compact and organized arrangement of spermatogonia, Sertoli cells, spermatocytes, spermatids, and Leydig cells was observed (Figures 5 and 6). In irradiated mice, testicular distortion of the seminiferous tubules, depletion of spermatogonia and other cell populations, including Leydig cells, and reduced gray value due to an empty tubular lumen were also observed (Figure 6.I.B). Three-dimensional analysis of the selected area in Figure 5 using ImageJ software revealed an increase in white intensity in the IR, indicating a loss of sperm cells from the lumen of the seminiferous tubule. (Figure 6 II). In the GTIN+ IR group of mice, it was observed that the gray value was enhanced and lowered the white color in 3D analysis, which indicated that GTIN could prevent such radiation-mediated testicular damage, as there was less distortion of the seminiferous tubule and less depletion of spermatogonia and other cell populations, including Leydig cells.

gossypetin preserved gamma radiation-mediated antioxidant perturbations in murine testis homogenate

Radiation exposure depleted endogenous antioxidant status in the testes of mice. Catalase activity, GSH level, SOD activity, and FRAP activity were depleted after radiation exposure. GTIN pre-treatment resulted in the significant amelioration of antioxidant parameters in the GTIN+IR group compared to the IR group (Figure 7)

DISCUSSION

Inspired by our previous investigations with GTIN, we now mechanistically show radioprotective effects on rapidly dividing radiosensitive organs against gamma (γ) radiation-induced oxidative stress in the testis and lymphocytes. In

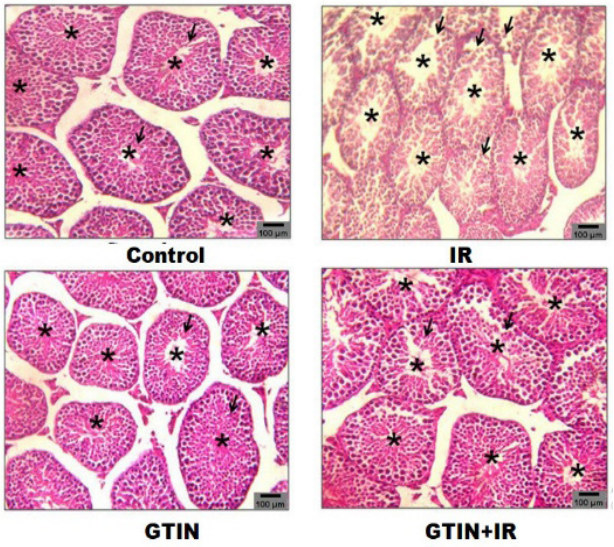

Figure 5: Photomicrographs of testis of mice from control, IR, GTIN and GTIN+IR groups stained by haematoxylin and eosin (Magnification, X 400). In the seminiferous tubules with control groups, the germ cells are organized in concentric layers (arrow) and the filled tubular lumen is observed (asterisks). The Sertoli cell nuclei (arrows) are positioned adjacent to the well defined peritubular tissue in which peritubular cells are observed (arrowheads). In IR groups, the altered seminiferous tubules show irregular shape, epithelial disorganization and detached germ cells with empty tubular lumen (asterisks). In some atrophied seminiferous tubules, loss of germ cells is observed (arrows)

