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Protective Effect Of Diosmin-Hesperidine Combination On Gamma Radiation-Induced Apoptosis In Liver Of Albino Rats. Molecular And Immunohistchemistry Studies

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Abstract

This study used radiation-induced damage in Wistar albino rats as a proxy for assessing the radio and antioxidant protective efficacy of diosmin-hesperidin, a natural plant citrus flavone of hesperidin derivative. Before being exposed to a cumulative dose (10Gy) of gamma radiation in the form of fractionated dose (2 Gy for 5 times every other day), rats were given diosmin-hesperidin dose (200 and 100 mg/kg body wt. correspondingly) orally for a month (every other day). The purpose of this study is to assess the diosmin-hesperidin combination's radioprotective and antioxidant efficacy. Apoptosis was evaluated using several methods, including real-time polymerase chain reaction (RT-PCR) analysis, biochemical analysis, histopathological changes, the comet assay, and caspase-3 activity. As shown by the findings, pretreatment with diosmin+hesperidine counteracted the detrimental effects of radiation on antioxidant indices (SOD, GPx, and GSH), lipid peroxidation, DNA damage, and apoptosis. Lipid peroxidation, DNA damage, and tissue damage were all mitigated by a diosmin+hesperidine dose (200 and 100 mg/kg body wt., respectively) that brought antioxidant status back to near normal. Histopathological analyses corroborated these findings, demonstrating that the livers of albino rats were protected from gamma-irradiation-induced damage when diosmin+hesperidine was given prior to the radiation exposure. So, the evidence suggests that diosmin+hesperidine can protect rats from radiation-induced harm. In addition, there were no signs of metabolic changes or DNA damage in the diosmin+hesperidine pretreatment group.

Keywords: Radioprotection, reactive oxygen species, Rt-PCR, DNA damage, and apoptosis are some of the terms used to describe the effects of gamma radiation on cells.

1. Introduction

Radiotherapy, medical product sterilization, and other uses of nuclear energy in the medical field, the nuclear energy business, agriculture, and food preservation all contribute to the rising radiation burden. Those who work with radioactive materials, radiation sources, and equipment are particularly vulnerable to radiation exposure, but the general public also faces risks from accidental ionizing radiation exposure (Weiss and Landauer, 2003; Shedid et al., 2019).

Ionizing radiation is harmful to living things, and it has been shown to cause structural and functional changes in important organs like the liver by producing reactive oxygen species (ROS) that harm crucial cellular targets (Kim and Jung, 2017). Jadeja et al. (2017), Li et al. (2015), and Muriel and Gordillo (2016) all agree that oxidative stress plays a significant role in liver damage. The lipids, proteins, and nucleic acids of hepatocytes are particularly

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vulnerable to reactive oxygen species (ROS). Lipid peroxidation damages the membrane (Niki, 2009), whereas protein oxidation disrupts DNA repair enzymes and signal transduction (Dalle-Donne et al., 2003). Additionally, there is the fact that DNA oxidation leads to mutation (Voulgaridou et al., 2011). Reactive oxygen species (ROS) damage is lessened by endogenous antioxidants such glutathione (GSH), which is considered the most important antioxidant molecule (Valko et al., 2007), superoxide dismutase (SOD), and GSH peroxidase. Radiosensitivity is found in non parenchymal hepatic cells like Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells. Radiation-induced changes in liver structure and function are exacerbated by the release of chemicals that stimulate fibrosis in the liver (Du et al., 2010). In response to the initial effects of radiation, such as DNA damage, oxidative stress, and ROS production, hepatocytes may also undergo apoptosis (Guicciardi et al., 2013). This process starts with the activation of a group of proteases called "Caspases" and continues through a complex cascade of events until cell death. Condensation of chromatin and DNA fragmentation are two of Caspase-3's main functions (Elmore, 2007).

However, the kidneys are extremely vulnerable to radiation. Those with abdominal cancers were doomed to have their kidneys irradiated (Cohen and Robbins, 2003; Ismail et al., 2016). It was demonstrated that even low-dose irradiation (4-5Gy) could cause serious kidney injury (Moulder and Cohen, 2014). Antioxidants like lipoic acid and vitamins A, C, and E are only a few examples of the radioprotective substances currently in use. They are doing their work by lowering tissue damage, lowering oxidative stress, and scavenging free radicals (Weiss and Landauer, 2003; Yuhas et al., 1980; Soule et al., 2007; Citrin et al., 2010). Several of these RCM compounds, however, have demonstrated either acute toxicity or a short biological half-life when used to counteract radiation (Arora et al., 2010).

As a result, natural compounds derived from plants have been tested to see if they may be used as RCM drugs. Plants high in flavonoids may prevent macromolecular damage in cells caused by radiation (Kuntic et al., 2014). Plants produce a wide variety of secondary metabolites, including polyphenols, a class of physiologically active chemicals. In addition to defending plants from radiation and poisons, these chemicals also help plants withstand a wide variety of microbial diseases. Polyphenols and their wide range of biological effects have been the subject of numerous recent investigations (Daglia, 2012; Quideau et al., 2011).

Several flavonoids have been reported as powerful antioxidants with radioprotective qualities (Hosseinimehr et al., 2003; Benkovic et al., 2008b), and this is because they may scavenge free radicals, sparing endogenous antioxidant enzyme systems and preventing their depletion (Nijveldt et al., 2001). In light of the fact that oxidative stress and radiation injury have many similarities, free radical scavenging is one of the most significant characteristics required for a chemical to be a good radioprotector. Extensive research on the radioprotective effects of various plant extracts has been conducted over the past decade (Sandeep and Nair, 2010), with positive results showing improved mouse survival, chromosome protection, and spermatogonal cell survival.

