

Hesperidin protects against diethylnitrosamine/carbon tetrachloride-induced renal repercussions via up-regulation of Nrf2/HO-1 signaling and attenuation of oxidative stress

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ARTICLE INFO

Article history:

Received on: 05/09/2017

Accepted on: 29/10/2017

Available online: 30/11/2017

Key words:

Hesperidin,
Diethylnitrosamine, Fibrosis,
Oxidative stress,
Nephrotoxicity.

ABSTRACT

The kidney plays a central role in detoxification and excretion of toxic metabolites, and therefore is susceptible to toxicity by xenobiotics. Hesperidin is a citrus flavonoid with multiple beneficial therapeutic effects. Here, we investigated the possible modulatory effect of hesperidin on diethylnitrosamine (DEN)/carbon tetrachloride (CCl₄)-induced nephrotoxicity in rats, pointing to the role of Nrf2/HO-1 signaling. Male Wistar rats received a single intraperitoneal injection of DEN. Two-weeks later, the DEN-induced rats received a subcutaneous injection of 3 ml/kg CCl₄ once/week for 16 weeks. DEN/CCl₄-induced rats were treated with 50 and 100 mg/kg hesperidin throughout the experiment. After 18 weeks, DEN/CCl₄-induced rats showed renal injury evidenced by the significant increase in circulating kidney function markers as well as histopathological alterations. Concurrent supplementation of hesperidin significantly ameliorated kidney function markers and prevented renal tissue damage induced by DEN/CCl₄. In addition, hesperidin treatment suppressed collagen deposition, lipid peroxidation and nitric oxide, and enhanced the antioxidant defenses in the kidney of DEN/CCl₄-induced rats. Hesperidin up-regulated the expression of Nrf2 and HO-1 in the kidney of DEN/CCl₄-induced rats. In conclusion, hesperidin prevents DEN/CCl₄-induced nephrotoxicity via attenuation of oxidative stress and alleviation of the antioxidant defense system. These effects are mediated via up-regulation of Nrf2/ARE/HO-1 signaling pathway.

INTRODUCTION

The kidney contains many xenobiotic metabolizing enzymes and plays a central role in metabolizing drugs and foreign compounds in the body. Kidney diseases represent a public health issue worldwide and can range from mild infection to dangerous kidney failure (Nasri, 2014). The kidneys receive approximately 25% of the cardiac output, and renal tubules have a high tendency for drugs uptake via transporter proteins or endocytosis. This can result in high intracellular levels of various drugs and substances that then metabolized, leading to formation

of reactive oxygen species (ROS) and toxic metabolites (Perazella, 2009). Excessive ROS production and oxidative stress have been demonstrated to play a role in drug-induced renal damage and tubular necrosis (Lopez-Novoa *et al.*, 2011; Mahmoud *et al.*, 2014, 2015; Abd El-Twab *et al.*, 2016). N-nitrosamines are chemical compounds produced by reactions of nitrosating compounds and organic amines (Rostkowska *et al.*, 1998). High concentrations of nitrosamines have been reported in processed meat because of the addition of nitrite to prevent the growth of *Clostridium botulinum* (Cho and Bratzler, 1970). Diethylnitrosamine (DEN) is a potent carcinogen found in soybean, cheese, tobacco smoke, processed meats, and a wide variety of foods (Verna *et al.*, 1996). The metabolism of DEN generates high levels of ROS leading to mutagenicity and carcinogenesis (Pradeep *et al.*, 2007; Ahmed *et al.*, 2015; Mahmoud *et al.*, 2017a). Therefore, attenuation of ROS generation can protect against DEN-induced cellular and tissue damage.

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Activation of the basic leucine zipper (bZIP) protein nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a major mechanism in the defense against oxidative stress. Nrf2 activates the expression of genes whose products are antioxidant and cytoprotective proteins involved in the detoxication and elimination of electrophilic agents and reactive oxidants (Nguyen *et al.*, 2009). We have recently demonstrated that activation of Nrf2 protects against drug-induced nephrotoxicity (Abd El-Twab *et al.*, 2016), hepatotoxicity (Mahmoud and Al Dera, 2015; Kamel *et al.*, 2016; Mahmoud *et al.*, 2017b,c,d) and hepatocarcinogenesis (Mahmoud *et al.*, 2017a), and oxidative stress induced by palmitate (Mahmoud *et al.*, 2017e,f) and ammonium chloride (Mahmoud *et al.*, 2017g). In this study, we demonstrated the protective effect of hesperidin against DEN/carbon tetrachloride (CCl₄)-induced nephrotoxicity and pointed to the role of Nrf2/heme oxygenase 1 (HO-1) signaling. Hesperidin is a citrus flavonoid belongs to a class of non-nutritive dietary substances that are widely distributed in vegetables, fruits and beverages (Mahmoud, 2012). Previous work from our laboratory showed multiple beneficial effects of hesperidin, including anti-oxidant (Mahmoud *et al.*, 2012), anti-inflammatory (Mahmoud, 2013, 2014), anti-diabetic (Mahmoud *et al.*, 2012) and hepatoprotective (Mahmoud, 2014). We have also demonstrated the nephroprotective effect of hesperidin in DEN/phenobarbital (PB)-induced rats (Ahmed *et al.*, 2015). Other studies have showed the protective effect of hesperidin against nephrotoxicity induced by several agents (Sahu *et al.*, 2013; Kamel *et al.*, 2014; Siddiqi *et al.*, 2015). However, the mechanisms underlying the nephroprotective effect of hesperidin are not fully elucidated. This study is a step toward exploring the protective mechanism of hesperidin against xenobiotics-induced renal injury.

