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RESEARCH ARTICLE

Evaluation of Antioxidant Potential of Noni (*Morinda citrifolia L.*) Fruit Juice against 3-Methyl-4-nitrophenol (PNMC)-Induced Acute Nephrotoxicity in Rats

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A B S T R A C T

The 3-methyl-4-nitrophenol (PNMC), found in pesticides and diesel exhaust particles (DEP), increases oxidative stress and induces tissue injury, while Noni (*Morinda citrifolia L*) is known for its antioxidant and anti-apoptotic properties. This study aimed to investigate the potential benefits of Noni juice on PNMC-induced nephrotoxicity in rats. A total of 56 adult male Sprague-Dawley rats were randomly divided into eight groups (n=7 each). These groups included: Control (PBS+0.05% Tween 80 S/C), Noni (2 ml Noni juice per rat), 1 mg/kg PNMC, 10 mg/kg PNMC, 100 mg/kg PNMC, Noni+1 mg/kg PNMC, Noni+10 mg/kg PNMC and Noni+100 mg/kg PNMC. Noni juice was given @ 2ml per rat through oral gavage, while PNMC was given through S/C injections. Treatments were given daily for five days. Then the rats were euthanized and blood samples were collected for serum biochemical analysis and assessing oxidative stress status. Kidneys were processed for histopathological and ultrastructural analyses and immunohistochemical demonstration of iNOS, eNOS, NGAL, active caspase-3 expressions and apoptotic index. The iNOS protein level was also assessed by ELISA. Results showed dose-dependent increasing degenerative changes in the kidneys with 1, 10 and 100 mg/kg of PNMC. The severity of these lesions was reduced in Noni+PNMC treatment groups. Mitochondrial damage occurred with 100mg/kg PNMC. A significant dose-dependent increase was recorded in iNOS, eNOS, NGAL and active caspase-3 expressions and apoptotic index in the kidneys with PNMC, while these parameters were decreased with Noni juice administration. Oxidative stress was highest with 100 mg/kg PNMC and decreased in Noni+PNMC groups. In conclusion, Noni juice efficiently attenuated PNMC-induced acute renal toxicity; however, its efficacy against PNMC induced chronic toxic effects need to be investigated.

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INTRODUCTION

Diesel exhaust particulates (DEP) are among the important causes of air pollution. Among their components, 3-methyl-4-nitrophenol (PNMC) plays the primary role. The PNMC is also a degradation product of phenythrothion, an organophosphate insecticide used in agriculture, veterinary medicine and for insect control. Residues of PNMC are found in the food, soil and water (Li *et al.,* 2009; Min *et al.,* 2016), increasing the risk of exposure for both humans and wildlife. Previous studies have documented the bioaccumulation of PNMC in, and its adverse effects on, biological systems, including the reproductive, endocrine, respiratory, cardio-vascular and immune systems. These adverse effects are associated with mechanisms like oxidative stress and apoptosis, with the renal tissues are particularly susceptible (Bu *et al.*, 2012; Fan *et al.*, 2022; Hu *et al.*, 2022).

Noni (*Morinda citrifolia L.*) is a fruit that grows in tropical regions worldwide and is rich in vitamins, minerals and antioxidants. Its nutritional and pharmacological properties, including anti-inflammatory and antioxidant effects, make it an important candidate for reducing adverse effects of reactive oxygen species (ROS). Noni fruit has been traditionally used for the treatment/control of diabetes, high blood pressure, inflammation and cancer. A recent study has shown the protective effects of Noni on the kidneys against xenobiotic-induced damage (Nuengchamnong *et al.*, 2023).

It is important to understand the possible adverse effects of PNMC on the kidney and the potential protective treatments for the potential risks associated with PNMC exposure. Therefore, aim of the present study was to investigate the possible protective effects of Noni fruit juice on PNMC induced kidney injury in adult male rats. These protective effects of Noni juice on PNMC-induced kidney injury were evaluated in terms of histological, immunohistochemical, oxidative stress and biochemical parameters.

MATERIALS AND METHODS

Chemicals and reagents: The 3-methyl-4-nitrophenol (PNMC, 2581-34-2) was purchased from the Sigma Chemical Co., USA. Noni fruit juice (99.5%) was purchased from a local private company (Alnoni Ltd., Antalya, Turkey).

Animals and experimental design: A total of 56 adult male Sprague-Dawley rats, weighing 200-280g, were obtained from the Istanbul University Experimental Animal Institute, Istanbul, Turkey. The animals were housed under a 12-hour light/dark cycle at 22±2°C and offered the standard rat diet and clean water *ad libitum.* The animals were allowed one week adaptation period before experiment. Experimental protocol was approved by the Istanbul University Local Ethics Committee for Animal Experiments (No: 2013/53).

Animals were randomly divided into eight groups, with seven rats in each group, and subjected to Noni juice and PNMC administration, as shown in Table 1. Each rat received 2 ml of Noni Juice via oral gavage following Temitope *et al.* (2011). Noni juice was given one hour before PNMC daily for five days. The doses of PNMC were prepared by dissolving PNMC in PBS with 0.05% Tween 80, as described by Yue *et al.* (2012).

Animals were euthanized by cervical dislocation under anesthesia 24 hours after the last treatment and their body weights were recorded. Blood was drawn from the heart of each rat, serum was separated and stored at -80°C. Kidneys were collected for morphological, histopathological and electron microscopy evaluation, with a small section stored at -80°C for ELISA. Tissues were fixed in 10% buffered formalin and 2.5% glutaraldehyde solution for histopathology and electron microscopy.

Morphological evaluation

Relative kidney weight (%): Kidney weights were recorded on the sixth day, 24h post final application) to calculate the percentage weight of organs relative to body weight, using the formula of Craig *et al.* (2015).

Histopathology and ultrastructural evaluations

Histopathological examination: Kidney samples were embedded in paraffin and sectioned at 4-5 µm. Sections were processed for routine staining with hematoxylin and eosin (H&E) and examined under a light microscope (Leica DM4000 B).

Electron microscopic evaluation: Sections of the kidneys were prepared for electron microscopic examination according to the method of Dittmayer *et al.* (2021). Ultra-thin sections (50 nm) from these regions were contrasted using 3% uranyl acetate and lead nitrate solutions and analyzed with a Jeol JEM 1011 transmission electron microscope (JEOL USA, Inc., MA, USA).

Immunohistochemical evaluation: Immunohistochemical sections of kidneys were prepared according to Abd-Elhamid *et al.* (2018). The antibodies were targeted to eNOS rabbit polyclonal antibody (1:100, RB-9279; Thermo Scientific, USA), iNOS rabbit polyclonal antibody (1:100, RB-9242-R7; Thermo Scientific, Fremont, USA), caspase-3 (active) (1:100, JM-3015-100; MBL Life, Woburn, USA) and anti-lipocalin-2 antibody /neutrophil gelatinase-associated lipocalin (NGAL) (1:250, ab41105; Abcam Cambridge, United Kingdom). The secondary antibody used was commercial kit (TP-125-BN, Thermo Scientific, USA) containing a biotinylated goat polyvalent antibody. Finally, Mayer's Hematoxylin was used to counterstain the sections.

