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Protective effect of *Nannochloropsis Oculata* against mercuric-induced histopathological alterations in the kidney of Nile tilapia

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ABSTRACT

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Objective: The present study was designed to evaluate the toxic effect of sublethal concentration of mercuric chloride (0.3 mg/L HgCl₂) on histopathological lesions in the kidney of Nile tilapia (*O. niloticus*) and the protective effect of microalgae, *Nannochloropsis oculata* (*N. oculata*) against the induced tissue alterations for 3 weeks.

Design: Randomized controlled study

Animals: Nile tilapia

Procedures: Fish were randomly assigned to 4 groups, group1: control (basal diet), group 2 (Hg/ exposed to HgCl₂ at a dose of 0.3 mg/L (1/4 of LC₅₀), and fed basal diet), group 3: (Hg+N5, similar to group2, but fed diet supplemented with *N. oculata* 5% and group 4 (Hg+N10, similar to group2, but fed diet supplemented with *N. oculata* 10 %). Two fish from each aquarium tank (6 fish/group) were sampled at weeks 1, 2, and 3 of the experiment. The posterior kidney was dried out in a graded ethanol series and then embedded in paraffin. Each block of tissue was cut into serial sections (5 µm thick) and stained with hematoxylin and eosin (H&E).

Results: Histopathological alterations were induced following mercuric exposure in a time-dependent manner. The kidney showed congestion, hemosiderosis, and hemorrhage with vacuolated tubular epithelium, hyaline droplet degeneration, and necrosis of the tubular epithelium. Supplementation with *N. oculata*, particularly at 10 %, succeeded in alleviating the histopathological induced lesions in the kidney.

Conclusion and clinical relevance: Our findings demonstrate that HgCl₂ has nephrotoxic properties that led to severe histopathological alterations in the kidney of Nile tilapia, while dietary supplementation with *N. oculata* was able to alleviate the induced kidney alterations.

Keywords: Fish, Heavy metals, Tissue alterations, Microalgae

1. INTRODUCTION

Aquaculture is one of the fastest-growing animal-food-producing sectors [1], which accounts for nearly half of the fish consumed across the world [2]. Tilapia (*Oreochromis niloticus*), is one of the most important farmed species commercially traded worldwide [3]. It is the most commonly inhabiting and consumed freshwater fish in Africa [4] and the second most farmed fish in the world [5]. Tilapia is commonly used as a pollution bioindicator in toxicological investigations [6-9].

In the last few decades, the increase in population growth, massive industrialization, and economic activities have resulted in increased wastes discharge in the aquatic environment with a wide range of pollutants, including heavy metals [10, 11]. Mercury (Hg) is one of the most toxic heavy metals entering water sources. It is highly toxic, non-biodegradable, and persistent in the environment with high potential to bioaccumulate and biomagnify through the food chain [12].

Mercury has been previously reported to induce deleterious damage to many body systems biomarkers, including the immune [13, 14], reproductive [15, 16],

hematological [17-19], and nervous biomarkers [20, 21]. Mercury induces its toxicity mainly through reactive oxygen species (ROS) production, which induces significant damage to the molecular components of proteins, lipids, and DNA of cells resulting in protein degeneration, lipid peroxidation and enzymatic inactivation [22]. Oxidative stress under mercuric toxicity has been numerous investigated [23-26].

Mercury is highly bio-accumulative. Its concentration in marine fish tissues can be significantly elevated to levels up to 100,000 ppm [27]. Organic Mercury has high bioaccumulation potential (up to 5000-times higher than its concentration in the surrounding water) [28].

ROS induced cellular damage and bioaccumulation of Hg eventually result in morphological alterations in fish tissues [11], which on the gross level can be visualized through histopathology. Liver, kidney, and gills are the main target organs for mercury accumulation [4, 29-31] and histopathological alterations [32-34]. Monitoring pathological alterations in tissues and organs dealing with bioaccumulation, biotransformation, and excretion are essential to understand the toxic effects of chemicals in fish [35].