MATERIALS AND METHODS

Chemicals

DEN, CCl₄, hesperidin, trichloroacetic acid, 1,1,3,3-tetramethoxypropane, thiobarbituric acid, 1-chloro-2,4-dinitrobenzene, reduced glutathione (GSH), 5,5'-dithiobis-(2-nitrobenzoic acid) and pyrogallol were purchased from Sigma (St. Louis, MO, USA). Antibodies against Nrf2, HO-1 and β -actin were supplied by Santa Cruz Biotechnology (USA). Assay kits for serum creatinine, urea and uric acid were purchased from Biosystems (Spain). Other chemicals were of analytical grade and were obtained from standard commercial supplies.

Experimental design and treatments

Thirty adult male Wistar rats weighing 140-160 g were used in the present study. The animals were obtained from the National Research Centre (NRC, Giza, Egypt), and were housed in well-aerated cages at normal atmospheric temperature and normal 12-hour light/dark cycle. The animals had free access to water and a standard diet of known composition. All efforts were done to reduce the number and suffering of animals and all animal procedures were in accordance with the recommendations of the animal ethics committee of Beni-Suef University (Egypt).

The experimental animals were divided into five groups as follows:

Group I (Control): rats received one intraperitoneal injection of 0.9% NaCl. Two weeks later, the animals received mineral oil subcutaneously (once/week) for 16 weeks. In addition, rats received 0.5% carboxymethyl cellulose (CMC) via oral gavage daily for 18 weeks.

Group II (100 mg HES): rats received 0.9% NaCl and mineral oil as Group I, and 100 mg/kg hesperidin (Ahmed *et al.*, 2015) dissolved in 0.5% CMC via oral gavage for 18 weeks.

Group III (DEN/CCl₄): rats received one intraperitoneal injection of DEN (200 mg/kg) dissolved in 0.9% NaCl (Banakar *et al.*, 2004). Two weeks later, the animals received 3 ml/kg CCl₄ diluted in mineral oil subcutaneously once/week for 16 weeks. In addition, rats received 0.5% CMC via oral gavage daily for 18 weeks.

Group IV (DEN/CCl₄ + 50 mg HES): rats received DEN and CCl₄ as Group III and 50 mg/kg hesperidin (Mahmoud *et al.*, 2012) daily for 18 weeks.

Group V (DEN/CCl₄ + 100 mg HES): rats received DEN and CCl₄ as Group III and 100 mg/kg hesperidin (Ahmed *et al.*, 2015) daily for 18 weeks.

At the end of the experiment, rats were sacrificed under mild anesthesia and blood samples were obtained for serum preparation. Kidneys were rapidly excised and immediately perfused with ice-cold saline. Samples from the kidney were homogenized in cold phosphate buffered saline (10% w/v), centrifuged and clear homogenate was separated and stored at -20°C. Other kidney samples were kept at -80°C for Western blotting while others were collected on 10% neutral buffered formalin for histological processing.

Assay of kidney function markers

Serum levels of creatinine, urea and uric acid were assayed using reagent kits purchased from Biosystems (Spain) according to the methods of Young (1995), Kaplan (1984) and Fossati *et al* (1980) respectively.

Assay of lipid peroxidation, nitric oxide (NO) and antioxidant defenses

Lipid peroxidation was determined in the kidney homogenate by assaying the level of malondialdehyde (MDA) according to the method of Preuss *et al* (1998). NO level was assayed following the method of Grisham *et al* (1996). GSH content was determined following the method of Beutler *et al* (1963) while superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were measured according to the methods of Marklund and Marklund (1974), Matkovics *et al* (1998) and Mannervik and Gutenburg (1981) respectively.

Histology

Kidney samples fixed in 10% neutral buffered formalin were dehydrated in ascending ethanol series, cleared in xylene, and embedded in paraffin wax. Blocks were prepared, and 4- μ m thick sections were cut by a sledge microtome. The paraffin embedded sections were deparaffinized, washed, and stained with hematoxylin and eosin (H&E) for histopathological examination under light microscope. To demonstrate collagen deposition in the kidney, paraffin sections were stained with Masson's trichrome.