Table 1: Experimental design, dosage and administration methods for Noni fruit juice and PNMC in rats of different groups

No	Group	Dosage and administration methods
1	Control	PBS (1 ml/kg b.w., s.c.) with 0.05% Tween 80
2	Noni	Noni fruit juice (2ml/animal, oral gavage)
3	1 mg/kg PNMC	PNMC (1 mg/kg, s.c.)
4	10 mg/kg PNMC	PNMC (10 mg/kg, s.c.)
5	100 mg/kg PNMC	PNMC (100 mg/kg, s.c.)
6	Noni+1 mg/kg PNMC	Noni fruit juice (2 ml/animal, oral gavage)+PNMC (1 mg/kg, s.c., 1 hr after Noni)
7	Noni+10 mg/kg PNMC	Noni fruit juice (2 ml/animal, oral gavage)+PNMC (10 mg/kg, s.c., 1 hr after Noni)
8	Noni+100 mg/kg PNMC	Noni fruit juice (2 ml/animal, oral gavage)+PNMC (100 mg/kg, s.c., 1 hr after Noni)

Scoring of immunoreactivity: The immunohistochemical staining process of iNOS, eNOS, caspase 3 (active) and NGAL was processed utilizing the histological score system, H-Score, which serves as a semiquantitative measurement of staining intensity and distribution (Bacus *et al.,* 1988). The percentage of positive cells was calculated, while the staining intensity score was divided into four levels: negative (0), weak (1), moderate (2) and strong (3). Finally, these two values were combined to generate the H-Score by the following formula:

H-Score = Σ Pi (i+1) where Pi; positive cell percentage, and i; intensity score.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL method): The TUNEL method was performed with an ApopTag Plus Peroxidase in situ Apoptosis Detection Peroxidase Kit (S7101, Chemicon International, Darmstadt, Germany) to detect apoptosis in kidney tissues. Rat thymus tissue served as a positive control, while distilled water substituted for the primary antibody was used as a negative control. The apoptotic index was evaluated by a Leica DM4000B light microscope, which was computer-assisted and set at x400 magnification. The apoptotic index was computed using the following formula (Yigit *et al.,* 2016):

Apoptotic index (AI) = (Number of apoptotic cells/Total number of cells i.e. non-apoptotic+apoptotic) x 100

Measuring oxidative stress parameters

Assessment of iNOS: The kidney samples were homogenized in an ice-cold 50 mM phosphate buffer (pH 7.4) and centrifuged at 3000g for 20 minutes at 4°C to obtain a clear supernatant. Protein concentrations of iNOS in kidney supernatants were quantified using an ELISA kit (YHB0574Ra; Shanghai YeHua Biological Technology Co, China) and expressed as ng/mg protein.

Evaluation of total oxidant (TOS) and antioxidant status (TAS): The serum oxidative stress panel was assayed using TOS and TAS reagents (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey). Oxidative stress index (OSI) was calculated per sample using: OSI (arbitrary unit) = TOS (µmol H_2O_2 Eq/L)/TAS (µmol Trolox Eq/L), following the protocol of Erel (2005).

Biochemical analysis: Blood samples were centrifuged at 2,500g for 15 min for serum separation, then aliquoted into 1.5 mL tubes and stored at -80°C until analysis. To minimize inter-assay variation, samples were assayed in a single batch. Parameters like urea, creatinine, albumin, AST, ALT and electrolytes (sodium, potassium and chloride) were quantified using an autoanalyzer (Architect c8000, Abbott Laboratories, IL, USA).

Statistical analysis: Statistical analysis was performed using one-way ANOVA, followed by Duncan's multiple means comparison, using SPSS version 13.0 for Windows (SPSS, Chicago, Illinois, USA). Data were expressed as mean±SE with statistical significance at $p \leq 0.05$.

RESULTS

Relative kidney weight: In our study, the group treated with Noni+1 mg/kg PNMC had significantly higher relative kidney weights compared to the control group ($p<0.05$), as shown in Table 2.

Histopathological and ultrastructural examinations

Histopathological evaluation: In all treatment groups, the dose-dependent histopathological changes due to PNMC included tubular degenerative changes, single-cell necrosis of the tubular epithelium, hyaline casts in tubular lumina and tubular lipidosis in both cortex and medulla. Mesangial cell proliferation, thickening of the basement membranes and glomerular lobulation in the glomeruli were also seen.

No pathological findings were observed in the control (Fig. 1a) and Noni juice groups (Fig. 1b). Lobulation of the glomeruli and mild tubular degenerative changes, including scattered pyknotic tubular cells (Fig. 1c), were the common histopathological findings with low dose (1 mg/kg) of PNMC. No prominent histopathological changes were noted in the Noni+1 mg/kg PNMC combination group (Fig. 1d). Lipidosis in the renal epithelium (Fig. 1e), mesangial cell proliferation and the thickening of the basement membranes were observed in the glomeruli with 10 mg/kg of PNMC. These tubular and glomerular degenerative changes were not seen in the Noni+10 mg/kg PNMC group (Fig. 1f). Histopathological changes were more severe in 100 mg/kg of PNMC; hyaline casts in the tubular lumina (Fig. 1g), lipidosis and single-cell necrosis were noted in the renal tubules. In addition, mild mononuclear inflammatory infiltration was recorded in all groups of PNMC. The histopathological alterations were not noted in the Noni+1 mg/kg PNMC group, while such findings were observed in the Noni+10 mg/kg PNMC and Noni+100 mg/kg PNMC (Fig. 1h) combination groups, but the severity of the lesions was decreased.

