

# Hesperidin mitigates DEHP-induced nephrotoxicity through anti-oxidant, anti-apoptotic, and anti-inflammatory pathway modulation: Evidence from Nrf2/HO-1/Keap-1, Bax/Bcl-2/Caspase-3, and TLR4/NF- $\kappa$ B axis activation

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## ABSTRACT

**Objective(s):** This study aimed to assess the protective role of hesperidin (HSP), a citrus flavonoid, against di-(2-ethylhexyl) phthalate (DEHP)-induced kidney toxicity in rats, focusing on oxidative stress, apoptosis, inflammation, and anti-oxidant defense pathways.

**Materials and Methods:** Thirty-five male rats were randomly assigned into five groups (7 per group): control group, DEHP-treated group (1 g/kg), DEHP + HSP (100 mg/kg) group, DEHP + HSP (200 mg/kg) group, and HSP-alone group, and treated orally for 30 consecutive days. Kidney tissues were collected for biochemical assays, including malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Gene expression of Nrf2, Keap1, HO-1, Bax, Bcl-2, Caspase-3, TLR-4, and NF- $\kappa$ B was analyzed using real-time PCR, and protein levels were evaluated using Western blotting.

**Results:** DEHP significantly increased oxidative damage and the expression of inflammatory and apoptotic markers, while decreasing anti-oxidant parameters. Co-treatment with HSP, particularly at 200 mg/kg, restored anti-oxidant balance, reduced lipid peroxidation, and down-regulated the expression of TLR-4, NF- $\kappa$ B, and Caspase-3. Moreover, HSP increased Bcl-2 levels and enhanced Nrf2/HO-1 signaling, as confirmed by both gene and protein expression data.

**Conclusion:** HSP demonstrates dose-dependent renoprotective effects against DEHP-induced nephrotoxicity in rats. The protective mechanism involves anti-oxidant enhancement and inhibition of oxidative stress-induced inflammation and apoptosis, supporting the therapeutic potential of HSP in managing phthalate-related renal injury.

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## Introduction

The increasing prevalence of environmental pollutants and their adverse effects on human and animal health has become a significant area of concern in recent years (1, 2). Among these pollutants, phthalates, particularly di(2-ethylhexyl) phthalate (DEHP), have garnered considerable attention due to their widespread use in various industrial applications, including plastic manufacturing and personal care products. DEHP is a plasticizer that enhances the flexibility and durability of polyvinyl chloride (PVC) products, making it a ubiquitous compound in modern life. However, its pervasive presence in the environment and potential toxicity has raised alarms regarding its impact on biological systems (3).

Research has revealed that DEHP exposure is associated with a range of toxicological effects, particularly in the kidneys. The nephrotoxic effects of DEHP can lead to acute and chronic kidney damage, characterized by alterations in

renal function, histopathological changes, and disruptions in cellular homeostasis. The mechanisms underlying DEHP-induced nephrotoxicity involve oxidative stress, inflammation, and apoptosis, which collectively contribute to renal dysfunction and tissue injury (1, 4, 5). Given the critical role of the kidneys in maintaining homeostasis, understanding the protective strategies against DEHP-induced nephrotoxicity is of paramount importance.

In this context, the exploration of natural compounds with potential nephroprotective properties has gained traction. One such compound is hesperidin, a flavonoid glycoside predominantly found in citrus fruits (6). Hesperidin has been shown to possess a variety of pharmacological properties, including anti-oxidant, anti-inflammatory, and cytoprotective effects (7). These properties suggest that hesperidin may mitigate the nephrotoxic effects of DEHP and promote renal health (6, 8). The potential protective mechanisms of hesperidin against DEHP-induced nephrotoxicity warrant thorough investigation to elucidate

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its role as a therapeutic agent. Hesperidin's potential as a nephroprotective agent has been explored in several studies, highlighting its ability to scavenge free radicals, reduce oxidative stress, and modulate inflammatory pathways (9). The anti-oxidant properties of hesperidin are attributed to its ability to enhance the activity of endogenous anti-oxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), while simultaneously reducing the levels of reactive oxygen species (ROS) and lipid peroxidation products (6–8). Additionally, hesperidin has been shown to inhibit pro-inflammatory cytokines and signaling pathways involved in the inflammatory response, further contributing to its protective effects on renal tissues (8, 10). The relevance of studying the protective effects of hesperidin against DEHP-induced nephrotoxicity extends beyond the realm of basic research; it has significant implications for public health and environmental policy. As awareness of the health risks associated with phthalate exposure continues to grow, there is an urgent need for effective strategies to reduce the burden of nephrotoxicity and promote renal health in populations at risk. The identification of natural compounds with nephroprotective properties could pave the way for the development of novel dietary interventions and therapeutic agents aimed at mitigating the adverse effects of environmental pollutants.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a ubiquitous transcription factor that plays a pivotal role in orchestrating inflammatory responses by regulating genes responsible for the production of proinflammatory mediators such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins, and cyclooxygenase-2 (3). Alongside NF- $\kappa$ B, toll-like receptor 4 (TLR-4) is a critical upstream activator that senses damage-associated molecular patterns and subsequently triggers NF- $\kappa$ B activation, amplifying inflammatory signaling (11). Di-(2-ethylhexyl) phthalate (DEHP) has been shown to stimulate TLR-4/NF- $\kappa$ B signaling, thereby contributing to systemic and tissue-specific toxic responses. For instance, Zhan et al. (2022) reported that DEHP exposure significantly up-regulated TLR-4 and NF- $\kappa$ B p65 expression in rat kidneys, promoting inflammatory cell infiltration and cytokine release (12). Likewise, mono-(2-ethylhexyl) phthalate (MEHP), the principal bioactive metabolite of DEHP, was shown to enhance NF- $\kappa$ B nuclear translocation and transcriptional activity in human placental cells, as demonstrated by Zhang et al. (13).

