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RESEARCH ARTICLE

Broccoli Partially Lowers Oxidative Stress, Histopathological Lesions and Enhances Antioxidant Profile of Mono Sex Tilapia Exposed to Zinc Oxide Nanoparticles

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A B S T R A C T

The environmental effluents negatively affect the health and biochemical functions of tresterial and aquatic animals. It is crucial to ascertain the mechanisms of induction of adverse effects and applications of different natural and syenthetic antioxidants to mitigate the negative impacts in target and not target animals. Therefore, in this trial broccoli (*Brassica oleracea*) was used to ameleiorate the negative effects of zinc oxide nanoparticles in fish. Mono sex Nile tilapia (*Tilapia niloticus),* fish reared in different groups were treated with variable doses of zinc oxide nanoparticles alone (T1-T2) mg/kg feed and the fish of groups (T4-T6) were fed with variable doses of nanoparticles and broccoli in combinations for a total duration of 28 days in cemented tanks having 200L water. Tissues (kidneys and brain) were obtained at different intervals of experiment for estimation of oxidative stress, severity of histopathological lesions and antioxidant profile. Results showed that oxidative profile in term of ROS and TBARS escalated significantly ($p<0.05$), while antioxidants enzymatic parameters like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were decreased significantly ($p<0.05$) in treated fish. The induction of oxidative stress and histo-architectural alterations were partially reduced in fish of groups (T4-T6). Various histo-architectural ailments in kidneys (atrophy and obliteration of renal tubule, nuclear hypertrophy, edema ceroid formation and deterioration of glomerulus) and in brain (edema, atrophy of neuron, microgliosis, neuronal degeneration, congestion and necrosis of neuron) were examined in treated fish. In conclusion, the findings of our trial unveiled that the supplementations of broccoli partially improved the antioxidant enzymes in tilapia by modifying the severity of inflammation.

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INTRODUCTION

Aquaculture is a vital protein source for humans and different other animals. The demand for acceptable production and the consumption of nutritious food has caused a rapid increase in human population across the globe (Vijayaram *et al.,* 2023). Aquatic animals including fish and other species cultivated through aquaculture play a crucial role in combating malnourishment and numerous other health benefits including anti-inflammation, cardio-protection, anti-oxidation, hepatoprotection, wound healing and neuroprotection (Chen *et al*., 2022; Holman *et al.,* 2023). Due to the industrial revolution and tremendous increase in human population, the demand for natural nutritional food (aquaculture) has greatly increased (Tan *et al.,* 2022). Optimum production and health of aquatic animals has become essential in response to growing pressure from human population (Vijayaram *et al.*, 2023). A healthy and perfect environment is of vital importance to obtain diverse nutrients offering several health benefits and substantial protein from rapid cultivation of diverse fish species within a short time (Akram *et al.*, 2021; Zubair *et al.*, 2022).

The aquaculture industry is facing unpleasant pressure due to the continual rise in human population and the indiscriminate utilization of pharmaceuticals along with various natural and synthetic chemicals (Vo *et al.*, 2022; Zubair *et al.*, 2022). Contamination of water bodies significantly impacts the physiological processes, growth characteristics, and regulation of immune system in aquaculture (Akram *et al.*, 2021). Assessment of detrimental effects of different toxicants prevalent in water bodies is crucial for the optimum physiological functions of different biological process including mechanism of osmoregulation, growth, and maintaining normal functions of gills, kidneys and other tissues of aquatic animals (Akram *et al.*, 2022; Malik *et al.*, 2022). Synthesis of different types of nanoparticles (NPs) from natural and synthetic compounds and their extensive application in biomedical fields, manufacturing of electrical devices, gas sensors, cosmetics, paints, and anti-dandruff shampoos is greatly contaminating aquatic and terrestrial environment across the globe (Jimenez-Carretero *et al.*, 2023; Ali *et al.*, 2024). Nanomaterials are extensively used to treat industrial waste water to mitigate environmental pollution (Jimenez-Carretero *et al.*, 2023). Nanoparticles after entering aquatic bodies cause huge risks and amplify oxidative stress via blood circulation in multiple organs in fish (Saddick *et al.*, 2017). Increased quantity of nanoparticles generates reactive oxygen species (ROS) in animals leading to damage to DNA, plasma membrane and disrupts cellular metabolism (Pérez-Labrada *et al.*, 2019). Due to their nano size, these chemical particles exhibit enhanced penetration capabilities, entering the body of fish through gills and alimentary canal with consumed food, ultimately accumulating in the muscles of fish (Sajjad *et al.*, 2023). The nanoparticle may cause toxic effects on exposed animals including public health due to high penetrating ability at different stage of synthesis, utilization, consumption and due to their inherent properties (Tortella *et al.*, 2020). Utilizing plant-derived antioxidants found in broccoli hold promise for enhancing fish quality.