Western blotting

Kidney samples were homogenized in RIPA buffer supplemented with proteinase inhibitors and the protein content was determined using Bradford reagent. Thirty μ g proteins were separated on SDS-PAGE, electrotransferred to nitrocellulose membranes and then blocked in 5% skimmed milk. The membranes were incubated with primary antibodies against Nrf2, HO-1 and β -actin (Santa Cruz Biotechnology, USA), followed by washing and incubation with the secondary antibodies. The blots were developed using enhanced chemiluminescence kit (BIORAD, USA), scanned and quantified using ImageJ (NIH, USA).

Statistical Analysis

Statistical analysis was performed using SPSS (v.16). Results were expressed as mean \pm standard deviation (SD) and all statistical comparisons were made by means of the one-way analysis of variance (ANOVA) test followed by Tukey's test post hoc analysis. A P value less than 0.05 was considered significant.

RESULTS

Effect of Hesperidin on kidney function markers in control and DEN/CCl₄-induced rats

Data summarized in Table 1 show the effect of hesperidin on kidney function markers in control and DEN/CCl₄-induced rats. The administration of DEN and CCl₄ produced a significant increase in serum creatinine ($P < 0.01$), urea ($P < 0.001$) and uric acid ($P < 0.001$) when compared with the control rats. Concurrent treatment of the DEN/CCl₄-induced rats with either 50 or 100 mg/kg hesperidin significantly ameliorated serum creatinine, urea and uric acid levels.

Table 1: Effect of hesperidin on kidney function markers in control and DEN/CCl₄-induced rats.

	Creatinine (mg/dl)	Urea (mg/dl)	Uric Acid (mg/dl)
Control	0.65 \pm 0.09	24.93 \pm 2.32	1.05 \pm 0.15
100 mg HES	0.66 \pm 0.04	29.93 \pm 2.68	1.17 \pm 0.11
DEN/CCl ₄	1.94 \pm 0.21***	94.69 \pm 7.19***	2.84 \pm 0.41***
DEN/CCl ₄ + 50 mg HES	0.70 \pm 0.03###	55.76 \pm 3.89###	1.51 \pm 0.07###
DEN/CCl ₄ + 100 mg HES	0.75 \pm 0.04###	41.07 \pm 1.94###	1.34 \pm 0.27###

Data are expressed as Mean \pm SD, (N=6). *** $P < 0.001$ versus Control and ### $P < 0.001$ versus DEN/CCl₄. DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; HES, hesperidin.

Rats received 100 mg/kg hesperidin for 18 weeks showed non-significant ($P > 0.05$) changes in serum levels of creatinine, urea and uric acid when compared with the control rats (Table 1).

Hesperidin prevents kidney injury induced by DEN/CCl₄ in rats

Microscopic examination of the kidney sections showed normal histological structure of the renal tissue in both control (Fig. 1A) and 100 mg/kg hesperidin supplemented rats (Fig. 1B).

