Gossypetin ameliorates ionizing radiation-induced oxidative stress in mice liver—a molecular approach

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Abstract
Radioprotective action of gossypetin (GTIN) against gamma (γ)-radiation-induced oxidative stress in liver was explored in the present article. Our main aim was to evaluate the protective efficacy of GTIN against radiation-induced alteration of liver in murine system. To evaluate the effect of GTIN, it was orally administered to mice at a dose of 30 mg/kg body weight for three consecutive days prior to γ-radiation at a dose of 5 Gy. Radioprotective efficacy of GTIN were evaluated at physiological, cellular, and molecular level using biochemical analysis, comet assay, flow cytometry, histopathology, immunofluorescence, and immunoblotting techniques. Ionizing radiation was responsible for augmentation of hepatic oxidative stress in terms of lipid peroxidation and depletion of endogenous antioxidant enzymes. Immunoblotting and immunofluorescence studies showed that irradiation enhanced the nuclear translocation of nuclear factor kappa B (NF-κB) level, which leads to hepatic inflammation. To investigate further, we found that radiation induced the activation of stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK)-mediated apoptotic pathway and deactivation of the NF-E2-related factor 2 (Nrf2)-mediated redox signaling pathway, whereas GTIN pretreatment ameliorated these radiation-mediated effects. This is the novel report where GTIN rationally validated the molecular mechanism in terms of the modulation of cellular signaling system instead of ‘This is the novel report where GTIN’. This is the novel report where GTIN is rationally validated in molecular terms to establish it as promising radioprotective agents. This might be fruitful especially for nuclear workers and defense personnel assuming the possibility of radiation exposure.

Keywords: gossypetin, gamma radiation, antioxidant, inflammation, lipid peroxidation, cell signaling

Introduction

Gamma (γ) radiation exerts its hazardous impacts on living beings through the generation of harmful reactive oxygen species (ROS). It induces cellular DNA damage leading to mutation and chromosomal damage [1,2]. Eventually, this leads to oxidation of proteins, and lipids with subtle and profound biological consequences [3]. γ-radiation creates oxidative stress in body mainly by radiolysis of water. It produces hydroxyl radical (OH·) which in turn initiates the generation of other ROS-like superoxide anion (O2•−), hydrogen radical (H·), hydroperoxyl (HO2·) radical, hydrated electron (e−aq), hydronium ion (H3O+), and hydrogen peroxide (H2O2) both in the extracellular and intracellular fluid [4].

Several synthetic compounds like lipioic acid, deoxyyspergualin, cysteine, cysteamine, and 2-mercaptopyrroloxylylglycine (2-MPG) were tested and recognized to be extraordinarily effective radioprotectors [5–8]. However, in most of the cases, they exert adverse side effects; therefore, the safety of such synthetic radioprotectors has not been much of success. Notwithstanding the above, the large potential of natural radioprotectors have aroused great interest among investigators that has resulted in increased research on such compounds—this sentence is inserted instead of the sentence—Recently, the potentialities of natural radioprotectors have gathered extensive attention [9,10]. Naturally occurring flavonoids, which are familiar for useful pharmacological properties including antioxidant activities, present an attractive prospect as natural radioprotectants [11–13]. Flavonoids and flavanols are effective free radical scavengers, enhance the endogenous antioxidant status, and thus provide protection against whole-body irradiation [14].

Gossypetin (GTIN) a 8-hydroxy analog of quercetin and has not been well explored for its biological or antioxidant properties. It is 3, 5, 7, 8, 3′, 4′-hexahydroxy flavone (Figure 1). Its glycosides occur in plants such as Hibiscus species. We previously demonstrated the biological and biochemical potential of GTIN and explained its chemical structure in favor of its functions [15].

For the purpose of maintaining systemic protection, liver remains a prominent choice, since it is a metaboli-
A. Khan et al.

...cally active organ and should reflect any systemic derangement upon radiation. The other major aspect of selection of liver is that liver cell regeneration is very slow and thus protection of the liver from any injury would remain a benchmark [16,17].

Thus, the present article is an evidence-based study where GTIN, a potential phytochemical, has prevented the radiation-induced effects in physiological as well as molecular levels. Thus, GTIN can be a safe candidate as radioprotectors in clinical uses. However, its application requires further evaluation through clinical trials.

Materials and methods

Chemicals

High-melting-point agarose (HMA), low-melting-point agarose (LMA), 2’,7’-dichlorofluorescein diacetate (DCFDA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4,6-tripyridyl-s-triazine (TPTZ), ethylenediaminetetraacetic acid (EDTA), Tris base, and ethanol were purchased from Merck India Ltd. (Mumbai, MH, India). All others chemicals used were of the highest analytical quality. GTIN was obtained by the extraction from the dried flowers of *Hibiscus vitifolius* as described previously [15,18].

