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Toxicology

Ameliorative Potential of Naringin Against Di-n-butyl phthalate-Induced Hepatorenal Toxicity in Rats

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ABSTRACT

Di-n-butyl phthalate (DBP), a ubiquitous plasticizer, is widely used in many industrial products and directly involved in numerous health issues. Naringin (NG) is a dietary flavonoid that possesses numerous health benefits. This study aimed to evaluate the ameliorative potential of NG against the hepatorenal toxicity of DBP. Rats were assigned into six groups, and treated orally, 3 times a week for 8 consecutive weeks. Control vehicle group received olive oil, NG group received NG (80 mg/kg), DBP 250 and DBP 500 groups received DBP 250 and 500 mg/kg, respectively, NG+DBP 250 and NG+DBP 500 groups received NG, an hour before DBP 250 and DBP 500administration, respectively. DBP evoked dose-dependent elevations in the serum aminotransferases activities, and urea, creatinine, and malondialdehyde levels, accompanied with a significant reduction in the serum total antioxidant capacity. Histopathologically, DBP 250 intoxication induced mitotic changes in some hepatocytes and mild hydropic degeneration in hepatocytes, decreased the size of the glomerular tuft, and increased the size of Bowman's space of the kidney. Moreover, DBP 500groupshowed mitotic changes in several hepatocytes and moderate hydropic degeneration in hepatocytes, sloughing of epithelial cells of the Bowman's space, and accumulation of proteinaceousmaterial in Bowman's space and renal tubules. Contrariwise, concurrent treatment with NG substantially attenuated the hepatorenal toxic effects of DBP, evidenced by the notable improvement in the serum biomarkers and histological architectures of the liver and kidneys. In conclusion, NG, by means of its natural antioxidant activity, could be a valuable phytochemical against DBP-inflicted oxidative hepatorenal damage.

Keywords: Di-n-butyl phthalate, Hepatorenal toxicity, Naringin, Oxidative stress, Total antioxidant capacity.

INTRODUCTION

Phthalates (PAEs) are plastic-modifying syntheticchemicals used in numerous industrial, agricultural, and medical applications, leading todailydirect or indirecthumans'exposure (Heudorf et al., 2007). Among PAEs, di-butyl phthalate (DBP) is a prevalent phthalate plasticizer, which is used in various daily consumer products (Abdul Majeed et al., 2021). Consequently, DBP is found in the food, water, and air in high concentrations, and is considered ubiquitous food and environmental contaminant (Wormuth et al., 2006; Gao et al., 2018).Once DBP is absorbed, itis biotransformedrapidly into its metabolites and eliminated in urine and feces. Although DBP does not persist in organs after short-term administration, special concerns have been focused on its potential chronic toxic effects (Zeng et al., 2013), and henceDBP has gained extensive attention as a global public health issue. Beside its endocrine-disrupting effect, DBP and its metabolites have been reported to cause damage to multiple organs, particularly liver and kidneys (Cheng et al., 2019; Radha and Mahaboob Basha, 2020). Accumulating evidence has reported that the liver and kidneys are among the target organs of DBP accumulation and the most sensitive organ for its toxic effects (Zeng et al., 2013; Cheng et al., 2019). The evidence from relevant *in vivo* and*in vitro*studies suggested oxidative stress as one of the main mechanismsof DBP-induced hepatic and renal damage (Cheng et al., 2019; Radha and Mahaboob Basha, 2020; Cui et al., 2021).This dedicates the substantial role of antioxidant supplementations to alleviate the DBP-induced oxidative tissues damage. Citrus by-products extracts contain various phenolic and flavonoid constituents, mainly hesperidin, neohesperidin, naringin, rutin and narirutin (Alam et al., 2014). Naringin (NG) is a natural flavone predominantly detected in a variety of citrus fruit. Once ingested, NG is rapidly converted by intestinal microflora to naringenin, a highly absorbed metabolite (Chen et al., 2014). Recent studies demonstrated the antimicrobial, anticancer, antioxidant, anti-inflammatory, neuroprotective, nephroprotective, hepatoprotective and cardioprotective activities of NG and its metabolite, naringenin (Adil et al., 2015; Chen et al., 2016; Gopinath and Sudhandiran, 2016; Salehi et al., 2019). However, many researchers have investigated the effect of DBP on the reproductive system, scanty literature focused on its adverse effect on liver and kidney. In addition, there are controversies regarding whether there is a dose-dependent relationship between DBP and hepatorenal damage. Accordingly, this study aimed to investigate the dose-dependent hepatorenal adverse effect of DBP and the prospective mitigating and ameliorativepotential of NG.

MATERIALS AND METHODS

Materials:

Di-n-butyl phthalate (CAS No.84-74-2; purity of 99%) andNaringin(CAS No. 10236-47-2; purity of ≥ 90%) were purchased from Sigma–Aldrich Company. Diagnostic kits for assessment of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities;urea and creatinine, malondialdehyde (MDA), total antioxidant capacity (TAC) levels were purchased from Biodiagnostic Company. Other chemicals

and reagents were of analytical grades and commercially available.