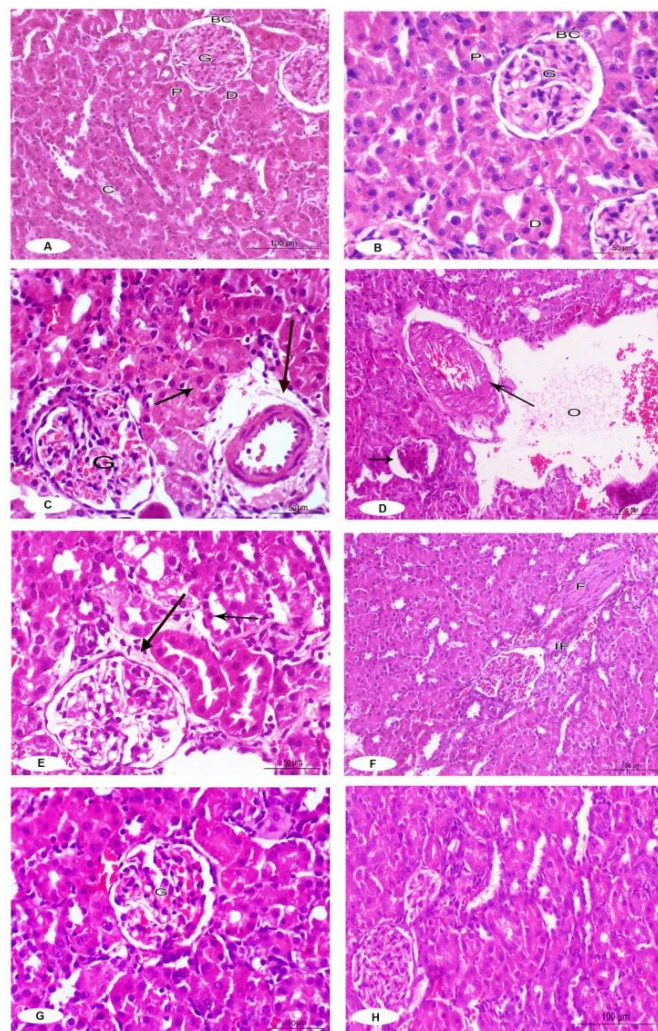


Fig. 1: Hesperidin prevents kidney injury in DEN/CCl₄-induced rats. Photomicrographs of H&E-stained kidney sections of (A) control and (B) hesperidin treated normal rats showing normal histological structures of renal parenchyma; the glomerulus (G), Bowman's capsule (BC), proximal tubules (P), distal tubules (D) and collecting tubules (C), (C-F) DEN/CCl₄-induced rats showing congestion of glomerular tuft (G), perivascular oedema (long arrow), convoluted tubules with cloudy swelling (short arrow) [C], atrophy of glomerular tuft (short arrow), thickening of the blood vessel (long arrow), edema (O) [D], pyknotic nuclei of epithelial lining renal tubules (short arrow), periglomerular infiltration (long arrow) [E], fibrosis (F) and inflammatory cells infiltration (IF) [F], (G) DEN/CCl₄-induced rats treated with 50 mg/kg hesperidin showing congestion of glomerular tuft (G) and nearly normal structure of tubules, and (H) DEN/CCl₄-induced rats treated with 100 mg/kg hesperidin showing nearly normal structures of renal corpuscles and renal tubules.

Rats received DEN and CCl₄ showed multiple histopathological changes, including congestion of glomerular tuft, atrophy of glomerular tuft, edema, cloudy swelling in convoluted tubules, thickening of the blood vessels, pyknosis, fibrosis and inflammatory cells infiltration (Fig. 1C-1F). Concomitant treatment of the DEN/CCl₄-induced rats with 50 mg/kg hesperidin showed marked amelioration in kidney tissues, however, congestion of glomerular tuft was observed (Fig. 1G). DEN/CCl₄-induced rats treated with 100 mg/kg hesperidin showed nearly normal structure of the renal corpuscles and renal tubules (Fig. 1H).

Hesperidin prevents collagen deposition in kidney of DEN/CCl₄-induced rats

Kidney sections of control (Fig. 2A) and 100 mg/kg hesperidin supplemented rats (Fig. 2B) stained with Masson's trichrome revealed normal amount and distribution of collagen fibers in the interstitium of renal cortex. In contrast, kidney sections of DEN/CCl₄-induced rats showed a massive accumulation of collagen fibers in between the glomeruli and the renal tubules (Fig. 2C & 2D). DEN/CCl₄-induced rats treated with 50 (Fig. 2E) and 100 mg/kg hesperidin (Fig. 2F) showed markedly decreased collagen when compared with the DEN/CCl₄-induced rats.

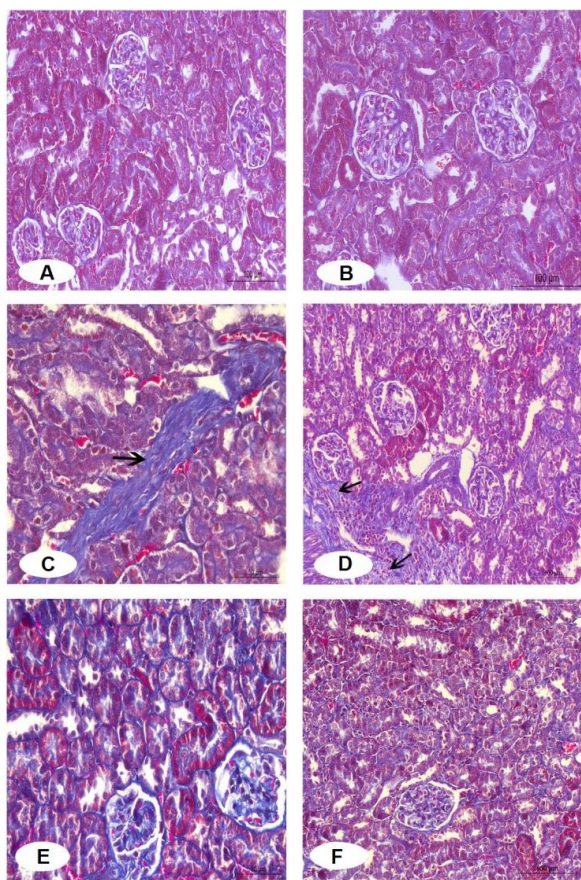


Fig 2: Hesperidin prevents collagen deposition in kidney of DEN/CCl₄-induced rats. Photomicrographs of Masson's trichrome-stained kidney sections of (A) control and (B) hesperidin treated normal rats showing normal content of collagen fibers in the interstitium of renal cortex, (C&D) DEN/CCl₄-induced rats showing heavy accumulation of collagen fibers in the interstitium of renal

cortex (arrows), (E) DEN/CCl₄-induced rats treated with 50 mg/kg hesperidin showing moderate content of collagen fibers in the renal corpuscles and tubules and (F) DEN/CCl₄-induced rats treated with 100 mg/kg hesperidin showing normal amount and distribution of collagen fibers.

