Egyptian Journal of Chemistry

The protective role of Diosmin, Hesperidine combination against heavy metals toxicity in Wistar albino rats: Biochemical, Immunohistochemical and Molecular Studies.

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Abstract

The benchmark of this study is to evaluate the antioxidant protective efficiency of Diosmin-Hesperidin combination, a natural citrus flavone of hesperidin derivative on heavy metals intoxication and Oxidative stress-induced damage in Wistar albino rats. Oral doses of diosmin-hesperidin in rats (200 and 100 mg/kg body weight, respectively) for a month (every other day) prior to heavy metals intoxication. Evaluation of the protective and antioxidant effects of the combination of diosmin and hesperidin, various Rt-PCR estimations, biochemical estimations, histopathological alterations as well as comet assay and caspase-3 activity for assessment of apoptosis were performed. Results indicated that heavy metals intoxication-induced decline in the levels of liver tissue P53 gene expression and increase of liver tissues of the apoptotic caspase-3 gene, also induced decline in the levels of liver tissue antioxidant parameters (SOD, GPx, and GSH), increased lipid peroxidation (MDA), DNA damage and apoptosis, these parameters were improved by pre-administration of diosmin+hesperidine. Diosmin+hesperidine dose (200 and100 mg/kg body wt. respectively) restored the p53 and caspase-3 genes near-normal values, antioxidant status to near normal and reduced lipid peroxidation, DNA, and tissue damage. These results were confirmed by histopathological examinations, which showed that pre-administration of diosmin+hesperidine protected the liver of albino rats against heavy metals intoxication-induced damage. Hence, it has been illustrated that diosmin+hesperidine might be an effective antioxidant and protector against heavy metals intoxication-induced damage in rats. Moreover, the diosmin+hesperidine alone pretreated group did not show any biochemical alterations, fold change of P53 or caspase-3 genes or DNA damage indicating the protective nature of the drug.

Keywords: Heavy metals intoxication, Diosmin+Hesperidine combination, Antioxidants, Reactive oxygen species, Rt-PCR, DNA damage, Apoptosis, Histopathology.

1. Introduction

Human activities play a major role in polluting the environment with toxic and carcinogenic metal compounds. There is evidence that these metals contaminate water sources and food chains and their compounds through accumulation. Hence, industrial pollution of the environment with metal compounds is becoming a serious problem. Unlike most organic pollutants, heavy metals will not degrade but will accumulate in the environment and food chain [1].

Cadmium contamination of the environment is a subject of serious international concern since the metal is known to enter the food chain and undergo bioaccumulation, endangering human health **[2]**. After absorption, cadmium is circulated in the blood, bound mainly to blood cells and albumin. It is almost all distributed to the liver and then redistributes progressively to the kidney as cadmium-metallothionein (Cd-MT). After distribution, close to 50% of the body burden is found in the liver and kidney **[3]**.

Mercury discovered thousands of years ago is one of the oldest known poisons **[4]**. Although in recent years, environmental and occupational exposures to mercury have been greatly reduced, this metal still remains a threat to human health from multiple sources: air, water, and food **[5]**. Once absorbed, mercury distributes widely to all tissues. The principal target organs of the inorganic mercury are kidney and liver **[6]**. Previous studies have revealed that HgCl2 caused histopathological and ultrastructural lesions in the liver evidenced by periportal fatty degeneration and cell necrosis **[7]**. Cadmium and mercury are considered among the most important toxic

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metals. Cadmium is present in the environment as a result of industrial and agricultural practices **[8].**

Oxidative stress is possibly involved in the pathology of some diseases and other inborn errors of lipids and protein metabolism and it was also affected liver and kidney functions **[9, 10]**. An "oxidative stress" is a consequence of an imbalance between pro-oxidant processes and effective antioxidant defense systems and finally the result of a disturbance of the "cellular redox homeostasis" **[11]**.

Plants are rich sources of polyphenols, which include anthocyanins, flavonoids, stilbenes, tannins, lignins, etc. **[12]**. Natural plant products are non-toxic with proven therapeutic benefits and have been utilized since ancient times for curing various ailments. About 60 % of the 1184 new drugs developed over the past 25 years owe their origin to natural sources **[13,14]**.
Diosmin (DS) (diosmetin 7-O-rutinoside), a natural flavone glycoside is readily obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in the pericarp of various citrus fruits **[15]**. DS possesses blood lipid lowering **[16]** and anticarcinogenic activities **[17]**. It enhances venous tone and microcirculation and protects capillaries **[18]**, mainly by reducing systemic oxidative stress **[19]**. Pharmacokinetic investigations have shown that DS is rapidly transformed by intestinal flora to its aglycone form, diosmetin. Diosmetin is absorbed and rapidly distributed throughout the body with a plasma half-life of 26–43 h. Diosmetin is degraded to phenolic acids or their glycine-conjugated derivatives and eliminated through the urine. The presence of degradation products such as alkyl-phenolic acids confirmed a metabolic pattern similar to other flavonoids. **[20]**. Chronic venous insufficiency and hemorrhoids are treated with Daflon 500, a diosmin formulation consisting of 90% diosmin and 10% hesperidin **[21]**. Diosmin has been shown to have beneficial effects on hypertension, hepatic ischemia, hepatic damage, hepatocarcinogenesis, and neurodegeneration **[22, 23, 24, and 25]**. In various trials, diosmin has also shown to protect against trichloroethylene-induced kidney damage in other studies **[26].**