Animals and experimental design:

The animals care and handling followed the ethical guidelines of the International Animal Care and Use Committee IACUC, Faculty of Veterinary Medicine, University of Sadat City (Approval No. VUSC-014-1-19). Generally, forty-two healthy male albino rats (180-200 g), of three months old were obtained from Alzyade Experimental Animals Production Center, Giza, Egypt. Rats were kept in the polypropylene cages at natural ventilated room under a standard laboratory condition (28±2 °C,50-65 % relative humidity and natural daily dark/light cycle), and allowed free access to standard commercial diet and clean tap water during the acclimatization period and throughout the experiment.

Rats were randomly divided into six groups (n= 7), and treated orally, 3 times a week for 8 consecutive weeks. Control vehicle group: Rats received olive oil, vehicle of DBP. Naringin (NG) group: Rats were administered 80 mg/kg bw NG dissolved in distilled water. Di-n-butylphthalat 250 (DBP 250) and Di-n-butylphthalat 500 (DBP 500) groups: Rats were administered 250 mg/kg bw and 500 mg/kg bw DBP dissolved in olive oil, respectively. Naringin and Di-n-butylphthalat 250 (NG+DBP 250) and Naringin and Di-n-butylphthalat 500 (NG+DBP 500) groups: Rats were administered NG (80 mg/kg), an hour before DBP (250 mg/kg bw) and (500 mg/kg bw) intoxication, respectively. The doses of NG and DBP were selected based on previous studies of Arumugam *et al.* (2016) and Yin *et al.* (2016), respectively.

Samples collection and preparation:

At the end of the experiment and 24 h after the last treatment, rats were fasted overnight, anesthetized by inhalation of isoflurane. Blood samples were collected from the retro-orbital plexus and centrifuged at 3000 rpm at 4°C for 15 min and the collected sera samples were kept at -80°C for serum biochemical analyses. After, animals were euthanized by cervical dislocation, liver and kidneys from each rat were collected and fixed in neutral-buffered formalin 10% for histopathological investigation.

Assessment of serum liver and kidney functions biomarkers:

- Serum ALT and AST (CAT.NO. AT1034) activities and urea (CAT.NO. UR2110) and creatinine (CAT. NO. CR1250) levels were assayed using commercial kits, following the manufacturer's instructions.
- Serum testosterone was determined using direct, competitive immunoassay kit (Feldman et al., 2002).
- Serum testosterone was determined using direct, competitive immunoassay kit (Feldman et al., 2002).

Assessment of serum oxidant/antioxidant biomarkers:

Serum MDA (CAT. NO. MD2529) level and TAC (CAT. NO. TA 25 13) were estimated following the manufacturer's instructions of the commercial kits.

Histopathological investigation:

Formalin- fixed liver and kidney tissue samples were routinely processed, embedded in paraffin wax, sectioned with a microtome (3-5µm thicknesses), stained with hematoxylin and eosin (H&E) stainaccording to Bancroft and Layton (2013), and photographed by using Lieca DMLB microscopes and Leica EC3 digital camera.

Statistical analysis:

The obtained data were subjected to ANOVA followed by Duncan's Multiple Range test for post hoc analysis using SPSS software, version 16 (released in 2007) and are presented as means ± S.E. Statistical differences were set at $P<0.05$.

RESULTS

Naringin mitigatedthe di-n-butyl phthalate-induced increase in serum liver function biomarkers in rats:

As presented in Fig. 1, no significant differences were observed in the serum ALT and AST activities between control and NG-treated groupsat *P<0.05*. Compared to the normal control values, rats orally intoxicated with DBP at dose levels of 250 or 500 mg/kg revealed a dose-dependent increase in the serum activities of ALT and AST. Oral administration of NG, one hour before DBP 250 or DBP 500 intoxication, significantly reducedthe serum ALT and ASTactivities when compared to DBP 250 orDBP 500 groups, respectively, and restored the normal control values.

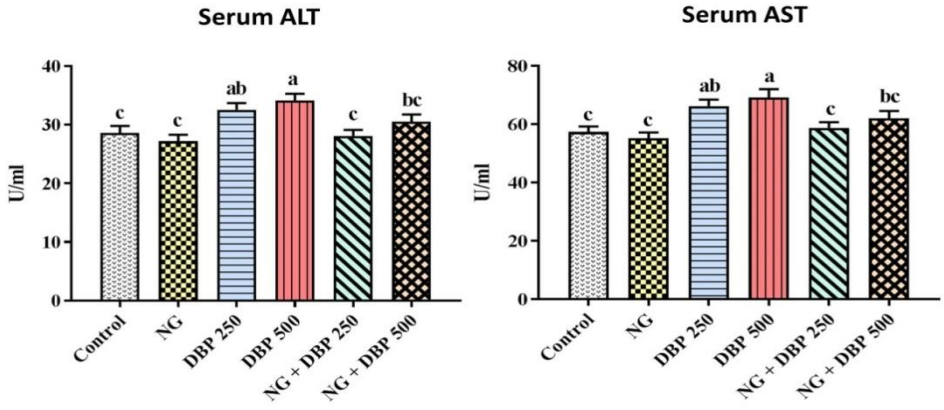

Figure (1): Serum alanine aminotransferase (ALT) andaspartate aminotransferase (AST) activities in control and different treated groups. Values are expressed as mean ± SE, n = 7. Bars with different letters (a, b, c) are significantly different at P<0.05. NG: naringin, DBP 250: di-n-butylphthalate (250 mg/kg), DBP 500: di-n-butylphthalate (500 mg/kg).