Hesperidin diminishes lipid peroxidation and NO in kidney of DEN/CCl₄-induced rats

DEN/CCl₄-induced rats exhibited a significant ($P < 0.001$) increase in kidney lipid peroxidation levels when compared with the control rats as depicted in Figure 3A. Treatment with 50 and 100 mg/kg hesperidin significantly ($P < 0.001$) decreased lipid peroxidation in the kidney of DEN/CCl₄-induced rats. Similarly, DEN/CCl₄-induced rats showed a marked ($P < 0.001$) increase in kidney NO levels when compared with the control rats, an effect that was significantly ($P < 0.001$) reversed following treatment with both doses of hesperidin (Fig. 3B).

Oral supplementation of 100 mg/kg hesperidin didn't affect levels of lipid peroxidation (Fig. 3A) and NO (Fig. 3B) in the kidney of normal rats.

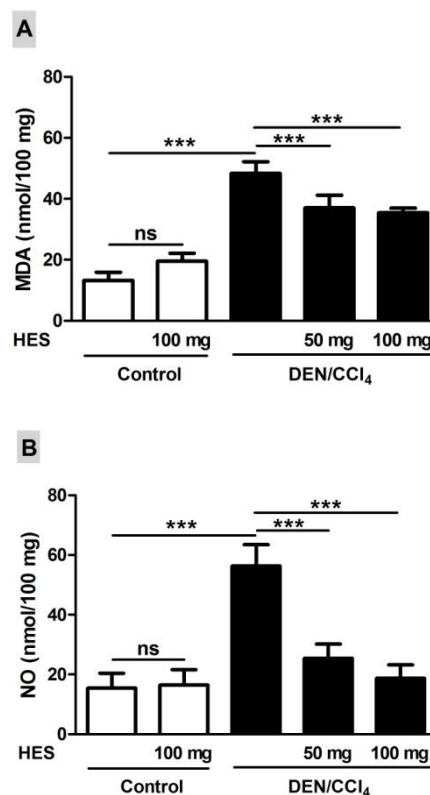


Fig. 3: Hesperidin diminishes (A) lipid peroxidation and (B) NO levels in kidney of DEN/CCl₄-induced rats. Data are expressed as Mean \pm SD, (N=6). *** $P < 0.001$. DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; HES, hesperidin; MDA, malondialdehyde; NO, nitric oxide; ns, non-significant.

Hesperidin enhances antioxidant defenses in kidney of DEN/CCl₄-induced rats

GSH content in the kidney homogenate of DEN/CCl₄-induced rats showed a significant ($P < 0.001$) decrease when compared with the control group as represented in Figure 4A. On the other hand, both doses of hesperidin markedly ameliorated GSH content in the kidney of DEN/CCl₄-induced rats. The activity

of SOD (Fig. 4B), GPx (Fig. 4C) and GST (Fig. 4D) was significantly ($P<0.001$) declined in the kidney of DEN/CCl₄-induced rats when compared with the corresponding control rats. Concurrent treatment of the DEN/CCl₄-induced rats with either dose of hesperidin significantly ($P<0.001$) alleviated the activity of SOD and GPx. The lower dose of hesperidin didn't affect the activity of GST which was significantly ($P<0.001$) increased in DEN/CCl₄-induced rats treated with the higher hesperidin dose.

Rats received 100 mg/kg hesperidin showed non-significant ($P>0.05$) changes in kidney GSH, SOD, GPx and GST when compared with the control rats.

Hesperidin activates Nrf2/HO-1 signaling in kidney of DEN/CCl₄-induced rats

DEN/CCl₄-induced rats exhibited significantly ($P<0.001$) down-regulated kidney Nrf2 (Fig. 5A) and HO-1 (Fig. 5B) when compared with the corresponding control group. DEN/CCl₄-induced rats treated with either 50 or 100 mg/kg hesperidin showed significant ($P<0.001$) amelioration of kidney Nrf2 and HO-1 expression. Normal rats treated with 100 mg/kg hesperidin showed significant ($P<0.001$) increase in kidney Nrf2 and HO-1 expression levels.