Hesperidin (hesperetin-7-rhamnoglucoside), a flavanone glycoside copiously found in sweet orange and lemon, is an inexpensive by-product of citrus cultivation **[27]**. HES is effectively used as a supplemental agent and helps to reduce edema or excess swelling in the legs due to fluid accumulation. It has been reported to possess a significant anti-inflammatory, analgesic, antifungal, antiviral antioxidant, and anticancer activity. Administration of HES maintained the levels of liver enzymes to be near the normal thereby restoring the membrane function [28, 29]. **Chopra *et al.*[30]** reported that HES due to its surefootedness in providing protective effect is efficient in combating free radicals that cause oxidative stress. HES on the other hand elevated the level of antioxidant enzymes to normal status, [31].

Hence, this study aimed to explore the antioxidant capacity of the combination of diosmin-hesperidin against heavy metal poisoning and oxidative stress damage in rats. To do this, lipid peroxidation and levels of antioxidant biomarkers, Rt-PCR of P53 and Caspase-3 genes of hepatic tissue, DNA damage, and apoptosis, serum hepatic injury markers as well as the liver tissues histopathology were evaluated.

2. Materials and methods
2.1. Chemicals
Diosmin (CAS number: 520-27-4) and Hesperidine (CAS number: 520-26-3) were a gift from Egypar Pharmaceutical Industries, El-Obor, Egypt. Di-potassium hydrogen orthophosphate (K_2HPO_4) and Potassium di-hydrogen orthophosphate (KH_2PO_4) were purchased from CARLO ERBA reagents and chemicals company, Paris-France. Assay Kits for liver (AST, ALT and ALP) markers were purchased from BioMED, Egypt. SOD, GSH and Lipid peroxide assay Kits were purchased from BIODIAGNOSTIC for diagnostic and research reagents, Egypt.

2.2. Experimental animals
Male Wistar albino rats, (150–200 g) were obtained from the Egyptian Organization for Biological Products and Vaccines. They were kept for about 15 days; before the onset of the experiment under observation to acclimatize the laboratory conditions. Rats were housed in specifically designed cages and kept under standard conditions of temperature (25 ± 5 °C), lighting (light/dark cycle), and humidity (60 percent). The animals were given standard food pellets.

2.3. Heavy metals (Cadmium and Mercury) intoxication:
Cadmium (II) Chloride (Cas No., 34330-64-8) is a white crystalline compound of cadmium and chlorine, with the formula $CdCl_2$. It is a hygroscopic solid that is highly soluble in water and Mercuric (II) Chloride (Cas No., 7487-94-7) is the chemical compound of mercury and chlorine with the formula $HgCl_2$. It is white crystalline solid and is a laboratory reagent and a molecular compound that is very toxic to humans.

2.4. Experimental design
40 male albino rats were divided into 4 groups (n = 10); diosmin dose (200 mg/kg body wt.) and hesperidin (100 mg/kg body wt.) dose was selected based on previous studies **[32]** and **[33]** respectively. **Group 1:** Rats orally administered the same volume of a vehicle only by oral tube every other day for a month, this group served as

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(control) group; **Group 2** (D200+H100): Rats were administered diosmin+hesperidin at 200+100mg/kg body weight respectively, orally every other day for a month and served as diosmin+hesperidine control group; **Group 3** Heavy metals: Rats were intoxicated with Cadmium chloride (CdCl2) 100 mg/Liter + Mercuric chloride (HgCl2) 25mg/Liter in drinking water for 2 months; Group 4 (D200+H100 + HM): Rats were intoxicated with Cadmium chloride (CdCl2) 100 mg/Liter + Mercuric chloride (HgCl2) 25gm/Liter in drinking water for 2 months after oral administration of diosmin+hesperidine at 200+100 mg/kg body wt., respectively, every other day for a month. One week following the last heavy metal intoxication dose, rats were slightly anesthetized with diethyl ether and blood was collected by heart puncture by disposable plastic syringes and transferred to sterile dry test tubes and allowed to coagulate at room temperature. Collected blood samples were allowed to clot for 30 min at 25 °C, centrifuged at 1200×g, and sera were separated and stored at -20°C pending biochemical analysis. Diosmin+hesperidine-protective effect against heavy metal-induce oxidative stress damage investigated using several biochemical assys, Rt-PCR of P53 and Caspase-3 gene, DNA fragmentation (Comit assay), Immunohistochemical assay for apoptosis evaluation and Histopatology of liver tissue.

2.5. Preparation of liver homogenate

Liver was excised, samples were taken, blood was cleared off, and then the samples were immediately placed in ice-cold containers containing 0.9 percent saline. A known amount of tissue was homogenized using appropriate phosphate buffer (according to the assay procedures) in a tissue homogenizer (Glas-Col®, Cat no.099C K6424, TERRE HAUTE USA). For performing biochemical assays, a 10% liver homogenate in 10 mM phosphate buffer was prepared, centrifuged at 10,000×g for 15 min at 4 °C and the supernatantwas collected and stored at −80 °C to perform GSH and totalthiobarbituric acid reactive substances (TBARS) determination tests.