Microalgae are autotrophic, photosynthetic microorganisms that have the ability to synthesize biologically complex components such as lipids, proteins, carbohydrates, pigments, and polymers [36]. Most microalgae have a high content of bioactive compounds, including protein, polysaccharides [37], and vitamins such as vitamins A, C, E, K, thiamine, pyridoxine, riboflavin, nicotinic acid, biotin, and tocopherol [38, 39]. *N. oculata* cellular composition is highly rich in bioactive compounds as proteins, polysaccharides and polyunsaturated fatty acids [40] along with its pigment content of violaxanthin with β -carotene, vaucherixanthin, all have antioxidant, anti-inflammatory, antimicrobial and immune-stimulant properties [41] making *N. oculata* a potential dietary supplement with high nutritional and immune stimulant values [42, 43]. However, their potential impact against the metals induced histopathological damage hasn't yet been investigated.

The present investigation has been carried out to investigate the histopathological alterations in the kidney following mercury intoxication and to elucidate the ameliorative role of the dietary supplementation of the microalgae *N. oculata* against mercury-induced toxicity in Nile tilapia.

2. MATERIALS AND METHODS

2.1. Fish maintenance

Nile tilapia were procured from a private fish farm in Kafr El-sheik. After 2 weeks acclimation period, a total of 120 fish (45-50 g) were allocated into 12 glass tanks filled with dechlorinated tap water, in triplicate (10 fish/tank, 30 fish/group). Fish were fed twice daily at 2% of their body weight. Daily water changes, and removal of fecal matter and wastes were carried out to maintain water quality. Water quality parameters were maintained during the experiment (24 ± 2 °C, dissolved oxygen 6.5–7.8 mg/L, pH 7.1–7.3). The photoperiod was 12 h light: 12 h dark.

2.2. Mercury exposure

A technical grade mercuric chloride (HgCl_2), (99% purity, El-Gomhoreya Chemical Company Cairo, Egypt) was used to induce fish toxicity. A stock solution of 1000 mg/L mercury was prepared by dissolving the calculated quantity of HgCl_2 in one Litre of distilled water, then the desired concentration in part per million (ppm) was prepared by adding a known volume of the stock solution into the glass aquaria. The control group was handled similarly, adding distilled water without Hg and under identical conditions like other groups.

2.3 Determination of median lethal concentration (LC_{50})

A 96-hr toxicity assay was performed according to Organisation for Economic Co-operation and Development (OECD) 203 guidelines for testing chemicals [44]. A preliminary series of static toxicity tests (0.3, 0.7, 1, 1.5, 3 mg/L HgCl_2) was applied to determine the appropriate range of Hg toxicity for Nile tilapia. Based on these preliminary tests, five concentrations (0.9, 1.2, 1.4, 1.5, and 1.7 mg/L) of HgCl_2 (each in triplicate, ten fish/tank, 30 fish/group) were

selected, while the control tank was kept without mercuric exposure and no food was supplied during the experiment to maintain water quality. Test solutions of the chosen concentrations were prepared by diluting a 1000 mg/L HgCl_2 stock solution. Mortalities were recorded at 24, 48, 72, and 96 hrs of exposure, and dead fish were removed regularly from the aquaria. The obtained data were statistically analyzed using Probit analysis for estimating the LC_{50} [45], and $\frac{1}{4} \text{LC}_{50}$ was taken as the safe Hg concentration [46].

2.4. *N. oculata* powder

N. oculata dried powder was purchased from the National Research Institute of Cairo, Egypt.

2.5. Diet preparation

Diets composition are presented in Table 1. All ration components were mixed with oil, and then water was added until a stiff dough is formed. Diet for each treatment was then extruded through a mincer forming strands, and allowed to dry in shadow, broken up, sieved into pellets, and stored in clean dried plastic bags at 4°C until use.

2.6. Experimental design

Fish were randomly assigned to four groups, namely group 1: control (basal diet), group 2 (Hg/ exposed to HgCl_2 at a dose of 0.3 mg/L ($\frac{1}{4} \text{LC}_{50}$), and fed basal diet), group 3: (Hg+NC5, similar to group 2, but fed diet supplemented with *N. oculata* 5%) and group 4 (Hg+NC10, similar to group 2, but fed diet supplemented with *N. oculata* 10%). Fish were fed twice daily at 2% of their body weight for 3 weeks. Water was changed daily up to 80%, with the addition of a new daily stock solution of HgCl_2 to the exposed aquaria, waste material, and fecal matter that were siphoned off daily to maintain water quality.

2.7. Sample collection

Six fish were sampled from each group (2 fish/ tank) at weeks 1, 2, and 3 of the experiment. Fish were euthanized with 200 mg/L of buffered tricaine methanesulfonate (MS222, Argent). The kidney was dissected out of fish and subjected to fixation in neutral buffered formalin for histopathological examination.