Animals

Swiss albino male mice (*Mus musculus*), 6–7 weeks old with body weight of 25 ± 2 g, were purchased from Chittaranjan National Cancer Institute, Kolkata, India. They were maintained according to the guidelines set by the Institutional Animal Ethics Committee, maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%), and a 12-h light–dark cycle. Animals were given standard mice feed and water *ad libitum*. The care and use of the animals reported in this study was approved by the Institutional Animal Ethics Committee of the University of Calcutta, Kolkata, India (IAEC/proposal/SD-4/2011 dated 4.4.2011).

Irradiation

Mice were irradiated with 60Co source of γ-radiation (5 Gy), at a dose rate of 1 Gy/min and a source-to-surface distance of 77.5 cm.

Experimental design

Swiss albino male mice were chosen from congenital colony and divided into four groups of eight animals each. The dose (30 mg/kg body weight) was selected after the survivality study by applying different doses of GTIN and determining the optimum protective dose. (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1053878.)

These groups were

- Control group: The mice of the control group were given distilled water through oral gavages once a day for three consecutive days.
- IR group: Mice were given distilled water for three consecutive days before exposing them to a single dose of 5-Gy 60Co γ-irradiation.
- GTIN group: GTIN was administered in mice (30 mg/kg body weight) orally for three consecutive days.
- GTIN + IR group: GTIN was administered in mice (30 mg/kg body weight) orally for three consecutive days. One hour after the administration of the last dose, the animals were exposed to a single dose of 5-Gy γ-irradiation.

All the animals were necropsied by cervical dislocation at 6 h of post-irradiation. The liver and serum were collected for the further experiments.

Isolation of hepatocytes from mice liver

Mice were sacrificed by cervical dislocation. Single cell of hepatocytes were isolated using the procedure described earlier [15,19] with slight modification. Briefly, the livers were mechanically disrupted by grinding with a syringe plunger on a cell strainer with 70-μm nylon mesh. Blood cells were lysed with a hypotonic red blood cell (RBC) lysis buffer (2.42 g Tris and 7.56 g ammonium chloride/L) in sterile distilled water, pH adjusted to 7.2), and a single-cell suspension was made in the complete RPMI 1640 medium supplemented with 100 Unit/mL penicillin and 100 μg/mL of streptomycin with 10% fetal bovine serum. The cell density used was 2.5 × 10^5 cells/mL; the viability of freshly isolated cells was consistently above 90% as indicated by a Trypan blue exclusion test.

Measurement of intracellular ROS

Intracellular accumulation of ROS level was evaluated after incubation of hepatocytes with a membrane-permeable fluorescent probe, DCFDA [15,20]. Briefly, the hepatocytes were incubated with PBS with saturating concentrations of DCFDA (1 μg/mL) in the dark for 30 min at room temperature. The fluorescence signal was measured using 480-nm excitation and 530-nm emission light on a FACS caliber instrument (BD Bioscience, Mountain View, CA, USA). For each sample, autofluorescence signal of
unstained hepatocytes was measured and used to adjust the fluorescence intensity of DCFDA-stained hepatocytes. Data were analyzed using Flow Jo software (version 7.6.5) attached with the flow cytometer. Analysis of fluorescence channel-1 (FL-1) fluorescence was performed with gating on the total unstained hepatocyte population to identify the live hepatocyte population. The same gate was used for all the samples to measure the FL-1 fluorescence intensity.

Biochemical estimations

Lipid peroxidation. Thiobarbituric acid-reactive substance (TBARS) in the liver tissue homogenate was estimated according to the previous protocol [21].

Superoxide dismutase activity. Superoxide dismutase activity was determined using the modified method [22].

Catalase activity. Catalase activity was evaluated by determining using the modified method [22].

Reduced glutathione was determined according to previous methods [25]. FRAP unit is equal with 100 μmol/dm³ Fe²⁺ [26].

Measurement of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase, and serum glutamic pyruvic transaminase levels

Serum alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) levels were measured using kits of Randox Laboratories Ltd by spectrophotometric assay. (Antrim, United Kingdom) according to the manufacturer’s instructions.

Histological analysis of liver tissue

For histological analysis, a small portion of liver tissue was cleaned and preserved in a fixative containing 10% buffered formaldeh. Liver slices were then processed and embedded in paraffin wax. Paraffin blocks were sliced with 5-μm thickness, processed, and stained with hematoxylin (H) and eosin (E) for histopathological evaluation of liver [27]. The stained slide for each group was observed under a light microscope (Olympus 207444, Tokyo, Japan) at 200 × magnifications. The photomicrograph was taken using the Camera Canon Power Shot S70.