Naringin lowered the di-n-butyl phthalate-induced elevation in serum kidney function biomarkers in rats:

Regarding the control values, rats administrated NG did not show any significant changes in the serum urea and creatinine levels at *P<0.05*. However, oral administration of DBP at dose levels of 250 or 500 mg/kg, induced a significant dose-dependent elevation in the serum urea and creatinine levels. Interestingly, co-administration of NG with DBP 250 or DBP 500 significantly decreased the serum urea and creatinine levels, compared to DBP 250 or DBP 500 groups, respectively,

and restored their normal control levels (Fig. 2).

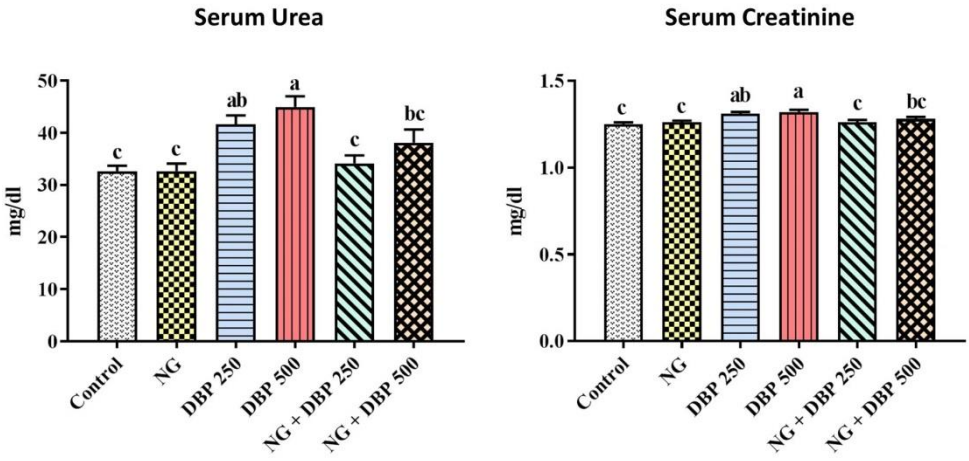

Figure (2): Serum urea and creatinine levels in the control and different treated groups. Values are expressed as mean ± SE, n = 7. Bars with different letters (a, b, c) are significantly different at P <0.05. NG: naringin, DBP 250: di-n-butylphthalate (250 mg/kg), DBP 500: di-n-butylphthalate (500 mg/kg).

Naringin improved the di-n-butyl phthalate-induced alterations in serum oxidant/antioxidant status in rats:

The alterations in the general oxidant/antioxidant status, including the serum MDA level and TAC are presented in Fig. 3. Compared to the control group, oral administration of NG induced no significant *(P<0.05*) variationsin theserum MDA level and TAC.In contrast, rats intoxicated with DBP 250 or DPB 500 exhibiteda significant dose-dependent increase in theserum MDA level concomitantly with significant reduction in the serum TAC, compared to the corresponding values of the control group. On the other hand, the concurrent administration of NG with either DBP 250 or DPB 500, significantly improvedthe DPB-induced alterations in the serum oxidant/antioxidant status, where it normalized the mean values of MDA level and TAC.