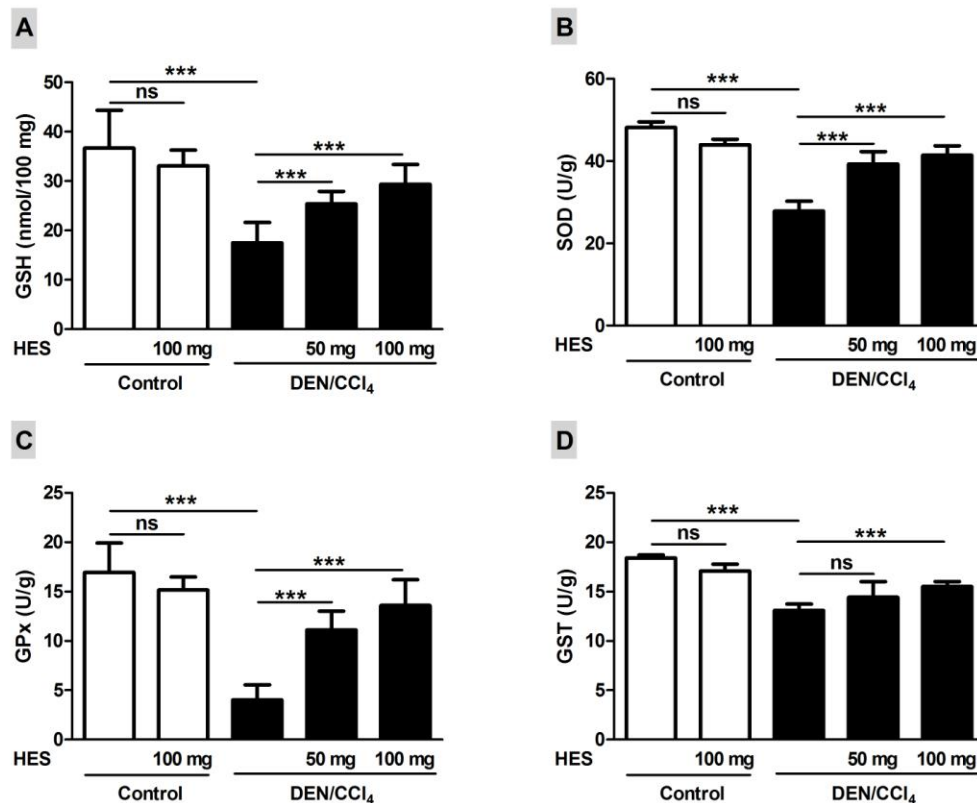


Fig 4: Hesperidin enhances antioxidant defenses in kidney of DEN/CCl₄-induced rats. Data are expressed as Mean \pm SD, (N=6). *** $P<0.001$. DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; HES, hesperidin; GSH, reduced glutathione; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; ns, non-significant.

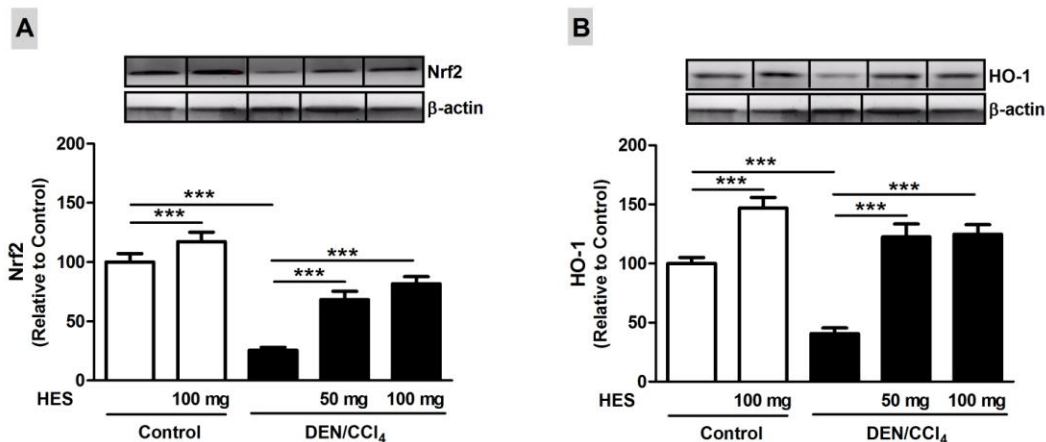


Fig. 5: Hesperidin activates Nrf2/ARE/HO-1 signaling in kidney of DEN/CCl₄-induced rats. Data are expressed as Mean \pm SD, (N=6). *** $P<0.001$. DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; HES, hesperidin; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO, heme oxygenase.