In parallel, oxidative stress mechanisms are also engaged in DEHP/MEHP-induced toxicity (14). Keap1 (Kelch-like ECH-associated protein 1), a cytosolic repressor of nuclear factor erythroid 2-related factor 2 (Nrf2), mediates redox-sensitive regulation by targeting Nrf2 for ubiquitination (15). Under oxidative conditions, MEHP and DEHP disrupt the Keap1–Nrf2 interaction, allowing Nrf2 to translocate to the nucleus and activate the transcription of cytoprotective genes such as HO-1 and NQO1 (16). A study by Dai et al. (2022) revealed that DEHP exposure in mice decreased Keap1 expression and enhanced Nrf2/HO-1 axis activation, suggesting a compensatory anti-oxidant response (17). Given the intertwined roles of TLR-4/NF- $\kappa$ B and Keap1/Nrf2 pathways in DEHP/MEHP-induced inflammation and oxidative stress, therapeutic strategies that target these molecular cascades may offer promising approaches for the prevention or attenuation of phthalate-related nephrotoxicity.

In conclusion, the nephrotoxic effects of DEHP represent a

critical public health concern, necessitating the exploration of protective strategies to safeguard renal health. Hesperidin, with its multifaceted pharmacological properties, emerges as a promising candidate for mitigating DEHP-induced nephrotoxicity. Future research should focus on elucidating the mechanisms underlying hesperidin's protective effects, optimizing its use as a therapeutic agent, and assessing its potential role in dietary interventions. By advancing our understanding of the interactions between environmental toxins and natural compounds, we can develop effective strategies to promote renal health and mitigate the impacts of nephrotoxicity in vulnerable populations.

## Materials and Methods

### Chemicals and reagents

Hesperidin (Catalog No. 17304, St. Louis, MO, USA), Phthalates standards (99.5% DEHP and 99% DBP) were purchased from Sigma-Aldrich and all other chemicals were obtained from Sigma-Aldrich Chemicals.

### Animal model and ethical approval

The experiment was carried out using 35 male Sprague-Dawley rats, each weighing between 220 and 250 grams and aged 10–12 weeks. The animals were obtained from the Experimental Research and Application Center of Atatürk University (Erzurum, Turkey). Following randomization, the rats were divided into five groups and housed in plastic cages under controlled conditions:  $24 \pm 1$  °C temperature,  $45 \pm 5\%$  relative humidity, and a 12-hour light/dark cycle. All animals had free access to standard rodent feed and water throughout the study. Prior to the start of the experimental procedures, the animals were allowed to acclimate to the laboratory environment for seven days. The study was approved by the Animal Experiments Ethics Committee of Atatürk University (Approval No: 2023/14/221) and conducted in compliance with the updated Guide for the Care and Use of Laboratory Animals. All animal experiments were conducted in accordance with EU Directive 2010/63 for the protection of animals used for scientific purposes and the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH, USA). Additionally, the study complied with the relevant Turkish regulations, including the Animal Protection Law No. 5199, the Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes (Official Gazette No. 28141, 13 December 2011), and the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees (Official Gazette No. 28914, 15 February 2014).

### ARRIVE guidelines compliance

The reporting of this study adheres to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines, ensuring transparency and reproducibility in the design, execution, and analysis of the animal experiments.

### Experimental groups

A total of 35 male rats were randomly divided into five groups, with seven animals in each group:

#### Control group

Rats received physiological saline orally once daily for 10 consecutive days.

#### HSP group (200 mg/kg)

Rats were administered hesperidin orally at a dose of 200

mg/kg body weight for 10 consecutive days (18).

#### DEHP group (1 g/kg)

Rats received DEHP orally at a dose of 1 g/kg body weight for 10 consecutive days to induce nephrotoxicity (19).

**Group DEHP+HSP 100:** Rats were administered DEHP orally at 1 g/kg body weight for 10 days, followed by hesperidin at 100 mg/kg body weight orally for 10 days.

#### Group DEHP+HSP 200

Rats were administered DEHP orally at 1 g/kg body weight for 10 days, followed by hesperidin at 200 mg/kg body weight orally for 10 days.

Twenty-four hours after the final hesperidin administration (on day 11), all rats were euthanized under general anesthesia by decapitation. The kidney tissues were carefully dissected, rinsed with physiological saline to remove blood and clots, gently dried on filter paper, and stored at  $-80^{\circ}\text{C}$  for subsequent advanced analyses.

The primary aim of the study is to evaluate the potential protective effects of hesperidin against DEHP-induced renal tissue damage using biochemical, molecular and protein expression analyses.

#### Determination of lipid peroxidation and parameters

Kidney tissues stored at  $-80^{\circ}\text{C}$  were pulverized using liquid nitrogen and weighed up to 0.5 g. The samples were homogenized in phosphate buffer (pH 7.4) at a ratio of 1:10 (w/v) using a QIAGEN TissueLyser LT. The homogenates were centrifuged at 11,000 rpm for 30 min at  $4^{\circ}\text{C}$  and the supernatant was collected for biochemical analysis.