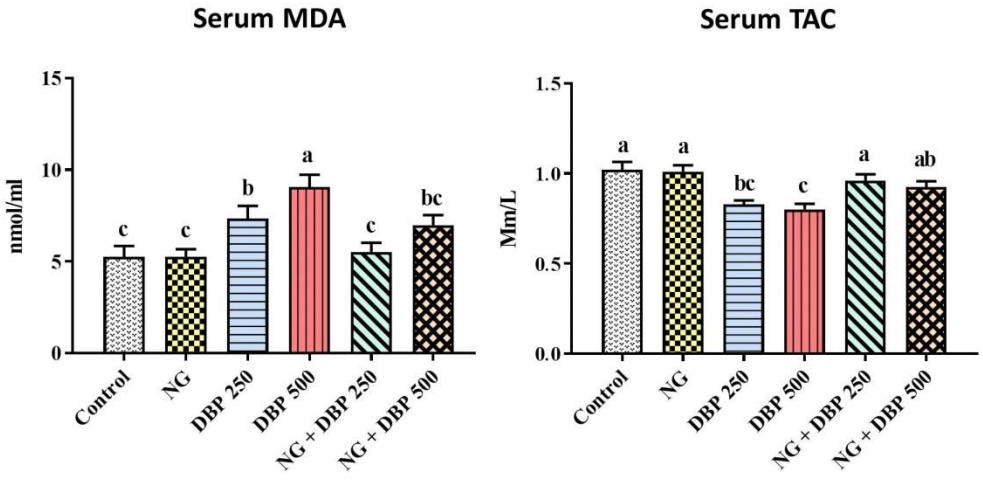

Figure (3): Serum malondialdehyde (MDA) level and total antioxidant capacity (TAC) in the control and different treated groups. Values are expressed as mean ± SE, n = 7. Bars with different letters (a, b, c) are significantly different at P <0.05. NG: naringin, DBP 250: di-n-butylphthalate (250 mg/kg), DBP 500: di-n-butylphthalate (500 mg/kg).

Naringin improved the di-n-butyl phthalate-induced alterations in liverhistoarchitectures:

Liver sections of both control (Fig. 4 A) and NG- treated (Fig. 4 B) groups showed normal histological architectures. Conversely, mitotic changes in some hepatocytes and mild vacuolar degeneration of hepatocytes were observed in liver sections of DBP 250- intoxicated rats (Fig. 4 C). Meanwhile, liver sections of DBP 500- intoxicated rats showed mitotic changes in many hepatocytes with moderate vacuolar degeneration in hepatocytes (Fig. 4 D & Fig. 5). On the other hand, hepatic tissues of rats co-administrated NG with DBP 250 showed few mitotic changes and mild vacuolar degeneration in hepatocytes (Fig. 4 E). While, hepatic tissues of rats co-administrated NG with 500 mg/kg of DBP showed some mitotic changes and moderate vacuolar degeneration in hepatocytes (Fig. 4 F).

