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RESEARCH ARTICLE

The Role of *Chlorella vulgaris* in Ameliorating of Neurobehavioral Impairments Induced by Copper Oxide Nanoparticles Subacute Toxicity in *Oreochromis niloticus*.

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ABSTRACT

Metal-oxide nanoparticles (NPs), including copper oxide (CuO-NPs), are being released uncontrollably into the environment, posing a significant threat to aquatic life. Chlorella vulgaris (ChV) demonstrated a protective effect against hazardous substances by considerably minimizing oxidative burden caused by many various compounds including NPs. This study planned to assess the effect of sub-acute exposure to CuO-NPs on neurobehavioral impairments in Oreochromis niloticus (O. niloticus) fish, and assess the ameliorative role of ChV against the induced neurotoxicity. A total of 144 O. niloticus was equally grouped into four groups as follows: control (C), ChV (30gm/kg diet), CuO-NPs (1/10 LC50, 5.72 µg/L), and CuO-NPs/ ChV groups. Our data proved that the exposure to CuO-NPs induced a significant increase of hyperpigmentations, ulcerations, tail corrosions and hemorrhages. A significant increase of surfacing, hiding, loss of equilibrium, laterality and motionless behaviors was recorded in CuO-NPs exposed fish. From the contrary side, exposure to CuO-NPs significantly decreased the number of crossings, feeding behaviors, escape and knocking reflexes. The CuO-NPs exposed fish revealed a significant reduction in total antioxidant capacity (TAC) level, and superoxide dismutase (SOD) activity, while malondialdehyde (MDA) and 8-hydroxyguanosine (8-OH2dG) levels significantly increased in brain tissue, in addition to a decline in acetylcholine esterase (AchE) activity in serum. The RT-PCR analysis demonstrated an up-regulation of nuclear factor kappa B (NF-κβ) and Caspase-3 mRNA expression levels, while b-cell lymphoma 2 (Bcl-2) mRNA expression level down-regulated in brain tissue. Moreover, histopathological alterations were observed in the brain tissue. Importantly, ChV significantly protected fish from neurobehavioral impairments induced by CuO-NPs. Our data demonstrated the neuroprotective effects of ChV in CuO-NPs exposed fish, promoting ChV's usage as a potential anti-inflammatory, anti-oxidant and neuroprotective agent.

Introduction

Nanoparticles (NPs) are materials that can be produced from a variety of macromolecules, whether they are synthetic or natural, using physical or chemical methods. They are typically 1–100 nm in size and can take on a variety of shapes, including a prism, rod, cube, sphere, or needle [1]. NPs are extensively employed as nanotechnology for medicinal purposes and nanocarriers of medications due to their tiny size and special properties [2]. However, their toxicity could also be attributed to their size, shape, surface functional groups, and dose-related features [3].

The increasing amount of NPs generated, consumed, and released into the environment puts aquatic systems and their biota at risk [4]. Pollution of the aquatic environment can occur because of air deposition, wastewater treatment, flushing from contaminated soil, and direct entrance of NPs into the water. They then interact with organisms via food, drink, and skin contact, ultimately entering cell walls and altering physiological activities [5]. Interestingly, in recent years, NPs have been extensively used in fish farming and seafood processing for purposes like food packaging and nanofiltration [6]. Following that, releasing of NPs, particularly metal NPs, into aquatic habitats and their harmful impacts on species is a critical concern contrary to protecting aquatic ecosystems [7].

Among metal oxide nanoparticles, CuO-NPs are commonly utilized in a variety of applications, including gas detectors, batteries, fabrics, catalytic organic conversions, electrocatalysis, processing of water, photocatalysis, solar cells, fuels, thermal transfer fluids, plasters, and paintings. The increasing use of CuO-NPs increases their discharge into the environment and contact with living beings. The tiny size and greater reaction rate of CuO-NPs enable them to diffuse directly through the cellular membrane [8], promote their interaction with biomolecules, impair membrane integrity, produce reactive oxygen species (ROS), cause oxidative stress (OS), lipid oxidation, DNA injury and cellular death [9]. Moreover, CuO-NPs contribute to the disturbance of antioxidant mechanisms [10]. Previous studies reported the oxidative injury of CuO-NPs including genotoxicity in *Allolobophora caliginosa* (earthworms) [11], and *Danio* (zebrafish) [12].

NPs can interact with tissue, blood, body fluid, and penetrate the central nervous system (CNS), which disturbs the functionality of the cardiac and cerebral systems [13]. Exposing zebrafish larvae and embryos to high dosages of CuO-NPs resulted in slower retinal neuronal development, suggesting neurotoxicity [14]. After exposure of *Danio rerio*, and *Caenorhabditis elegans* to CuO-NPs, they displayed behavioral changes, including an increase in mucus secretion, loss of balance, and the reduction of swimming ability and feeding behavior [15, 16]

Cu-NPs enter neural cells and target mitochondria, which may result in an increase in OS and decline the cell viability or metabolic activity [17]. Also, CuO-NPs can cross blood brain barrier and can interrupt its permeability inducing neurotoxicity [18].

Chlorella vulgaris (ChV) is a fascinating green unicellular microalga that grows in fresh water, it has a various biological and pharmacological properties and is commonly utilized in aquaculture [19]. The natural antioxidant components, including chlorophyll, polyphenol, vitamins, and sulfur-containing substances, which can neutralize oxidative free radicals, may be

responsible for ChV's protective function [20]. Dietary ChV can enhance disease resistance and lessen stress [21]. Also, according to Wu *et al*. [22], ChV has an antioxidant and anti-inflammatory properties, which may be crucial for animal health.

Antioxidants were increased when the *O. niloticus* were fed a diet containing ChV [23]. Likewise, ChV effectively mitigated colitis induced by acetic acid in rats by inhibiting the expression of the *NF-κB* and *Caspase-3* genes [24]. Similarly, ChV meals greatly enhanced the antioxidant status, and adjusted the hepatorenal functioning, stress levels, and neurotransmitter levels of acrylamide-exposed *O. niloticus* [25]. Moreover, supplementation with 10% ChV may protect *O. niloticus* from penoxsulam sub-acute toxicity by improving growth performance, oxidant/antioxidant status, and liver state [26].

Based on the foregoing, this study aimed to evaluate the potential of *C. vulgaris* in mitigating the adverse effects of CuO-NP exposure on antioxidant defenses, inflammatory responses, and neurobehavioral functions in *O. niloticus*.

Material and methods

Assessed compounds

Biological synthesis of copper oxide nanoparticles (CuO-NPs)

Pseudomonas florescence MT20 isolate was inoculated in F-Base medium and incubated at 37℃ for 24 h. The supernatant was gathered by centrifugation at 6000 rpm for 15 min. To optimize the reaction, 30 mL of bacterial supernatant was mixed with 70 mL of $CuSO_4 \cdot 5H2O$ (0.2 mM) (Sigma-Aldrich Chemical Co.; St. Louis, MO, USA) (CAS Number: 7758-99-8). The optimal conditions were pH 7, temperature 37℃, concentration of $CuSO4 \cdot 5H_2O$ (0.2 mM), reaction time of 90 min, and agitation speed of 200 rpm. The outcome of the mixture's color shifted from blue to dark green, indicating CuO-NP synthesis and manufacturing according to El-Saadony *et al.* [27].