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels using the method described by Placer *et al.* (1966). In brief, thiobarbituric acid (TBA) was added to the homogenate, the mixture was boiled in a water bath, and the resultant pink chromogen was extracted with n-butanol. Absorbance was measured at 532 nm, and MDA levels were expressed as nmol/g tissue (20).

#### Determination of anti-oxidant defense parameters

GSH content was determined following the method of Ball (1966), based on Ellman's reaction (21). This assay measures the formation of 2-nitro-5-mercaptobenzoic acid through the reduction of disulfide reagents. The absorbance of the resulting yellow complex was read at 412 nm and results were expressed as nmol/g tissue. Total protein concentration in the samples was quantified using the Lowry method (22) for enzyme activity normalization. GPx activity was determined according to Rotruck *et al.* (1973) and expressed as U/g tissue (23). SOD activity was measured based on the method of Sun *et al.* (24), and CAT activity was evaluated using the protocol of Góth (25); both were expressed as U/g tissue.

#### Evaluation of Bax, Bcl-2, caspase-3, Nrf2, HO-1, TLR-4, and NF- $\kappa$ B mRNA expression by real-time PCR

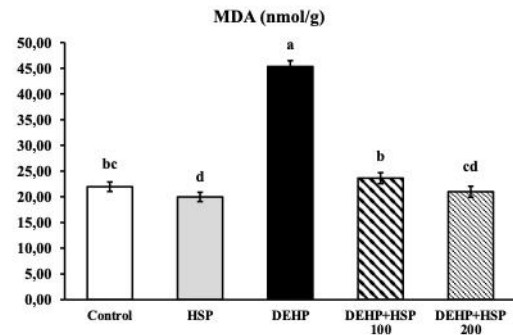
Primers for Keap-1, Nrf2, NF- $\kappa$ B, HO-1, Bax, Bcl-2, caspase-3, and TLR-4 genes were designed using the Primer-BLAST tool from NCBI (Table 1). Tissue samples (50 mg) were homogenized in 1 ml QIAzol reagent (QIAGEN). Chloroform (200  $\mu$ l) was added, and samples were centrifuged at 12,000  $\times$ g for 20 min at  $4^{\circ}\text{C}$ . The aqueous phase was mixed with 500  $\mu$ l isopropanol and centrifuged at 12,000  $\times$ g for 10 min. The pellet was washed with 75% ethanol and centrifuged at 7,500  $\times$ g for 5 min. RNA was dissolved in RNase-free DEPC-treated water and quantified by measuring absorbance at 260/280 nm. RNA samples were treated with DNase I (Thermo Scientific) to eliminate genomic DNA contamination. cDNA synthesis was performed using the miScript Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. cDNA quality and concentration were measured at 260/280 nm and standardized before  $-20^{\circ}\text{C}$  storage. mRNA expression levels were determined using a QIAGEN real-time PCR system. GAPDH was used as the reference gene. The reaction mix included SYBR Green ROX Dye Master Mix (QIAGEN), gene-specific primers, cDNA templates, and nuclease-free water. Reactions were run in triplicate. Gene expression was quantified using the  $2^{-\Delta\Delta Ct}$  method (26).

**Table 1.** Primer sequences used for quantitative real-time PCR analysis of target genes

Gene	Sequences (5'-3')	Accession No
Bax	F: TTTCATCCAGGATCGAGCAG R: AATCATCCTCTGCAGCTCCA	NM_017059.2
Bcl-2	F: GACTTTGCAGAGATGTCCAG R: TCAGGTACTCAGTCATCCAC	NM_016993.2
Caspase-3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA	NM_012922.2
HO-1	F: ATGTCCCAGGATTTGTCCGA R: ATGGTACAAGGAGGCCATCA	NM_012580.2
NF- $\kappa$ B	F: AGTCCCGCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	NM_001276711.2
Nrf-2	F: TTTGTAGATGACCATGAGTCGC R: TCCTGCCAACTTGCTCCAT	NM_031789.3
TLR-4	F: ACCGTTCTGTCATGGAAGGA R: ACCGTTCTGTCATGGAAGGA	NM_019178.2
GAPDH	F: GAGTATGTCGTGGAGTCTAC R: CAGGATGGATTGCTGACAAT	NM_017008.4

### Western blot detection of Bax, Bcl-2, caspase-3, Nrf2, HO-1, Keap1, TLR4, and NF- $\kappa$ B proteins

To prepare tissue homogenates, ice-cold RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc., Texas, USA) was added to the samples, and homogenization was performed using a tissue homogenizer. The homogenates were then centrifuged at 16,000  $\times$ g for 20 min at 4 °C, and the resulting supernatants were collected for protein analysis. Total protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Rockford, IL, USA) according to the manufacturer's protocol. Equal amounts of protein (25  $\mu$ g per well) were mixed with Laemml sample buffer (containing bromophenol blue, Tris-HCl pH 6.8, glycerol,  $\beta$ -mercaptoethanol, and SDS) and separated on a 10% SDS-polyacrylamide gel (SDS-PAGE). Dual color protein markers were used to verify the efficiency of the protein transfer. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry blotting system (Thermo blotting apparatus, Rockford, IL, USA). To block non-specific binding, membranes were incubated in 5% bovine serum albumin (BSA) prepared in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1.5 hr at room temperature. Membranes were then washed three times (5 min each) with TBS-T. Following the washing steps, the membranes were incubated overnight at 4 °C on a shaker with primary antibodies diluted in TBS-T, targeting the following proteins:  $\beta$ -tubulin (sc-47778), Bax (sc-20067), Bcl-2 (sc-7382), caspase-3 (sc-56053), Keap-1 (sc-365626), Nrf2 (sc-365949), HO-1 (sc-390991), TLR4 (sc-293072), and NF- $\kappa$ B (sc-8008) (Santa Cruz Biotechnology, USA). After incubation with primary antibodies, membranes were washed five times with TBS-T for 5 min each and then incubated for 1.5 hr at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:1000 dilution, sc-2005, Santa Cruz Biotechnology, USA) diluted in TBS-T. Following the secondary antibody incubation, membranes were washed again five times with TBS-T. The protein bands were visualized using Trideont femto Western HRP Substrate (GeneTex GTX14698) prepared as a 1:1 working solution. As