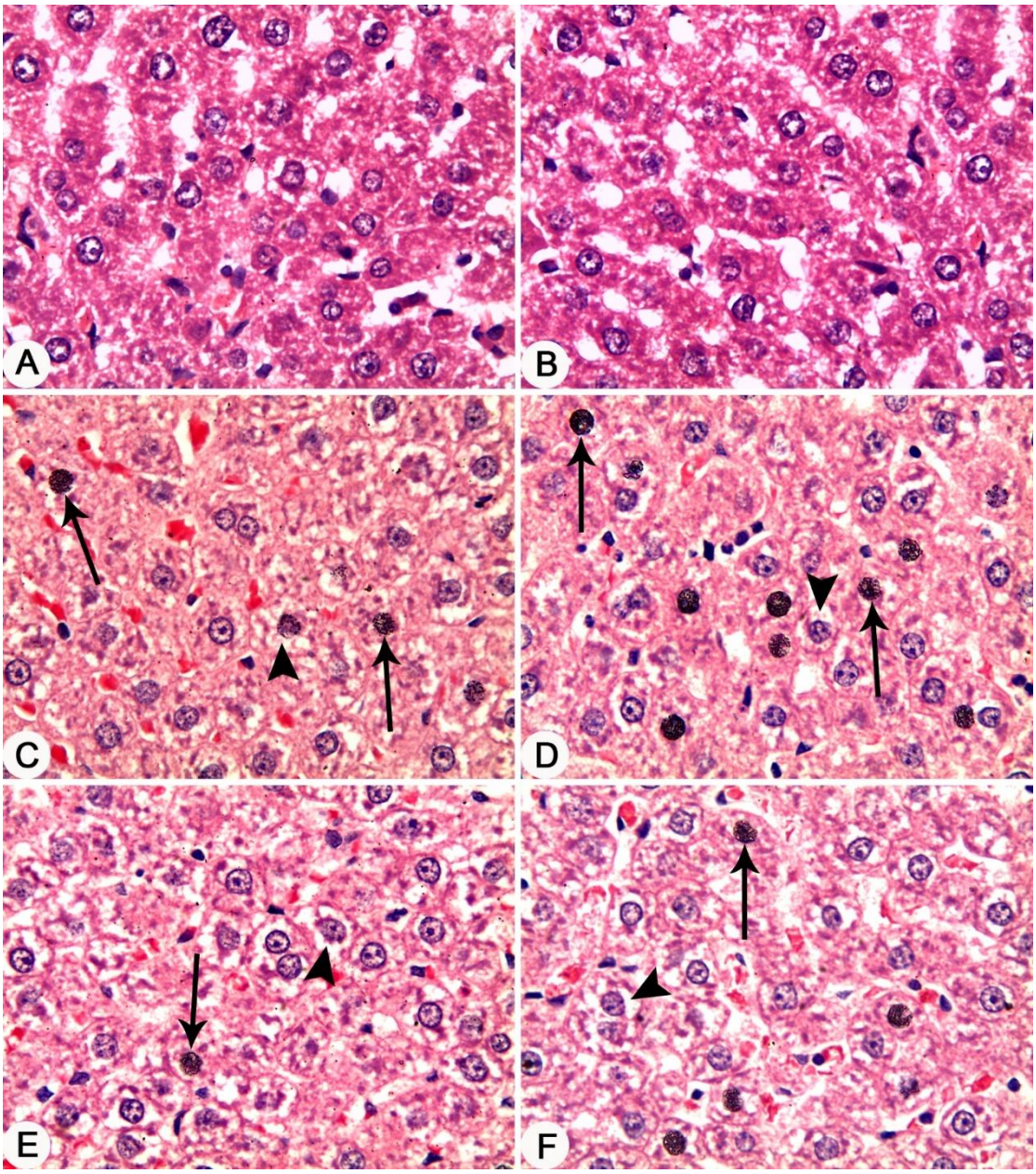

Figure (4): Liver, Rat: **A)** control vehicle group and **B)** Naringin treated group: showing normal histological architectures. **C)** DBP 250-intoxicated group: showing mitotic changes in some hepatocytes (arrows) and mild vacuolar degeneration of hepatocytes (arrowhead). **D)** DBP 500-intoxicated group: showing mitotic changes in several hepatocytes (arrows) and moderate vacuolar degeneration of hepatocytes (arrowhead). **E)** NG+DBP 250-treated group: showing mitotic changes in a few hepatocytes (arrows) and mild vacuolar degeneration of hepatocytes (arrowhead). **F)** NG+DBP 500-treated group: showing mitotic changes in some hepatocytes (arrows) and moderate vacuolar degeneration of hepatocytes (arrowhead). H&E stain, X 400.

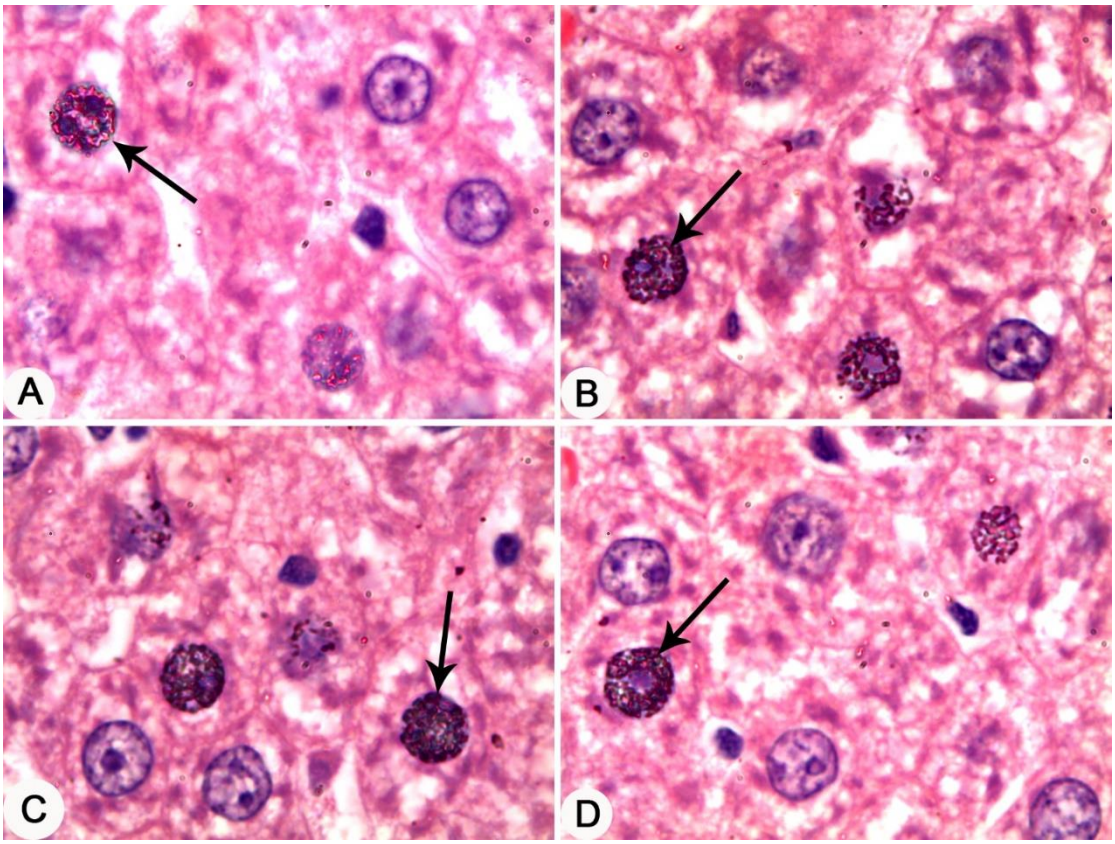

Figure (5): Liver, Rat: Dibutyl phthalate 500 mg treated group: **A)**Showing the nucleus of hepatocytes in the early interphase stage of mitosis (arrow). **B)**Showing the nucleus of hepatocytes in the late interphase stage of mitosis (arrow). **C)**Showing the nucleus of hepatocytes in the early prophase stage of mitosis (arrow). **D)**Showing the nucleus of hepatocytes in the late prophase stage of mitosis (arrow). H&E stain, X 1000.