‘Bioavailability of GTIN by High-performance liquid chromatography High-performance liquid chromatography

To estimate the GTIN concentration in mice liver, high-performance liquid chromatography (HPLC) was carried out using liver homogenate according to the previous method [16]. For this analysis, mice were orally administered with GTIN (30 mg/kg body weight) for three consecutive days. Animals were sacrificed after 1 and 7 h of GTIN administrations (as in this study, mice were irradiated 1 h after the last dose of GTIN administration and sacrificed 6 h after irradiation). Liver tissues were collected and homogenized with 10 mM sodium phosphate buffer (pH: 7.4) containing 10 mM MgCl₂ in ice. Liver homogenate (1.5 mL) was mixed with 555 μL of antioxidant solution (containing 0.2 g/ml ascorbic acid and 1 mg/ml EDTA) and 30 μl of o-phosphoric acid. The mixture was vortexed, centrifuged, and supernatant was collected for HPLC. Solvent A consisted of 4% acetic acid in water and solvent B composed of acetic acid/methanol/water (1:25:25). These solutions were eluted as follows: 0–1.5 min, 100% A; 1.5–10 min, 100% A to A:B (50:50); 10–12 min, A:B (50:50) to 100% B. The retention time for GTIN was about 30 min at 276 nm.

Alkaline single-cell gel electrophoresis (comet assay)

Radiation-induced DNA double strand breakage in hepatocytes was evaluated using single-cell gel electrophoresis based on the method of Singh et al [28]. In brief, frosted slides were covered with 1% NMA in PBS and allowed to solidify. It was followed by the addition of a second layer of LMA containing approximately 2% NMA in PBS and immediately cover slips were placed on the slides. 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 of 10, 1% DMSO, and 1% Triton X100) was applied for overnight at 4°C. After removal of the slides from the lysing solution, they were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na₂-EDTA, and 0.2% DMSO, at pH ≥ 13.0). The slides were equilibrated with the same buffer for 20 min before electrophoresis at 25 V, 180mA for 20 min. After electrophoresis, the slides were washed with 0.4 M Tris–HCl buffer, pH: 7.4, to remove the alkali. Slides were stained with 50 μl of EtBr (20 μg/ml) and observed under microscope with bright field phase contrast and epifluorescence facility (Leica DC 300 FX, Wetzler, Germany) using 400 × magnifications. The quantification of the DNA strand breaks of the images were done using the Comet Score software by which % DNA in tail, tail length, tail moment, and Olive tail moment were determined.

Measurement of cytokine levels

The levels of murine serum tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) were measured using a sandwich ELISA Kit purchased from Endogen Inc. (Rockford, IL, USA) [17].

Immunofluorescence

Immunofluorescence was performed according to the method described by Das et al. [29]. After fixing of liver.
tissue in 4% buffered formalin, the tissue was embedded in paraffin, serially sectioned at 5 μm. Immunohistochemical staining for nuclear factor kappa B (NF-κB) (p65) was carried out on paraffin sections with anti-NF-κB (p65) antibody (Imgenex, San Diego, CA, USA). Briefly, sections were deparaffinized in xylene and made penetrable by treating with 0.1% Triton X100. Thereafter, antigens were unmasked by heating the sections at 90°C for 10 min in 10 mM citrate buffer, pH: 6. The sections were then allowed to cool at room temperature for 20 min. Next, sections were incubated with diluted primary antibodies overnight at 4°C and washed with PBS after incubation. The bound primary antibodies were incubated with fluorescent isothiocyanate (FITC)-tagged secondary antibody. Nuclei were stained using 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescent signals were observed under a microscope (Olympus IX 81) and if there is any nuclear translocation of NF-κB, the color of FITC was observed into the DAPI-stained nuclei. Quantification of NF-κB nuclear translocation was done by evaluating the color intensity using “Image J software.”