For SOD test, 0.5 ml of ice-cold extraction reagent "provided with SOD kit" was added to 1 ml of supernatant, vortex for 30s. Then centrifuged at 4000×g for 10 min at 4 °C and the aqueous upper layer was collected and kept at 4 °C for immediate assay.

2.6. Antioxidant evaluation

To evaluate lipid peroxidation, the method described by **Ohkawa et al. [34]** was used to measure the hepatic tissue content of malondialdehyde (MDA), 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. [35]**.
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.

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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: The Oligonucleotide Primers Sequence:

Gene	Primer sequence	
p53 **Longxi et al. [36]**	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

Central fragments from liver were crushed, transferred to 1 ml icecold phosphate buffered saline (PBS). This suspension was stirred for 5 min then filtered. 100 μl of Cell suspension was mixed with 600 μl of low-melting agarose. On pre-coated slides, 100 μl of this mixture was dispersed. After 15 minutes in lysis buffer, the coated slides were electrophoresed for 2 minutes at 100 mA. At 4 °C, stain with 20 μg/ml ethidium bromide (EtBr). Observations of EtBr-stained DNA were made using a 40x objective on a fluorescent microscope for visualization of DNA damage; a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera was used to determine the quantitative and qualitative level of DNA damage in the cells by measuring the length and percentage of of DNA migration. The program then estimated the tail moment. In most cases, 50 to 100 cells were randomly selected and evaluated per sample **[37].**

2.9. Quantitative assessment of apoptosis by immunohistochemistry to active Caspase-3 in liver

Using polyclonal anti-rabbit Caspase-3 antibody CPP32, Ab-4 (Cat. #RB-1197-R7 ready-to–use for immunohistology), ThermoScientificTM, Fremont, CA 94539, USA), and a detection kit DAKO LSAB® System-HRP, immunolocalization for caspase-3 was performed on 3-4m thickness liver sections as described by **Krajewska et al.** (1997). (DAB, DAKO, Denmark). By treating the specimen with hydrogen peroxide, the natural peroxidase activity was technically inhibited. The specimen was then treated with a specific primary diluted antibody, followed by successive incubations with biotinylated link antibodies containing peroxidase labelled streptavidin and anti-rabbit immunoglobulins. After 5–10 minutes of incubation with the substrate chromogen DAB, the staining was complete, resulting in a brown-colored precipitate at the antigen sites. 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.[38].**

2.10. Histopathological examination

The liver tissues from each group were promptly isolated after blood sampling. A little amount of hepatic tissue from the left lateral lobe's anterior section, were removed and washed with normal saline. The tissues were then sliced into minute sections (3–5 mm thick and 1 cm long), placed in labelled tissue cassettes, and preserved in 10% buffered formalin. Fixed tissues were treated as usual, fixed in paraffin and sliced into 5 μm thick sections for hematoxylin and eosin staining. A binocular digital microscope (SCO Tech GmbH, Germany) was used to view and photograph all liver sections in order to determine pathophysiological alterations. A certified pathologist from Theodor Bilharz Research Institute's pathology and electronic microscopy department analyzed the blinded sections. In each sample, at least three different parts were evaluated.

2.11. Statistical analysis

Data were represented as mean ± SEM. Significant differences between groups were tested using GraphPadInStat (Graph software Inc., V 3.05, Ralph Stahlman, Purdue University, Lafayette, IN). Appropriate graphs were plotted using Prism 5 software, version 5.00 (GraphPad Software, San Diego, CA, USA). To make comparisons between groups, the ANOVA test was performed. A statistically significant P value was less than 0.05.

3. Results

3.1. Effect of diosmin+hesperidine on the hepatic oxidative/antioxidant criterion.

Table 1 show the toxicity effects of heavy metal intoxication which cause significant rise (p < 0.01) in hepatic MDA (129.4%) as compared to control group, and a significant decrease (p < 0.01) in GSH (-32.9%), SOD (-33.7%) and GPx (-28.8%) as compared to control group.

The amolurative effects of diosmin+hesperidine on the hepatic oxidative/antioxidant criterion. Oral administration of diosmin+hesperidine at a dose of 200+100 mg/kg body wt. respectively, for a month (every other day) caused results to near to normal as showin in the hepatic MDA was significantly increased by **(36.8%)** compared to control untreated rats. On the other hand, decreased in hepatic GSH **(-14.8%)**, SOD **(-10.9%)** and GPx **(-15.1%)** in compared to control untreated rats.