Naringin improved the di-n-butyl phthalate-induced alterations in kidney histoarchitectures:

Normal renal architectures were observed in kidney sections of both control and NG-treated groups (Figs. 6 A, B). However, rats intoxicated with DBP at both doses of 250 mg/kg (Fig. 6C) and 500 mg/kg (Fig. 6D) showed decreasing the size of the glomerular tuff and increase the size of Bowman's space. Additionally, sloughing of few epithelial cells and accumulation of proteinous material in Bowman's space and presence of protein cast in renal tubules were observed in rats intoxicated with DBP 500 mg/kg. Conversely, co-administration of NG either with DBP 250 (Fig. 6E) or DBP 500 (Fig. 6F) showed normal renal histological architectures.

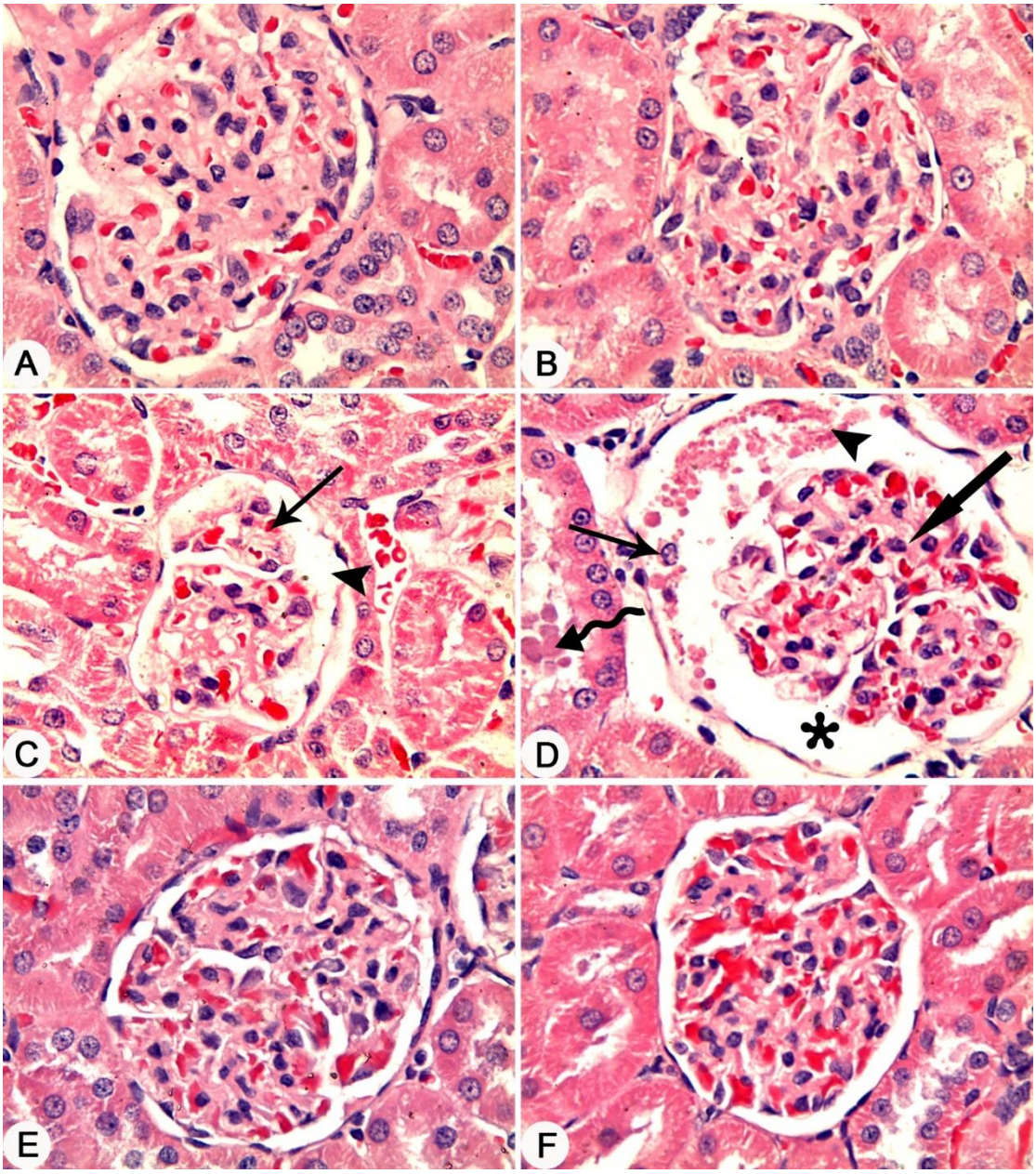

Figure (6): Kidney, Rat: **A)** control vehicle group and **B)** Naringin treated group: showing normal histological architectures. **C)** DBP 250-intoxicated group: showing decreasing the size of the glomerular tuff (arrow) and increase the size of Bowmans space (arrowhead). **D)** DBP 500-intoxicated group: showing decreasing the size of the glomerular tuft (thick arrow), increase the size of Bowmans space (asterisk), sloughing of few epithelial cells in the Bowmans space (thin arrow), accumulation of proteinous material in Bowmans space (arrowhead) and protein cast in renal tubules (bended arrow). **E)** NG+DBP 250-treated group: showing normal histological architectures. **F)** NG+DBP 500-treated group: showing normal histological architectures. H&E stain, X 400.