Chlorella vulgaris (ChV)

Identification of ChV was authenticated by Prof. Dr. Abo El-Khair Badawy El-Sayed (Head of Algal Biotechnology Unit National Research Centre Cairo Egypt). It is a green powder, soluble in water but insoluble in other organic solvents.

Fish care and the preparation of tested diets

A total of 144 *Oreochromis niloticus* (35 ± 0.40 g body weight) were obtained from El-Abbassa Fish Hatchery, Sharkia Province, Egypt. Prior to the experiment, fish were adjusted for two weeks in glass aquaria (80 × 40 × 30 cm) full of 60 L of dechlorinated tap water and fed a baseline diet without the use of supplementary nutrients. The aquaria were maintained under stable conditions, with a temperature of 26 ± 1.5℃, pH of 6.9 ± 0.5, dissolved oxygen levels of 5.5 ± 0.5 mg/L, and ammonia concentrations of 0.035 ± 0.01 mg/L. A regulated photoperiod of 10 hours light: 14 hours dark was applied in the laboratory. Throughout the experiment, water quality was routinely monitored twice per week.

The experimental diet was prepared by combining ChV at a rate of 30gm/kg diet with the homogeneously mixed basal diet ingredient (Table 1) and then pelleted employing a pellet machine. The pellets were properly dried at ambient temperature (26 ℃ for 48 hours), packaged in dry plastic bags, then placed in a refrigerator at 4 ℃ till be used.

Table 1: Ingredients concerning the diets tested (%) for *Oreochromis niloticus.*

Diet ingredients (%)	*R-D
Fish meal (65.4% CP)	40
Soybean meal (44%)	20
Yellow corn	13
Wheat flour	15
Wheat Bran	2
Fish oil	7
Monocalcium phosphate	2
(1) Vitamin mixture	0.45
(2) Mineral mixture	0.55
Chlorella vulgaris	30
Chemical analyses (% DM)	
Crud Protein	38.90
Crude fat	10.50
Ash	5.84

***R-D**= Control reference diet [28]

(1) **Vitamin mix (IU or mg kg diet):** vitamin A, 16000 IU; vitamin D, 8000 IU; vitamin K, 14.72; thiamin, 17.8; riboflavin, 48; pyridoxine, 29.52; cyanocobalamin, 0.24, tocopherols acetate, 160; ascorbic acid (35%), 800; niacinamide, 79.2; calcium-D- pantothenate,73.6; folic acid, 6.4; biotin, 0.64 L-carnitine, 100.

(2) **Mineral mix (mg kg diet):** Cu (CuSO4), 2.0; Zn (ZnSO4), 34.4; Mn (MnSO4), 6.2; Fe (FeSO4), 21.1; I (Ca (IO3)2), 1.63; Se (Na2SeO3), 0.18; Co (CoCl2), 0.24; Mg (MgSO4.H2O), 52.7.

Experimental protocol

The experimental protocol was reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) (Approval number ZU-IACUC/2/F/217/2023).

O. niloticus was evenly allocated into 4 groups (36 fish/group). Fish groups were subdivided as (3 replicates/group), (12 fish/replicate). In the control group (C), fish received a basic diet devoid of additives and housed in aquaria with pure water. In the ChV group, fish received basal diet enriched with 3% ChV (30 gm ChV /kg). The CuO-NPs group was exposed to 1/10 LC_{50} (5.72 µg L^{-1}) (The estimated LC_{50} was 57.20 mg/L, unpublished data), by the addition of the CuO-NPs to water and fed a basal diet for 21 days. The CuO-NPs+ ChV group was exposed to 1/10 LC_{50} (5.72 µg L^{-1}) CuO-NPs and fed a baseline diet enriched with 3% ChV for 21 days.

During the time of the experiment, fish received food three times a day (7:00 a.m., 11:00 a.m., and 4:00 p.m.) at a dietary level equal to 3% of the fish biomass; feed needs were established weekly dependent on the fish's growing weight, and toxicity was inspected.

Behavioral investigations

For 21 days, a stop watch and a video camera were used to monitor behavior every day between 9:00 a.m. and 3:00 p.m. based on Altmann *et al.* [29]. The behavioral habits and mean frequency were determined for 15 min to 8 hours per week. Fish were watched for any abnormal clinical symptoms, including hyperpigmentation (coloration), which was documented weekly by counting the number of fish with evident melanin

pigments in all groups, hemorrhages, ulcers on the body, and tail corrosions. The noticed behavior habits were described as follows:

- *Number of midline crossings*: The tank was split by an external midline, and the number of midline crossings from fish through 3 min was recorded in every aquarium [30].
- *Surfacing behavior*: It is looking for air around the water surface because of the low oxygen concentration in the tank [31].
- *Feeding behavior*: Performance of fish during feeding [32].
- *Laterality*: It is measured by the number of fish that are shown on lateral motion at the bottom for 3 min per day [33].
- *Loss of equilibrium*: The failure of fish keep their balance inside the water column for one time per day [34].
- *Hiding*: Number of fish that shelter in tank sides for 3 min per day [35].
- *Loss of reflexes* (knocking on a single side of the aquaria and get away when attempting to capture fish with a net) [36].
- *Motionless*: Remaining inactive in a category at the bottom of the pond for 3 min per day [37].

Sampling

Blood samples were taken from caudal vessels of three fish per replicate (6 samples per group) using a sterile syringe in BD Vacutainer PST II Tubes, then coagulated and centrifuged at 3000 rpm for 15 min to isolate sera. Serum was stored at -20°C until AChE activity was determined. The brain tissue was obtained from fish and assigned into three groups; the first set was dissected, immersed in approximately 5 vol of RNAlater® solution, stored at -80°C for RT-PCR procedures, the second set was homogenized by a WiseTis HG-15D homogenizer for the measurement of oxidative stress (OS) and antioxidants biomarkers and the specimens from the last set were immediately fixed for 48 h in 10% neutral buffered formalin for histopathological studies.

Biochemical measurements of AChE and oxidative stress -related biomarkers

Commercial fish ELISA kits supplied by MyBioSource, San Diego, USA were used to estimate AChE (ng/ml), SOD (U/ml), MDA (nmol/ml), 8-OHdG (pg/ml) (Catalog No: MBS705766, MBS705758, MBS1601664, and MBS2700257, respectively) according to the manufacturer's guidelines. Total antioxidant capacity level was estimated calorimetrically by specific diagnostic kits (Bio diagnostic Co., Giza, Egypt). (Catalog No: TA 25 13).

Transcriptional analysis of (Bcl-2, Caspase, NF-κβ) in brain tissue using quantitative real-time PCR

The transcriptional expression analysis of tested genes was performed as previously reported in Abou-Zeid *et al.* [38].The oligonucleotide-specific primers were shown in Table (2). The target gene expression levels were adjusted by comparing them to the mRNA expression of a known housekeeping gene, B-actin. The findings are expressed as fold-changes versus the control group, using the $2^{-\Delta\Delta}$CT technique [39].