Diosmin (diosmetin 7-O-rutinoside), a naturally occurring citrus lavones of hesperidin derivative, has been investigated for its anti-mutagenic, anti-inflammatory, anti-hyperglycemic, anti-allergic, and antioxidant capacities (Srinivasan and Pari, 2012; Kartup et al., 1987). The citrus fruit pericarp is rich in diosmin because it comes from the Rutaceae family (Imam et al., 2015). Dehydrogenation of hesperidin flavonoid yields this compound, which was initially discovered in 1925 from the leaves of Scrophularia nodosa L., (Scrophulariaceae) (Bogucka-Kocka et al., 2013). Diosmin formulations with names like Daflon 500 (containing 90% diosmin and 10% hesperidin) are utilized for treating hemorrhoids and chronic venous insufficiency (Meyer, 1994). Diambarasan and Raja (2012), Tahir et al. (2013)b, Tanrikulu et al. (2011), Dholakiya and Benzeroual (2011), Golbabapour et al. (2013), myocardial infarction (Golbabapour et al.), neurodegeneration (Dholakiya and Benzeroual 2011), and hepatocarcinogenesis (Tahir et al., 2013a) are merely a few of the studies that have proven that diosmin has numerous beneficial impacts on hypertension. Diosmin has been shown to protect against trichloroethylene-induced kidney damage in several investigations (Rehman et al., 2013).

Hesperidine (hesperetin-7-rhamnoglucoside) is a cheap by-product of citrus farming (Garg, et al., 2001). It is a flavanone glycoside abundant in sweet orange and lemon. Edema, or excessive swelling in the legs caused by fluid accumulation, can be efficiently treated with HES when administered as a supplementary treatment. Anti-inflammatory, analgesic, anti-fungal, antiviral, antioxidant, and anti-carcinogenic properties have all been attributed to it. Tommasini et al. (2005) and Park et al. (2008) found that HES administration restored membrane function by keeping liver enzyme levels close to normal. According to Chopra et al. (2006), HES is effective in reducing oxidative stress by neutralizing the free radicals that contribute to it. Conversely, HES restored normal levels of antioxidant enzymes (Wilmsen et al., 2005).

Therefore, the current investigation was conducted to investigate whether or not the combination of diosmin and hesperidine may provide radioprotection against the damages generated by total-body gamma-irradiation in rats. Lipid peroxidation, antioxidant biomarker levels, serum hepatic injury markers, DNA damage, relative transcript quantification (Rt-PCR) of liver P53 and Caspase-3 gene expression, apoptosis, and liver tissue samples were analyzed.

2. Materials and methods

2.1. Chemicals

Diosmin (CAS: 520-27-4) and Hesperidine (CAS: 520-26-3) were gifts from the Egyptian company Egypar Pharmaceutical Industries at industrial zone, El-Obor. Both di- and potassium di-hydrogen orthophosphate (K2HPO4 and KH2PO4) were bought from Carloerba-France. We bought AST, ALT, and creatinine and urea assay kits for the liver and kidneys from BioMED in Egypt. For use as diagnostic and research tools, Egypt's BIODIAGNOSTIC sold SOD, GSH, and lipid peroxide assay kits.

2.2. Experimental animals

We purchased male Wistar albino rats from the Egyptian Organization for Biological Products and Vaccines. They weighed between 150 and 200 g. Before the trial began, they were kept under observation for roughly 15 days to get used to the lab environment. Rats were housed in specially constructed cages with regular parameters such as temperature (25 ± 5 °C), humidity (60%) and proper lighting (cycle of light and dark). The animals were provided with an endless supply of water and regular food pellets. All procedures involving animals were in compliance with the European Community Council Directive of 24 November 1986, and ethical approval was granted by the Theodor Bilharse Instatement's animal ethics committee (No. 29 12 2014,)

2.3. Gamma irradiation of animals

Gamma Cell-40 was used in the irradiation process, which was accomplished by Egypt's National Center for Radiation Research and Technology (NCRRT), located in Cairo. Atomic Energy of Canada Limited produces the caesium-137 irradiation unit known as the gamma cell-40. At the time of the experiment, the dose rate was 0.54 Gy/min. 10Gy of radiation were administered in 5 doses of 2Gy each, spaced every other day. Fractionation is often utilized in preclinical studies to create animal models with high single fraction doses that are as close to human exposure as feasible (Constanzo et al., 2017). These sub-lethal dosages were chosen to allow for the investigation of the risk effect of whole-body radiation without causing the rats under study to perish.

2.4. Experimental design

40 male albino rats were divided into 4 groups (n = 10); diosmin dose (200 mg/kg body wt.) and hespiridine (100 mg/kg body wt.) dose were selected based on previous studies (Jain et al., 2014; Perumal et al., 2015) and (Sarhan, 2016). Group 1: For a month, rats received the same volume of vehicle solely orally via an oral tube every other day; this group acted as the control group; Group 2 (D200+H100): Rats were given 200+100 mg/kg body weight of diosmin and hesperidine, respectively. orally every other day for a month, acting as a control group for diosmin+hesperidine.; Group 3 (10Gy): For a month, rats were given oral vehicle only every other day and exposed to cumulative doses of 10 Gy (fractionated into 5 doses of 2 Gy each). The rats served as a 10 Gy irradiated control group.; Group 4 (D200+H100 + 10Gy): Rats were given oral diosmin+hesperidine at 200+100 mg/kg body weight, respectively, every other day for a month, followed by cumulative doses (10Gy);

Rats were put to sleep with ether anesthesia, and blood samples were collected from the retro-orbital

venous plexus one week after the final dose of radiation. After being allowed to clot for 30 minutes at 25 °C, collected blood samples were centrifuged at 1200 g, separated into sera, and stored at -20 °C pending biochemical analysis. Diosmin-hesperidine as radioprotective effect was investigated using liver excision, various biochemical studies, P53 and Caspease -3 gene expression evaluation, histological abnormalities, comet assay, and apoptosis assessment.