**Immunoblot assay**

Liver was homogenized using tissue homogenizer (Sono Plus, Germany) in ice-cold 0.2 mM phosphate buffer (pH: 7.4) containing protease inhibitors (0.1 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.3% NP-40, and 1 g/mL pepstatin A) to obtain a 10% tissue homogenate. The tissue homogenate (1.5 mL) was centrifuged at 12,000g (30 min at 4°C) in a cold centrifuge (Sorvall, USA). The supernatant was separated and centrifuged again at 15,000g (30 min at 4°C). The supernatant was taken for analysis of cytoplasmic fractions. The pellet procured after the first spin (12,000g) was washed thrice with PBS at 900g (10 min, 4°C) and resuspended in 0.5 mL PBS containing protease inhibitors. The suspension was centrifuged at 100,000g for 1h at 4°C using an ultracentrifuge (Hitachi, Japan). The separated supernatant was used for analysis of nuclear protein. Protein concentration was determined using Lowry method [30]. Equal amounts of protein (50 μg) in each lane were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to a nitrocellulose membrane. After completion of gel electrophoresis, proteins were electrotransferred (100 V, 1 h) to a nitrocellulose membrane using a mini-trans blot assembly (Bio-Rad, USA). The nitrocellulose membrane was blocked using blocking solution (3%, w/v, BSA in TBS) for 2 h at room temperature. Protein expressions were analyzed by probing with the respective mouse monoclonal primary antibodies (1:1000 dilutions) against the former (Imgenex, San Diego, USA). Following three washes of 15 min each in washing buffer (TBS, 0.2% Tween 20), membranes were incubated in TBS containing 1:10,000 dilutions of goat anti-mouse IgG alkaline phosphate-conjugated secondary antibodies. The membranes were again washed (three times each for 15 min) with washing buffer and then treated with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoly1-phosphate (NBT-BCIP) reagent for 20 min. The protein bands obtained were further subjected to densitometric analysis using Gel Documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

The values were given as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Tukey’s post hoc test was done for statistical evaluation of the data and for the determination of the level of significance in various groups. In all cases, a value of p < 0.05 was considered significant.

**Results**

**GTIN scavenged the radiation-induced intracellular ROS**

To determine whether GTIN can protect the liver cells from γ-radiation-mediated oxidative stress by altering the level of intracellular ROS, hepatocytes were incubated with DCFDA and then the intensity of oxidized fluorescent product of DCFDA was monitored by flow cytometric analysis (Figure 2). The analysis showed that in the IR group, intracellular ROS was higher as revealed from the fluorescence intensity which was significantly higher than non-irradiated control. In case of GTIN + IR group, the ROS level reduced significantly by 55% as the intensity reduced than the IR group (p < 0.05). These observations indicated that γ-irradiation enhanced the intracellular ROS level and GTIN pretreatment scavenged these ROS in murine hepatocytes.

**Biochemical estimations**

**Lipid peroxidation**

γ-radiation (5 Gy) caused significant (p < 0.05) enhancement by 112% in the TBARS amounts (7.03 ± 0.002 nmol of TBARS/g tissue) compared with control group (3.17 ± 0.15 nmol of TBARS/g tissue). In GTIN + IR group, pretreatment with GTIN ameliorated the effect of radiation exposure as TBARS level significantly (p < 0.05) reduced by 57% (4.03 ± 0.15 nmol of TBARS/g tissue) as compared with irradiated group (Figure 3A). Thus, GTIN elicited radiation-induced hepatic lipid peroxidation.

**Glutathione**

γ-radiation prompted significant (p < 0.05) 55% reduction (0.166 ± 0.002 μmol/mg of protein) in GSH level compared with control group (0.37 ± 0.003 μmol/mg protein). Treatment with GTIN resulted in significant augmentation (0.38 ± 0.002 μmol/mg protein) in GSH level compared with the control group (p < 0.05). Preadministration of GTIN to radiation-exposed mice showed 57% enhancement (0.37 ± 0.004 μmol/mg protein) in GSH content compared with irradiated group (Figure 3B). Therefore, hepatic glutathione content was restored after radiation exposure by the GTIN pretreatment.
Figure 2. Effect of GTIN on radiation-induced intracellular ROS level of hepatocytes was determined by flow cytometry. Hepatocytes were suspended in PBS with saturating concentration of H$_2$DCFDA in the dark for 30 min at room temperature. The DCFDA fluorescence signal was detected using 480-nm excitation and 530-nm emission light. For each sample, autofluorescence signal of unstained hepatocytes was measured and used to adjust the fluorescence intensity of DCFDA-stained hepatocytes. Data were analyzed using FlowJo software (version 7.6.5). Analysis of FL-1 fluorescence was performed with gating on the total unstained hepatocyte population to identify the live hepatocyte population. The same gate was used for all the samples to measure the FL-1 fluorescence intensity. (I) (A) Control group; (B) Irradiated group (IR); (C) mice pretreated with GTIN (30 mg/kg body wt) for 3 days (GTIN); and (D) GTIN treated plus irradiated (GTIN+IR). (II) Represents the statistical comparison of fluorescence of four different groups for three individual experiments (Error bars are SEM and n = 8): *control vs. IR, ^IR vs GTIN/IR. p < 0.05 was considered significant.