**Figure 1.** Effects of DEHP and hesperidin on rat renal MDA levels. Data are presented as mean  $\pm$  S.E. (n = 7). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. MDA: Malondialdehyde; DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin

the substrate is light sensitive, chemiluminescent detection was carried out in the dark. Band images were captured using a GelDoc XR documentation system (Bio-Rad, USA), and the relative protein expression levels were quantified using Image Lab 6.1 software (Bio-Rad, USA) (27).

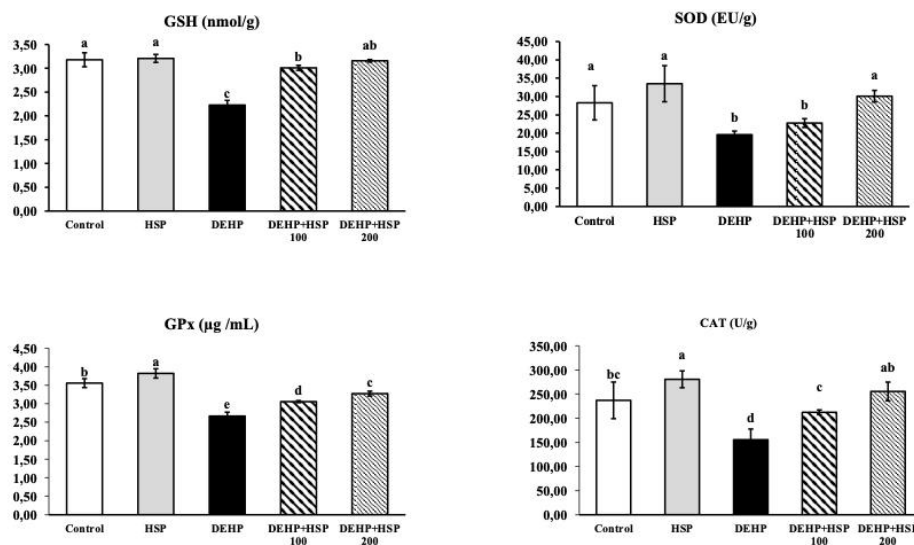
### Statistical analysis

Data were tested for normality using the Shapiro-Wilk test. Since the data showed a normal distribution, parametric statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Results are expressed as mean  $\pm$  S.E., and  $P < 0.05$  was considered statistically significant.

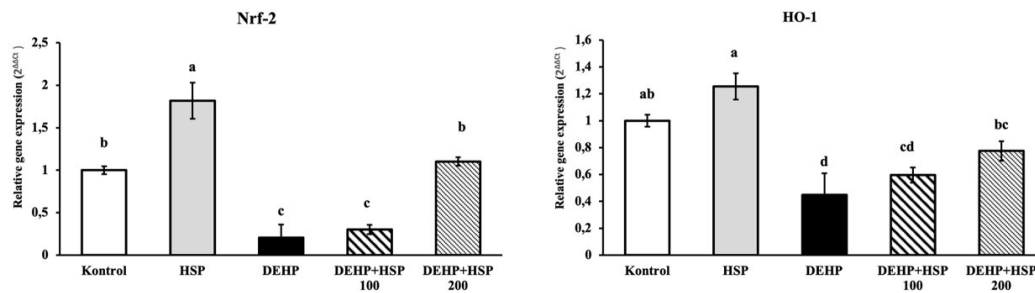
## Results

### Oxidative stress parameters in response to DEHP and hesperidin treatment

Compared to the control group, DEHP exposure (1 g/kg) led to a significant increase in MDA levels ( $P < 0.001$ ), indicating elevated lipid peroxidation (Figure 1), along with a marked decrease in anti-oxidant defense parameters such as GSH, SOD, GPx, and CAT ( $P < 0.001$ ) levels (Figure 2),



**Figure 2.** Effects of DEHP and hesperidin on rat renal anti-oxidant parameters (GSH, SOD, CAT, and GPx). Data are presented as mean  $\pm$  S.E. (n = 7). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin



**Figure 3.** Relative mRNA expression levels of HO-1 and Nrf2 in rat kidney tissues across the experimental groups, as determined by RT-qPCR. Data are presented as mean  $\pm$  S.E. (n = 7). Gene expression levels were normalized to GAPDH and expressed as fold change relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin. Groups sharing at least one common letter are not significantly different.

confirming the induction of oxidative stress in renal tissue. Rats treated with hesperidin following DEHP exposure showed significantly improved anti-oxidant status. In both DEHP + HSP 100 mg/kg and DEHP + HSP 200 mg/kg groups, MDA levels were significantly reduced, and anti-oxidant parameters (GSH, SOD, GPx, and CAT) were significantly increased compared to the DEHP-only group ( $P < 0.001$ ). The protective effect was more pronounced in the DEHP + HSP 200 group, where anti-oxidant levels approached those of the control group. Hesperidin administration alone (HSP 200 group) did not significantly alter oxidative stress markers compared to the control, suggesting its safety and lack of pro-oxidative effects under physiological conditions. These findings suggest that hesperidin exerts a dose-dependent protective effect against DEHP-induced oxidative renal damage.