Table 2: The oligonucleotide-specific primers were synthesized by Sangon Biotech (Beijing, China)

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession no.
β-actin	GCAGGAGTACGATGAGTCCG	CTCTGCGCCTGAGTTGTGTA	XM_003443127.5
NF-κβ	TCGGTGTAGCAGGCTTTTGT	GCTGCAGAGATGTGGGTGAT	XM_013277333.3
Bcl-2	ATGCAAAGAGAAGGTCGCCA	CAAAACAGGCTGGTTCCGTG	XM_003437902.5
Caspase-3	TTCTTTGGTACGGACGGCTC	CCTCTGCAAGCCTGGATGAA	NM_001282894.1

Histopathological investigation

The fixed specimens from brain tissue were processed and stained with hematoxylin and eosin (H&E), then become ready for examining microscopically [40]. All section photographs were shot via a Swift microscope coupled with a Swift digital camera.

Statistical analysis

Statistical analysis was performed on all experimental data through applying a One-way Analysis of Variance (ANOVA) using SPSS version 20 (IBM, USA), followed by Tukey's multiple comparisons post hoc test. The statistical significance was approved at *($p < 0.05$).*

Results

Effects of CuO-NPs, ChV, and their combinations on clinical signs in O. niloticus

Fish exposed to CuO-NPs exhibited hyperpigmentations, ulcerations, tail corrosions and hemorrhages in significant manner (Figure 1), when compared with those of the control and ChV groups. However, co-supplementation of ChV induced a remarkable reduction of the boosted hyperpigmentations and hemorrhages, while non-significantly reduced the elevated ulcerations and tail corrosions in CuO-NPs+ ChV exposed-group, when compared with those of the CuO-NPs-intoxicated group (Table 3).

Effects of CuO-NPs, ChV, and their combinations on behavioral responses of O. niloticus

In ChV exposed-group, there was non-significant decreases of surfacing, hiding and motionless behaviors, loss of equilibrium, laterality, when compared with those of the control group. On contrary, it generated a substantial rise in fish feeding, number of crossings behaviors, escape and knocking reflexes, when contrasted with those of the control group.

In the CuO-NPs-intoxicated group, a significant increase of surfacing, hiding, loss of equilibrium, laterality and motionless behaviors was recorded, in contrast to those of the control group. Combining treatment with ChV decreased elevated levels and modulated such behaviors, where the loss of equilibrium, laterality, and motionless behaviors showed non- significant difference from those of control, indicating that they normalized to the control values. On the other hand, both surfacing and hiding behaviors, still showed a significant increase in comparison with the control group, but did not attain the control values.

Exposure to CuO-NPs induced a significant decrease of fish feeding, number of crossings behaviors, escape and knocking reflexes, when compared with those of the control group. Co-exposure to ChV and CuO-NPs

moderated aforementioned behaviors by elevating the observed behavioral reductions. The raise in the reduced feeding, number of crossings behaviors and knocking reflex, in CuO-NPs+ChV exposed-group, was significantly different from those of CuO-NPs exposed group. Furthermore, the modified levels of crossing behaviors and knocking reflexes did not reach control values, although feeding behavior did, compared with those of the control group. Concerning the escape reflex, co- supplementation of ChV could not improve the altered behavior, as there was no significant increase, in CuO-NPs+ChV exposed-group, when compared with that of the CuO-NPs-intoxicated group (Table 3).

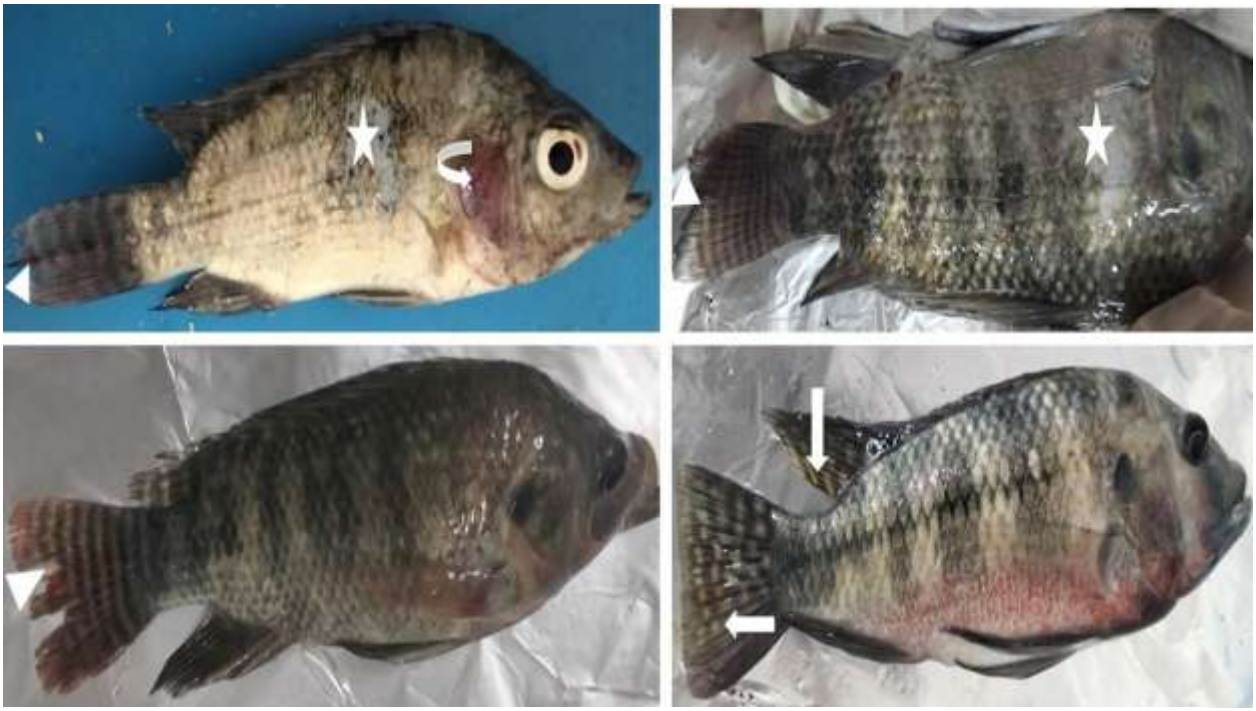

Figure 1. *O. niloticus* exposed to (1/10 LC_{50}) of CuO-NPs exhibited hyperpigmentations (thick arrow), ulcerations (star), tail corrosions (arrow head), and hemorrhages (curved arrow), in significant manner.

Table 3: Effects of CuO-NPs (CuO-NPs: 5.72 mg/L), ChV (ChV: 30 gm/kg), and their combinations on clinical signs and behavioral observations of *O. niloticus*.