Figure 3. Lipid peroxidation and antioxidant parameters. Bar 1: control group, Bar 2: mice irradiated with 5-Gy γ-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), Error bars are SEM for n = 8, p < 0.05 was considered as level of significance. In all these cases Statistical comparison: *Control vs IR, ^Control vs GTIN, #GTIN vs GTIN + IR. (A) Effect of GTIN on radiation (5 Gy) induced LPO of liver tissue homogenate in terms of TBARS (nanomoles of TBARS/mg of protein). (B) Effect of GTIN on radiation (5 Gy)-induced alteration of reduced glutathione (nmol/mg protein) in mouse liver homogenates. (C) Effect of GTIN on radiation (5 Gy)-induced alteration of catalase activity (μmol H$_2$O$_2$/min/mg of protein) in mouse liver homogenates. (D) Effect of GTIN on radiation (5 Gy)-induced alteration of SOD activity (unit/mg protein) in murine liver homogenates. (E) Effect of GTIN on radiation (5 Gy)-induced alteration of FRAP Unit in murine liver homogenates.
The systemic toxicity after hepatic toxicity

The catalase activity liver homogenate from control and IR group were 31.08±0.61 Unit and 17.29±0.33 Unit. The unit of catalase activity was defined as μmol H2O2 reduced/mg protein" is inserted instead of deleted word. Therefore, irradiation caused the 44% diminution of catalase activity than control groups. In GTIN + IR group activity was 27.25±0.38 Unit. Thus, in GTIN + IR catalase group, the catalase activity was significantly superior (57%) to irradiated mice (Figure 3C).

Superoxide dismutase activity

The liver homogenate of control group showed superoxide dismutase (SOD) activity of 2.99±0.17 unit/mg of protein. After exposure of mice with 5-Gy γ-radiation it showed a significant 23% reduction of (2.29±0.11 unit/mg protein) of SOD level in comparison to control. SOD activity was restored (2.77±0.03 unit/mg protein) in the GTIN + IR group which was significant (p<0.05) compared with IR group (Figure 3D). Therefore, GTIN was able to conserve the SOD activity of mice liver homogenate even after the radiation exposure.

Ferric reducing antioxidant power

γ-radiation (5 Gy) induced a 74% decrease (0.58±0.05 ferric reducing antioxidant power (FRAP) Unit) in FRAP level compared with control group (2.29±0.12 FRAP unit). Interestingly, preadministration of GTIN prior to radiation resulted in significant (p<0.05) increase in FRAP level (1.45±0.08 FRAP Unit) when compared (87%) with the irradiated group (Figure 3E). Thus, GTIN restored the total antioxidant pool which was reduced after irradiation.

GTIN prevented gamma-radiation-mediated hepatic toxicity

The systemic toxicity after γ-radiation was assessed by liver toxicity markers. The liver function status was assessed by estimating the serum level of liver enzymes GPT, GOT, and ALP (Table I). It was observed that γ-radiation elevated SGPT, SGOT, and serum ALP levels. In case of GPT it was observed that there was significant difference between control (9.45±0.27 IU/L) and IR (19.26±0.24 IU/L) group. A significant difference existed between IR and GTIN + IR (11.74±0.22 IU/L) values.

In case of SGOT, the level significantly increased in IR group (40.5±0.89 IU/L) than control (28.78±0.91 IU/L), whereas in GTIN + IR group (27.38±1.09 IU/L) the level significantly decreased than IR group. The serum ALP elevated significantly in IR group (12.36±0.52 KA unit) than control (8.64±0.11 KA unit). The ALP level remained significantly low in GTIN + IR group (8.52±0.41 KA unit) than IR group.

GTIN pretreatment ameliorated gamma-radiation-mediated hepatic alterations

The histological investigations of the liver sections in IR group showed that radiation exposure resulted in significant morphological changes characteristic of inflammation. Compared with control, these morphological changes include inflammatory cell infiltration, intensive inflammatory response around the central vein, and hematoxylin-rich nuclei. Some of the hepatocytes were found to be swelled and membranes appeared disrupted resulting in necrosis and sinusoidal space augmentation is inserted. These were the indications of liver damage as a result of inflammation. GTIN treatment prior to radiation restored the radiation-mediated hepatic alterations (Figure 4).