Table 2: Relative organ weights and biochemical parameters for rats of different groups (Means±SE)

Parameters	Control	Noni	1mg/kg PNMC	10 mg/kg PNMC	100 mg/kg PNMC	Noni+ 1 mg/kg PNMC	Noni+ 10 mg/kg PNMC	Noni+ 100 mg/kg PNMC
Relative kidney weight (g/g bw)	0.76±0.22^{a}	0.75±0.20^{a}	0.74±0.13^{a}	0.72±0.18^{a}	0.79±0.69^{ab}	0.88±0.53^{b}	0.79±0.28^{ab}	0.72±0.26^{a}
Albumin (g/dL)	1.13±0.20	1.12±0.84	1.18±0.31	1.03±0.23	1.04±0.43	0.92±0.11	0.99±0.48	0.88±0.58
Creatinine (mg/kg/dL)	0.46±0.13	0.45±0.15	0.49±0.19	0.48±0.15	0.47±0.09	0.48±0.13	0.46±0.12	0.48±0.12
Urea (mg/kg/dL)	16.3±0.46	17.6±0.55	20.0±2.11	22.1±2.60	22.1±0.26	18.1±1.52	16.0±1.82	17.6±0.55
Sodium (mmol/L)	1.43±2.81^{bc}	1.41±1.55^{c}	1.47±1.01^{ab}	1.43±0.76^{bc}	1.49±1.08^{a}	1.46±1.44^{abc}	1.47±1.47^{ab}	1.45±0.79^{abc}
Potassium (mmol/L)	5.38±0.37^{b}	5.50±0.24^{b}	5.10±0.23^{b}	5.61±0.31^{b}	5.41±0.70^{b}	6.65±0.43^{a}	5.63±0.20^{b}	6.61±0.46^{a}
Chloride (mmol/L)	1.02±0.74^{c}	1.02±1.05^{c}	1.04±0.40^{abc}	1.02±0.84^{c}	1.06±1.11^{a}	1.05±1.24^{ab}	1.03±0.84^{bc}	1.02±0.44^{c}
ALT (U/L)	81.8±7.76	76.6±7.57	70.6±4.71	81.0±10.43	71.8±5.41	65.3±10.09	67.8±4.91	73.1±7.51
AST (U/L)	142.5±6.8^{a}	132.3±11.7^{a}	143.8±8.40^{a}	144.8±16.0^{a}	256.5±40.8^{c}	156.6±14.6^{ab}	131.6±8.14^{a}	207±19.5^{bc}

Values with different superscript letters within a row indicate a statistically significant difference ($p<0.05$).

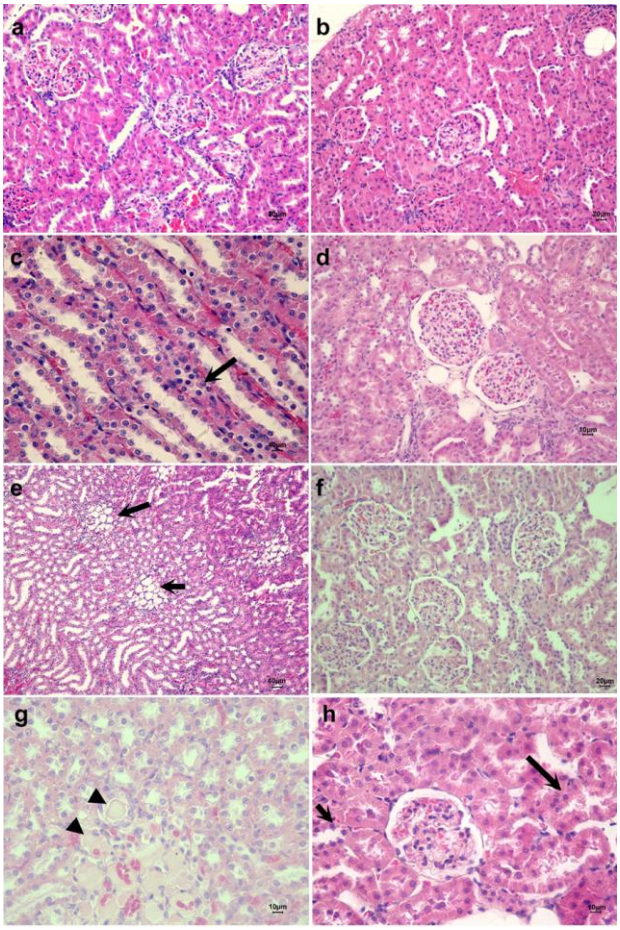

Fig. 1: Histopathology of the kidneys of rats of experimental groups showing: Control (a) and Noni juice (b): Normal histomorphology in the tubules and glomeruli. Scale bar: 20 µm (x200). 1 mg/kg PNMC (c); Pyknosis of the tubular epithelial cells (arrow). Scale bar: 10 µm (x400). Noni+1 mg/kg PNMC (d); Renal corpuscles and tubules reveal normal morphology except for mild congestion. Scale bar: 20 µm (x200). 10 mg/kg PNMC (e); Tubular lipidosis (arrowheads). Scale bar: 40 µm (x100). Noni+10 mg/kg PNMC (f): Diffuse and moderate degenerative changes in the tubular epithelium (arrows). Scale bars: 20 µm (x200). 100 mg/kg PNMC (g); Hyaline casts (arrowheads) in the medullary tubular lumina. Scale bar: 10 µm (x400). Noni+100 mg/kg PNMC (h): Single-cell necrosis (arrows) accompanying tubular degenerative changes. Scale bars: 10 µm. (x400; H&E)