Groups / Parameters	Control	ChV	CuO-NPs	CuO-NPs + ChV
Hyperpigmentation	0.00±0.00	0.00±0.00	6.33±0.21*	4.00±0.37*#
Ulcer	0.00±0.00	0.00±0.00	2.67±0.76*	2.33±0.84*
Tail corrosion	0.50±0.22	0.00±0.00	5.00±0.45*	4.33±0.21*
Hemorrhage	0.50±0.50	0.00±0.00	4.67±0.33*	1.33±0.21*#
Surfacing	1.50±0.31	0.83±0.22	8.00±0.37*	6.33±0.56*#
Hiding	0.50±0.22	0.33 ±0.21	3.00 ±0.37*	2.00±0.37*
Number of crossings	7.00±0.37	8.50±0.22*	2.00 ±0.37*	4.00±0.45*#
Loss of equilibrium	0.50±0.22	0.00±0.00	1.50±0.22*	1.00±0.00
Laterality	0.67±0.21	0.00±0.00	2.00 ±0.45*	1.00±0.37
Motionless	3.33±0.92	2.33±0.56	9.00±0.45*	5.33±0.56#
Feeding	11.00±1.32	14.00±0.45*	2.67±0.21*	9.50±0.22#
Escape reflex	10.33±1.17	13.00±0.73*	4.00±0.45*	4.67±0.21*
Knocking reflex	8.83±0.40	13.33±0.56*	3.00±0.45*	6.00±0.36*#

(CuO-NPs), copper oxide nanoparticles, **(ChV)** *Chlorella vulgaris*. Values are means ± SEM of six fish per experimental group. *P value < 0.05* Vs control. #P value < 0.05 Vs CuO-NPs group.

Effects of CuO-NPs, ChV, and their combinations on AChE activity and antioxidant /oxidative stress-related indices of O. niloticus

Regarding the AchE activity, ChV supplementation resulted in a non-significant rise in the ChV-exposed group compared to the control group. On the other side, exposure to CuO-NPs significantly decreased the AchE activity in the CuO-NP-intoxicated group as compared to the control group. When compared to the CuO-NPs-intoxicated group, co-administration of ChV exhibited no substantial impact on the lowered serum levels of AchE, which remained significantly different from the control value (Figure 2).

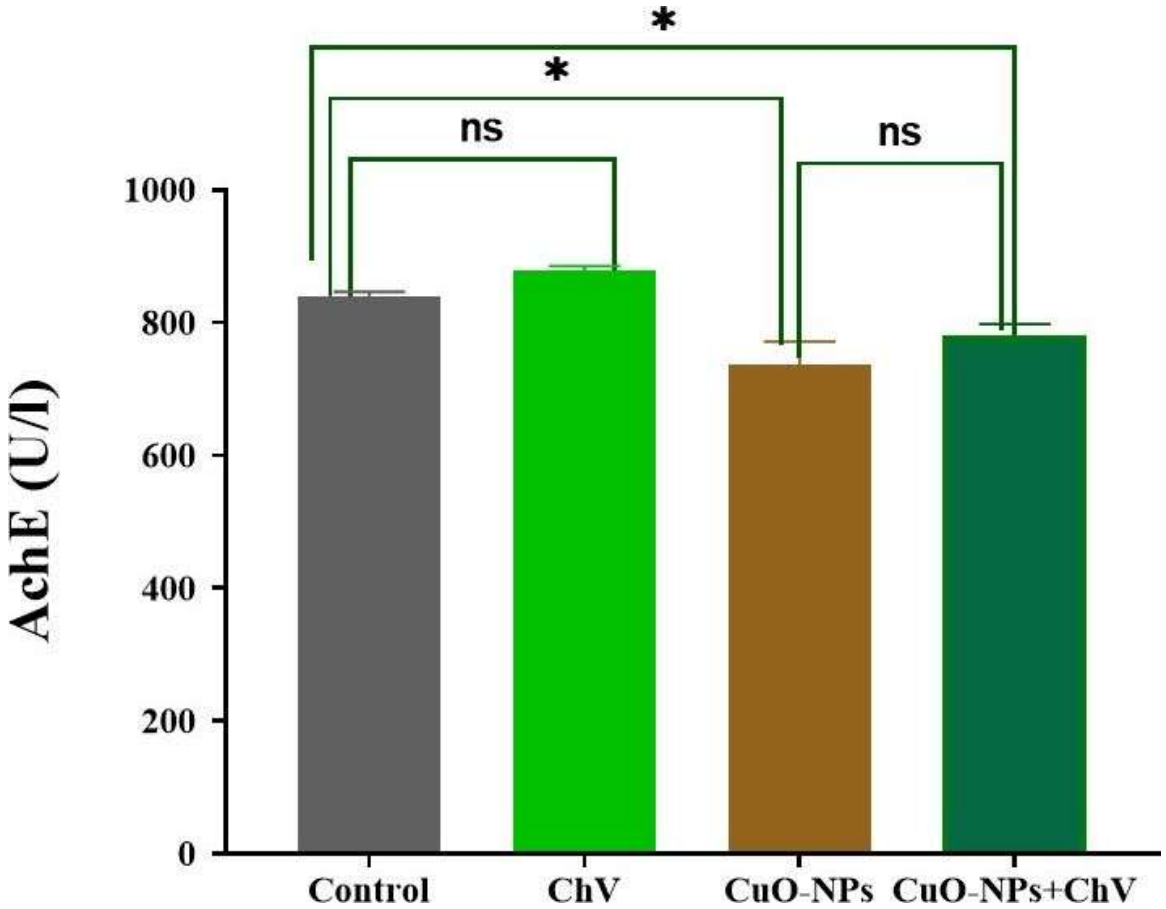

Figure 2. Effects of CuO-NPs, ChV and their combinations on acetylcholine esterase (AchE) activity in serum of *O. niloticus*. Values are means ± SEM of six fish per experimental group. $^{*}P$ *value* < *0.05* Vs control. $^{\#}P$ *value* < *0.05* Vs CuO-NPs group.

Supplementation of ChV induced a significant increase of TAC level and non-significant increase of SOD activity in brain tissue in the ChV exposed group, in contrast to those of the control group. Exposure to CuO-NPs induced a significant decrease of both TAC levels and SOD activity in the CuO-NPs-intoxicated group, in contrast to those of the control group.

Co-supplementation with ChV had no influence on the lowered level of TAC in the CuO-NPs+ChV exposed group, which exhibited non- significant rise when compared to the CuO-NPs-intoxicated group. It significantly elevated the lowered level of SOD activity in the CuO-NPs+ChV-exposed group as compared to the CuO-NPs-intoxicated group, but did not reach the control value.

From the other side, supplementation of ChV induced a non-significant decrease of MDA and 8-OH2dG levels in the ChV-exposed group when contrasted with those of the control group. Exposure to CuO-NPs induced a remarkable rise in the levels of MDA and 8-OH2dG in the brain tissue of CuO-NPs-intoxicated group when compared with those of the control group. The addition of ChV significantly decreased the levels of MDA and 8-OH2dG in CuO-NPs+ChV exposed-group although they remained higher than control values (Figure 3).