Bioavailability of GTIN

The native form of GTIN in liver was determined from HPLC analysis using GTIN standard. After 1st hour and 6th hour of GTIN administration, liver GTIN concentrations were 2.26 mg/g of liver tissue and 7.14 mg/g of liver tissue, respectively (Figure 5A and B). The percentages of bio-absorption of free GTIN were 8% after 1 h and 24% after 6 h. It was also observed that after 24 h, no free GTIN was available in liver. Therefore, free GTIN was available to combat the oxidative stress during these time periods. On the basis of this, the mice were irradiated 1 h after last dose of GTIN administration and sacrificed 6 h after irradiation, so that during this time period free GTIN remains available in liver. Thus, based on the above experiments, we further designed the time of administration of GTIN and the sacrifice time of animal after radiation.

GTIN ameliorated radiation-mediated DNA damage

γ radiation exposure (5 Gy) significantly increased all the comet parameters, that is, tail length, % DNA

| Parameters | Control | IR | GTIN | GTIN + IR |
|------------|---------|----|------|-----------|
| ALP (KA)   | 8.64±0.11 | 12.36±0.52* | 8.28±0.24 | 8.52±0.41* |
| SGPT (IU/L)| 9.45±0.27 | 19.26±0.24* | 8.79±0.41 | 11.74±0.22* |
| SGOT (IU/L)| 28.78±0.91 | 40.5±0.89* | 24.64±1.14 | 27.38±1.09* |

*Control vs IR, *GTIN + IR vs IR, *Q + IR vs IR.
in tail, tail moment, and Olive tail moment compared to control (Figure 6). Tail length was significantly increased in IR (50.33 ± 5.03) than control (4.33 ± 1.33) but significantly reduced in GTIN + IR (10.67 ± 3.04) than IR group. In IR group (1.54 ± 0.09), % of DNA content significantly increased than control (0.14 ± 0.05), but decreased significantly at GTIN + IR (0.26 ± 0.02) than IR group. Similar results were observed in case of tail moment and Olive tail moment. Tail moment remained significantly higher in IR (0.54 ± 0.03) than that in control (0.03 ± 0.01), whereas it decreased significantly in GTIN + IR (0.07 ± 0.02) group. In case of Olive tail moment, IR group (0.84 ± 0.11) showed significant enhancement than control (0.09 ± 0.003), whereas GTIN + IR group had significantly lower value (0.22 ± 0.01) than IR group (p < 0.05).

GTIN ameliorated gamma-radiation-mediated serum TNF-α and IL-6 level

In IR group, IL-6 level was significantly elevated in IR (137.78 ± 5.52 pg/ml) group than in control (90.23 ± 7.42 pg/ml) group, whereas in case of GTIN + IR (92.81 ± 7.45 pg/ml) serum level was significantly decreased in IR group (Figure 7A). Similar trend was observed in case of TNF-α level. GTIN pretreatment ameliorated the TNF-α level as the value significantly decreased in GTIN + IR (120.82 ± 2.32 pg/ml) than in IR group (152.76 ± 2.39 pg/ml) (Figure 7B).

GTIN prevented the gamma-radiation-mediated nuclear translocation of NF-κB (p65)

Maximum nuclear localisation of NF-κB was observed in radiation-exposed mice. The nuclear entry of p65 was lessened in GTIN ± IR than in IR. From the immuno blot data it was revealed that the expression of cytosolic NF-κB (p65) protein in irradiated group was significantly (p < 0.05) declined than that in control. In case of GTIN + IR, the expression of cytosolic NF-κB (p65) significantly augmented than IR group. In case of nuclear NF-κB (p65) expressions of the proteins were reciprocally present in the cytosol and nucleus (Figure 8).

The immunofluorescence study confirmed the NF-κB localization in the cytoplasm of the control hepatocytes as well as radiation-induced nuclear translocation of NF-κB. In case of IR, 47 nuclei were p65 positive out of 60 nuclei in the selected region (Figure 9) whereas 14 nuclei were p65 positive out of 60 nuclei in GTIN + IR slide. Therefore, in the GTIN + IR group, nuclear translocation of p65 was less in comparison to the IR group.

Effect of GTIN on radiation-induced expression of Cdc42, phosphorylated stress-activated protein kinase/c-Jun NH2-terminal kinase, and Bax protein in liver

Our data revealed that Bax- and Cdc42-phosphorylated SAPK/JNK expressions were significantly activated in IR group than that in control. GTIN reduced radiation-mediated Cdc42 activation followed by SAPK/JNK phosphorylation and Bax activation (Figure 10). This suggested
that GTIN can modulate radiation-induced mitochondrial-dependent apoptotic pathways.