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Table 1: Hepatic oxidative/antioxidant parameters of male rat groups:

Group		MDA (nmol/g tissue)	GSH (mg/g tissue)	SOD (U/g tissue)	GPx (U/mg protein)
					Parameters
Control	Mean	89.1	23.7	10.12	23.43
	±SE	±5.62	±1.92	±0.7	± 1.86
$D_{200}+H_{100}$	Mean	82.9	24.55	10.18	24.02
	±SE	±5.18	±2.16	±0.84	± 1.95
	% of change	(-7.0%)	(+3.2%)	(+0.6%)	(+2.53%)
Heavy metals	Mean	204.4 a'b'e'f'	15.95 a'b'	6.71 a'b'e'	16.7 a'b'
	±SE	±9.5	±1.0	±0.48	± 0.86
	% of change	(+129.4%)	(-32.9%)	(-33.7%)	(-28.8%)
$(D_{200}+H_{100})+$ Heavy metals	Mean	121.9 abc'd'	19.91	9.02	19.91 b
	±SE	±9.8	±1.52	±0.68	± 1.68
	% of change	(+36.8%)	(-14.8%)	(-10.9%)	(-15.1%)

- *Each value represents the mean ± SE of 10 Rats in each group.*
a= P<0.05 & a'= P< 0.01; Significantly different from control group.
b= P<0.05 & b'= P< 0.01; Significantly different from D&H Group.
c= P<0.05 & c'= P< 0.01; Significantly different from heavy metals group.
d= P<0.05 & d'= P< 0.01; Significantly different from D&H + heavy metals group.
% of change; change of results of different groups from control group.

3.2. Assessment of liver injury markers
The toxicity effects of heavy metal intoxication has affected the liver. This was denoted through a significant increase (P < 0.01) in the activity of serum AST (96.0%), ALT (104.5%), ALP (139.4%), and GGT (91.7%) liver enzymes, as compared to their respective values in the control rats.

Administration of diosmin+hesperidine prior to heavy metal toxicity of rats has induced a significant improvement in the liver serum injury biomarkers. Moreover, diosmin+hesperidine dose (200+100 mg/kg body wt. respectively) control group didn't show any significant change in liver injury biomarkers, (Table 2).

3.3. Comet assay
The alteration in the levels of comet attributes analyzed in the liver of different groups of rats is shown in (table 3) (Fig. 1). The results indicated a prominent increase in the levels of all comet attributes (tail length, tail moment, % DNA in the tail) in the heavy metal groups compared to diosmin+hesperidine (200 + 100 mg/kg body wt., respectively) pre-administered groups. $D_{200}+H_{100}$

modulated the effects of heavy metal intoxication by decreasing the levels of comet attributes to a significant level. In diosmin+hesperidine alone pre-administered group, we observed no significant increase in the comet formation when compared to control group.

3.4. Effect of diosmin+hesperidin and heavy metal intoxication on P53 and Caspase-3 gene expression in the rat liver tissue by Rt-PCR:
Table 4 shows that heavy metal intoxication caused a significant elevation in hepatic mRNA of caspase 3 (p<0.001), compared to the control group. On contrary, mRNA levels of p53 were significantly reduced (p<0.001) in heavy metal intoxicated rats, compared to normal non-heavy metal toxicated rats.

Multiple comparison analysis showed that pretreatment of heavy metal intoxicated rats with a combination of diosmine and hesperidine caused a more pronounce reductions in hepatic mRNA levels of caspase 3 (3.9-fold), while more pronounced elevation was recorded in mRNA level of p53 which reached 1.9-fold, compared to the heavy metal intoxicated group.
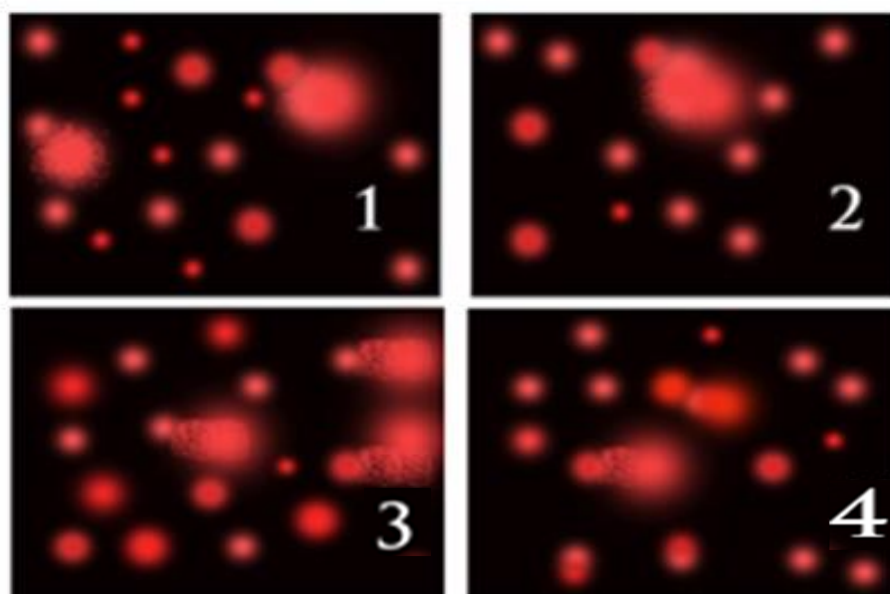
Table 2: Liver injury markers for all rat groups:

Group		AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)
					Parameters
Control	Mean	33.66	26.6	72.8	45.4
	±SE	±2.1	±1.92	±4.6	±3.78
$D_{200}+H_{100}$	Mean	35.09	26.2	71.8	43.6
	±SE	±2.8	±2.16	±5.2	±3.66
	% of change	(4.3%)	(-1.7%)	(-1.4%)	(-3.9%)
Heavy metals	Mean	66.0 a'b'e'f'	54.4 a'b'e'f'	174.0 a'b'ce'f'	87.1 a'b'ce'f'
	±SE	±5.2	±4.7	±14.5	±6.33
	% of change	(96.0%)	(104.5%)	(139.4%)	(91.7%)
$(D_{200}+H_{100})+$ Heavy metals	Mean	44.2 abc'd'	35.3 abc'd'	111.4 a'b'c'd'	62.0 a'b'c'd'
	±SE	±3.8	±3.12	±8.7	±4.99
	% of change	(31.3%)	(32.6%)	(53.8%)	(36.5%)

Legends as in table (1).

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Table 3: Effect of diosmin+hesperidin and heavy metal (Cd+Mg) of the DNA fragmentation (Comet assay) in the liver tissue:

Group		Parameters				
		Tailed %	Untailed %	Tail length μm	Tail DNA %	Tail moment UNIT
Control	Mean	5.0	95	2.39	2.87	6.28
	±SE	± 0.4	± 8.9	± 0.19	± 0.23	± 0.48
$D_{200}+H_{100}$	Mean	4.3	95.7	1.93	2.36	4.24 a
	±SE	± 0.39	± 9.1	± 0.16	± 0.20	± 0.37
	% of change	(-14%)	(0.7%)	(-19.3%)	(-17.7%)	(-32.4%)
Heavy metals	Mean	21.5 a'b'c'd'	78.5	6.0 a'b'e'f'	5.57 a'b'	30.53 a'b'd'
	±SE	± 1.9	± 6.8	± 0.51	± 0.45	± 2.5
	% of change	(330%)	(-17.4%)	(150.4%)	(94.2%)	(386.1%)
$(D_{200}+H_{100})+$ Heavy metals	Mean	14.75 a'b'c'	85.25	4.15 a'b'c'd'	4.91 a'b'	18.6 a'b'c'
	±SE	± 1.3	± 7.9	± 0.36	± 0.41	± 1.5
	% of change	(195%)	(-10.3%)	(73.3%)	(71.2%)	(196.1%)

Legends as in table (1).

Fig 1: Photomicrographs of DNA fragmentation (Comet assay) in rat liver tissue: Picture: 1, control rats & Picture: 2, DSM & HES group & Pictures: 3 heavy metals & Pictures 4 DSM&HES + heavy metals

Table 4: **Hepatic mRNA Levels of P53 and Caspase 3 in Different Studied Groups (Mean±SE).**

Group		Parameters	
		Relative Fold Change in Hepatic P53 gene	Relative Fold Change in Hepatic Caspase-3 gene
Control	Mean	1.59	0.075
	±SE	±0.52	±0.005
$D_{200}+H_{100}$	Mean	1.43	0.086
	±SE	±0.20	± 0.006
	% of change	(-16%)	(11%)
Heavy metals	Mean	0.46a'b'd	0.69a'b'd
	±SE	±0.38	± 0.16
	% of change	(-113%)	(615%)
$(D_{200}+H_{100})+$ Heavy metals	Mean	1.02a'b'c	0.23a'b'c
	±SE	±0.04	± 0.02
	% of change	(-57%)	(155%)

Legends as in table (1).

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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. 2), initiation of apoptosis by 55% following intoxication of rats with cadmium+mercury, then appoptotic cell was alleviated to near 35% in groups treated with diosmin+hesperidine prior to heavy metal intoxication as compared to control rats.

Table 5. Apoptotic indices (%) of caspase-3 positive cells in the liver tissue:

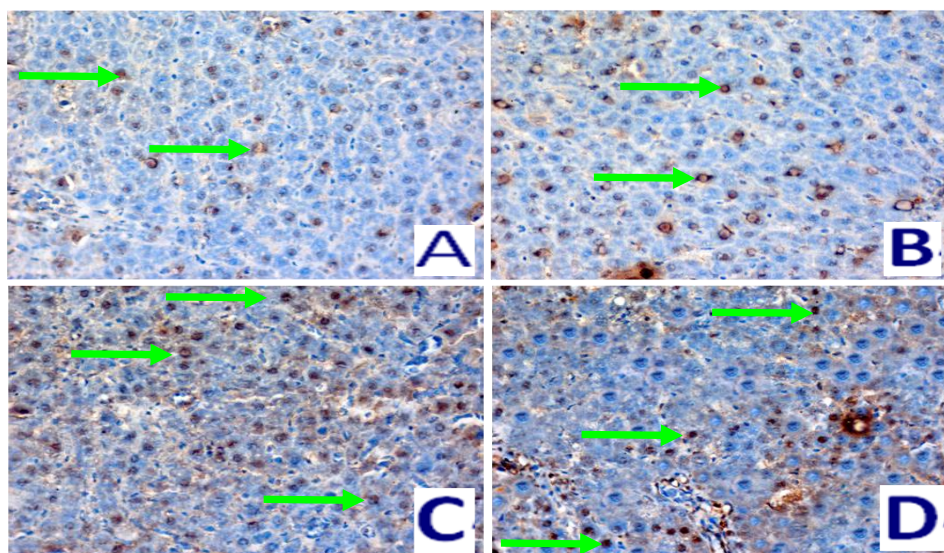| Group		Parameter
		Apoptotic Index (%)
Control	Mean ±SE	7.9±0.65
$D_{200}+H_{100}$	Mean ±SE	8.4±0.76 a
Heavy metals	Mean ±SE	55.7 ±5.2 a'b'd
$(D_{200}+H_{100})$+ Heavy metals	Mean ±SE	35.8 ±3.48 a'b'c

Legends as in table (1).