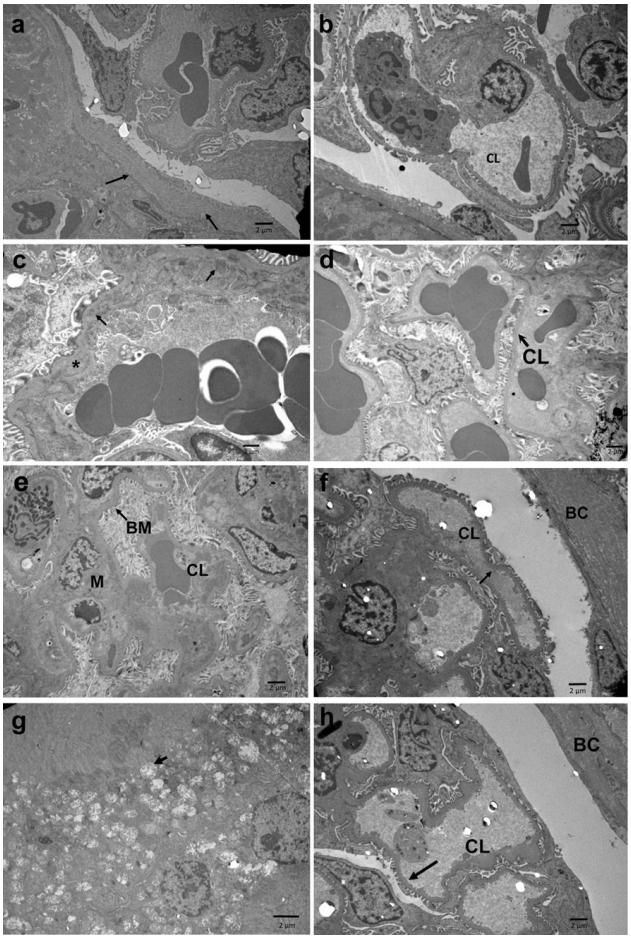

Fig. 2: Electron micrographs of the kidneys of rats from different groups showing: Control (a): Ultrastructurally normal glomeruli. The basement membrane thickness is within normal range, and its borders are regular (arrows). Noni juice (b): Glomerular structure and segments are normal; the capillary lumina (CL) are open and mesangium (M) reveals normal morphology. Scale bars: 2 µm. 1 mg/kg PNMC (c): The cortical region shows slight thickening of the basement membranes of the glomeruli, irregularity in the inner borders and sparse, scattered electron-dense deposits (arrows); slight microvillous transformation and partial pedicel obliteration in the epithelial cells and partial obliteration and endothelial interposition (*) in the fenestra of the capillary endothelium. Noni+1 mg/kg (d): The pedicles of the epithelial cells of the glomeruli are unremarkable, and the filtration slits are preserved. The lumina of the capillaries (CL) and endothelial fenestrae are clear (arrow). Scale bars: 2 µm. 10 mg/kg PNMC (e): Increased mesangial matrix (M), thickening of the basement membrane (BM), subendothelial electron-dense deposits, and microvillus transformation in epithelial pedicels; electron-dense deposits, obliterating (CL) the capillary lumina. Noni+10 mg/kg PNMC (f): Basement membrane borders are slightly irregular (arrows), and capillary lumina (CL) are open. Slight lamelarization of the Bowman's capsule (BC) is observed. Scale bars: 2 µm. 100 mg/kg PNMC (g): Deletion of the mitochondrial cristae in all tubules (arrow). Noni+100 mg/kg PNMC (h): Irregularity in the basement membrane borders and lamellae in the lamina densa of the capillary basement membrane, including Bowman's capsule (BC) and interstitial vessels (arrow). Endothelial fenestrae are preserved, and capillary lumens (CL) are visible. Scale bars: 2 µm.

Electron microscopic evaluation: Electron microscopy of kidneys revealed normal morphology in the glomeruli and proximal and distal tubules of the control (Fig. 2a) and Noni juice groups (Fig. 2b). Irregularities at the basement membrane boundaries of the glomeruli and alterations at the organelle level were observed in groups administered with 1 (Fig. 2c), 10 (Fig. 2e) and 100 mg/kg PNMC (Fig. 2g). Mitochondrial damage manifested by the deletion of the mitochondrial cristae was also noted in the 100 mg/kg PNMC group (Fig. 2g).

The tissue damage induced, particularly with median and high doses of PNMC, was not detected in the Noni+1 mg/kg PNMC group, and the ultrastructure was similar to that of the control at the glomerular level (Fig. 2d). In the Noni+10 mg/kg (Fig. 2f) and 100 mg/kg PNMC (Fig. 2h) combination groups, the changes induced by PNMC were milder than in PNMC groups.

Immunohistochemistry: In the kidney tissue, the iNOS H-Score was significantly increased in the 1, 10 and 100 mg/kg PNMC groups in a dose - dependent manner compared to control and Noni juice groups ($p<0.001$), with the highest value for 100 mg/kg of PNMC group (Fig. 5). Control (Fig. 3a) and Noni (Fig. 3b) groups showed weak iNOS immunoreactivity in renal tubules. In correlation with the PNMC doses, the intensity of iNOS staining increased with higher doses, reaching its peak at 100mg/kg PNMC dose (Fig. 3c). Considering all Noni+PNMC combination groups, an inverse relation was observed in a dose - dependent manner with respect to

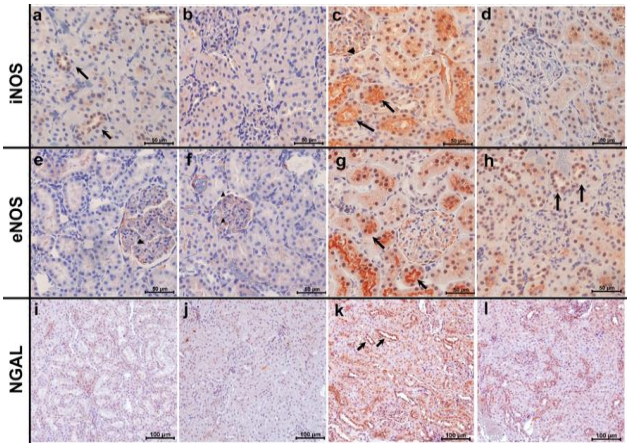

Fig. 3: Immunohistochemical staining for iNOS, eNOS, NGAL in the kidneys of experimental rats showing: iNOS: Control (a) and Noni (b): Weak iNOS immunoreactivity in the renal tubules (arrows). 100 mg/kg PNMC (c): Strong iNOS positivity, increasing in a dose-dependent manner in the renal tubules (arrows) and glomeruli (arrowhead). Noni+100 mg/kg PNMC (d): The iNOS staining intensity of the renal tubules and glomeruli decreases in an inverse correlation with the dose. Scale Bars: 50 µm (x400): eNOS: Control (e) and Noni (f): Weak eNOS immunoreactivity in the glomeruli (arrowhead). 100 mg/kg PNMC (g): Strong eNOS immunoreactivity in the renal tubules (arrows). Noni+100 mg/kg PNMC (h): Marked reduction in eNOS-positive staining in the renal tubules (arrows) and glomeruli. Scale bars: 50 µm (x400): NGAL: Control (i) and Noni (j): Very weak NGAL-positive staining. 10 mg/kg PNMC (k): Intense positive staining for NGAL in the tubular cells (arrows). Noni+10 mg/kg PNMC (l): Reduced NGAL immunoreactivity in the tubular cells. Scale bars: 100 µm (x200).

immunoreactive cells positive for iNOS. Additionally, Noni+1 mg/kg PNMC demonstrated no change in this parameter, like the levels observed in the control and Noni-only groups. A reduction in iNOS immunostaining was observed in the Noni+10 and 100 mg/kg PNMC combination groups. In Fig. 3d, a decrease in iNOS immunostaining is seen with the combination of Noni+100 mg/kg PNMC.

In the kidney tissue, a significant increase was noted in the eNOS H-Score of the 10 and 100 mg/kg PNMC groups compared to the control and Noni groups, while this parameter was significantly decreased ($p<0.001$) in the Noni+1 and 10 mg PNMC groups compared to Noni+100 mg/kg PNMC (Fig. 5). However, no difference was noted between the 100 mg/kg PNMC and Noni+100 mg/kg PNMC groups. The control (Fig. 3e) and Noni group (Fig. 3f) immunohistochemically showed weak eNOS expression in the glomeruli, while 1, 10 and 100 mg/kg PNMC groups showed strong eNOS immunoreactivity in the renal tubules and glomeruli. In Fig. 3g, the strongest eNOS reactivity is demonstrated in the 100 mg/kg PNMC group. On the other hand, Noni+1, 10 and 100 mg/kg PNMC groups revealed a decrease in the eNOS immunoreactivity in the renal tubules and glomeruli. This decrease was most notably observed in the Noni+100 mg/kg PNMC group (Fig. 3h).