**GTIN activated Nrf2-driven Mn-SOD via PI3K and Akt phosphorylation**

As shown in Figure 11, exposure to radiation decreased the phosphorylation of Akt and PI3K in IR-treated groups than that in control groups significantly. Inhibition of the PI3K/Akt pathway by IR reduced the NF-E2-related factor 2 (Nrf2) translocation and Mn-SOD protein levels. In our present study, we observed that GTIN pretreatment significantly upregulated PI3K and Akt phosphorylation leading to Nrf2 nuclear translocation and Mn-SOD activation when compared with IR group.

**Discussion**

In the present study, the strategies were to find whether IR perturbs the metabolic oxidative balance. Thus *in vivo* model for IR induced stress was developed. Liver is the major metabolic organ. If liver experiences stress and strain, then it will lead to serious physiological consequences in the entire organism. The present analysis involved the assessment of damage using key signaling molecules of cellular metabolism as well as inflammatory development. With the purpose of establishing an agent that potentially prevents the physiological stresses after radiation exposure, we hypothesized the GTIN is an effective compound. Due to its unique chemical structure, it effectively combats the reactive species after radiation. Moreover, due to its bioavailability for wide period of post absorption, it can mediate its effects in the metabolic balances.

In our study, it was shown that 5-Gy γ-radiation diminished the endogenous antioxidant system [31]. Increased level of LPO confirmed the radiation-mediated membrane damage of liver. Furthermore, radiation caused enhanced leakage of liver function enzymes like GPT, GOT, and ALP in IR groups. These enzyme markers are the indicators of liver toxicity [32]. The underlying mechanism of these effects leading to liver toxicity is that radiation generates free radicals, damaging the cell membrane and
Radioprotective action of gossypetin

thereby releasing the cytosolic enzymes [16,33]. Overall
tissue damage in IR groups was evident from our histo-
logical study. The enhancement of the comet parameters
in IR group in indicated radiation-mediated DNA damage,
which may be the cause of the overall architectural dam-
age of the liver tissue.

From immunofluorescence and Immuno blot analysis,
we demonstrated that γ-radiation augmented the nuclear
translocation of NF-κB (p65) in murine hepatocytes. How-
ever, NF-kB pathway is considered as a prototypical pro-
inflammatory signaling pathway. Ionizing rays would not
be an exception to activate redox-sensitive transcription
factor NF-κB due to the generation of ROS [34]. Moreover,
NF-kB is responsible for expression of proinflammatory
genes including cytokines, chemokines, and adhesion mol-
ecule [35] which was supported by the enhanced TNF-α
and IL-6 expression in IR groups in our study. It was
reported that oxidative stress and SAPK/JNK maintain a
balance and modulate NF-κB activity, which determines
stress response and other inflammatory phenomena [36].
Activated Cdc42 triggered JNK pathway resulting in ini-
tiation of apoptosis regulating the activities of preexisting
Bcl-2 family proteins [37,38,39]. In our present study, it
was evident that radiation enhanced intracellular ROS
which leads to activation of Cdc42 and further activation
of SAPK/JNK. Results also revealed the increased level of
Bax protein in the IR-treated group, indicating that the Bax
caused the outer mitochondrial membrane to become leaky,
which helped in releasing cytochrome-c into the cytosol
thereby triggering apoptosis [36].

One major system that reacts with oxidative stress to
restore the redox balance involves genes coordinately

Figure 6. (I): Damage of hepatocyte DNA after radiation determined by alkaline comet assay (single-cell gel electrophoresis); (II): Different
comet parameters of Figure 6 (I). (A) Tail length, (B) % of DNA in tail, (C) Tail moment, and (D) Olive tail moments. Errors are SEM
(n = 8). p < 0.05 was considered as level of significance. *IR vs control, IR vs GTIN + IR.

Figure 7. Panel (A) and panel (B) showing the serum IL-6 and TNF-α levels, respectively. Error bars are SEM (n = 8). In all these cases
statistical comparison: *control vs IR; IR vs GTIN + IR. p < 0.05 was considered significant.
Figure 8. NF-κB (p65) expression was determined by Immuno blot analysis. Lane 1: control group of mice, lane 2: mice irradiated with 5 Gy (IR), lane 3: mice treated with GTIN (30 mg/kg body weight) for three days, respectively, and lane 4: mice treated with GTIN (30 mg/kg body weight) for three days plus irradiated (GTIN + IR). Error bars are SEM (n = 8). In all these cases statistical comparison: *control vs IR; ^IR vs GTIN + IR. p < 0.05 was considered significant.