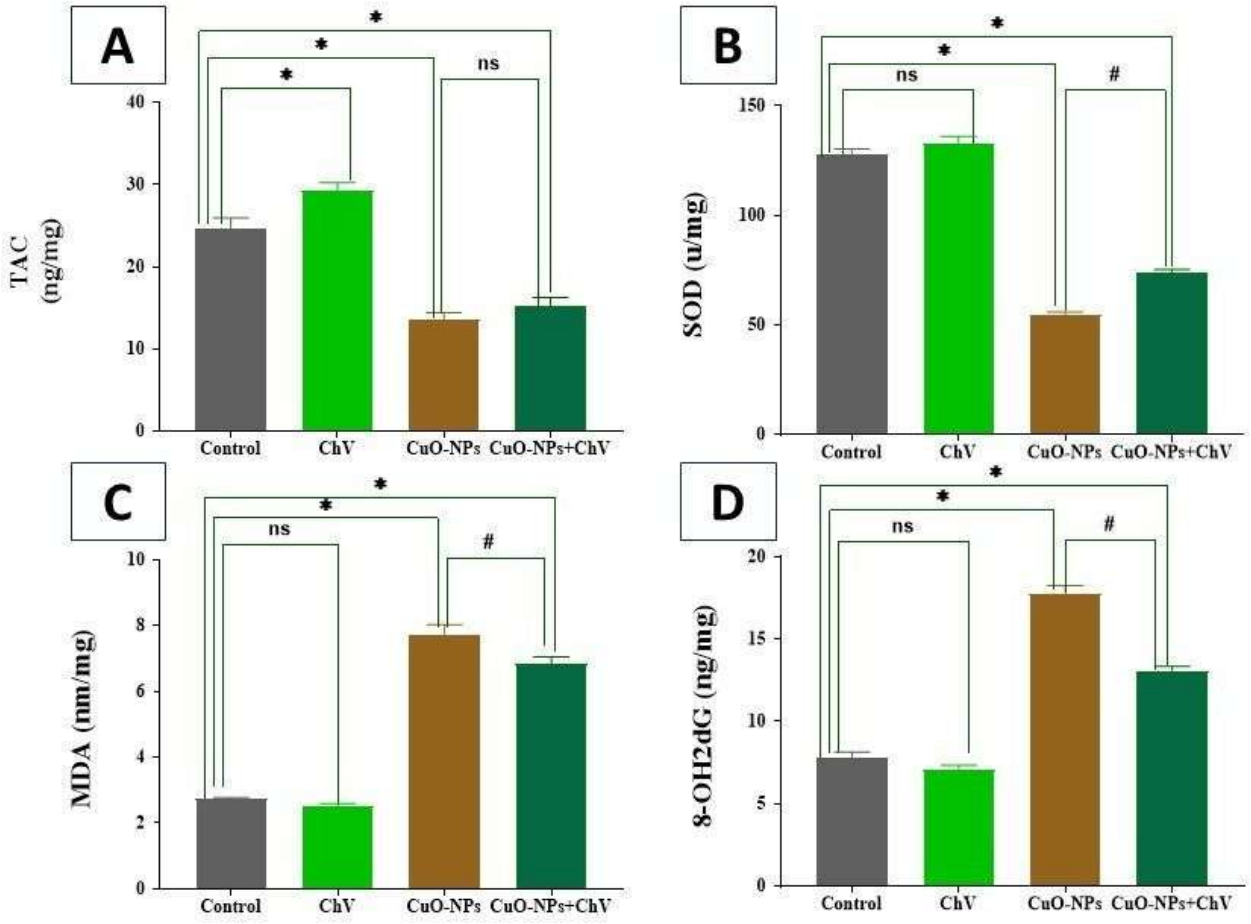

Figure 3. Effects of CuO-NPs, ChV and their combinations on antioxidant/ OS related indices in brain tissue of *O. niloticus.* **(A)** total antioxidant capacity (TAC), **(B)** superoxide dismutase (SOD), **(C)** malondialdehyde (MDA), and **(D)** 8-hydroxyguanosine (8-OH_2dG). Values are means ± SEM of six fish per experimental group. **P value < 0.05* Vs control. $^{\#}$*P value < 0.05* Vs CuO-NPs group.

Effects of CuO-NPs, ChV, and their combinations on expression profile of Bcl-2, Caspase-3, NF-κβ in brain tissue of O. niloticus

Supplementation of ChV non-significantly down-regulated *NF-κβ* and *Caspase-3* and non-significantly up-regulated mRNA expression levels of *Bcl-2* in ChV exposed-group when compared with those of the control fish. Exposure of CuO-NPs induced a significant up-regulation of their expression levels in CuO-NPs intoxicated group, in opposition to those of the control group. In contrast, co- supplementation of ChV significantly down-regulated the *NF-κβ* and *Caspase-3* mRNA expression levels, in CuO-NPs+ChV exposed-group, when compared with those of the CuO-NPs-intoxicated group. Exposure of CuO-NPs induced a significant down-regulation of *Bcl-2* mRNA level in CuO-NPs intoxicated group, in opposition to the value of control fish. This level was not significantly up-regulated in CuO-NPs+ChV exposed-group, compared with that of the CuO-NPs-intoxicated group (Figure 4).

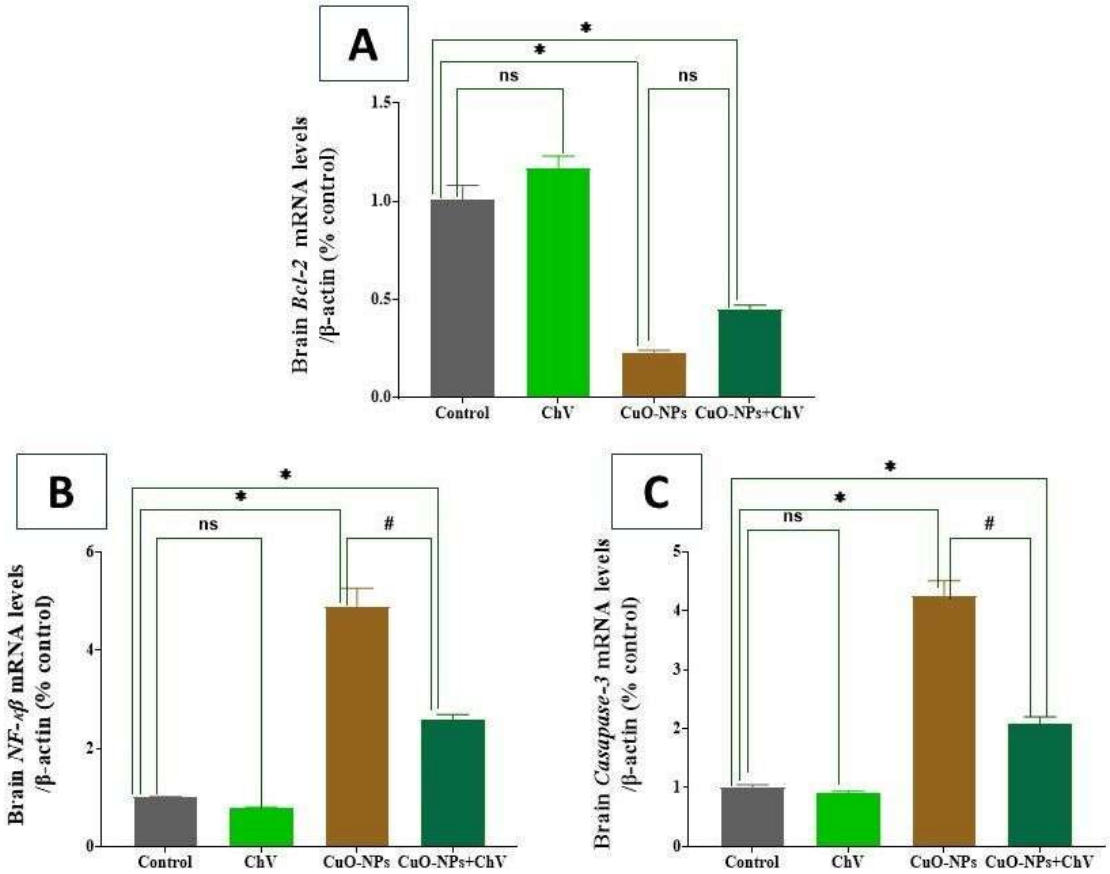

Figure 4. Effects of CuO-NPs, ChV and their combinations on *(Bcl-2, Caspase-3, NF-κβ)* mRNA expression levels in the brain tissue of *O. niloticus.* **(A)** b-cell lymphoma 2 (*Bcl-2*), **(B)** nuclear factor kappa B (*NF-κβ*), **(C)**, and *Caspase-3.* Values are means ± SEM of six fish per experimental group. **P value < 0.05* Vs control. $^{\#}$*P value < 0.05* Vs CuO-NPs group.