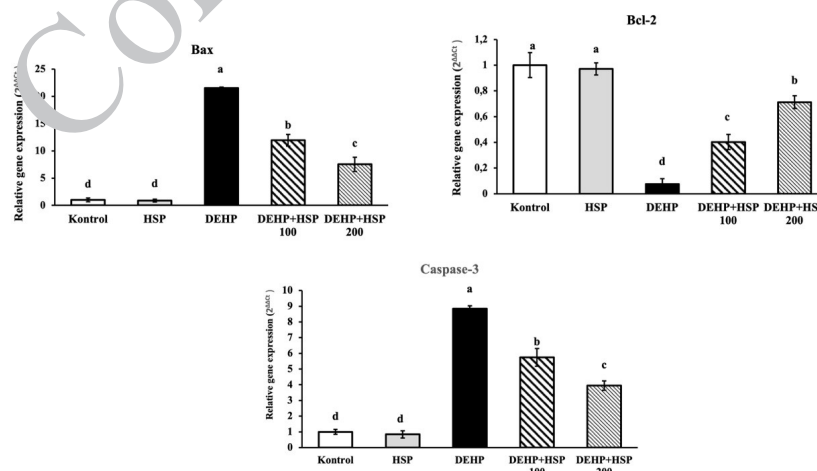
#### Effects of hesperidin on DEHP-induced alterations in Nrf2 and HO-1 gene expression in renal tissue

The mRNA expression levels of Nrf2 and HO-1 were assessed by RT-PCR, as shown in Figure 3. DEHP exposure significantly suppressed Nrf2 ( $P < 0.001$ ) and HO-1 ( $P < 0.001$ ) mRNA expression levels in renal tissue compared to the control group. In contrast, treatment with hesperidin at both 100 mg/kg and 200 mg/kg following DEHP exposure significantly reversed these effects. Specifically, Nrf2 and HO-1 expression levels were significantly up-regulated in

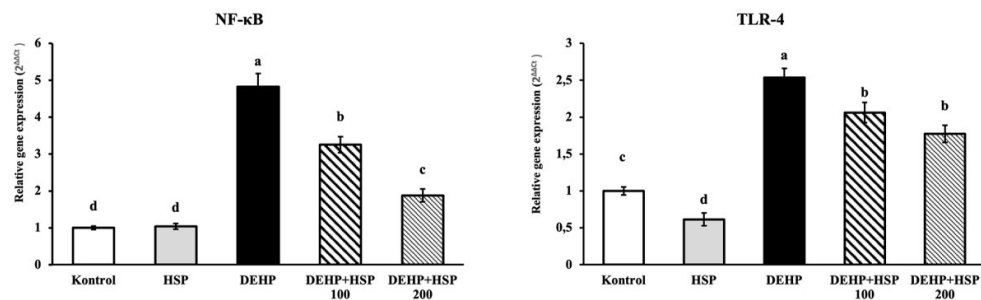
both DEHP + HSP groups compared to the DEHP group ( $P < 0.001$ ). The modulation of gene expression was more prominent in the DEHP + HSP 200 group, which showed Nrf2 and HO-1 expression levels nearing those of the control group. No significant difference in the expression of Nrf2, HO-1 was observed between the HES 200 group and the control group, suggesting that hesperidin alone does not dysregulate these genes under normal conditions. These results demonstrate that hesperidin restores redox balance in the kidney by activating the Nrf2/HO-1 pathway thereby counteracting DEHP-induced oxidative stress at the transcriptional level.

#### Effects of hesperidin on Bax, Bcl-2, and caspase-3 gene expression in DEHP-induced renal apoptosis

The mRNA expression levels of Bax, Bcl-2 and caspase-3 were assessed using RT-PCR, as shown in Figure 4. DEHP administration significantly altered the expression of apoptosis-related genes in renal tissue. Specifically, Bax and caspase-3 mRNA levels were markedly increased ( $P < 0.001$ ), while Bcl-2 expression was significantly decreased ( $P < 0.001$ ) compared to the control group, indicating enhanced pro-apoptotic activity and suppressed anti-apoptotic signaling. In the groups treated with hesperidin following DEHP exposure, a significant reversal of these alterations was observed. Both DEHP+HSP 100 mg/kg and DEHP+HSP 200 mg/kg groups showed decreased Bax



**Figure 4.** Relative mRNA expression levels of Bax, Bcl-2, and caspase-3 in rat kidney tissues across the experimental groups, as determined by RT-qPCR. Data are presented as mean  $\pm$  S.E. (n = 7). Gene expression levels were normalized to GAPDH and expressed as fold change relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin.