Figure 9. NF-κB (p65) activation in mice was determined by immunofluorescence. Control: without any treatment, IR: mice irradiated with 5-Gy γ-radiation, GTIN: mice treated with GTIN (30 mg/kg body weight) for three days, respectively, GTIN + IR: GTIN treated plus irradiated. NF-κB positive nuclei were estimated from the fixed region.
Radioprotective action of gossypetin

regulated by transcription through the antioxidant response element (ARE). This is activated primarily by binding of the transcription factor Nrf2 [40]. We observed that the irradiation caused the significant reduction of Nrf2 nuclear translocation. Mn-SOD is a mitochondrial matrix enzyme that scavenges ROS and protects the cell against the insults of oxidative stress. It mediates a key role in cell survival and is necessary for the maintenance of mitochondrial integrity in cells exposed to oxidative stress [40,41]. Therefore, radiation-induced downregulation of nuclear translocation of Nrf2 caused the reduction in Mn-SOD level also, which was confirmed by our Western blot data. The molecular mechanism of perturbation leading to mitochondrial damage was further confirmed by showing several signaling pathways, including PI3K/AKT which was involved in the induction of Nrf2/ARE-driven gene [37,39].

In our study, GTIN treatment prior to radiation was found to protect liver from radiation-induced liver inflammation by inhibiting the nuclear translocation of NF-kB and reducing lipid peroxidation. Thus, the membranes remained protected from leaking liver function enzymes into the extracellular matrix. The maintenance of the endogenous antioxidant level in GTIN + IR-treated groups can cause to combat the liver cell against radiation-mediated oxidative stress. GTIN prevented apoptosis induction in GTIN + IR groups by reducing intracellular ROS and inhibition of the upstream SAPK/JNK, recruitment of Cdc42, reduced expression of Bax, and promoting survival of the liver against radiation. Protective action of GTIN against radiation-mediated DNA damage was crucial to protect the liver from apoptosis because DNA damage is one of the major indicators of cellular apoptosis.

It was revealed that GTIN upregulated Mn-SOD expression by increasing the nuclear translocation of Nrf2 which were attenuated by radiation. The possible mechanisms leading to nuclear translocation of Nrf2 include its release from Keap1 into the cytosol and enhancing ARE-binding activity of Nrf2. Our results demonstrated that GTIN increased the nuclear translocation of Nrf2, suggesting that GTIN may indirectly enhance the Mn-SOD in this way. It is known that several signaling pathways, including PI3K/AKT are involved in the induction of Nrf2/ARE-driven gene [39]. The activation of the PI3K/Akt pathway is a key step in diverse biological processes, including cell proliferation, growth, and survival. Therefore, our results

Figure 10. (A) Effect of GTIN on radiation-induced Cdc42; total and phosphorylated SAPK/JNK; Bax expressions were determined by Immuno blot analysis. Band density values were compared with the β-Actin loading control. (B) Densitometric analysis of Cdc42 with respect to β-Actin. (C) Densitometric analysis of SAPK/JNK with respect to β-Actin. (D) Densitometric analysis of phosphorylated Bax with respect to β-Actin. Statistical comparison: *Control vs IR, IR vs GTIN + IR. Values were presented as mean ± SE (n = 3). p < 0.05 was considered significant.
demonstrated that the Nrf2-mediated increase in Mn-SOD protein induced by GTIN is dependent on the activation of PI3K/Akt pathway. Thus, GTIN prevented radiation-induced systemic and cellular oxidative stress. The protective action may depend on either the ability of the native GTIN to act as a scavenger of radiation-induced reactive species or by interacting with cell-signaling cascades either by itself or its metabolized form (Figure 12).

In conclusion, here we report for the first time that GTIN mediated a vital role in protecting the liver from radiation hazards. It was responsible for preventing the radiation-mediated oxidative stress. Therefore, our present result confers the evidence to the usefulness of GTIN-rich diet in preventing higher levels of radiation-induced inflammation, lipid peroxidation, and oxidative stress. It may remain an important justification for nuclear workers or defense personnel assuming possibility of nuclear exposure. However, further studies are warranted to find out its dose and toxicity profile and its protective effects on normal cells vis-a-vis tumor cell protection in radiation exposures being put into clinical use.

Acknowledgment

The authors are thankful to Professor M. Nandi of SINP, Kolkata for providing necessary animal irradiation facilities. SD acknowledges CSIR, GOI, for fellowship to KM, WB DBT, Kolkata, India for fellowship support to DD, CPEPA, UGC for fellowship to SBK & AS, UGC-DAE to MS, and LSRB to UD. The authors acknowledge Dr Deba-
sish Bandhopadhyay of CGCRI, Kolkata for his editorial comments on manuscripts.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1053878.