2.5. Preparation of liver homogenate

Blood was removed from the liver samples, and they were then immediately transferred to ice-cold containers containing 0.9% saline. In accordance with the assay procedures, a known quantity of tissue was homogenized in a tissue homogenizer (Glas-Col®, Cat. No. 099C K6424, TERRE HAUTE USA) using the proper phosphate buffer. For the purpose of performing assays for the detection of total thiobarbituric acid reactive compounds (TBARS) and GSH, the supernatant was collected and stored at –80 °C. A 10% liver homogenate in 10 mM phosphate buffer was produced for biochemical assays. This homogenate was centrifuged at 10,000 g for 15 min at a temperature of – 4 °C.

For the SOD test, mix 1 ml of supernatant with 0.5 ml of the ice-cold extraction reagent "provided with SOD kit" and vortex for 30 seconds. The aqueous upper layer was then collected and stored at 4 °C for immediate analysis after being centrifuged at 4000×g for 10 min at 4 °C.

2.6. Analysis of antioxidants

The method of Ohkawa et al. (1979) was utilized to measure the amount of malondialdehyde (MDA) in the hepatic tissue in order to assess lipid peroxidation. using Assay Kits (BIODIAGNOSTIC, Egypt). Briefly, 0.2 ml of liver homogenate or standard was mixed with 1 ml chromogen solution (Thiobarbituric acid + detergent + stabilizer) and heated in a boiling water bath for 30 min.

TBARS were determined by measuring the absorbance at 534 nm and expressed as MDA (nmol/g tissue).

Tissue levels of SOD, GPx and GSH were determined by Assay kit (BIODIAGNOSTIC, Egypt.), according to the provider instructions. SOD assay relies on the ability of the enzyme to inhibit the phenazine methosulphate (PMS)-mediated reduction of nitro blue tetrazolium (NBT) dye. The assay mixture contained: 1 ml of working reagent (Phosphate buffer + NADH + NBT), 0.1 ml of sample or dist. water for the control. The reaction was initiated by addition of 0.1 ml PMS and the increase in absorbance was measured at 560 nm for 5 min for control (A control) and sample (A sample) at 25 °C.

The % inhibition was calculated from (ΔA control – ΔA sample/ ΔA control) x 100.

SOD activity (U/g tissue) = % inhibition x 3.75 × 1 / g tissue used

Determination of GSH-Px activity according to the method of Rotruck et al. (1973).

The procedure is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG). This reaction is catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (b-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPx activity.

Levels of GSH in liver homogenates were assayed by mixing 0.5 ml of homogenate with 0.5 ml TCA and centrifugation at 3000 rpm for 15 min. Then, 0.5 ml of supernatant was mixed with 1 ml of phosphate buffer (pH 8) and 0.1 ml of 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB). The yellow-colored substance formed was measured at 405 nm. The results were expressed as GSH mg/g tissue.

2.7. Liver sampling and processing for Rt-PCR

Part of liver rinsed with shield saline, weighed and rapidly frozen in liquid nitrogen then stored at -70ºc until assayed. RNA was extracted from hepatic tissue homogenate using Rneasy Purification Reagent (Qiagen, Valencia, CA). The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene, USA). cDNA was generated from 5µg of total RNA extracted with 1µl (20 pmol) antisense primer of each gene and 0.8µl superscript AMV reverse transcriptase for 60 min at 37˚c. For PCR, 4µl cDNA were incubated with 30.5µl water, 4µl 25mM MgCl2, 1µl dNTPs (10mM), 5µl 10x PCR buffer, 0.5µl (2.5U) Taq polymerase and 2.5µl of each primer containing 10pmol (primer sequences were shown in table 1). The reaction mixture was

subjected to 40 cycles of PCR amplification as follows: denaturation at 95˚c for 1 min, annealing at 67˚c for 1 min and extension at 72˚c for 2 min. A real time- PCR mixture was then prepared as follows: 25µl SYBR Green Mix (2x), 0.5µl cDNA, 2µl primer pair mix (5pmol/µl each primer), 22.5µl H2O. According to the amplification procedure, relative expression of each studied gene was calculated according to the following the formula: densitometrical units of each studied gene/densitometrical units of β-actin. Beta actin was amplified with the same run of tested genes as housekeeping gene to detect RNA integrity.

Table 1: The Oligonucleotide Primers Sequence

Gene	Primer sequence
p53 **(Longxi *et al*., 2011)**	Forward 5′-GTTCCGAGAGCTGAATGAGG-3′ Reverse 5′-TTTTATGGCGGGACGTAGAC-3′
caspase 3 (A062449)*	Forward 5′-ATGGACAACAACGAAACCTC-3′ Reverse 5′-TTAGTGATAAAAGTACAGTTCTT-3′
β actin (NM_017008)*	Forward 5′ TGCTGGTGCTGAGTATGTCG 3′ Reverse 5′ TTGAGAGCAATGCCAGCC 3′

* Gene bank accession

2.8. Comet assay

Liver pieces from the center were broken up and placed in one milliliter of ice-cold phosphate buffered saline (PBS). After stirring the suspension for five minutes, it was filtered. 600 µl of low-melting agarose were combined with 100 µl of cell suspension. This combination was applied in 100 µl to slides that had already been coated. The coated slides were electrophoresed for two minutes at 100 mA after being submerged in lysis buffer for fifteen minutes. Etidium bromide (EtBr) staining at 4 °C (20 µg/ml). Using a 40x objective on a fluorescent microscope, observations of EtBr-stained DNA were made in order to visualize DNA damage. The Komet 5 image analysis software, created by Kinetic Imaging, Ltd. (Liverpool, UK), was connected to a CCD camera in order to measure the length of DNA migration and the percentage of migrated DNA, which allowed for the quantitative and qualitative assessment of DNA damage in the cells. The tail moment was finally estimated by the software. For each sample, 50–100 randomly chosen cells were examined (Singh et al., 1988).