2.8. Histopathological examination

The posterior kidney was dried out in a graded ethanol series and then embedded in paraffin. Each block of tissue was cut into serial sections (5 μm thick) and stained with hematoxylin and eosin (H&E) according to the method described by Bancroft and Gamble [47].

3. RESULTS

3.1. Determination of LC_{50}

LC_{50} was estimated to be 1.2 mg/L Hg, and $\frac{1}{4} \text{LC}_{50}$ (0.3 mg/L) was taken as safe sublethal Hg concentration.

Table 1. Ingredients of basal and experimental diets.

Diet ingredients (g/kg diet)	Control	NC 5	NC 10
Yellow corn	126	146	133
Soya bean meal	203	200	190
Fish meal	200	160	150
Corn gluten	10	30	0
Gelatine	20	20	20
Oil	30	35	45
Wheat bran	400	350	350
Minerals and vitamins premix	5	5	5
Salt	3	3	3
Dicalcium phosphate	1	1	1
Methionine	2	3.2	3.2
Algae	0	50	100

*Trace minerals & vitamins premixes were prepared to cover the levels of the microminerals & vitamins for tilapia fish. Vitamins premix (IU or mg/kg diet); vit. A 5000, Vit. D3 1000, vit. E 20, vit. k3 2, vit. B1 2, vit. B2 5, vit. B6 1.5, vit. B12 0.02, Pantothenic acid 10, Folic acid 1, Biotin 0.15, Niacid 30. Mineral mixture (mg/kg diet); Fe 40, Mn 80, Cu 4, Zn 50, I 0.5, Co 0.2 & Se 0.2.

3.2. Histopathology of the kidney

Kidney at all-time points in the control group showed normal glomeruli, renal tubules, and normal capillary system in the interstitium (Plate.1). Higher magnification of the control kidney showed well developed renal corpuscles with normal glomeruli surrounded by Bowman's capsule, normal renal tubules with contact round or oval nuclei, and characteristic tall columnar epithelium. Also, interstitial hemopoietic tissue appeared normal (Plate.2). However, the kidney of Hg exposed group was in-time dependent manner, being more severe at Week 2 and 3 compared to week 1, where congestion and hemorrhage in the interstitial tissue were observed at week 1, but, more progressive lesion, including congestion and necrosis, were more evident at week 2 and 3 (Plate.1). Higher magnification of the kidney at week 1 showed congestion, hemosiderosis, and hemorrhage with vacuolated tubular epithelium. The lesions became more progressive than earlier at weeks 2 and 3 showing vacuolated tubular epithelium, hyaline droplet degeneration, and necrosis of the tubular epithelium at week two while, at week three the kidney showed vacuolated tubular epithelium

with edema, hemorrhage, hemosiderosis and severe necrosis (Plate.2).

Supplementation with *N. oculata* at 5 % showed less severe lesions in the kidney, including congestion in the interstitial tissue at weeks 1 and 2 only (Plate.1). Higher magnification of the kidney showed vacuolated tubular epithelium and congestion in the interstitial tissue during the first two weeks, while at week 3, the kidney showed vacuolated tubular epithelium and hyaline droplet degeneration of the tubular epithelium (Plate.2). Noteworthy that the kidney of the fish supplemented with *N. oculata* at 10 % showed milder degrees of congestion at weeks 1 and 2 (Plate.1). Higher magnification of renal sections showed milder degrees of congestion with tubular degeneration and hyaline droplet degeneration (Plate.2).

4. DISCUSSION

Histopathological examination is one of the most essential tools in the diagnosis of heavy metals toxicity. Heavy metals usually accumulate in specific target organs such as liver, kidney, and gills [4, 29-31], and their presence causes tissue damage that could be emphasized by histopathological examination [11].

The kidney plays a vital role in maintaining internal body stability regarding electrolytes, water balance, and the elimination of nitrogenous metabolites. It is the leading trophic site for mercuric chloride bioaccumulation in chronic exposures [48]. The kidney has been described as a target organ affected by mercuric toxicity.