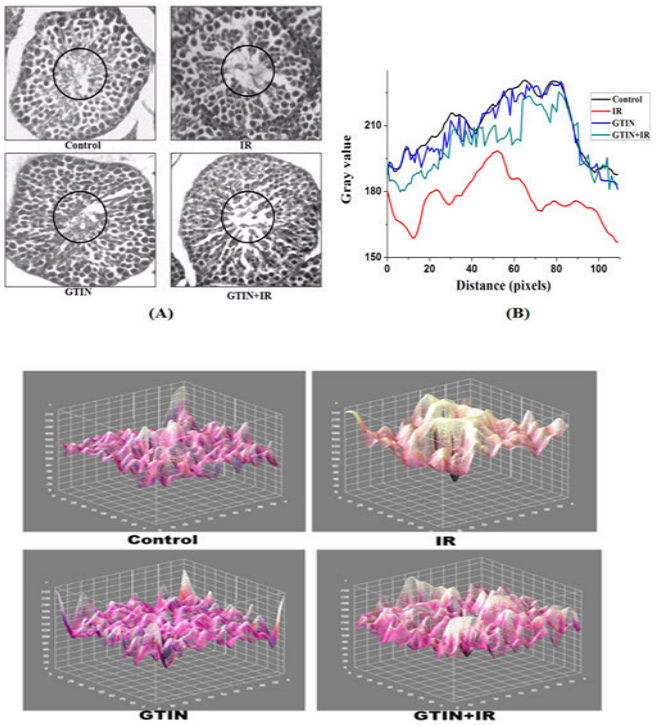

Figure 6: (I) (A) Photomicrographs of seminiferous tubule of mice from control, IR, GTIN and GTIN+IR groups stained by haematoxylin and eosin (Magnification, X 400). The picture is converted into the gray scale for quantification. (B) The Gray value is calculated from the selected area of Figure 6A by ImageJ software. We further analyzed the photomicrographs in the selected areas for quantitative analysis. In IR groups, reduction of the gray value indicates the loss of sperm cells in the central lumen of seminiferous tubules. In GTIN+IR groups, enhancement of gray value indicates the intactness of the seminiferous tubule lumen which is chiefly occupied by sperm cells. (II) Three dimensional analysis of Figure 6 (I) from the selected area by ImageJ software. Increasing the intensity of white area indicates the loss of sperm cells from the lumen of the seminiferous tubule

this mouse model, GTIN was administered at 30 mg/kg body weight for three consecutive days before exposure to 5 Gy of γ radiation. The protective efficacy of GTIN was evaluated using biochemical assays, comet and DNA fragmentation analyses, and histopathology.

To assess how GTIN mitigates radiation-induced stress *in vivo*, the current focus was on tissues particularly susceptible to radiation. Thus, hematological parameters, including red blood cell (RBC) counts, hemoglobin concentration, and WBC counts, were evaluated. Additionally, we investigated the protective effects of GTIN on the testis, a rapidly dividing organ highly vulnerable to radiation-induced damage. Our selection of GTIN was based on its demonstrated safety and compatibility with normal mammalian organs, tissues, and cells, as reported in our previous research [7, 18]. Building on our prior work with phytocompounds, we hypothesized that GTIN could serve as a viable countermeasure agent owing to its chemical structure, bioavailability, and potent free-radical-scavenging capacity. With this foundation, we assessed GTIN's ability to protect the genome in mouse models.

Radiation exposure can significantly impair vital hematological parameters, primarily by damaging the hematopoietic system [34,35]. In our study, we had a clear advantage over the irradiated whole-body models, which showed a substantial decrease in these parameters compared to the control group. This decline may be attributed to direct bone marrow damage, precursor cell suppression, or cell loss from circulation due to hemorrhage or capillary leakage in the presence of free radicals or their derivatives. Whole-body irradiation also led to a decrease in hemoglobin concentration, likely due to increased membrane lipid peroxidation (LPO) in RBCs, as previously shown [36]. Elevated LPO results in reduced membrane elasticity, increased membrane permeability, and the leakage of hemoglobin from RBCs [37]. The alkaline comet assay revealed significant DNA damage following γ-irradiation, which explains the decrease in WBC count. However, GTIN pre-treatment effectively mitigated this radiation-induced damage to the hematopoietic system.

The presence of hydroxyl groups in GTIN served as effective scavengers of free radicals, as previously reported [7,38]. This study confirmed that GTIN pre-treatment *in vivo* protected lymphocytes against radiation-induced DNA damage by scavenging free radicals.

Radiosensitivity varies among cells, tissues, and organs, depending on cell division rate and differentiation level. The testis is a radiosensitive organ, with its radiosensitivity arising