2.9. Quantitative evaluation of apoptosis in the liver using immunohistochemistry for active Caspase-3

According to Krajewska et al. (1997), the polyclonal anti-rabbit Caspase-3 antibody CPP32, Ab-4 (Cat. #RB-1197-R7 ready-to-use for immunohistology), Thermo ScientificTM, Fremont, CA 94539, USA), and the DAKO LSAB® System-HRP detection kit (DAB, DAKO, Denmark) were used to perform the immunolocalization technique for caspase-3 on liver sections with a thickness of 3–4 µm. The specimen was incubated with hydrogen peroxide in order to technically extinguish the endogenous peroxidase activity. After that, the sample was incubated with a primary antibody that had been suitably described and diluted. This was followed by successive incubations with biotinylated link antibodies that contained anti-rabbit immunoglobulins and streptavidin that had been labeled with peroxidase. After 5-7 minutes of incubation with the substrate chromogen DAB, which produced a brown precipitate at the tissue's antigen sites, the staining process was finished. All immunostained slides were analyzed using Zeiss microscope with high resolution (Axioscope, Germmany) at power x400 in 10 successive high-power fields. Caspase-3 antigens were expressed as brown nuclear staining. Apoptotic index (%) = active caspase-3 immunopositive cells number x100/total cells number of the field according to (Duan et al., 2003).

2.10. Histopathological evaluations

Following a blood sample, each group's liver tissue was promptly extracted. A tiny section of liver tissue from the anterior region of the left lateral lobe were removed, and cleaned with normal saline. Subsequently, 3-5 mm thick and 1 cm long tissue fragments were cut, placed in tissue cassettes with labels, and preserved in 10% buffered formalin. Fixed tissues underwent standard processing, were embedded in paraffin, and sectioned into 5 µm thick pieces. These sections were then placed on slides for staining with hematoxylin and eosin. A binocular digital microscope (SCO Tech GmbH, Germany) was used to inspect and take pictures of every part of the liver and kidney in order to identify any pathological alterations. A certified pathologist from Theodor Bilharz Research Institute's pathology and electronic microscopy department performed a blind analysis of

the sections. Every sample has at least three distinct parts evaluated.

2.11. Analytical statistics

The data was shown as mean ± SEM. With GraphPad InStat (Graph software Inc., V 3.05, Ralph Stahlman, Purdue University, Lafayette, IN), significant differences across groups were assessed. Prism 5 software, version 5.00 (GraphPad Software, San Diego, CA, USA), was used to plot appropriate graphs. The ANOVA test was employed to make group comparisons. A P value of less than 0.05 was deemed statistically noteworthy.

3. Results

3.1. Impact of the diosmin+hesperidine combination on the antioxidant/oxidative criteria in the liver

Fig. 1, demonstrate the protective effects of diosmin+hesperidine and the harmful effects of gamma radiation on the liver oxidative/antioxidant criteria. Hepatic MDA was considerably reduced by (-7.0%) when oral diosmin+hesperidine at a dose of 200+100 mg/kg body weight, respectively, was administered for a month (every other day) in comparison to untreated control rats. On the other hand, rise in hepatic GSH (+3.2%), SOD (+0.6%) and GPx (+3.4%) in compared to control untreated rats.

Male albino rats exposed to a cumulative dosage of 10 Gy of gamma radiation showed a substantial rise ($p < 0.001$) in MDA of approximately (+116.2%), while cause, decline ($p < 0.05$) in hepatic GSH (-29.7%) SOD (-30.5%) and GPx ($p < 0.001$) (-28.8%), with significant compared to control untreated group.

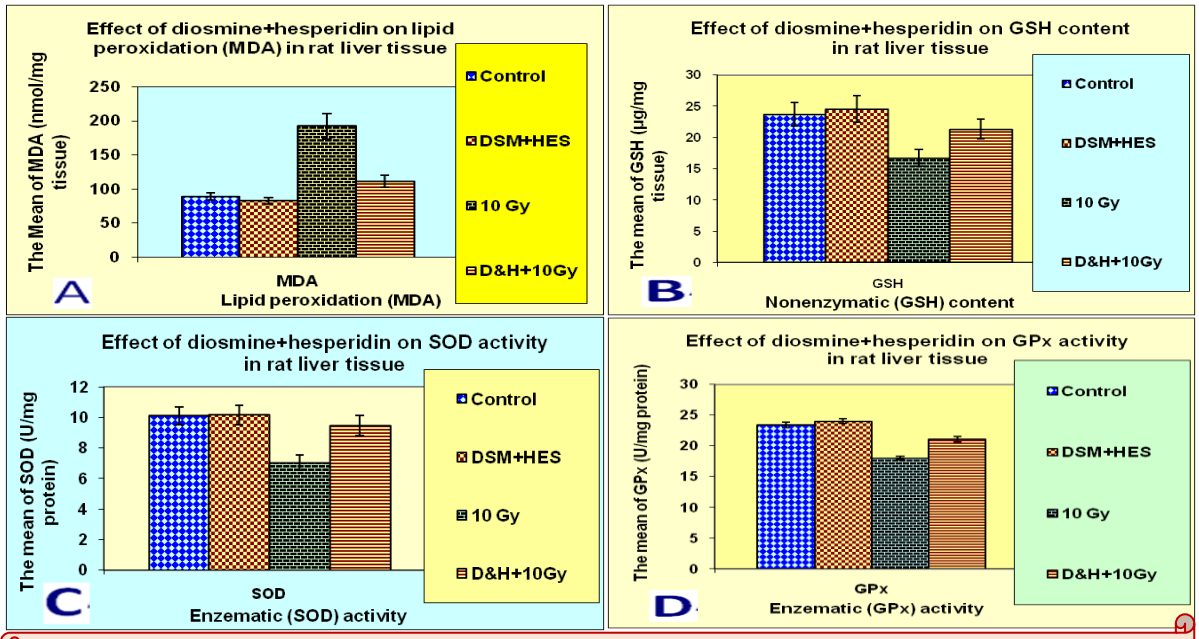


Fig 1: Showing the effect of diosmine + hesperidine on the mean ±SE of, (A) lipid peroxidation (MDA), (B) GSH content, (C) SOD activity, and (D) GPx activity in rat liver tissue.