Histopathological investigation

Control and ChV groups showed normal histological structures of neurons, normally distributed glia cells and neuropil (Figure 5A and B). Exposure to CuO-NPs showed vacuolated neuropil, numerous numbers of degenerated neurons, congested cerebral blood vessels, and increase number of glia cells particularly at perivascular area (Figure 5C). Meanwhile, co-exposure of ChV and CuO-NPs revealed neuroprotective effect with few numbers of pyknotic neurons surrounded by glia cells (Figure 5D).

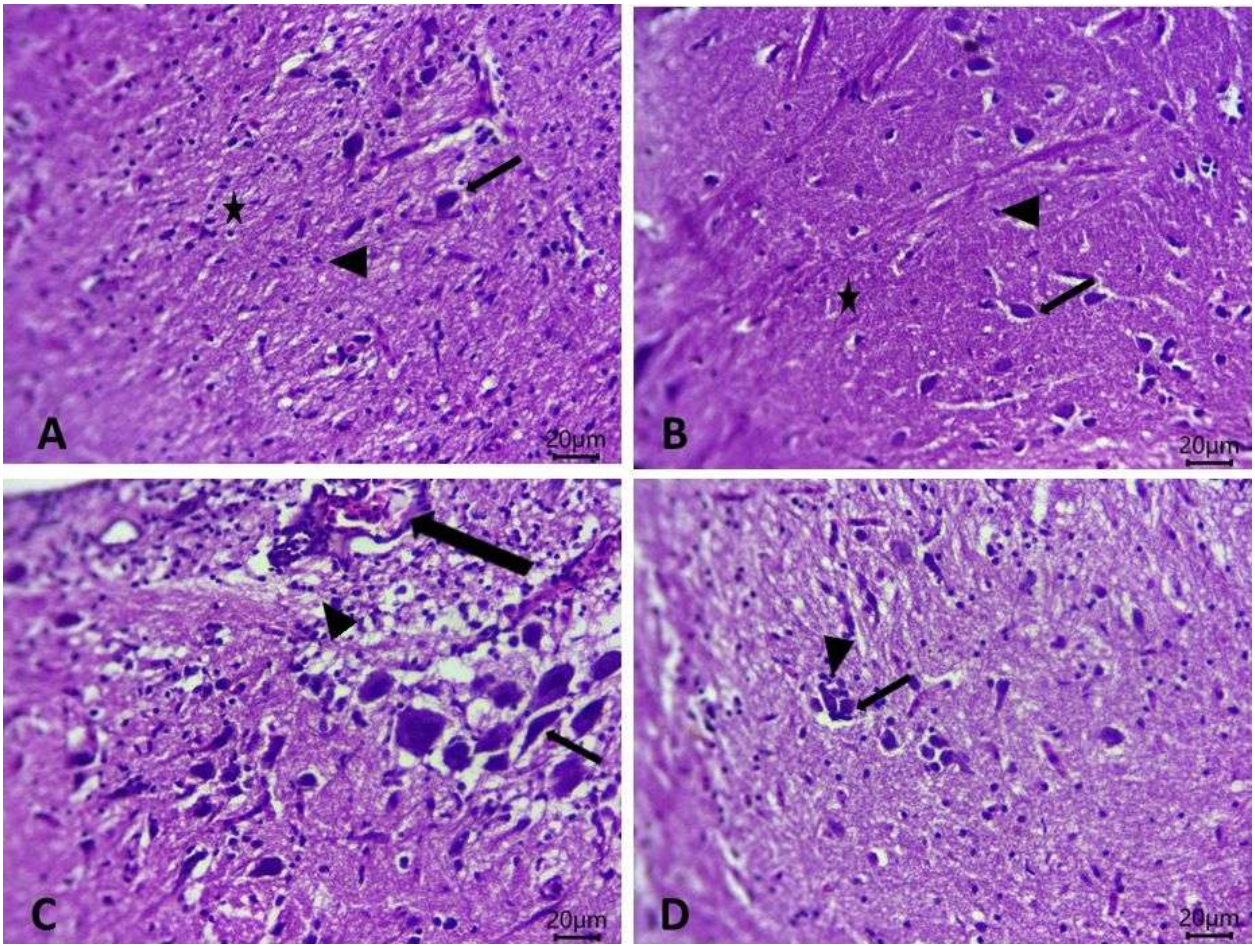

Figure 5. Representative photomicrograph of H&E-stained brain tissue sections. Control and ChV groups showing; A and B: normal histological structures of neurons (arrow), normally distributed glia cells (arrowhead) and neuropil (star). CuO-NPs-intoxicated group showing; C: numerous numbers of degenerated neurons (arrow), congested cerebral blood vessels (thick arrow), and increase number of glia cells particularly at perivascular area (arrowhead). CuO-NPs+ChV exposed group showing; D: few numbers of pyknotic neurons (arrow) surrounded by glia cells (arrowhead) (Scale bar at 20 µm).

Discussion

The current study's findings demonstrated that CuO-NPs toxicity caused an impressive rise in hyperpigmentations, ulcerations, tail corrosions and hemorrhages. The observed dyspigmentation on the skin of fish were related to the malfunction of the endocrine pituitary gland, which particularly under the stress of toxin [35]. Noga *et al.* [41] demonstrated that even very transiently high blood cortisol harms skin epithelial structure. The observed tail corrosions in our study in response to exposure stress may associated with deficiency in nutrition due to the loss of appetite represented by the alteration of eating behavior [42].

Previous NPs toxicity investigations in several fish species exhibited similar effects as those shown in Zebrafish embryos and larvae treated with TiO2-NPs, which displayed lack or aberrant pigmentation organization [43]. Kim *et al.* [44] found that exposing Zebrafish to AuNPs led to pigmentation, behavioral

abnormalities, and nervous system impairment. *O. mossambicus* treated with ZnO-NPs developed skin discoloration and ulceration [45]. Furthermore, African catfish exposed to AgNPs developed skin darkening and bleeding [46].

Here, the supplementation with ChV has decreased the incidence of skin lesions. As ChV has anti-inflammatory characteristics, including its usefulness in controlling ulcerative colitis [24]. As well, ulcerative lesions was decreased in fish exposed to penoxsulam and fed on 10% ChV-supplemented diet [26].