**Figure 5.** Relative mRNA expression levels of NF-κB and TLR-4 in rat kidney tissues across the experimental groups, as determined by RT-qPCR. Data are presented as mean  $\pm$  S.E. (n = 7). Gene expression levels were normalized to GAPDH and expressed as fold change relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin

and caspase-3 expression levels ( $P < 0.001$ ) and increased Bcl-2 levels ( $P < 0.001$ ) compared to the DEHP-only group. The DEHP+HSP 200 group demonstrated the most pronounced anti-apoptotic profile, approaching expression levels seen in the control group. No statistically significant differences in the expression of Bax, Bcl-2, or caspase-3 were observed between the HSP 200 group and the control group, suggesting hesperidin alone does not interfere with apoptotic homeostasis under physiological conditions. These findings indicate that hesperidin mitigates DEHP-induced renal apoptosis by modulating key apoptotic regulators at the transcriptional level.

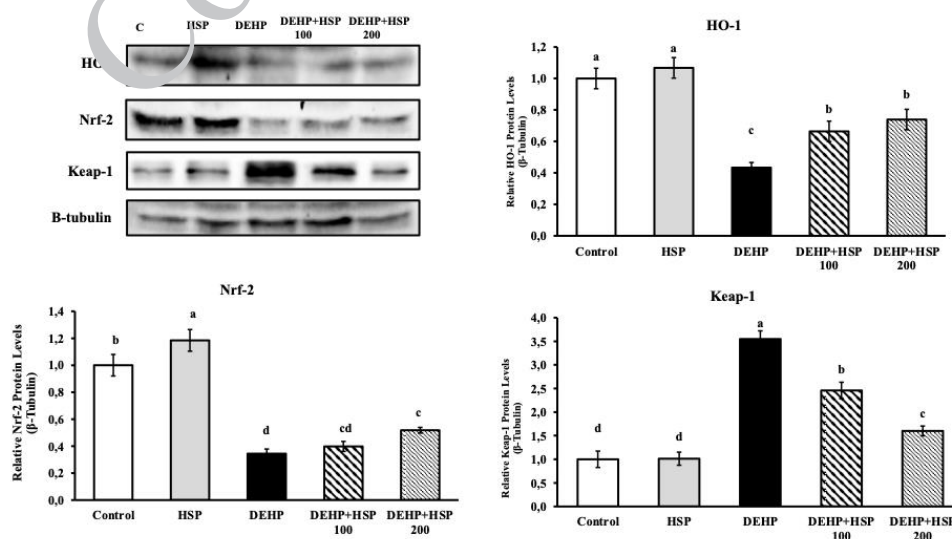
#### Effects of hesperidin on NF-κB and TLR-4 gene expression in DEHP-induced renal inflammation

The mRNA expression levels of NF-κB and TLR-4 were assessed using RT-PCR, as shown in Figure 5. DEHP exposure significantly elevated the mRNA expression levels of NF-κB and TLR-4 in renal tissue compared to the control group ( $P < 0.001$ ), indicating the activation of inflammation-related signaling pathways. Administration of hesperidin following DEHP treatment markedly reduced the expression of both inflammatory markers. The DEHP

+ HSP 100 mg/kg and DEHP + HSP 200 mg/kg groups exhibited significantly lower NF-κB and TLR-4 mRNA levels than the DEHP-only group ( $P < 0.01$  to  $P < 0.001$ ), with the higher dose demonstrating a more substantial anti-inflammatory effect. The gene expression levels in the DEHP+HSP 200 group approached those observed in the control group. Furthermore, the HES 200 group alone showed no significant differences in NF-κB or TLR-4 expression compared to controls, indicating that hesperidin does not elicit an inflammatory response in normal renal tissue. These findings highlight the anti-inflammatory potential of hesperidin in DEHP-induced renal damage through modulation of NF-κB and TLR-4 gene expression.

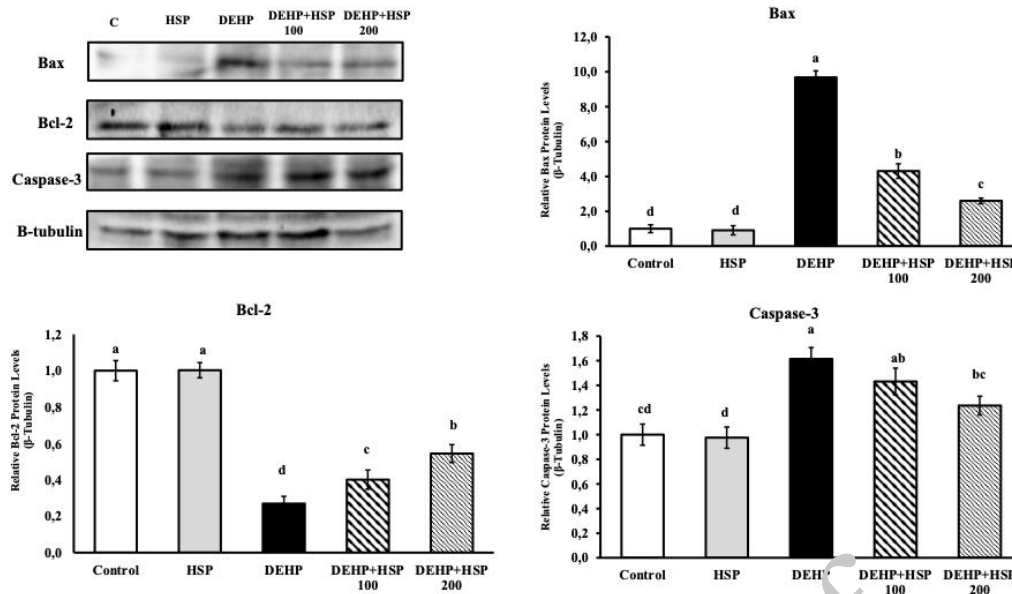
#### Modulatory effects of hesperidin on protein expression of Bax, Bcl-2, caspase-3, Nrf2, HO-1, Keap1, NF-κB, and TLR-4 in DEHP-Induced renal damage