3.2. Evaluation of indicators associated with liver injury

The findings showed that rats' livers have been impacted by a 10Gy radiation exposure. The activity of the liver enzymes ALP, GGT, AST, and ALT in the blood significantly increased ($P < 0.05$) in comparison to the control rats' corresponding levels. Rats that received diosmin+hesperidine (200+100 mg/kg body weight, respectively) before being exposed to radiation showed a marked improvement in the indicators for liver serum injury (Fig. 2).

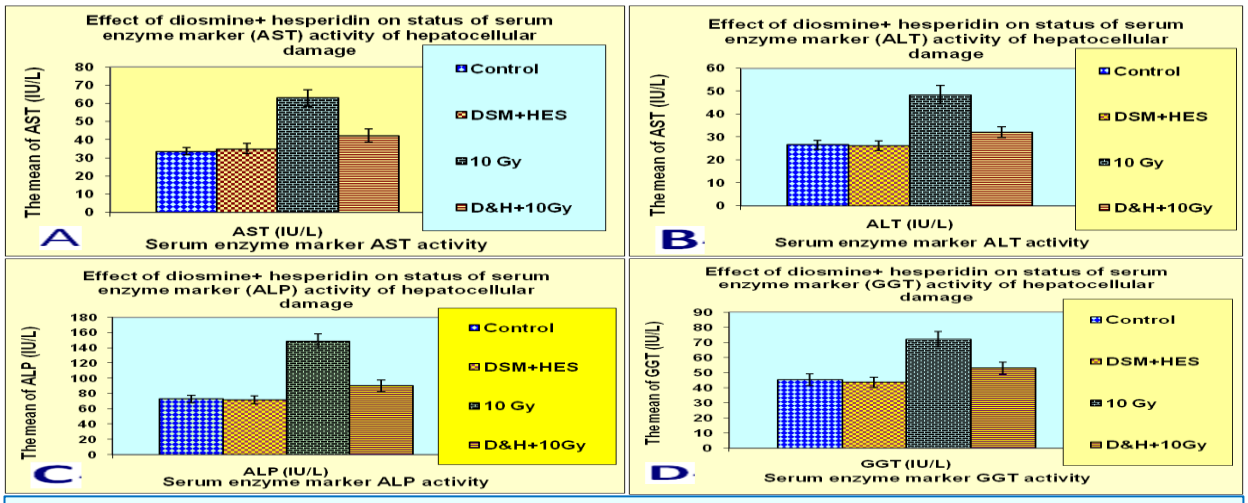


Fig 2: Showing the effect of diosmine + hesperidine on the mean±SE of liver enzyme markers indicative of hepatocellular damage (A) AST, (B) ALT, (C) ALP and (D) GGT.

3.3. Comet assay

Figures 3A–H and 4 depict the changes in the comet attribute levels that were examined in the livers of several groups of rats. The findings showed that the radiated groups had significantly higher levels of all comet features (tail length, tail moment, and percentage of DNA in the tail) than the pre-administered diosmin+hesperidine (200 + 100 mg/kg body weight, respectively) groups. D200+H100 significantly reduced the comet characteristics, which modified the radiation effects. When comparing the diosmin+hesperidine alone pre-administered group to the control group, we found no discernible increase in comet formation.

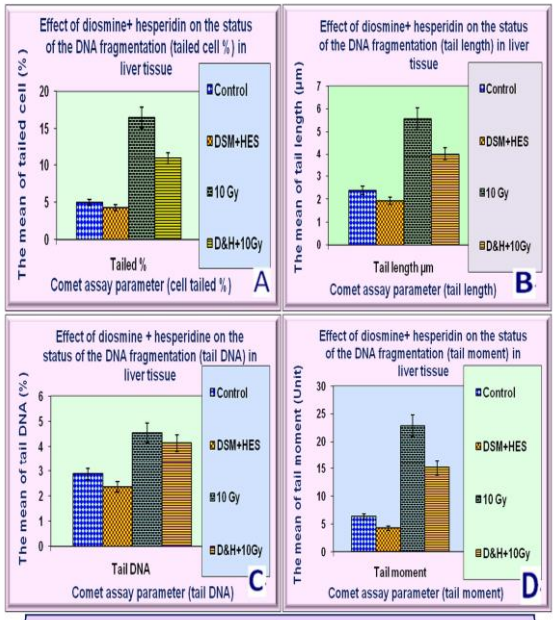


Fig 3: Showing the effect of diosmine + hesperidine on the mean±SE of DNA fragmentation (Comet assay) parameters. (A) tailed cell %, (B) tail length µm (C) tail DNA % and (D) tail moment (Unit).