Exposure to CuO-NPs induced a significant increase of surfacing, hiding, loss of equilibrium, laterality and motionless behaviors. On the other hand, exposure to CuO-NPs induced a significant decrease of number of crossings, escape reflex, knocking reflex and feeding behavior. Meanwhile, co-exposure to ChV and CuO-NPs significantly modulated these alterations. Sovová *et al.* [47] found a relative decrease in numbers of midline crossings by juvenile rainbow trout (*Oncorhynchus mykiss*) by $CuSO_4$ and Cu-NPs treatments. Further studies have reported that CuO-NPs exposures induce behavioral changes in the form of enhanced rate of opercular activity, loss of balance, and increased surfacing activity [48]. In a similar manner, all strains of *C. elegans* isolates displayed greater sensitivity to CuO-NPs, as indicated by eating behavior [16]. Similar behavioral responses were also reported in *Cyprinus carpio* which exposed to various dosages of waterborne Cu-NPs and CuO resulted in some behavioral modifications like frequent opercular movements, greater mucus production and lessened fish motions [49]. Additionally, *Cyprinodon variegatus* which exposed to CuO-NPs showed an increase in mucus secretion, and a loss in equilibrium [50].

In truth, the impacts of environmental contaminants on fish behavior may be a direct consequence of raised ROS levels [51]. Also, neurodegenerative study revealed that the high Cu content was responsible for considerable neuronal damage, where NPs can enter the brain and may be linked to neurodegenerative impairments [52].

It is realistic that eating demotivation may be related to biological factors that affect fish feeding behavior. Given that serotonin is a crucial neurotransmitter known to affect a variety of tilapia behaviors, including feeding behavior, it may be linked to alterations in serotonin levels brought about by ZnO-NPs [53]. Undoubtedly, a number of factors could contribute to these alterations, including disruptions to the neuronal pathways involving important brain areas in the animal' serotonergic system [51]. Additionally, diminished eating motivation with exposure to ZnO-NPs may be due to olfactory loss (anosmia) in the animals [54].

Surfacing behavior may be attributed to respiratory distress and altered osmotic balance resulting in fish surfacing, sluggish and deaths due to direct exposure of fish gills to poisonous NPs which release Cu into water inducing changes gills function and lowers the uptake of O_2 [55]. Also these data are in accordance with Radhaiah and Rao *et al.* [56] who noticed the surfacing behavior of fish owing to $CuSO_4$ exposure; this phenomenon may be attributable to the fish's hypoxic condition. Concerning the loss of equilibrium, it is possible that the region in the brain linked with the keeping of homeostasis may have been disrupted [57, 58].

The adjusting of behaviors in CuO-NPs-exposed fish and fed with ChV diet could be related to its protective role on brain tissue and ameliorating the stress state in the CuO-NPs-exposed fish, due to its antioxidant attributes, subsequently altered the neurotransmitter level and subsequently modified the behaviors of the fish intoxicated with CuO-NPs [25].

Zhao *et al.* [59] reported that CuO-NPs exposure significantly reduced the AChE activity in juvenile carp brain, revealed that CuO-NPs in water have neurotoxic ability to carp. Likewise, our results were showed that exposure to CuO-NPs induced a significant decrease of the AchE activity. Inhibition of the AChE enzyme caused acetylcholine to accumulate within synapses, resulting in excessive stimulation of cholinergic nerves and impaired neuro-muscular synchronization, as evidenced by behavioral changes in eating and movement [60]. The behavioral changes in fish point to a strong correlation between these behaviors and the AchE decreases seen in intoxicated fish brains [42].

The inhibition in ChE activity during CuO-NPs exposure was recorded in various fish species including adult zebrafish and *O. niloticus* [61, 62]. Regarding CuO-NPs, Since CuO-NPs might disintegrate while being transported through the fish body, the majority of the Cu in the brain was in the form of Cu ions. Thus, it is likely that free Cu^{2+} ions dissolved from CuO-NPs inside the fish body were the primary cause of ChE inhibition in the brain [59]. Additionally, as cortisol is the main factor in the stress response and is important for fish welfare, it may be the cause of behavioral changes in fish due to elevated serum cortisol levels, which alter fish brain function [63].

Concerning the mitigating role of ChV, our data are in harmony with Mansour *et al.* [64] who mentioned that the addition of ChV to the diet of oxyfluorfen-exposed catfish increased the level of serum AchE. Also, Edrees *et al.* [25] who fed *O. niloticus* on the ChV-enriched diet against acrylamide toxicity.

Exposure to CuO-NPs induced a significant decrease of TAC and SOD activities and increased the MDA and 8-OH2dG levels in brain tissue. Meanwhile, co-exposure of ChV and CuO-NPs significantly modulated the decreased SOD activity and elevated MDA and 8-OH2dG levels in brain tissue. The disturbance in antioxidant status was previously recorded in *O. niloticus* [65]. Moreover, the elevation of oxidative injury marker was also previously documented in adult zebrafish [61], Catfish [66]. The reduced TAC value due to CuO-NP exposure can be attributed to the potential of CuO-NP to utilize the power of antioxidant defense mechanisms in blood serum [67].

CuO-NPs generate ROS and hinder the antioxidant defense system as SOD, enhancing the oxidation damage. Also, Cu ions are known to be hazardous to aquatic creatures, where, Cu assists in Fenton and Haber-Weiss reactions, which encourage the production of ROS and OS [68]. According to Lapresta-Fernández *et al.* [69], ROS damages the cell's membrane and cellular organelles and changes cell integrity. With the breakdown of membrane integrity, NPs can enter the cell, finally causing cell death.

The ROS produced worsens inflammation and damage proteins, DNA, and lipid membranes [70]. He *et al.* [71] showed that the generated Cu^{2+} from CuO-NPs is the primary source of oxidative stress and excess superoxide anions in cells.

The antioxidant potency of ChV was recorded previously against various pollutants in fish studies including *O. niloticus* intoxicated with penoxsulam, heavy metals, diazinon, deltamethrin, and cadmium [25, 72, 73], African Catfish exposed to oxyfluorfen and polystyrene nanoplastics [64]. As well as, common carp intoxicated with imidacloprid [74].

The presence of SOD in microalgae is the reason for the notable rise in SOD that occurs when microalgae are added to the diet. Ascorbate peroxidase, SOD, CAT, and a non-specific peroxidase are among the antioxidant enzymes that microalgae like ChV may express [75]. ChV's bioactive components, such as lutein flavonoids, carotenoids, chlorophyll, tocopherols, and polyphenols, may be responsible for its protective impact against oxidative damage caused by CuO-NPs [76]. Additionally, ChV has a lot of astaxanthin, a carotenoid that is regarded as super vitamin E because of its natural antioxidant properties [77].

Exposure to CuO-NPs induced a significant up-regulation of *NF-κβ* and *Caspase-3* mRNA expression and down-regulated *Bcl-2* mRNA levels in brain tissue. Meanwhile, co-exposure to ChV and CuO-NPs modulated the above-mentioned altered mRNA expression genes levels in brain tissue.