DISCUSSION

Di-n-butyl phthalate is a well-known environmental pollutant, which is widely used as a plasticizer in various industrial products and implicated in numerous health problems (Cheng et al., 2019).The

indiscriminate use of plasticizershas raised concerns abouttheir adverse health impacts. Naringin, a bioflavonoid found in citrus fruit peel, has many pharmacological properties (Ahmed et al., 2017). The role of the liver in detoxification andthe kidneys in excretion of xenobiotics predisposes these organs to the structural and/or functional damages. Thus, evaluation of liver and kidney functions markers, along with the histopathological alterations have a higher predictive value in assessment of the hepatic and renal toxic effects of environmental pollutants and may help in evaluation of the potential ameliorative role of the natural antioxidants against such toxic insults. Therefore, this study aimed to evaluate the prospective ameliorative role of NG as a natural therapeutic and protective alternativeagainst the hepatorenal toxicity of DBP in male rats.

Over the long term exposure, DBP could accumulate primarily in the liver and kidneys due to their curial role in the biotransformation and detoxification of xenobiotics (Zeng et al., 2013), resulting in adverse hepatic and renal toxic effects (Gao et al., 2018; Praveena et al., 2018; Cheng et al., 2019; Cui et al., 2021).The current study indicated the hepatorenal toxic effect of DBP, evidenced by the alterations of serum liver and kidney functions and oxidant/antioxidant biomarkers, along with the histopathological findings in liver and kidney architectures.

Our findings demonstrated that oral administration of rats with DBP at dose levels of 250 or 500 mg/kg for two months significantly increased, in a dose-dependent manner, the serum activities of ALT and AST with mitotic changes and hydropic degeneration of hepatocytes. Similarly, Cheng et al., 2019reported significant increases in the serum AST/ALT ratio, with obvious edema of the liver cells, narrowing of the hepatic sinusoids, and dilated central vein in mice following exposure to DBP at a dose level of 50 mg/kg/day for 28 consecutive days. A recent study of Radha and Mahaboob Basha (2020) on rats exposed to DBP for threegenerations demonstrated increase in serum transaminase activities, associated with congestion and dilatation of sinusoids and remarkable loss of hepatic architecture. Abdul Majeed et al. (2017) reported significant increase in serum ALT activity of rats fed50 mg/kg DBP/day for 13 weeks. Conversely, no significant effects were observed in serum ALT and AST activities of rats exposed to lower doses (10, 50, 100 mg/kg) of DBP in feed for 4 weeks (Abdul Majeed et al., 2021).

Serum transaminases, ALT and AST, are the most reliable indicators for hepatocellular damage. The degenerationof hepatocytes and the changes in the cell membrane permeability result in the release of these enzymes into the bloodstream and the elevation in their serum activities (Kasarala and Tillmann, 2016).Thus, the recorded elevation in serum ALT and AST activities in DBP-intoxicated rats may reflect the degeneration and the cellular damage of hepatocytes,which wereobserved in the histopathological findings.

Concerning the renal toxic effect of DBP, impairment in the kidney functions was recorded in DBP-exposed rats, evidenced by a dose-dependent increase in serum urea and creatinine levels, beside the recorded pathological changes in renal architectures. Consistent with these results, mice exposed

to 50 mg/kg DBP for 28 days exhibited significant elevation in the serum urea and creatinine levels (Cheng et al., 2019; Lianget al., 2021), with remarkable pathological changes in the glomeruli and renal tubules (Cheng et al., 2019). Serum urea and creatinine levels are valuable indicators for kidney dysfunction, where elevation of urea level reflects impairment in renal tubular reabsorption, and the increment of in serum creatinine level indicates impairment of glomerular filtration rate (Adedara et al., 2012). Herein, the recorded impairment in renal function and the alterations of renal tissue architecture suggest the adverse toxic effect of DBP on kidney.