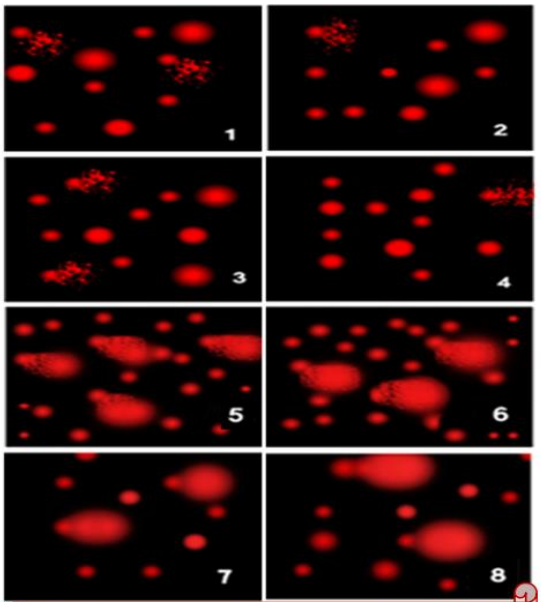


Fig 4: Photomicrographs of DNA fragmentation (Comet assay) in rat liver tissue: Pictures1, and 2 control rats & Pictures 3 and 4DSM & HES group & Pictures 5 and 6 10 Gy radiation & Pictures 7and 8 DSM&HES + 10 Gy radiation

3.4. Effect of diosmin+hesperidin and γ-irradiation on P53 and Caspase-3 genes expression in the rat liver tissue by Rt-PCR

The effect of DSM+HES administration and radiation exposure on the gene expression of two of the appoptotic genes is shown in fig. 5. The mRNA expression of P53 and caspase-3 was measured by real time PCR.

The present results illustrate a high significant down-regulation ($p< 0.01$) of P53 gene expression in irradiated rats with 10 Gy as compared to the control, DSM+HES and (DSM&HES)10Gy groups. On the other hand, a high significant up-regulation ($p< 0.01$) of Caspase-3 gene expression in irradiated rats with 10 Gy as compared to the control and DSM+HES and (DSM&HES)10Gy groups. Our results demonstrated that, the group that administrated the diosmine and hesperidine showed amelurated results neer that of control and DSM+HES groups.

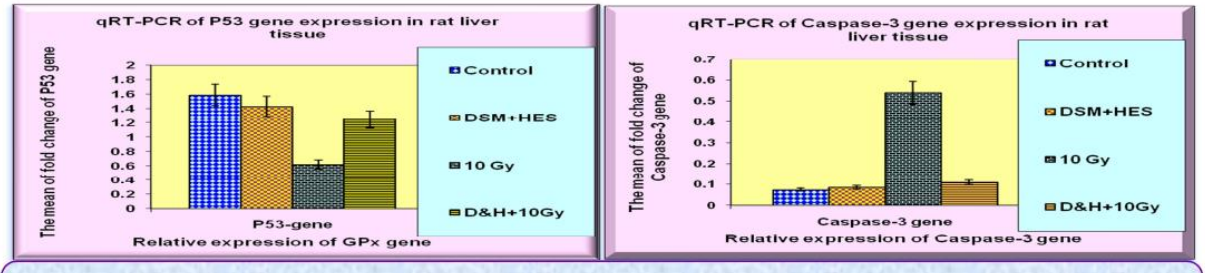
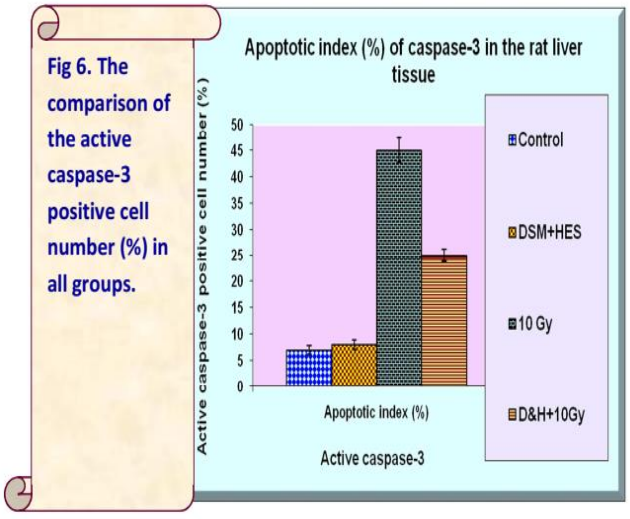


Fig 5. Showing the effect of diosmine + hesperidine on the mean ±SE of relative expression of (A) P53 gene and (B) Caspase-3 gene in the liver of control and treated rats. The results depicted are normalized to levels of β-actin gene.

3.5. Quantitative assessment of apoptosis in liver

Cells with activated caspase-3 for identification and quantitation of apoptotic cells in liver tissues were quantified by immunolocalization technique. As shown in (Fig. 6), initiation of apoptosis by 48% and 65% following irradiation of rats with (10Gy) was alleviated in groups treated with diosmin+hesperidine prior to radiation exposure.



3.6. Histopathological studies

Histopathological examination of the control group showed a normal hepatocyte pattern, (Fig. 7A). A similar structure was observed for the diosmin+hesperidine control (D200+H100) group (Fig. 7B) indicating the protective nature of the drug. The results indicated that during radiation treatment with the 10Gy cumulative dose, there was some histological changes in the hepatocytes, (Fig. 7C).

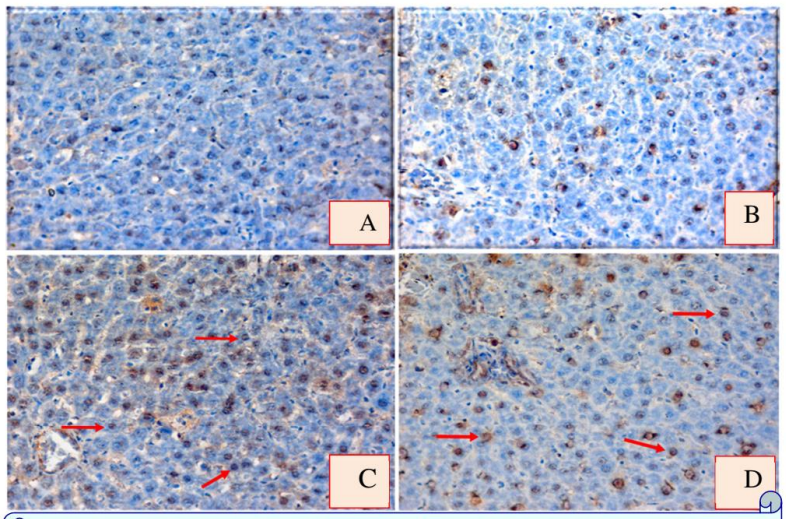


Figure 7: A liver section from (A) control rat showing few hepatocytes; 6% are a positive reaction for caspase-3, (B) liver section from DSM+HES group (+ve Control) showing few hepatocytes; 7% are a positive reaction for caspase-3 (nuclear brown), (C) liver section from 10Gy (F.D) group showed moderate number positive nuclei with expression of caspase-3 (apoptotic index about) 45% (arrows) and (D)a rat administered DSM+HES and then irradiated with 10Gy (F.D) showing 25 % positive hepatocytes for caspase-3 as nuclear brown stain, (arrows), (IHC, DAB x400).