DISCUSSION

The kidney is highly susceptible to damage by toxicants because of the high volume of blood flowing through it and the filtration of large amounts of toxins which can be concentrated in the kidney tubules (Perazella, 2009). Herein, we demonstrated the protective effect of the citrus flavonoid hesperidin against DEN/CCl₄-induced nephrotoxicity in rats. Our results showed the ability of hesperidin to prevent DEN/CCl₄-induced kidney injury and oxidative stress via up-regulation of the Nrf2/HO-1 signaling pathway.

DEN/CCl₄-induced rats in the present study showed renal damage as evidenced by the significant increase in circulating levels of creatinine, urea and uric acid. Serum creatinine level has been reported to reveal glomerular function and its increase is an indicator of renal failure (Stevens and Levey, 2005). Urea is a by-product of protein metabolism and is used as a marker of kidney injury (Stevens and Levey, 2005), and uric acid has been proposed as a potential risk factor for new-onset kidney diseases (Kanda *et al.*, 2015). These findings are in agreement with our previous studies where we reported increased serum levels of creatinine, urea and uric acid in DEN/PB-induced rats (Ahmed *et al.*, 2015; Mahmoud *et al.*, 2015). Renal injury induced by DEN/CCl₄ was further confirmed by the observed histological alterations, including congestion and atrophy of glomerular tuft, edema, cloudy swelling in convoluted tubules, thickening of the blood vessels, pyknosis, fibrosis and inflammatory cells infiltration. In this context, we have shown dysplastic renal tubules with karyomegalic nuclei, atrophy of glomerular tuft, and inflammatory cells infiltration in the kidney of rats treated with DEN/PB (Ahmed *et al.*, 2015; Mahmoud *et al.*, 2015). Treatment of the DEN/CCl₄-induced rats with hesperidin significantly prevented kidney damage. These findings support our previous study where hesperidin prevented DEN/PB-induced nephrotoxicity in rats (Ahmed *et al.*, 2015). The nephroprotective effect of hesperidin has been previously reported in animal models of cisplatin and trichloroethylene-induced rats (Sahu *et al.*, 2013; Kamel *et al.*, 2014; Siddiqi *et al.*, 2015).

Renal fibrosis caused by excessive accumulation of collagen in the kidney is considered the principal process involved in the progression of chronic kidney diseases (Pradère *et al.*, 2008). The presence of kidney fibrosis seemed mostly to be viewed as an endpoint or marker of tissue or organ failure and loss of function (Cohen, 1995). In addition, Khubchandani *et al.* (2010) reported that the normal glomerular basement membrane composed of type IV collagen which has an important function in the process of filtration, therefore, increased collagen production plays a key role in the development and progression of glomerular sclerosis. Moreover, Yang *et al.* (2010) stated that the development of interstitial fibrosis was a secondary process that resulted from defective epithelial repair and could be regarded as a default mechanism of inadequate regeneration. Here, kidney sections of DEN/CCl₄-induced rats showed a massive accumulation of collagen fibers in the interstitium of renal cortex. To the best of

our knowledge, reports showing the fibrogenic effect of DEN in kidney of rats are scarce, but several studies demonstrating increased collagen deposition after CCl₄ administration (Ogeturka *et al.*, 2005; Hamed *et al.*, 2012) add support to our findings. Interestingly, treatment with hesperidin prevented excessive collagen deposition in the kidney of DEN/CCl₄-induced rats, demonstrating the anti-fibrogenic activity of hesperidin. The antifibrotic activity of hesperidin against liver fibrosis in rats has been reported in the studies of Elshazly and Mahmoud (2014) and Pérez-Vargas *et al.* (2014).

DEN has been reported to induce the generation of ROS and eventually resulting in oxidative stress and cellular injury (Pradeep *et al.*, 2007; Ahmed *et al.*, 2015; Mahmoud *et al.*, 2017a). Trichloromethyl free radicals, produced from CCl₄ metabolism, combine with cellular lipids and proteins in the presence of oxygen forming trichloromethyl peroxy radicals which attack lipids in the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethyl peroxy free radical elicit lipid peroxidation and destruct Ca²⁺ homeostasis, resulting in cell death (Matei *et al.*, 2008). ROS provoke peroxidation of native membrane lipids, leading to altered membrane functions through decreasing its fluidity and changing the activity of its bounding enzymes and their receptors. Since ROS have been implicated in the mechanisms that lead to tubular necrosis (Lopez-Novoa *et al.*, 2011), reduction of oxidative stress may be a good target for prevention and treatment of renal toxicity. This explains the significantly increased lipid peroxidation levels in the kidney of DEN/CCl₄-induced rats as we previously demonstrated (Ahmed *et al.*, 2015; Mahmoud *et al.*, 2015).