The protein expression levels of Keap1, Nrf2, and HO-1 are presented in Figure 6, while the apoptotic markers Bax, Bcl-2, and caspase-3 are shown in Figure 7. Additionally, the inflammatory mediators NF-κB and TLR4 are depicted in Figure 8. Western blot analysis supported the gene expression findings, showing that DEHP administration

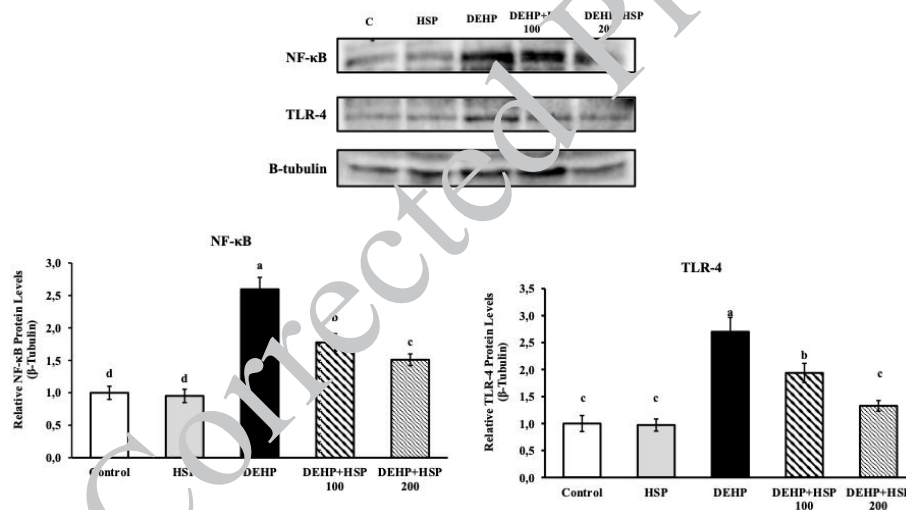


**Figure 6.** Representative Western blot bands and densitometric quantification of HO-1, Nrf2, and Keap1 protein expression in rat kidney tissues across the experimental groups

Data are presented as mean  $\pm$  S.E. (n = 7). Protein expression levels were normalized to  $\beta$ -tubulin and expressed relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin



**Figure 7.** Representative Western blot bands and densitometric quantification of Bax, Bcl-2, and caspase-3 protein expression in rat kidney tissues across the experimental groups. Data are presented as mean ± S.E. (n = 7). Protein expression levels were normalized to β-actin and expressed relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin



**Figure 8.** Representative Western blot bands and densitometric quantification of NF-κB and TLR-4 protein expression in rat kidney tissues across the experimental groups. Data are presented as mean ± S.E. (n = 7). Protein expression levels were normalized to β-actin and expressed relative to the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ( $P < 0.05$ ). Groups sharing at least one common letter are not significantly different. DEHP: Di-(2-ethylhexyl) phthalate; HSP: Hesperidin

significantly increased the protein levels of Bax, caspase-3, Keap1, NF-κB, and TLR-4, while markedly reducing Bcl-2, Nrf2, and HO-1 levels compared to the control group ( $P < 0.001$  for all), indicating enhanced apoptosis, oxidative stress, and inflammation at the protein level. Hesperidin treatment following DEHP exposure significantly reversed these alterations. Both DEHP + HSP 100 mg/kg and DEHP + HSP 200 mg/kg groups exhibited increased levels of Bcl-2, Nrf2, and HO-1, along with decreased expression of Bax, caspase-3, Keap1, NF-κB, and TLR-4 proteins compared to the DEHP group ( $P < 0.001$ ). The 200 mg/kg dose of hesperidin showed a more pronounced restorative effect, with protein levels approaching those observed in the control group. No significant changes were observed in

any of the target protein expressions in the HSP 200 group compared to the control group, confirming that hesperidin alone does not disrupt renal cellular homeostasis under physiological conditions. These findings reinforce the protective role of hesperidin in counteracting DEHP-induced renal toxicity through modulation of key apoptotic, oxidative, and inflammatory proteins.

**Discussion**

The nephrotoxic effects of DEHP have garnered significant attention in recent years, primarily due to its widespread use in various industrial applications and its prevalence in the environment. DEHP is known to disrupt endocrine functions and has been implicated in a range

of adverse health outcomes, particularly concerning renal function (12). In this study, we explored the protective effects of hesperidin, a flavonoid predominantly found in citrus fruits, against DEHP-induced nephrotoxicity. Our findings suggest that hesperidin exhibits significant nephroprotective properties, warranting further investigation into its potential therapeutic applications. The findings are consistent with existing literature that documents the nephrotoxic effects of DEHP, which is believed to occur through oxidative stress, inflammation, and apoptosis (1, 3, 12). Mechanistically, DEHP exposure leads to excessive generation of ROS, which overwhelms the renal anti-oxidant defense system and initiates lipid peroxidation, mitochondrial dysfunction, and DNA damage. This oxidative imbalance acts as an upstream trigger for inflammatory signaling and apoptotic cascades, ultimately resulting in renal cellular injury and functional impairment (1).