The liver of control (A) and D+H (B) showed intact lobular hepatic architecture and normal morphological appearance as thin hepatic plates (red arrow), dilated congested central vein (black arrow). (H&E x400). The rats irradiated with cumulative dose 10Gy (C) showed intact lobular hepatic architecture and hepatocytes arranged in thin plate (red arrow), dilated congested sinusoids (black arrow), hepatocytes with binucleated nuclei (yellow arrow). (H&E x400) (Fig. 8). Rats of group D200+10Gy (D) showed preserved lobular hepatic architecture and normal morphological appearance as thin hepatic plates (red arrow), central vein (black arrow), sinusoids (yellow arrow) (H&E x400) (Fig. 8).

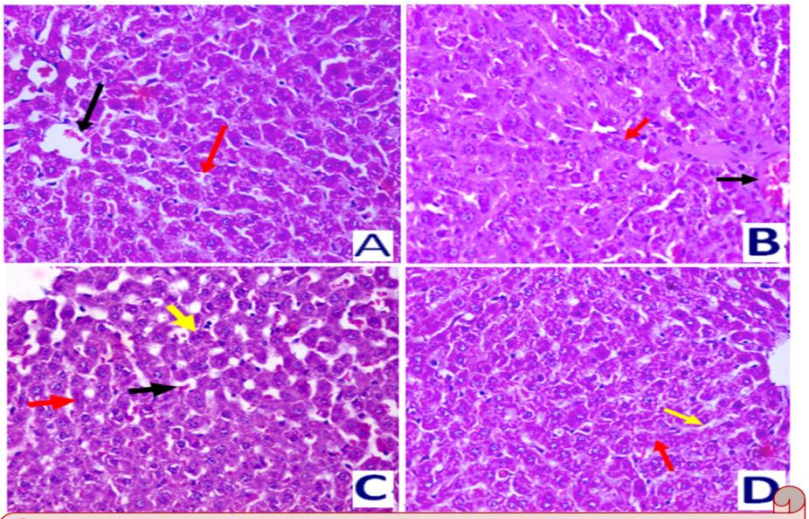


Figure 8: liver section from (A) normal control group (-ve) showed preserved (intact) lobular hepatic architecture and normal morphological appearance as thin hepatic plates (red arrow), dilated congested central vein (black arrow), (B) DSM+HES showed preserved (intact) lobular hepatic architecture and almost normal morphological appearance (red arrow), dilated congested central vein (black arrow), (C) 10Gy irradiation group demonstrated preserved (intact) lobular hepatic architecture and normal morphological appearance as thin hepatic plates (red arrow), central veins (black arrow), and sinusoids (yellow arrow) (H&E,x200,x400). Hepatocytes were arranged in thin plates (red arrow), dilated congested sinusoids (black arrow), and hepatocytes with binucleated nuclei (yellow arrow).

4. Discussion

Our findings emphasized that cumulative whole-body irradiation in rats at 10Gy doses significantly produced oxidative damage and decreased liver antioxidant measures. However, we found that diosmin+hesperidine pretreatment (200+100 mg/kg body wt., respectively) greatly reduced oxidative damage in radiation-exposed rats. While high single-dose radiation therapy might be more effective in treating cancer, as a side consequence it could injure healthy tissues more (Logani et al., 1995). Since the entire radiation dose is divided into smaller doses over the course of several days in fractionated radiation therapy, the toxicity to healthy cells is reduced while the cancer is effectively treated. This increases radiation's efficacy on cancer while sparing healthy cells (Nahum, 2015).

Radiation accidents are mainly concerned with single large doses of radiation. Oxidative stress is induced in a variety of tissues after ionizing radiation exposure (Saada et al., 2010; Sm et al., 2018). When reactive oxygen species (ROS) interact with biological membranes, a highly destructive process known as lipid peroxidation occurs. It generates highly hazardous metabolites in addition to altering the membranes' structure and function (Srinivasan et al., 2009). An increase in lipid peroxidation markers (MDA) was seen in this study after rats were exposed to gamma radiation throughout their bodies. According to Kalpana et al. (2009), this was caused by the interaction of hydroxyl radicals that were produced when water underwent radiolysis after being exposed to ionizing radiation. These radicals destroyed proteins, membrane lipids, and DNA, resulting in the formation of physical and chemical tissues as well as the eventual death of cells. The levels of SOD and GSH decrease significantly after radiation exposure, which is accompanied by an increase in oxidative products. Antioxidants could be running out because of all the work being done to reduce reactive oxygen species (ROS) (Jagetia and Reddy, 2005).