The kidney of fish from mercury exposed group was severely damaged showing congestion, hemosiderosis, hemorrhage, and vacuolated tubular epithelium at weak one and the lesions became more progressive at weak 2 and 3 showing vacuolated tubular epithelium, hyaline droplet degeneration and necrosis of the tubular epithelium at weak two, while at weak three the kidney showed vacuolated tubular epithelium with edema, hemorrhage, hemosiderosis, and severe necrosis.

Mercury has a higher affinity towards sulfhydryl groups on the cell membrane, causing a disturbance in active transport and cell functions [48]. Therefore, the kidney has been reported to be target organs for mercury bioaccumulation and increased concentration [48-50]. All these reasons contribute to the damage induced by mercury in the function and morphology of the kidney.

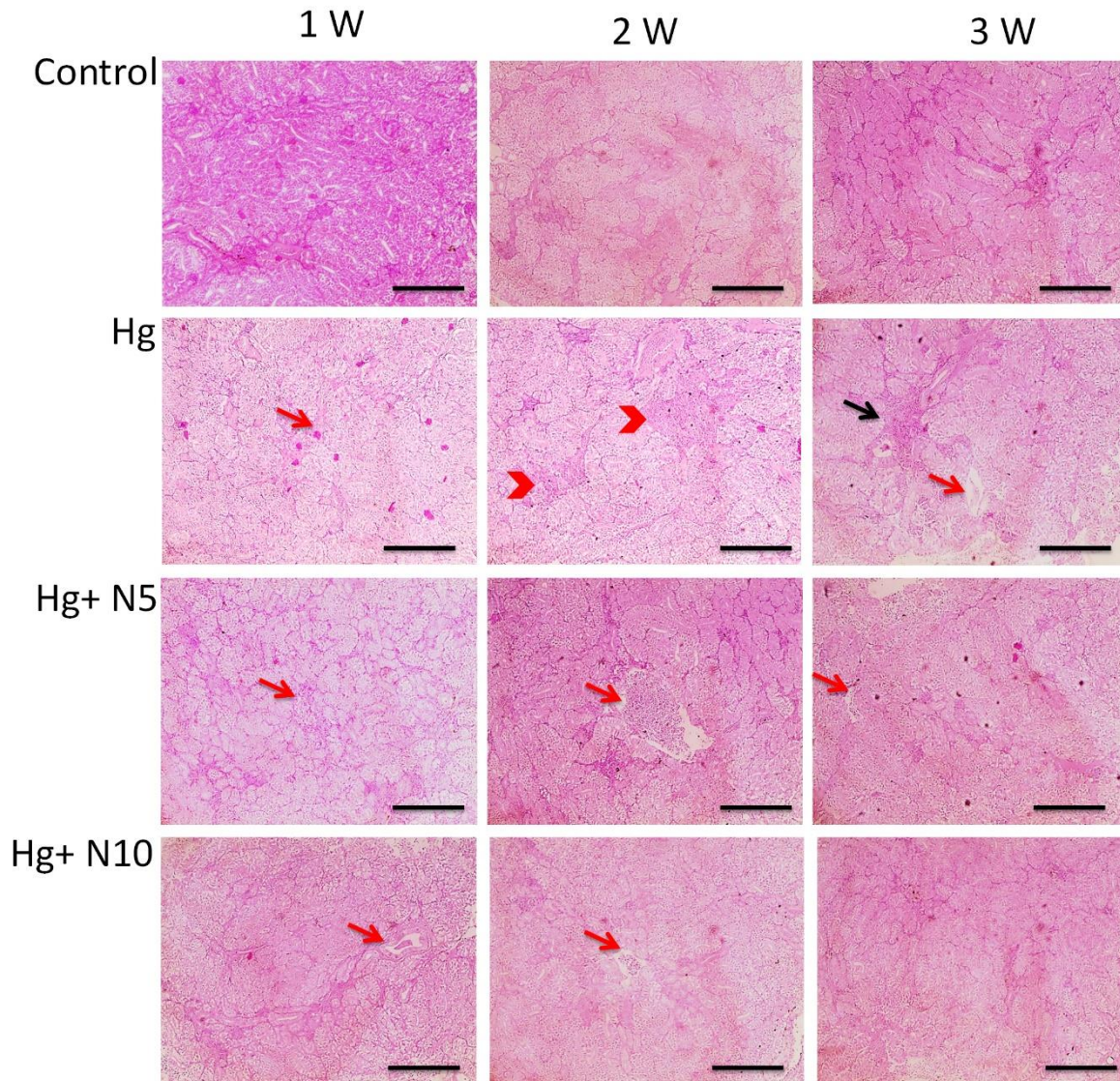


Plate.1. Microscopic pictures of H&E stained sections of kidneys showing normal glomeruli, tubules, hemopoietic tissue (control group) at weak 1 (column A), 2 (column B) and 3 (column C). Renal sections from Hg exposed group show congestion (red arrows) and hemorrhage (red arrowheads) in interstitial tissue at weak 1 (column A) and weak 2 (column B), congestion (red arrows) and necrosis (black arrows) at weak 3 (column C). Renal sections from Hg+N5 show congestion in interstitial tissue (red arrows) at weak 1 (column A) and 2 (column B) only. Milder degrees of congestion (red arrows) appear in renal sections from Hg+N10 at weak 1 (column A) and 2 (column B) only. X: 100 bar 100.

Kidney damage on the histopathological level was also reported in Nile tilapia exposed to $2 \mu\text{g/g}$ HgCl_2 through semi-static exposure where the kidney showed hydropic degeneration, necrosis in the tubular epithelium with hyaline droplets and deposition of the pleomorphic crystal [51]. Additionally, the kidney of Indian Major Carp (*Labeo rohita*) exposed to 0.1 mg/L HgCl_2 for 30 days showed desquamated epithelium, shrunk glomerulus, necrosis and pyknosis of the nuclei with hypertrophied cells in renal tubules [48], while the kidney of juvenile zebra seabream exposed to $2 \mu\text{g/L}$ HgCl_2 for 28 days showed vacuolar and hydropic degeneration of tubular epithelium and pigment deposits around the tubules.

Large necrotic areas in posterior kidney and eosinophilic material filled the tubular lumen [50].

In our study, supplementation with *N. oculata*, particularly at 10%, was able to diminish histopathological alterations in the kidney. This might be attributed to its bioactive constituents that have antioxidant, immunostimulant, and metal chelating activities, particularly violaxanthin and provitamin A (β -carotene [52], α -linolenic acid [40], and content of complex anionic sulfated heteropolysaccharides, particularly mannans and sulfated heterorhamnan [53-56].