Figure 2: A liver section from (A) control rat group showing few hepatocytes; 8% are a positive reaction for caspase-3, (B) liver section from DSM+HES group (+ve Control) showing few hepatocytes; 9% are a positive reaction for caspase-3 (nuclear brown), (C) liver section from heavy metals group showed moderate number positive nuclei with expression of caspase-3 (apoptotic index about) 56% (arrows) and (D) liver section from a rat administered DSM+HES and then intoxicated with heavy metals, DSM+HES & heavy metals group showing 36 % positive hepatocytes for caspase-3 as nuclear brown stain, (arrows), (IHC, DAB x400).

3.6. Histopathological studies

Histopathological examination of the control group showed a normal hepatocyte pattern, (Fig. 3A). A similar structure was observed for the diosmin+hesperidine control (D200+H100) group (Fig. 3B) indicating the protective nature of the drug. The results indicated that during intoxication with the heavy metals (mercuric and cadmium), there was some histological changes in the hepatocytes, (Fig. 3C). On the other hand, the histological changes in heavy metal intoxicated rats group was alleviated to near normal hepatic architecture and normal morphological appearance in heavy metal intoxicated rats group that treated with diosmine+hesperidine.

The liver of **control (A)** showed preserved (intact) lobular hepatic architecture and normal morphological appearance as thin hepatic plates (black arrow), **D+H (B)** showed preserved (intact) lobular hepatic architecture and almost normal morphological appearance, with congested central veins (black arrows), **heavy metals (C)** showed partial loss of hepatic architecture, hepatocytes show mild apoptotic changes (yellow) and necrotic changes (black),Rats of group **D200+H100 & heavy metals (D)** showed preserved hepatic lobule architecture and mild hydropic degeneration (black arrow) and mild spotty necrosis (red arrow).
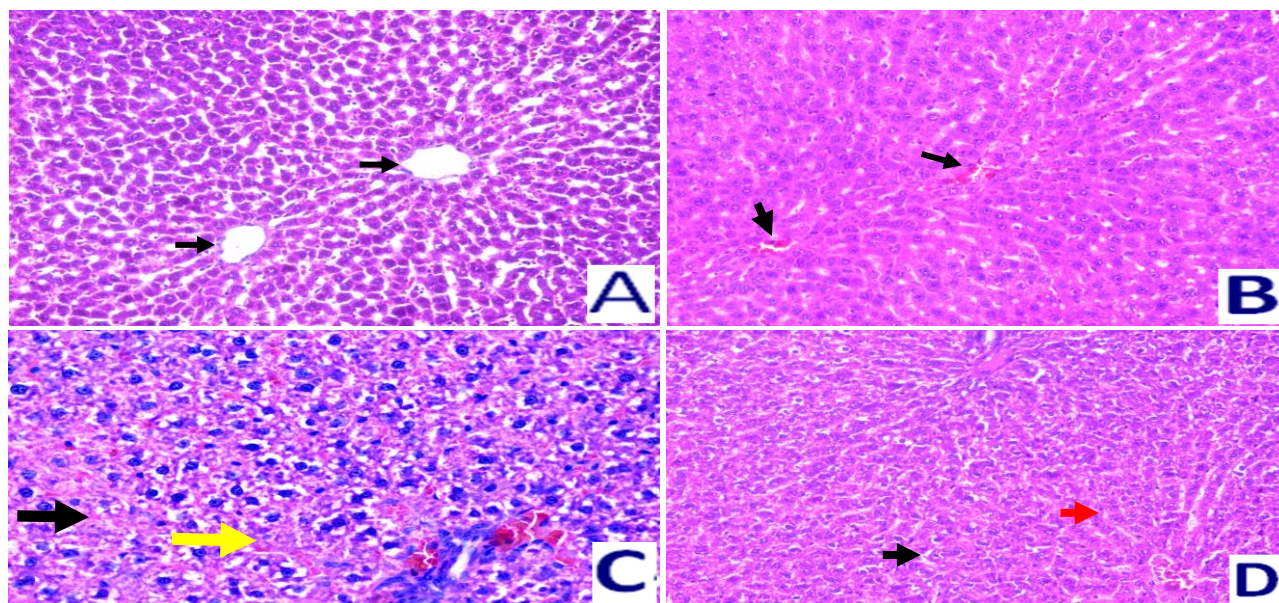
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Figure 3: liver section from (A) **normal control group (-ve)** showed preserved (intact) lobular hepatic architecture and normal morphological appearance as thin hepatic plates (black arrow), **(H&E, x200),** (B) **DSM+HES group** showed preserved (intact) lobular hepatic architecture and almost normal morphological appearance, with congested central veins (black arrows) (H&E, x200). (C) **Heavy metals group** showed partial loss of hepatic architecture, hepatocytes show mild apoptotic changes (yellow) and necrotic changes (black) (H&E, x400), and (D) **DSM&HES + Heavy metals group** showed preserved hepatic lobule architecture and mild hydropic degeneration (black arrow) and mild spotty necrosis (red arrow) (H&E, x200).