Our results are in agreement with studies conducted for evaluation of CuO-NPs exposure Abdel-Latif *et al.* [78] noticed a significant upregulation of *Caspase-3* gene in gills and liver of *O. niloticus*. Furthermore, an obvious rise in the mRNA expression level of *Caspase-3* genes in the liver of *T. fasciatus* [79]. Also, the transcript of *Caspase-3* was rapidly and significantly raised in monocyte/macrophage of *O. niloticus* [80]. *O. niloticus* exposed to Cu toxicity showed a remarkably down-regulation in *Bcl-2* [32].

Caspases are effective markers and mediators for detecting stress-induced apoptosis [81] CuO-NPs caused the exposed *O. niloticus* tissues to undergo apoptosis [78]. Also, CuO-NPs enhanced *caspase-3* activity, inducing apoptosis [82].

Exposure to Cu caused severe oxidative cellular damage by generating ROS, which is associated with a strong proinflammatory response, via activating *NF-κB* and creating damage in DNA [24]. ROS and pro-inflammatory mediators contribute to the initiation of apoptosis [83]. The increase in *NF-κβ* gene expression indicated that CuO-NPs increased oxidative stress [84].The induced *NF-κβ* suppressed by antioxidants [85].

The recorded protective function of ChV in modulating the expression pattern of tested genes was in accordance with results obtained by Yu *et al.* [86], where, *NF-κB* and *caspase-3* gene expressions downregulated, while *BCL2* expression level increased by ChV supplementation in largemouth bass. Aboumosalem *et al.* [24] demonstrated that ChV inhibited *NF-κB* and *Caspase-3* genes expressions in rat. The modulation of inflammatory markers can be accomplished by lowering ROS generation and down-regulating the COX-2 gene, which are key mediators that change the expression of multiple genes linked to inflammation [87]. Along the same line, the bioactive elements in microalgae can reduce inflammation in fish skin through their anti-inflammatory properties and lessen the expression of pro-inflammatory genes [88]. Actually, the anti-inflammatory effect of ChV may be linked to carotenoids especially, violaxanthin that has strong antioxidant

features, minimizing ROS production, and improving endothelial function [89].

Also, our data demonstrated that ChV has anti-apoptotic properties via modulating *Caspase-3* and *Bcl-2* expression. This outcome is in agreement with Ibrahim *et al.* [90] who tested the anti-apoptotic properties of ChV. The potential effect of ChV in suppressing apoptosis could be connected to a reduction of lipid peroxidation and inflammation [24]. Also, algae inhibit apoptosis by up-regulating the *Bcl-2* protein production. *Bcl-2* overexpression may allow cells to cope better with ROS by permitting boosts in endogenous antioxidant enzymes that counteract the ROS-induced drop in *Bcl-2* and prevent cellular death [91].

Histopathological finding demonstrated that the exposure to CuO-NPs showed vacuolated neuropil, numerous numbers of degenerated neurons, congested cerebral blood vessels, and the increased number of glia cells particularly at perivascular area. Meanwhile, co-exposure of ChV and CuO-NPs revealed neuroprotective effect. Similarly, exposure to $CuSO_4$ or CuO-NP in *O. niloticus* has been shown to induce degeneration of the neuropil along with pyknotic nuclei [92]. Also, our outcomes are in accordance with Habotta *et al.* [42] who studied that the effects of $CuSO_4$ on *O. niloticus* showed neuronal vacuolation, necrosis with inflammatory infiltrations, hemorrhage and edema. These modifications indicate that Cu has a fatal toxic effect on brain tissue and can accumulate in those tissues.

Concerning, the mitigating role of ChV, our findings are in consistency with results noticed by Zahran *et al.* [93] who mentioned that ChV dietary supplementation ameliorated histopathological alterations and reduced the frequencies of lesions in gills and liver of *O. niloticus* after arsenic exposure. Also, the detrimental changes in the brain, liver and spleen tissues decreased and its histological lesion scores improved in *O. niloticus* against acrylamide toxicity [25].

Conclusion

The current study provides information concerning the neurobehavioral toxicity induced by the exposure to CuO-NPs in *O. niloticus*, including a significant neurobehavioral alteration, oxidative injury and brain tissue damage. On the other hand, ChV co-administration with CuO-NPs, elicited a palliative potency against these deleterious effects. This was presented by a substantial decrease in the levels of oxidative injury markers, modulation of behavioral indices, and alleviating the morphological perturbation observed in the brain tissues of fish.

Conflicts of interest

The authors declare no conflict of interest.

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الملخص العربى

في تحسين الاضطرابات العصبية السلوكية الناتجة عن السمية تحت الحادة Chlorella vulgaris دور الطحلب الأخضر لجسيمات أكسيد النحاس النانوية في أسماك البلطي النيلي

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جسيمات أكسيد المعادن النانوية (NPs) بما في ذلك أكسيد النحاس (CuO-NPs) تطلق بشكل غير منضبط في البيئة، مما يشكل تهديدًا خاصًا للكائنات الحية في البيئة المائية. أظهرت طحالب الكلوريلا (ChV) تأثيرًا وقائيًا ضد المواد الضارة من خلال تقليل الاجهاد التأكسدي الناجم عن عديد من المركبات المختلفة بما في ذلك الجسيمات النانونية. هذه الدراسة قد خططت الي تقييم تأثير التعرض شبه الحاد لجزيئات CuO-NPs على الاضطرابات السلوكية العصبية في اسماك البلطي، وتقييم الدور التحسيني لـ ChV ضد السمية العصبية. تم تقسيم 144 سمكة بالتساوي إلى أربع مجموعات ، كما يلي: مجموعة التحكم، مجموعة ChV (30غرام/كغ من النظام الغذائي)، مجموعة CuO-NPs (10/1 LC50، 5.72 ميكروغرام/لتر)، ومجموعات CuO-NPs/ChV. أثبتت النتائج أن تعرض CuO-NPs أدى إلى زيادة كبيرة في التصبغات المفرطة، والتقرحات، وتآكل الذيل، والنزيف. تم تسجيل زيادة كبيرة في السطحية، الاختباء، فقدان التوازن، الجانبية والسلوكيات الثابتة في الأسماك المعرضة. من ناحية أخرى، أدى تعرض الأسماك لجزيئات أكسيد النحاس النانوية إلى انخفاض كبير في عدد العبور، وسلوكيات التغذية، وردود الفعل على الهروب والطرق. أظهرت الأسماك المعرضة انخفاضًا كبيرًا في TAC ، ونشاط إنزيم SOD، بينما زادت مستويات MDA و8-OH2dG بشكل كبير في أنسجة المخ، بالإضافة إلى انخفاض نشاط إنزيم AChE في المصل. أظهرت تحليل تفاعل البلمره المتسلسل زيادة في مستويات التعبير عن mRNA لـ NF-κβ وCaspase-3، بينما انخفض مستوى التعبير عن mRNA لـ Bcl-2 في نسيج المخ. علاوة على ذلك، لوحظت تغييرات هيستوباثولوجية في نسيج مخ الأسماك. من المهم أن ChV حمى بشكل ملحوظ ضد الاضطرابات العصبية السلوكية التي تسببها CuO-NPs. أظهرت النتائج التأثيرات الحامية لـ ChV في الأسماك المعرضة لجزيئات CuO-NPs، مما يدعم الاستخدام المحتمل لـ ChV كعامل مضاد للالتهابات، مضاد للأكسدة وحامي للأعصاب.