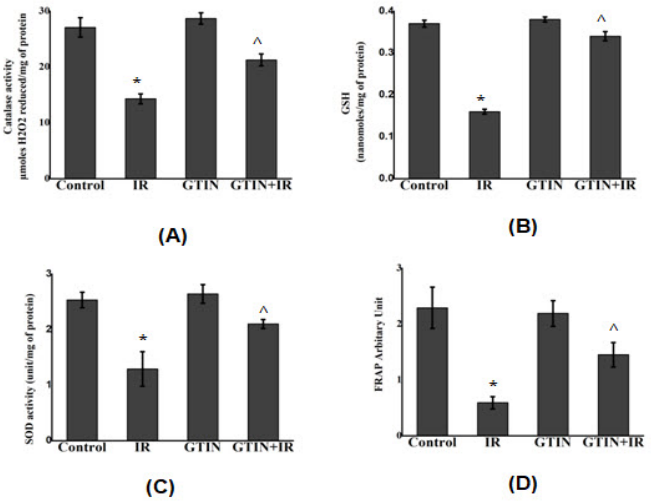

Figure 7: Antioxidant parameters of mice testis homogenate. In these four panels Bar 1: control group, Bar 2: mice irradiated with 5 Gy gamma radiation (IR), Bar 3: mice pretreated with GTIN (30 mg/kg body wt) for 3 days (GTIN), Bar 4: GTIN treated plus irradiated (GTIN+IR). Bars are mean ± standard deviation (SD) for n=8. $p<0.05$ was considered as level of significance. In all these cases Statistical comparison: *Control vs IR, ^ IR vs GTIN+IR (A) Effect of GTIN on radiation (5 Gy) induced alteration of catalase activity (μmol H_2O_2 reduced/mg protein) in murine testis homogenate. (B) Effect of GTIN on radiation (5 Gy) induced alteration of reduced glutathione (nmol/mg protein) in the mouse testis homogenate. (D) Effect of GTIN on radiation (5 Gy) induced alteration of superoxide dismutase activity (unit/mg protein) in murine testis homogenate. (E) Effect of GTIN on radiation (5 Gy) induced alteration of Ferric Reducing Antioxidant Power (FRAP Unit) in murine testis homogenate

from polyunsaturated fatty acids (PUFAs) in cell membranes, rendering it highly susceptible to oxidative damage and lipid peroxidation [39, 40]. Our investigation revealed elevated acid phosphatase (ACP) activity in irradiated testes, likely due to lysosomal membrane breakdown and enzyme release. Alkaline phosphatase (ALP) activity, associated with germinal cells, decreased post-irradiation, possibly contributing to the decline in germ cell population.

The observed post-irradiation architectural alterations in the testis were indicative of radiation-induced damage [41,42]. GTIN pre-treatment effectively defended against radiation-induced biochemical changes, including reduced lipid peroxidation, increased ACP activity, and improved ALP activity. Furthermore, histopathological analysis showed that GTIN prevented germinal epithelial distortion and spermatogonia depletion. Therefore, this study underscores the crucial role of GTIN in protecting cell membranes, preventing enzyme leakage, and reducing radiation-induced oxidative stress in radiosensitive tissues. There was an *ex vivo* study that showed the protective action of one of the derivatives of GTIN (3,5-di-O-Methyl Gossypetin) against oxidative stress in human keratinocytes [43]. Our findings highlight the potential benefits of a GTIN-rich diet in mitigating radiation damage to radiosensitive tissues, underscoring the need for further clinical trials before considering its introduction into clinical practice. This research represents a significant milestone in identifying suitable countermeasures for radiation-induced damage. GTIN mitigated these radiation-induced alterations in both lymphocytes and testis. GTIN's distinctive chemical structure-function properties may support superlative radioprotective actions by quenching the active free radicals produced by ionizing radiation. This exploration of the biochemical role of GTIN further reiterated, providing a rational foundation for comprehensive physiological participation in rapidly dividing organs.

The mechanism of action of this protection lies, in fact, in the lone pair of electrons in the free radicals generated by radiolysis that were well quenched by the C ring consisting of GTIN. In the chemical structure of hexahydroxy flavones, the C ring functions as a sink for unpaired electrons [44, 45]. In conclusion, GTIN demonstrates significant radioprotective potential in shielding rapidly dividing radiosensitive organs from radiation-induced oxidative stress. Future studies could investigate the impact of GTIN on additional radiosensitive organs, such as the gut, bone marrow, and ovaries, particularly given its purported broad-spectrum protective effect on rapidly dividing tissues. This study advances our understanding of GTIN's biochemical mechanisms and properties, paving the way for potential applications in radioprotection and advancing the field.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.