4. Discussion

Our results highlighted the fact that intoxication with heavy metals significantly induced oxidative injury and reduced the hepatic antioxidant parameters. On the other hand, we demonstrated that pretreatment of rats with diosmin and hesperidin at (200 and 100 mg/kg body wt. respectively) significantly ameliorated oxidative injury induced by heavy metals toxicity. Notably, 200 mg of diosmin +100 mg of hesperidine was more effective in reducing heavy metals-induced injuries than that treatment with each substance in a single form. Heavy metal ions exhibit cytotoxicity and significantly affect on the health of the human [39]. They may increase the release of reactive oxygen species (ROS) that results in cell damage due to oxidative stress [40].

The present study was performed in order to discern the effects of combined exposure to cadmium and mercury on liver function and histopathological alterations in male adult Wistar rats. Cadmium (100 mg/l) and mercury (25 mg/l) were combined and given orally for 8 weeks in the current study. The reason for investigating cadmium and mercury is that they are usually found in the same polluted environments.

High mercury concentrations in the tissues led to a significant depletion of the liver [41]. The mechanism of oxidative damage associated with increased lipid peroxidation, thiobarbituric acid reactive substances, and

the loss of protein-bound thiols. Mercury can bind to sulfhydryl groups of proteins, which leads to a decrease in thiol EEl activity [42]. This may be due to the distortion of metal-responsive transcription factor-1 (MTF-1). MTF-1, also metal regulatory transcription factor-1, is a pluripotent transcriptional regulator involved in cellular adaptation to various stress conditions, primarily exposure to heavy metals but also to hypoxia or oxidative stress [43]. The interaction between cadmium (Cd) and mercury (Hg) can be a good example. In fact, cadmium and mercury have proved to be extremely toxic to mankind while their usage in various industries has increased rapidly in this century [2].

Cadmium pollution in the environment is a serious international problem because it is well known that cadmium enters the food chain and undergoes bioaccumulation, thereby endangering human health [2]. After absorption, cadmium circulates in the blood and mainly binds to blood cells and albumin. It is almost all distributed to the liver and then redistributes progressively to the kidney as cadmium-metallothionein (Cd-MT). After distribution, close to 50% of the total body burden is found in the liver and kidney [3].

Mercury identified thousands of years ago is one of the oldest toxicants known [4]. Although in recent years, environmental and occupational exposures to mercury have been greatly reduced, this metal still remains a threat

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to human health from several sources: air, water, and food [5]. Once absorbed, mercury distributes widely to all tissues. The principal target organs of the inorganic mercury are the kidney and liver [6]. Previous studies have revealed that HgCl2 caused histopathological and ultrastructural lesions in the liver evidenced by periportal fatty degeneration and cell necrosis [7].

Due to human activities, such as mining and smelting, metal pollution is becoming a major risk to many ecosystems. Among the metals that cause pollution, cadmium and mercury are considered non-essential elements and have no known physiological functions. They are extremely toxic to plants and animals, have a long half-life, and are extremely persistent in the environment [44]. Through this study, we investigated some effects of simultaneous coexposure to inorganic cadmium and mercury in the function of the liver and kidney and their histological structure in the rat. Both substances are hepato- and nephrotoxic, but they affect these organs in different ways [45].

Liver damage, followed by cadmium [2] and mercury exposure [46] is well established by the elevated levels of serum hepatic marker enzymes indicating the cellular leakage and loss of functional integrity of hepatic membrane architecture. High levels of aspartate transaminase (AST) and alanine transaminase (ALT) are the crucial parameters to detect liver damage [47]. In the present study, the results demonstrated that cadmium chloride alone increased biochemical parameters such as ALT and AST. [48] have also observed a similar type of results in rat serum when treated with cadmium. Other studies also observed that the liver damage in Cd-treated mice was mainly due to the elevation of AST and ALT levels in serum [49].

Diosmin and hesperidine has various biologic properties confirmed by numerous in vitro and in vivo studies. It acts as an antioxidant, antihyperglycemic, anti-inflammatory, antimutagenic, and antiulcer agent [21]. Diosmin and hesperidine, like most flavonoids, has the ability to scavenge oxygen-free radicals [50], and hence, they should also decrease oxidative stress markers. A closer look at this study, oral treatment with diosmin+hesperidine prior to cadmium and mercury intoxication has reversed the heavy metals induced decreased activity of the antioxidant enzyme; SOD that could be a result of diosmin+hesperidine stimulated diminished oxidative damage. Apart from the enzymatic antioxidants, non-enzymatic antioxidants such as GSH play an excellent role in protecting the cells from oxidative threats.