Oxidative stress is the impairment of cellular oxidant/antioxidant status due to the overproduction of ROS and the depletion of the intracellular antioxidants, resulting in lipid peroxidation and alternations in membrane permeability (Lopez et al., 2007). In the present study, the recorded biochemical and pathological changes may be as a result of oxidative stress that is indicated by the significant dose-dependent elevation of serum MDA level andthe significant reduction of serum TAC in DBP-intoxicated rats. In the same line, the results recorded by Radha and Mahaboob Basha (2020) also pointed toward the involvement of oxidative stress in the hepatorenal toxic effects of DBP, resulting in disturbances in their serum function biomarkers. Accumulating evidence suggested that DBP could increase the intracellular ROS levels (Cheng et al., 2019; Wang et al., 2020; Liang et al., 2021), leading to changes in the cell membrane permeability and loss of cellular homeostasis, which may probably explains the liver and kidney dysfunctions and tissue damage. Meanwhile, ROS have been reported to be implicated in signal transduction pathways and can induce necrosis and/or apoptosis, which ultimately lead to pathological changes and organ dysfunction. In addition, recent studies of Cheng et al. (2019) and Liang et al. (2021) proved that DBP-induced hepatorenal dysfunctions were likely to be related to the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway, which play a crucial role in cellular oxidative stress pathway, and consequently enhance oxidative tissue damage.

According to previous studies, the hepatorenal protective effect of the phytochemicals is related, at least in part, to their anti-inflammatory and antioxidant properties (Abd Eldaim et al., 2020; Saleh et al., 2022). Antioxidants can prevent oxidative stress-mediated tissue injuries directly, by scavenging of ROS, and indirectly, by enhancing the intracellular antioxidant defense system (Kurutas, 2016). Flavonoids, are natural phenolic compounds found in various vegetables and fruit, which have been considered as potent antioxidants and free radical scavengers (Panche et al., 2016). Naringin, a natural flavonoid richly present in citrus fruit peel, possesses numerous therapeutic potential and has been demonstrated to exert antioxidant, anti-inflammatory, nephroprotective, hepatoprotective properties (Adil et al., 2015; Chen et al., 2016).

Regarding the cytoprotective and ameliorative effects of NG against DBP-induced hepatorenal adverse effects,the current results revealed that concomitant

treatment with NG at a dose level of 80 mg/kg, one hour before DBP 250 or 500-intoxication mitigated the observed biochemical and pathological alterations induced by DBP. This was evidenced by the marked reduction in serum ALT and AST activities and urea and creatinine levels, and the improvement in liver and kidney architectures, confirming its potent hepato- and reno-protective effects.Our findings are in agreement with previous findings of Adil et al. (2015), who reported that oral administration of NG as dose levels of 40 and 80 mg/kg for 28days alleviated the hepatic and renal toxic effects induced by sodium arsenite via decreasing the serum ALT and AST activities, hepatic and renal MDA level and increasing their GSH content and SOD activity, along with marked improvement in hepatic and renal tissues architectures. Similarly, Adil et al. (2016) confirmed that NG pretreatment significantly decreased serum transaminases, urea and creatinine values, significantly restored the altered MDA, NO, GSH, SOD levels, beside the improvement of the histological alterations induced by acetaminophen in the liver and kidney.The hepatorenal protective mechanisms of NG may involve the elimination of free radicals, up-regulation of the antioxidant genes, down-regulation of the inflammatory cytokines, and modulating the apoptotic/antiapoptotic genes (Dong et al., 2015; Amini et al., 2019; Elsawy et al., 2021). Taken all, the notable improvement in liver and kidney functions and structures in DBP- intoxicated rats pretreated with NG could be attributed to its combined pharmacological actions, mainly antioxidant, anti-inflammatory, and anti-apoptotic activities (Chen et al., 2016; Amini et al., 2019; Elsawy et al., 2021).

CONCLUSION

The aforementioned results demonstrated the potent ameliorative effect ofNG against DBP-induced hepatorenal toxic effects, reflected by the improvement of liver and kidney functions biomarkers that correlate to the recorded improvement in general oxidant/antioxidant status and histological structure of liver and kidney. Eventually, NG may provide an efficient strategy to counteract the side effects DBP, possibly via its curcial antioxidant properties.

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AUTHORS' CONTRIBUTIONS

Conceptualization: Salah S. El-Ballal, Hany A. Amer, Nermeen B. El-Borai; Methodology: Sameh H. El-Nady, Nermeen B. El-Borai; supervision: Anis Anis, Salah S. El-Ballal, Hany A. Amer, Nermeen B. El-Borai; investigation: Anis Anis, Sameh H. El-Nady, Salah S. El-Ballal, Hany A. Amer; data analysis: Sameh H. El-Nady, Nermeen B. El-Borai; writing original draft: Anis Anis, Sameh H. El-Nady, Nermeen B. El-Borai; writing—review & editing: Anis Anis, Sameh H. El-Nady, Salah S. El-Ballal, Hany A. Amer, Nermeen B. El-Borai.

STATEMENTS & DECLARATIONS

Ethics approval and consent to participate:

Ethics approval and consent to participate this study was approved by the International Animal Care and Use Committee IACUC, Faculty of Veterinary Medicine, University of Sadat City (Approval No. VUSC-014-1-19).

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Consent to Publish:

All the authors have given their consent to publish this manuscript.

Competing Interest:

The authors declare no competingof interest.

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