In contrast, hesperidin treatment significantly ameliorated these deleterious effects by reducing renal oxidative stress, inflammation, and apoptosis markers. The anti-oxidant action of hesperidin appears to be a primary protective mechanism, as it not only scavenges ROS directly but also enhances endogenous anti-oxidant enzyme activities, thereby restoring redox homeostasis in renal tissues. The observed increases in SOD, CAT, GPx, and GSH levels suggest that hesperidin reinforces the intrinsic anti-oxidant defense system disrupted by DEHP exposure (28). This aligns with previous studies that have highlighted the role of flavonoids in mitigating oxidative stress-related injuries in various organ systems (29, 30).

Another aspect worth discussing is the potential role of hesperidin in modulating apoptosis. DEHP-induced oxidative stress promotes mitochondrial membrane permeabilization, leading to the release of cytochrome c and activation of caspase-dependent apoptotic pathways, particularly through increased Bax expression and caspase-3 activation alongside suppression of the anti-apoptotic protein Bcl-2 (3). In the present study, hesperidin reversed these molecular alterations by down-regulating Bax and caspase-3 expression while up-regulating Bcl-2, indicating suppression of the mitochondrial apoptotic pathway and preservation of renal cell viability (31). Our study observed a decrease in markers of apoptosis in the hesperidin-treated groups, further supporting the hypothesis that hesperidin can prevent DEHP-induced cell death.

Nrf2 is a central transcription factor responsible for regulating the cellular anti-oxidant defense system through the transcription of genes encoding detoxifying and cytoprotective molecules, such as HO-1 and GSH. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by its inhibitory partner Keap1, which facilitates its ubiquitination and subsequent proteasomal degradation (32). Exposure to oxidative stressors such as DEHP disrupts this regulatory interaction; however, sustained or excessive oxidative burden may paradoxically suppress Nrf2 signaling, as observed in DEHP-treated renal tissues (33, 34). For example, a decrease in both Nrf2 expression and HO-1 protein levels has been observed in the kidneys after DEHP exposure (35). Similarly, in mouse models, DEHP administration led to down-regulation of renal Nrf2 signaling and a decrease in its downstream targets (36).

It is also essential to consider the implications of our findings in the context of public health and environmental exposure. Given the ubiquitous nature of DEHP and its

potential health risks, the identification of protective agents such as hesperidin is particularly relevant. As consumers increasingly seek natural alternatives to synthetic chemicals, the incorporation of hesperidin-rich foods into the diet may offer a feasible strategy for mitigating DEHP-related health risks. Public health campaigns aimed at educating individuals about the benefits of dietary flavonoids could play a significant role in preventing DEHP-induced nephrotoxicity. However, it is crucial to acknowledge the limitations of our study. While our results are promising, further research is needed to elucidate the precise mechanisms underlying hesperidin's protective effects. Importantly, hesperidin treatment restored Nrf2 nuclear activation and up-regulated HO-1 expression, suggesting that activation of the Keap1–Nrf2–HO-1 axis represents a key molecular mechanism underlying its nephroprotective effect. HO-1, beyond its role in heme catabolism, exerts potent anti-oxidant and anti-inflammatory effects by reducing free iron availability, limiting ROS production, and generating cytoprotective metabolites such as biliverdin and carbon monoxide (37). Additionally, HO-1 has been shown to inhibit the NF- $\kappa$ B pathway both directly and indirectly, further reducing the production of proinflammatory cytokines (35). The interplay between Nrf2/Keap1 signaling and NF- $\kappa$ B activity is particularly relevant in DEHP-induced renal toxicity, where oxidative stress and inflammation coexist and reinforce tissue injury (38). Thus, therapeutic modulation of the Keap1–Nrf2–HO-1 axis may offer a promising strategy to counteract DEHP-mediated renal damage.

Despite the promising findings, this study has several limitations that should be acknowledged. First, the experiment was conducted using only male rats; therefore, potential sex-related differences in DEHP-induced nephrotoxicity and hesperidin-mediated protection were not evaluated. Second, the study focused on a relatively short exposure period (10 days), which may not fully reflect the effects of chronic DEHP exposure commonly encountered in environmental and occupational settings. Third, although key oxidative stress, inflammatory, and apoptotic pathways were investigated at both gene and protein levels, histopathological examination of kidney tissues was not included, which could have provided additional structural confirmation of renal injury and protection. Furthermore, the pharmacokinetics and bioavailability of hesperidin were not assessed, limiting conclusions regarding its optimal dosing and translational relevance. Finally, extrapolation of these findings to humans should be made with caution, as animal models may not fully replicate human renal responses to phthalate exposure.

## Conclusion

Our study provides compelling evidence for the nephroprotective effects of hesperidin against DEHP-induced nephrotoxicity. The anti-oxidant, anti-inflammatory, and anti-apoptotic properties of hesperidin suggest that it could serve as a potential therapeutic agent for individuals exposed to nephrotoxic agents. As the prevalence of DEHP in the environment continues to raise concerns regarding public health, the exploration of natural compounds like hesperidin offers a promising avenue for future research and intervention strategies. Further investigations are warranted to fully understand the mechanisms of action, optimal dosing, and long-term

effects of hesperidin in the context of nephroprotection.

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### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### Authors' Contributions

T D and B AY conceived and designed the study. T D and BAY performed the experiments, provided reagents, and contributed to data interpretation. T D, B AY, and F Y analyzed and interpreted the data. T D drafted the manuscript. All authors critically reviewed and approved the final version of the manuscript.

### Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this manuscript.

### Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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Corrected Proof