Treatment with diosmin+hesperidine before heavy metal intoxication brought GSH to near-normal levels, which could be as a result of decreased membrane damage as evidenced by decreased lipid peroxidation. These findings support prior research that found diosmin+hesperidine to be effective in preventing oxidative stress. The present findings support prior researchs that stated that diosmin+hesperidine are effective in preventing oxidative stress [21]. The observed histopathological results confirmed that oral administration of diosmin prior to radiation exposure preserved the integrity of tissues and reduced the damage caused by the irradiation effect due to the free radical scavenging properties and antioxidant nature of diosmin and hesperidine.

DNA is a target for oxidative stress because reactive oxygen species caused double-stranded breaks which can lead to mutation or cell death [15]. The comet assay is a simple method for determining DNA damage, strand breakage, and repair in single cells [51].Comet assay of murine tissues as bone marrow and spleen of the oxidative stress mice showed increased comet parameters indicating reactive oxygen species induced damages like the formation of single and double-strand breaks [52]. In our Study, this damage was evident in the intoxicated rats with both cadmium and mercury in combination form. Decreased DNA fragments were observed in rats pretreated with diosmin + hesperidin. These results indicated that diosmin+hesperidinereduced the susceptibility of DNA to oxidative damage and protected DNA from deleterious effects of heavy metal intoxication and hence it might be useful to prevent cadmium+mercury-induced genomic damage insults.

This was in line with diosmin and hesperidin's antimutagenic characteristics. Diosmin and hesperidin antimutagenic effect against hebatic, colon and esophageal, has been revealed in several animal experiments. Diosmin'santimutagenic activity was attributed to the downregulation of inflammatory markers and inhibition of cellular proliferation [25, 53, 54, and 55].

In the present study, rats exposed to cadmium+mercury intoxication resulted in overexpression of caspase 3 genes. Reactive oxygen species, which were originally characterized in terms of their harmful effects on cells are increasingly implicated in various cell fate decisions and signal transduction pathways [56]. Reactive oxygen intermediates induced by oxidative stress as a result of cadmium+mercury toxicity can trigger mitochondrial pathways to release caspase-activating factors. Hence, oxidative stress may play a direct role in heavy metal-induced apoptosis [57]. **Vaseva and Moll** revealed that in normal mice exposed to heavy metal Cadmium+mercury mitochondrial p53 accumulation occurs in oxidative stress-sensitive organs like thymus, spleen, and testis, but not in radio-resistant organs like liver and kidney [58]. These results are in line with our results which show a decrease in the mRNA level of p53 in cadmium +mercury

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intoxicated rats. In fact, the choice of response to p53 is strongly influenced by the cell type, the cellular environment, and the type of signal.

Komarova *et al.* demonstrated that the upstream signals that direct the induction of p53 is controlled in a tissue-specific manner [59]. **Marchenko** *et al.* demonstrate that during p53-dependent apoptosis, a fraction of stress-stabilized wild-type p53 rapidly translocates to the mitochondrial outer membrane [60]. Alternatively, p53 upregulates the expression of Bax, a regulator of mitochondrial membrane permeabilization, and induces apoptosis by the release of cytochrome c from the mitochondria [61]. This translocation occurs during p53-dependent apoptosis, however, there are still many experiments that show that heavy metal-induced apoptosis occurs independently of the function and presence of p53 [62, 63].

Additionally, diosmin and hesperidin may have an anti-apoptotic impact by inhibiting caspase-3 production [26, 33]. Caspases are important enzymes in the apoptosis process, which occurs when damaged cells are exposed to cytotoxic stress (e.g., heavy metals) systematically destroy their own cellular components to commit suicide [64]. Caspase-3 is an executioner caspase related to the initiation of the "death cascade." Thus, it is an important marker of the cell's entry point into the apoptotic pathway [65]. Activation of caspase-3 after intoxication with cadmium+mercury induces cell death through the apoptotic pathway [66].

Diosmin+hesperidin treated rats showed decreased caspase-3 activities and decreased apoptosis, consistent with comet assay results that showed decreased heavy metal-induced DNA damage in the diosmin+hesperidin treated group. In the current study, the diosmin+hesperidin dose (200+100 mg/kg body wt. respectively) showed a more significant beneficial effect against oxidative stress, DNA, and tissue damage. Moreover, this dose showed no signs of toxicity in the healthy treated rats. These findings are consistent with **Meyer,** who showed the perfect safety of Daflon 500 mg in animals with LD50 greater than 3000 mg/kg. He also showed that Daflon 500 mg in the clinical trials was well tolerated with rare and mild side effects over long-term treatment up to one year [21].

5. conclusion
According to the results obtained in this study, we have shown that diosmin+hesperidin exerted its antioxidant protective effects by attenuating oxidative stress, inhibiting heavy metals-induced DNA damage and apoptosis, increasing the antioxidant status, and anti-peroxidative potential thus might exert a beneficial impact on heavy metals-induced tissue damage. Hence, diosmin+hesperidin may be a promising drug that can

prevent heavy metals from causing damage to normal tissues, and at the same time, heavy metals that are highly toxic in the environment. It also can provide protection against occupational heavy metals intoxication.

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