In contrast, DEN/CCl₄-induced rats showed a significant decline in kidney GSH content. The declined GSH might be due to nicotinamide adenine dinucleotide phosphate (NADPH) depletion or increased consumption of GSH in non-enzymatic removal of oxygen-radicals (Gumieniczek, 2005). In addition, reduced activity of the antioxidant enzymes SOD, GPx and GST was observed in the kidney of the DEN/CCl₄-induced rats. The reduced activity of antioxidant enzymes could be explained in terms of increased consumption of these enzymes to act upon the free radicals. Therefore, the induced renal damage in this study occurred via both stimulation of oxidative stress and abolishment of antioxidant defense system. In agreement with these findings, we have reported increased lipid peroxidation and NO along with declined GSH, SOD, GPx and GST in kidney and liver of DEN/PB-induced rats (Ahmed *et al.*, 2015; Mahmoud *et al.*, 2015, 2017a).

Treatment of the DEN/CCl₄-induced rats with hesperidin significantly attenuated lipid peroxidation, decreased NO and enhanced antioxidant defenses in the kidney. Therefore, we assume that hesperidin exerts a nephroprotective effect against DEN and CCl₄ via its antioxidant potential. In this context, we have reported the ability of hesperidin to attenuate oxidative stress in animal models of diabetes (Mahmoud *et al.*, 2012), nephrotoxicity (Ahmed *et al.*, 2015) and hepatotoxicity

(Mahmoud, 2014). In the study of Tirkey *et al* (2005), hesperidin successfully attenuated the effects of CCl₄ on GSH content and SOD activity in kidney tissues.

To explore the mechanism behind the attenuated oxidative stress and enhanced antioxidants in DEN/CCl₄-induced rats treated with hesperidin, we determined the expression levels of Nrf2 and HO-1. Nrf2 is a basic leucine zipper protein that activates the expression of antioxidant and defensive proteins involved in the detoxication and elimination of electrophilic agents and reactive oxidants (Nguyen *et al.*, 2009). Under homeostatic conditions, Nrf2 is kept at low levels via binding to a homodimer of Kelch-like ECH-associated protein 1 (Keap1) through the DC domain of either one of the Keap1 subunits. This binding leads to Cullin3/Rbx1-catalyzed polyubiquitination followed by proteasomal degradation of Nrf2 (Cullinan *et al.*, 2004; Katoh *et al.*, 2005). In cases of oxidative/electrophilic or xenobiotic stress, the Cullin3/Rbx1-dependent polyubiquitination of Nrf2, resulting in the accumulation of Nrf2 and its translocation to the nucleus (Cullinan *et al.*, 2004). Within the nucleus, Nrf2 binds to the antioxidant response element (ARE) and activates the expression of genes of antioxidant proteins, including HO-1, nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1 (NQO1), SOD, GPx and GST (Hayes and Dinkova-Kostova, 2014). Therefore, activation of Nrf2 enhances the antioxidant defenses and subsequently prevent oxidative stress.

In this study, DEN/CCl₄-induced rats exhibited declined Nrf2 and HO-1 expression, an effect that was significantly reversed in hesperidin treated groups. Although Nrf2 is induced by oxidative/xenobiotic stress, DEN/CCl₄-induced rats showed a decrease in Nrf2 expression. This decline is a direct result of excessive ROS production as we have demonstrated in different models of surplus ROS production (Mahmoud and Al Dera, 2015; Kamel *et al.*, 2016; Abd El-Twab *et al.*, 2016; Mahmoud *et al.*, 2017b-g). In addition, we have recently reported decreased expression of Nrf2 and HO-1 in the liver of DEN/PB-induced rats (Mahmoud *et al.*, 2017a). Interestingly, DEN/CCl₄-induced rats treated with hesperidin showed a significant increase in kidney Nrf2 and HO-1 expression. In support of our findings, hesperidin has been reported to up-regulate Nrf2 expression in a model of gentamicin-induced nephrotoxicity (Subramanian *et al.*, 2015). However, our study is the first to show the ability of hesperidin to up-regulate Nrf2/HO-1 signaling in the kidney of DEN/CCl₄-induced rats.

In conclusion, our results demonstrate the protective efficacy of hesperidin against DEN/CCl₄-induced oxidative stress, kidney injury and fibrosis in rats. Hesperidin diminished lipid peroxidation and enhanced the antioxidant defenses GSH, SOD, GPx and GST in the kidney of DEN/CCl₄-induced rats. These effects are mediated via up-regulation of Nrf2/ARE/HO-1 signaling pathway.

ACKNOWLEDGEMENT

The authors wish to thank the Scientific Research

Development Unit at Beni- Suef University for funding this study.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest

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How to cite this article:

Aly MS, Galaly SR, Moustafa N, Mohammed HM, Khadrawy SM, Mahmoud AM, Hesperidin protects against diethylnitrosamine/carbon tetrachloride-induced renal repercussions via up-regulation of Nrf2/HO-1 signaling and attenuation of oxidative stress. *J App Pharm Sci*, 2017; 7 (11): 007-014.