The NGAL H-Score was significantly increased in 1, 10 and 100 mg/kg PNMC compared to the control and Noni groups ($p<0.001$), while it was decreased in Noni+1, 10 and 100 mg/kg PNMC groups. However, this decrease was statistically significant only in the Noni+1 mg/kg PNMC compared to all PNMC groups (Fig. 5). While the control (Fig. 3i) and Noni (Fig. 3j) groups showed very weak NGAL-positive staining, the 1, 10 (Fig. 3k) and 100 mg/kg PNMC groups exhibited intense positive staining for NGAL in the tubular cells. The intensity of NGAL immunoreactivity in the renal tubular cells was decreased in all Noni+PNMC combination groups, with being significant in Noni+1 and 10 mg/kg (Fig. 3l) PNMC groups. Regarding the active caspase 3 H-Score in the kidney tissue, a significant increase was detected in all PNMC (1, 10 and 100 mg/kg) groups compared to the control group. However, the 1 mg/kg PNMC group did not differ significantly from the Noni group. Additionally, Noni+PNMC combination groups did not show significant differences compared to control group, with the exception of Noni+100 mg/kg PNMC group. However, a statistically significant decrease was observed in the Noni+1 mg/kg PNMC group compared to all PNMC groups (Fig. 5).

In the control (Fig. 4a) and Noni (Fig. 4b) groups, weak activity of caspase 3 immunoreactivity was observed in the tubular epithelial cells. Also, the staining intensity for active caspase-3 was reduced in the Noni+10 mg/kg PNMC (Fig. 4d) and Noni+100 mg/kg PNMC groups (Fig. 4f) compared to 10 (Fig. 4c) and 100 mg/kg PNMC groups (Fig. 4e).

Among all eight groups, the apoptotic index was significantly increased only in the kidney tissues of 1, 10 and 100 mg/kg PNMC groups compared to control and Noni juice groups. In contrast, the apoptotic cell index was significantly decreased in all Noni+PNMC combination groups ($p<0.001$) compared to only PNMC groups (Fig. 5). The TUNEL-positive cells with weak staining were observed in the control (Fig. 4g) and the Noni (Fig. 4h) groups. The TUNEL-positive cells were increased in the renal tubules of 10 mg/kg PNMC (Fig. 4i) and 100 mg/kg PNMC (Fig. 4k) groups, and decreased in the groups treated with Noni, specifically in Noni+10 mg/kg PNMC (Fig. 4j) and Noni+100 mg/kg PNMC (Fig. 4l) groups.

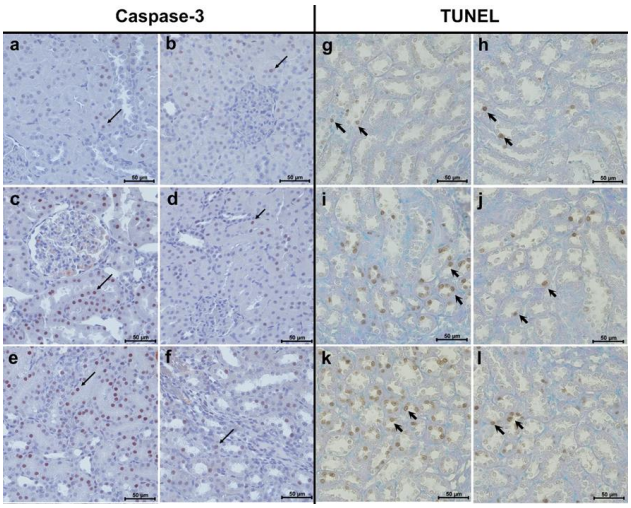

Fig. 4: Immunohistochemical staining for active Caspase-3 and (TUNEL) of apoptosis in the kidneys of experimental rats showing: Control (a) and Noni (b): Weak active Caspase-3 immunoreactivity in the tubular epithelial cells (arrows). 10 and 100 mg/kg PNMC (c, e): Increased active Caspase -3 immunoreactivity in a dose-dependent manner. Noni+10 and 100 mg/kg PNMC (d,f): Reduced immunostaining intensity in the renal tubular cells (Arrows). Scale bars: 50 µm (x400): Control (g) and Noni (h): Weakly stained TUNEL-positive cells (arrows) in the tubular epithelium. 10 and 100 mg/kg PNMC (i, k): Dose dependently increased TUNEL-positive cells (arrows). Noni+10 and 100 mg/kg PNMC (j, l): Significantly decreased TUNEL-positive cells (arrows) in a direct correlation with the decreasing dose of PNMC. Scale bars: 50 µm (x400).