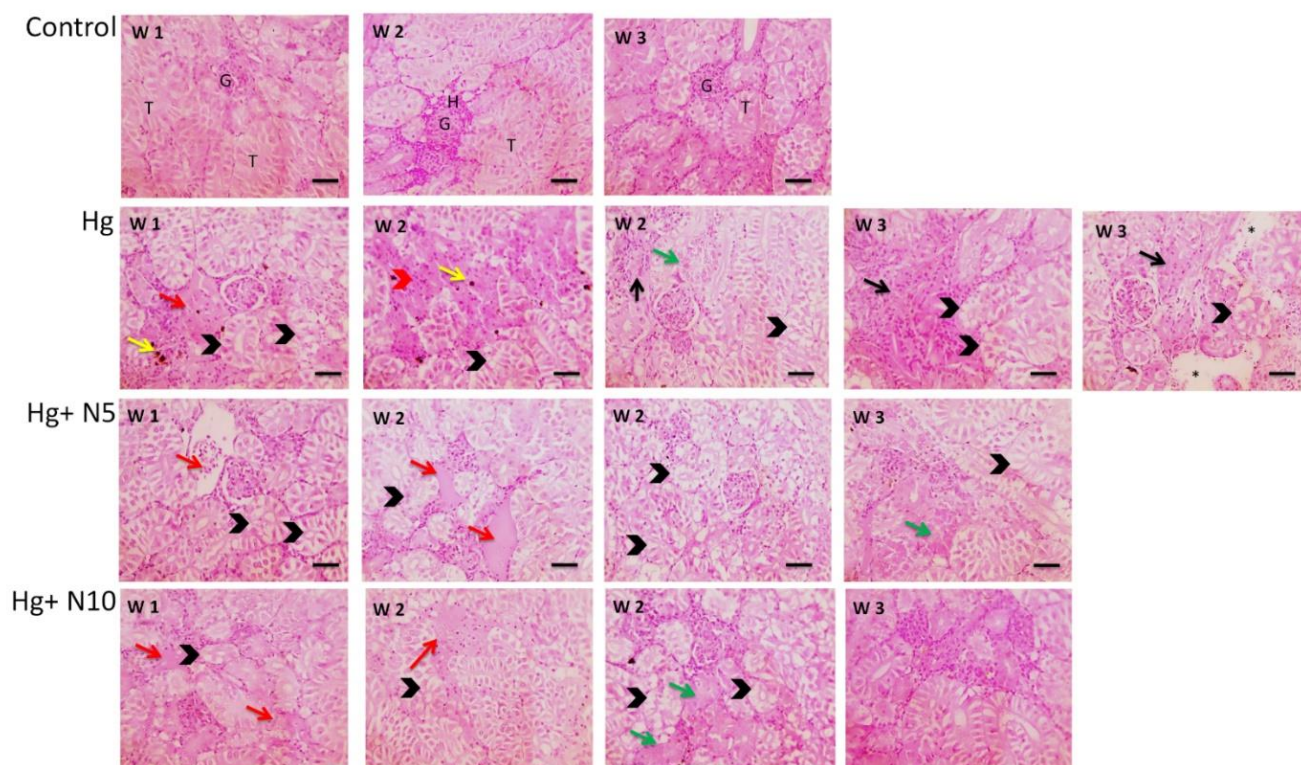


Plate.1. Hg exposed group show congestion (red arrows), hemosiderosis (yellow arrows) with vacuolated tubular epithelium (black arrowheads) at weak 1, vacuolated tubular epithelium (black arrowheads), hyaline droplet degeneration (green arrows) and necrosis (black arrows) at weak 2, vacuolated tubular epithelium (black arrowheads), edema (asterisks), hemorrhage (red arrowheads), hemosiderosis (yellow arrows), necrosis (black arrows) at weak 3. Renal sections from Hg+N5 after show vacuolated tubular epithelium (black arrowheads), congestion in interstitial tissue (red arrows) at weak 1 and 2, vacuolated tubular epithelium (black arrowheads) and hyaline droplet degeneration (green arrows) at weak 3. Milder degrees of congestion (red arrows) appear in renal sections from Hg+N5 with tubular degeneration (black arrowheads) and hyaline droplet degeneration (green arrows) at weak 1 and 2 only. X: 100 bar 100.

The presence of phenolics [57] and flavonoid compounds [55], carotenoids, namely, violaxanthin, astaxanthin, lutein, zeaxanthin, chlorophylls a and b and β -carotene contributes at a major extent to the antioxidant activity of *N. oculata* [53]. Phenolic compounds and flavonoids are electron donor substances that exhibit an essential role in reduction capacity [58] and antioxidant activity [55]. They also have metal chelating activities [53, 55], which help to lower Hg accumulation and the resulting damage. Also, *N. oculata* have high cellular content of ω -3 poly unsaturated fatty acids (PUFAs, α -linolenic (ALA, C18:3 ω 3) and eicosapentaenoic (EPA, C20:5 ω 3)) which constitute about 32 % of the total fatty acids [40], along with ω 6 fatty acids. These components exert vital action in the activation of the immune system; they act as ligands to immune cells [59].

Ameliorating effects of microalgae against histopathological alterations induced by pollutants have been reported on many occasions, where dietary supplementation with 2% *Chlorella pyrenoidosa* (*C. pyrenoidosa*) was able to reduce histopathological alterations in the kidney of Prussian carp (*Carassius gibelio*) exposed to 10 mg/L CdCl₂ for 21 days [60]. Similarly, *C. vulgaris* decreased the intensity of the histological lesions in the kidney of Nile tilapia intoxicated with sodium arsenite (NaAsO₂) at 7 mg/L for 21 days [61]. Additionally, dietary supplementation with *Thunbergia*

laurifolia leaf at a dose of 0.2 and 2 mg extract/g food managed to decrease morphological alterations in the kidney of Nile tilapia exposed to 45 mg/L of lead nitrate (Pb(NO₃)₂) [62].

Conclusion

Mercuric has nephrotoxic properties, and its toxicity elicited severe damage and histopathological alterations in the kidney of Nile tilapia, while dietary supplementation with *N. oculata* succeeded in ameliorating mercuric induced toxicity and relieved the histopathological lesions in the kidney, particularly at level of 10%.

Conflict of interest

Authors declare that they have no conflict of interest

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Author' contributions

A.M. performed the experiment and drafted the MS; E.Z. reviewed and edited MS; E.Z. F.M. and V.Z. supervised the

whole research work.

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