Numerous in vitro and in vivo research have validated diosmin's varied biological features. Silambarasan and Raja (2012) and others have noted its antioxidant, antihyperglycemic, antiinflammatory, antimutagenic, and antiulcer effects. It seems to reason that diosmin, like most flavonoids, would reduce oxidative stress markers due to its ability to scavenge oxygen-free radicals (Pietta, 2000). Closer inspection of this study reveals that diosmin, when administered orally prior to irradiation, restored the antioxidant enzyme SOD's activity, possibly as a result of diosmin's stimulation of reduced oxidative damage. Non-enzymatic antioxidants, such as glutathione (GSH), play an important role in defending cells against oxidative stress in addition to the enzymatic antioxidants. Diosmin treatment prior to radiation exposure resulted in GSH levels close to normal, which may have been due to less membrane damage as shown by lower lipid peroxidation. These findings corroborate the prior research that found diosmin to be an effective antioxidant (Silambarasan and Raja, 2012).

The histopathological findings showed that the antioxidant and free radical-scavenging qualities of diosmin+hesperidine, when taken orally before radiation exposure, preserved tissue integrity and reduced radiation-induced damage.

Ionizing radiation causes the most significant sort of damage, mutations or cell death, by inducing double stranded breaks in DNA (Srinivasan et al., 2008). The comet test is a sophisticated technique for monitoring single-cell DNA damage and repair (Collins, 2004). Increased comet characteristics in the bone marrow and spleen of mice subjected to whole-body irradiation (Nair and Nair, 2013) indicated radiation induced damages such as the production of single and double strand breaks. Our research showed that this type of damage was present in rats exposed to a cumulative dose of 10Gy of radiation. Irradiated rats that had been pretreated with diosmin+hesperidine showed a decrease in DNA fragment size. These results suggested that by reducing DNA's vulnerability to oxidative damage and shielding DNA from the harmful effects of ionizing radiation, diosmin+hesperidine may be useful in minimizing radiation-induced genomic damaging shocks.

This was in line with the established non-mutagenic potential of diosmin (Tanaka et al., 1997c). Diosmin has been shown in several animal studies to have an antimutagenic effect against esophageal (Tanaka et al., 1997a), hepatic (Tahir et al., 2013a), colon, and buccal pouch cancers (Rajasekar et al., 2016). Diosmin's antimutagenic action has been associated with inhibition of cellular proliferation and downregulation of inflammatory

markers (Tahir et al., 2013a; Tanaka et al., 1997b).

Overexpression of caspase 3 genes was observed in this study in rats subjected to whole-body -irradiation. Once thought to only have deleterious effects on cells, reactive oxygen species are now thought to have a role in a wide variety of cell fate decisions and signal transduction pathways (Holmström and Finkel, 2014) [20]. Ionizing radiation produces reactive oxygen intermediates, which can set off the mitochondrial pathway, releasing caspase-activating factors. Radiation can cause apoptosis, and oxidative stress may play a direct part in this process (Shinomiya, 2001) [21].

Mitochondrial p53 increase occurs in radiosensitive tissues like the thymus, spleen, and testis in normal mice exposed to whole body -irradiation (5 or 10 Gy), but not in radioresistant organs like the liver and kidneys (Vaseva and Moll, 2009, p. 23). Consistent with these findings, we found that the mRNA level of p53 decreased in irradiated rats.

Cell type, cellular environment, and signal type all play a role in determining the p53 response that is ultimately activated. Tissue-specific regulation of the upstream signals that direct p53 induction was shown by Komarova et al. (2000) [24]. Marchenko et al. (2000) [25] show that a subset of stress-stabilized wild-type p53 rapidly translocates to the outer mitochondrial membrane during p53-dependent apoptosis. However, p53 can also cause apoptosis by causing the release of cytochrome c from the mitochondria (Vuong et al., 2012)[26] by increasing the expression of Bax, a regulator of mitochondrial membrane permeabilization. While this relocation takes place during p53-dependent apoptosis, many studies have shown that radiation-induced apoptosis occurs regardless of the function and presence of p53 (Narti et al., 1998; Shostak et al., 1999; [27], [28]).

It has been suggested that diosmin has an anti-apoptotic impact via inhibiting caspase-3 expression (Rehman et al., 2013). Damaged cells that have been subjected to cytotoxic stress (such as radiation) commit suicide by progressively destroying their own cellular components via an enzyme caspase (Taylor et al., 2008). Caspases, or executioner caspases, are linked to the start of the "death cascade". Because of this, it is an essential marker for determining when a cell has begun the apoptotic process (Nicholson et al., 1995). Caspases 3 and 4, when activated by radiation exposure, cause cell death through the apoptotic pathway (Marini et al., 2009). Caspase-3 activity and apoptosis were reduced in diosmin-treated rats, correlating with the reduced radiation-induced DNA damage seen in the comet assay. The current investigation found that the diosmin+hesperidine dose (200+100 mg/kg body wt.) was much more effective than the (100 mg/kg body wt.) in preventing oxidative stress, DNA damage, and tissue necrosis. In addition, the treated healthy rats showed no toxicity indications at this level. Meyer (1994) showed that Daflon 500 mg was completely safe for use in animals with LD50 values more than 3000 mg/kg, which is in line with these findings. He also showed that, even after a year of treatment, 500 mg of Daflon was well tolerated in clinical trials, with just mild side effects recorded.

Finally, by reducing oxidative stress, averting DNA damage and apoptosis, and increasing antioxidant status and anti-peroxidative potential, the results of this study imply that diosmin+hesperidine may have a protective impact against radiation-induced tissue damage. Diosmin+hesperidine might be a useful medication to prevent radiation-induced damage to healthy tissues because larger radiation doses are utilized in clinical radiotherapy to better control cancer or in the event of a radiation mishap. It can also shield workers from radiation hazards in the workplace.

5. Conclusion and recommendation

The results of the study show that using protective doses of diosmin and hesperidin before exposure to radiation doses has a protective role against the harmful effects of radiation. Therefore, the study recommends the use of diosmin and hesperidin for people who are exposed to radiation in their workplaces as a type of natural products have radioprotectors and antioxidants that protects against radiation and its harmful effects.

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7. Declaration of competing interest

The authors declare that none of the work reported in this study could have been influenced by any known competing financial interests or personal relationships.

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