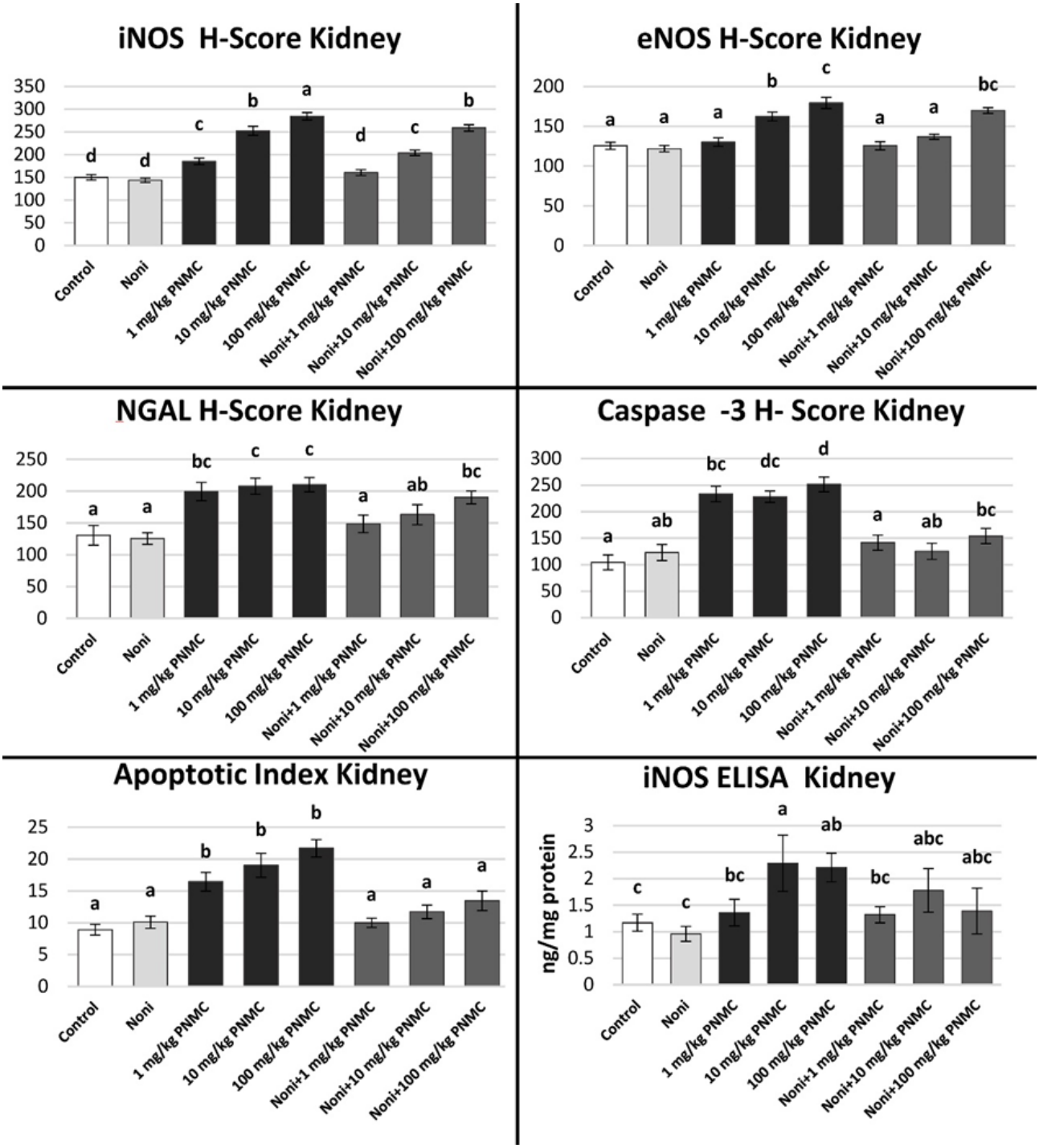

Fig. 5: iNOS, eNOS, NGAL, Caspase-3 H-Score, Apoptotic index (%) and iNOS ELİSA protein levels in the kidney tissue of experimental rats. Different letters above the bars for each parameter indicate significant differences between groups ($p<0.05$).

Oxidative stress parameters: ELISA results revealed that renal iNOS protein levels were significantly higher in 10 and 100 mg/kg PNMC groups than in the control, Noni juice and 1 mg/kg PNMC groups ($p<0.05$). Moreover, the decrease in iNOS levels in the Noni+1 mg/kg PNMC group was statistically significant compared to the 10 mg/kg PNMC group (Fig. 5).

There were non-significant differences among the eight groups for serum TAS values. The highest mean TOS value was observed in 100 mg/kg PNMC group ($p<0.01$). When 1 mg/kg PNMC was compared with Noni+1 mg/kg PNMC, a significant decrease in TOS was found in the latter group. Mean OSI value was significantly increased in 100 mg/kg PNMC group compared to control and Noni groups ($p<0.05$). Furthermore, OSI values decreased in Noni+PNMC combination groups compared to different PNMC doses, but this decrease was not statistically significant (Table 3).

Biochemical analysis: The 100 mg/kg PNMC group had the highest levels of sodium chlorine and AST ($p<0.05$). There were non-significant differences in the levels of ALT, albumin, urea and creatinine across all groups (Table 2). Serum potassium levels were significantly higher in the Noni+1mg/kg and 100 mg/kg PNMC combination groups than in the control and all other groups ($p<0.05$).

Table 3: Comparison of the oxidative stress parameters in rats of different groups (Mean±SE)

Parameters	Control	Noni	1 mg/kg PNMC	10 mg/kg PNMC	100 mg/kg PNMC	Noni+ 1 mg/kg PNMC	Noni+ 10 mg/kg PNMC	Noni+ 100 mg/kg PNMC
TAS (mmol Trolox Eq/l	1.27±0.56	1.28±0.12	1,27±0.41	1.19±0.46	1.20±0.41	1.12±0.53	1.11±0.55	1.22±0.58
TOS (μmol H_20_2 Eq/L)	14.02±3.05^{c}	14.62±1.02^{c}	26.08±3.67^{ab}	18.83±2.67^{bc}	30.99±2.87^{a}	15.38±1.6^{c}	18.73±1.8^{bc}	25.66±5.09^{ab}
OSI (Arbitrary Unit)	11.82±1.63^{b}	10.32±2.10^{b}	19.16±3.84^{ab}	19.25±3.01^{ab}	24.17±2.07^{a}	17.54±3.03^{ab}	18.67±2.0^{ab}	16.80±3.07^{ab}

TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index; Values with different superscript letters within a row indicate a statistically significant difference ($p<0.05$).

DISCUSSION

The health benefits of Noni juice, including anti-inflammatory and antioxidant properties, have been previously documented (Dussossoy *et al.*, 2011; Nuengchamnong *et al.*, 2023). Noni fruit juice was also reported to have protective effects on the kidney against xenobiotics such as drugs and pesticides (Marinho *et al.*, 2020). We did not observe any histopathological change in the kidneys of the rats of groups that received Noni juice, thereby supporting its beneficial effects.

In the toxicity evaluation, the relative organ weight measurement (organ to body weight ratio) has been suggested to be a valuable tool when the body weight is affected (Michael *et al.*, 2007). In our study, although neither PNMC nor Noni juice alone caused any significant changes in the relative weight of the kidneys, a significant increase was observed in the kidney relative weight with 1 mg/kg PNMC administration in combination with Noni juice. In a previous study carried out in rats, non-significant difference was reported in the relative kidney and overall body weights with the administration of 1, 10 and 100 mg/kg/day of PNMC subcutaneously for five days (Yue *et al.*, 2012). Likewise, when 28-day-old rats were injected subcutaneously with PNMC (1, 10 and 100 mg/kg body weight) daily for five days, the kidney weight was not affected (Li *et al.*, 2007). Although these results are consistent with those of our study, the current data do not allow for the establishment of any link between the effects of PNMC alone or in combination with Noni juice on relative organ weight and further studies are required in this regard.

Histologically, our research revealed that PNMC induced toxic effects at the cellular level in the renal tissue showed a dose-dependent increasing severity. Granular degenerative changes of the tubular epithelium in the cortex and medulla, mesangial cell proliferation, thickening of the glomerular basement membrane, increased glomerular lobulation, hyaline casts in the tubular lumina and lipidosis of the medullary tubular epithelium were observed in rats given different concentrations of PNMC. Single-cell necrosis was also detected in the tubular epithelial cells, especially at a dose of 100 mg/kg PNMC. Parallel with our findings, Yue *et al.* (2012) showed that 1, 10 and 100 mg/kg/day PNMC given subcutaneously for five days caused intense necrosis in the medullary tubular epithelium of the rat kidney. In the same study, 10 mg/kg PNMC caused irregularity in the basement membrane of the renal tubule, while 100 mg/kg PNMC dose resulted in the thickening of the basement membrane and disintegration in the glomeruli. In our study, the acute renal effects of PNMC were consistent with the tissue damage induced by PNMC (Yue *et al.*, 2012). The severity of the renal impairment due to the acute PNMC toxicity was observed at the cellular level in the Noni+10 mg/kg and 100 mg/kg PNMC combination groups; however, it was lower compared to all PNMC treated groups. Similarly, our electron microscopic findings revealed irregularities in the basement membrane borders, basement membrane enlargement, and organelle-level damage, particularly in mitochondria, within the renal glomeruli in all groups treated solely with PNMC (1, 10 and 100 mg/kg). These abnormalities were reduced in the Noni+PNMC combination groups, confirming our histopathological findings.

Metabolic changes cause structural alterations in the mitochondrial cristae, known as “remodeling." This change initiates the apoptotic process with the release of cytochrome C, which induces the downstream caspase activity, leading to cell death (Kondadi *et al.*, 2019). Therefore, in our study, ultrastructurally demonstrated mitochondrial damage, such as the deletion of the cristae, is considered to be an indicator of renal damage in the PNMC treated groups, which was supported by our findings on apoptosis and oxidative stress parameters.

Toxic effects of PNMC on the body organs are revealed by increased oxidative stress levels. Oxidative stress caused by PNMC was reported to have been reduced by antioxidant components like walnut polyphenol extract and quercetin (Bu *et al.*, 2012). Noni fruit contains nearly 200 active components, including phenolic compounds, vitamin C, lignans, polyphenols, anthraquinones, alpha-tocopherol and beta-carotene, revealing antioxidant and anti-inflammatory benefits against scavenging free radicals and preventing lipid peroxidation (Fontes *et al.*, 2023). Noni fruit also showed a protective effect against lipid peroxidation on the renal cells (Marinho *et al.*, 2020). In our study, the H-Scores of iNOS and eNOS in kidney tissue were increased by different doses of PNMC. However, in all Noni+PNMC combination groups, these values were significantly reduced. Similarly, the OSI values, which increased with different doses of PNMC administration (especially with 100 mg/kg), decreased numerically in all Noni+PNMC combination groups. The decrease in oxidative markers by the Noni juice can be attributed to the antioxidant properties of this juice.

Noni is known to inhibit oxidative stress-induced apoptosis in many cells exhibiting anti-apoptotic properties (Ol *et al.*, 2017). According to our results, 5-day PNMC exposure increased apoptotic cell number and caspase-3 expression in a dose-dependent manner; however, these parameters were decreased in all Noni+PNMC combination groups, which is compatible with the findings of Yue *et al.* (2012). These results indicate that PNMC induced renal toxicity was mainly through apoptosis via oxidative stress. The decrease in PNMC-induced apoptotic activity in the Noni+PNMC combination groups was associated with the antioxidant

and anti-apoptotic compounds present in the Noni juice. We believe that Noni juice treatment had a protective impact on cellular toxicity induced by PNMC, which was also supported by our ultrastructural, oxidative stress and apoptosis findings, revealing that the mitochondrial damage in the PNMC treated groups was reduced in the presence of Noni juice.

According to Bellomo *et al.* (2004), serum creatinine is an unreliable marker during the acute phase of kidney injury. This is due to various factors such as age, gender, muscle mass, metabolism, medications and hydration status, which can cause changes in serum creatinine concentrations (Baxmann *et al.*, 2008). Therefore, serum creatinine concentrations may not fully reflect the extent of loss of renal function. Our study suggests that PNMC-induced cellular damage in the kidney did not result in significant differences in serum urea and creatinine levels. This is consistent with the previous conclusions drawn by Bellomo *et al.* (2004) and Baxmann *et al.* (2008).

The NGAL is one of the early-phase and most strongly induced proteins released in the kidney after ischemic or acute nephrotoxic injury in animal models (Frydman *et al.*, 2023). In our study, PNMC (1, 10 and 100 mg/kg) increased the NGAL expression in the distal and proximal tubules; however, this parameter decreased in the presence of Noni juice given with 1 and 10 mg/kg of PNMC, suggesting that Noni exhibits a protective effect on tubular damage during mild to moderate PNMC toxicity but failed to be effective at high PNMC concentrations. In a previous study based on a rat model of cisplatin-induced nephrotoxicity (Nemmar *et al.*, 2014), DEP which is an essential source of PNMC, prominently increased the renal NGAL expression after a 10-day intra-tracheal exposure. This is compatible with our findings indicating the significance of NGAL as a biomarker in the acute phase of renal injury.

It is well known that AST is not a liver-specific enzyme and it is also expressed in the renal tubular cells to a lesser degree (Chatterjee *et al.*, 2000). While the mean serum AST activity was significantly increased in the 100 mg/kg PNMC treated rats compared to controls and low dose PNMC treated groups, non-significant differences were noted among all groups regarding serum ALT activity and albumin levels. This increase in the serum AST activity in the 100 mg/kg PNMC treated rats showed that 100 mg/kg of PNMC may cause tissue damage, most likely in the renal tubular cells. According to Elhalwagy *et al.* (2008), fenitrothion changed the serum biochemical profile in albino rats, characterized by increased serum AST, ALT, albumin, urea and creatinine levels. Although PNMC is a metabolite of fenitrothion, there may be differences related to the specific individual effects of this metabolite. Some workers linked the increase in the serum AST activity with renal tissue damage, not the liver, in renal ischemia-reperfusion models (Avlan *et al.*, 2006; Camara-Lemarroy and Guzman, 2009). In our study, the highest concentration of PNMC caused necrosis in the kidney along with diffuse tubular degenerative changes and the increase in serum AST activity, which is supported the data of Chatterjee *et al.* (2000).

According to Yue *et al.* (2012), the disintegration of the glomerular basal membrane, ultrastructurally demonstrated by electron microscopy, might be the indicator of impaired renal functions through the deterioration of glomerular filtration and reabsorption with different doses (1,10 and 100 mg/kg) of PNMC administration. Parallel with the findings of Yue *et al.* (2012), we also suggest that the increase in serum sodium and chlorine levels in the 100 mg/kg PNMC treated group was due to the disruption of the glomerular basement membranes, as shown on electron micrographs, hampering bidirectional ion transport between the intracellular and extracellular environments. Furthermore, the increased serum potassium levels in the Noni+1 mg/kg and Noni+100 mg/kg PNMC combination groups were associated with the high potassium content of Noni juice (Mueller *et al.*, 2000), generating excessive potassium concentration in blood in addition to the minimal potassium excretion due to the impaired glomerular filtration as a result of PNMC-induced thickening of the basement membrane.

Conclusions: Our study showed that Noni (*Morinda citrifolia L.*) fruit juice exhibits a protective role against PNMC-induced tissue injury in Sprague-Dawley adult male rats, reducing oxidative stress and apoptosis in the renal tissue. However, it is necessary to conduct more research to understand the mechanism of these effects and to investigate its potential effects on other body organs in domestic animals.

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Authors' contributions: FY and EİA conceived the study design and were involved in conceptualization. FY, MBT, ABS and EİA developed and executed the methodology. FY, MBT and ABS analyzed and interpreted the data. FY, MBT, AA and ABS were responsible for writing the original draft and reviewing and editing the manuscript. OBBE, AA, GYO, EGG, SS, BİB, IO and HHB contributed to the development and execution of the methodology. All authors critically reviewed the manuscript for important intellectual contents and approved the final version.

REFERENCES

Abd-Elhamid TH, Elgamal DA, Ali SS, *et al.*, 2018. Reno-protective effects of ursodeoxycholic acid against gentamicin-induced nephrotoxicity through modulation of NF-κB, eNOS and caspase-3 expressions. Cell Tissue Res 374:367-87.

Avlan D, Tamer L, Ayaz L, *et al.*, 2006. Effects of trapidil on renal ischemia-reperfusion injury. J Pediatr Surg 41:1686-93.

Bacus S, Flowers JL, Press MF, *et al.*, 1988. The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. Am J Clin Pathol 90:233-39.

Baxmann AC, Ahmed MS, Marques NC, *et al.*, 2008. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin. Clin J Am Soc Nephrol 3:348-54.

Bellomo R, Kellum JA and Ronco C, 2004. Defining acute renal failure: Physiological principles. Intensive Care Med 30:33-37.

Bu TL, Jia YD, Lin JX, *et al.*, 2012. Alleviative effect of quercetin on germ cells intoxicated by 3-methyl-4-nitrophenol from diesel exhaust particles. J Zhejiang Univ Sci B 13:318-26.

Camara-Lemarroy CR and Guzman GFJ, 2009. Effect of sulfasalazine on renal ischemia/reperfusion injury in rats. Ren Fail 31:822-28.

Chatterjee PK, Cuzzocrea S, Brown PA, *et al.*, 2000. Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. Kidney Int 58:658-73.

Craig EA, Yan Z and Zhao QJ, 2015. The relationship between chemical-induced kidney weight increases and kidney histopathology in rats. J Appl Toxicol 35: 729–36.

Dittmayer C, Goebel HH, Heppner FL, *et al.,* 2021. Preparation of samples for large-scale automated electron microscopy of tissue and cell ultrastructure. Microsc Microanal 27:815-27.

Dussossoy E, Brat P, Bony E, *et al.*, 2011. Characterization, anti-oxidative and anti-inflammatory effects of Costa Rican noni juice (*Morinda citrifolia L.*). J Ethnopharmacol 133:108-15.

Elhalwagy MEA, Darwish NS and Zaher EM, 2008. Prophylactic effect of green tea polyphenols against liver and kidney injury induced by fenitrothion insecticide. Pestic Biochem Phys 91:81-89.

Erel O, 2005. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 38:1103-11.

Fan S, Zhao X, Xie W, *et al.*, 2022. The effect of 3-methyl-4-nitrophenol on the early ovarian follicle development in mice by disrupting the clock genes expression. Chem Biol Interact 363:110001.

Fontes RF, Andrade JKS, Rajan M, *et al.*, 2023. Chemical characterization of different parts of noni (*Morinda citrifolia*) fruit and its freeze-dried pulp powder with emphasis on its bioactive compounds and antioxidant activities. Food Sci Technol 43: e103722; doi.org/10.1590/fst.103722.

Frydman S, Freund O, Zornitzki L, *et al.*, 2023. Indexed neutrophil gelatinase associated lipocalin: a novel biomarker for the assessment of acute kidney injury. J Nephrol 1-7; doi: 10.1007/s40620-023-01800-y.

Hu S, Liu H, Tao S, *et al.*, 2022. The environmental pollutant 3-methyl-4-nitrophenol reduces the regulatory T cells in the intestine. Toxicology 482:153356.

Kondadi AK, Anand R and Reichert AS, 2019. Functional interplay between cristae biogenesis, mitochondrial dynamics and mitochondrial DNA integrity. Int J Mol Sci 20:4311.

Li C, Taneda S, Suzuki, *et al.*, 2007. Effects of 3-methyl-4-nitrophenol in diesel exhaust particles on the regulation of testicular function in immature male rats. J Androl 28:252-58.

Li X, Li C, Suzuki AK, *et al.*, 2009. Endocrine disruptive effect of 3-methyl-4-nitrophenol isolated from diesel exhaust particles in Hershberger assay using castrated immature rats. Biosci Biotechnol Biochem 73:2018-21.

Marinho AD, Silveira JA, Chaves-Filho AJ, *et al.*, 2020. Protective effects of a lipid transfer protein isolated from *Morinda citrifolia* seeds in gentamicin-induced nephrotoxicity in rats. Rev Bras Farmacogn 30:568-76.

Michael B, Yano B, Sellers RS, *et al.*, 2007. Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices. Toxicol Pathol 35:742-50.

Min J, Lu Y, Hu X, *et al.*, 2016. Biochemical characterization of 3-methyl-4-nitrophenol degradation in Burkholderia sp. strain SJ98. Front Microbiol 7:791.

Mueller BA, Scott MK, Sowinski KM, *et al.*, 2000. Noni juice (*Morinda citrifolia*): Hidden potential for hyperkalemia? Am J Kidney Dis 35:310-12.

Nemmar A, Beegam S, Yuvaraju P, *et al.*, 2014. Potentiation of cisplatin-induced nephrotoxicity by repeated exposure to diesel exhaust particles. Exp Biol Med 239:1036-44.

Nuengchamnong N, Saesong T, Ingkaninan K, *et al.*, 2023. Antioxidant activity and chemical constituents identification by LC-MS/MS in bio-fermented fruit drink of *Morinda citrifolia* L. Trends Sci 20:6498-98.

Ol K, Kanbak G, Ilhan A, *et al.*, 2017. *Morinda citrifolia* (noni) and low dose aspirin prevent apoptotic cell death and oxidative stress on isoproterenol induced myocardial infarction in rats. Erciyes Med J 39:165-70.

Temitope AA, Tolulope OA, Funlola CT, *et al.*, 2011. Protective effect of Tahitian noni juice on the reproductive functions of male Wistar rats treated with cyclophosphamide. Iran J Pharm Therap 10:39-43.

Yigit F, Gurel-Gurevin E, Isbilen-Basok B, *et al.*, 2016. Protective effect of *Spirulina platensis* against cell damage and apoptosis in hepatic tissue caused by high fat diet. Biotech Histochem 91:182-94.

Yue Z, She RP, Bao HH, *et al.*, 2012. Necrosis and apoptosis of renal tubular epithelial cells in rats exposed to 3-methyl-4-nitrophenol. Environ Toxicol 27:653-61