Utilizing plants as a defense mechanism is advantageous for health because many plants serve as a rich source of nutritious foods containing anti-oxidants (Chen *et al.*, 2022; Holman *et al.*, 2023). These antioxidants can combat various diseases and age-related disorders by reacting with the free radicals produced during oxidative stress, protecting multiple health issues (Chen *et al.*, 2022). Brassica family has gained popularity owing to its phytochemical and bioactive substances among different plants and is well known regarding its anticancer and antimicrobial effects (Holman *et al.*, 2023). Brassica vegetables are favored for their low-fat content, high mineral and vitamins, phenolic and glycosylate chemicals well-known for their numerous health benefits (Salehi *et al.*, 2021). Brassica vegetables are rich in flavonoids which exhibit diverse biological effects such as anti-diabetic, anti-inflammatory, anti-cancer, and anti-microbial properties (Le *et al.*, 2020). To counteract the oxidative process, naturally occurring plants with antioxidant properties are frequently used across the globe (Chen *et al.*, 2022).

Considering the crucial role of broccoli in mitigating the toxic effects of nanoparticles in fish, this study was design to investigate the antioxidant potential of broccoli-based diet in toxicity induced by zinc oxide nanoparticles in different organs of Mono sex tilapia.

MATERIALS AND METHODS

Approval of the study: Prior to start and execution of trial, the research was approved by the Advanced Studies and Research Board, Bahauddin Zakariya University Multan (No. Acad/Scholar's file/1932. Dated;10-02-2020).

Cultivation and phytochemical analysis of broccoli: During the month of November, 2020 seeds of a hybrid type of *Brassica oleracea* were purchased and grown. Healthy florets were picked during the month of April, 2021 and dried at 60^{o}C before grinding and powder formation. The broccoli powder was mixed with a commercial fish feed containing 23% protein at 1:3 ratios. Phytochemical properties of broccoli were assessed by using the AACC (2000) standard techniques. Phytochemical analysis of broccoli indicated low moisture contents (3.13±0.43%), proteins (26.40±0.7%), carbohydrates (39.26±0.58%), fibers (15.26±0.42%), fats (8.63±1.02%) and ash contents (6.11±0.45%). Different minerals contents like phosphorus (586.26±1.01 mg/100g), potassium (3211.7±1.53 mg/100g), calcium (531.23±0.72 mg/100g), magnesium (231.45±0.35 mg/100g), iron (8.88±1.15 mg/100g), copper (0.75±0.10 mg/100g) and zinc (6.01±0.53 mg/100g) were determined by using the earlier procedure (Moreno *et al.*, 2006). The total phenolic compounds (87.6±3.8 GAE/100g) and other antioxidants capacity of dried broccoli were estimated by inhibition of 2, 2- diphenyl-1- picryl hydrazyl (451.29±0.60 mg/100g) while Ferric Reducing Antioxidant Potential (319.77±3.19 mg/100g) and 3-Ethylbenzothiazoline-6-sulphonic acid (764.31±6.62 mg/100g) were estimated using the previous protocol (Benzie and Strain, 1999).

Fish management and experimental protocol: ZnO NPs were synthesized and different concentrations like 150, 300 and 450mg/kg were used in diets in this trial. Approximately, 140 samples of tilapia (mono sex) were purchased, transported in plastic bags containing oxygen and were reared in cemented tanks having 200L water capacity at Bahauddin Zakariya University Multan, Pakistan. Fish were treated in seven different tanks labeled as T0 (control), T1 (ZnO NPs; 150mg/kg), T2 (ZnO NPs;300mg/kg), T3 ZnO NPs; 450mg/kg), T4 (ZnO NPs 150mg/kg + Broccoli), T5 (ZnO NPs 300mg/kg + Broccoli), T6 (ZnO NPs 450mg/kg + Broccoli). Prior to the start of trial, all specimens were acclimatized and were offered with special diet for 15 days. All feed ingredients were carefully mixed and converted to pellet form by using a pelleting machine prior to administration in tanks for feeding to fish @ 3% of their body weight twice a day throughout this experimental trial. Physiochemical characteristics of water quality parameters were carried out at 0, 14, 21 and 28th days of the trial with average values indicated in Table 1.

Table 1: Physico-chemical analysis of experimental water.

Parameters with units	Values
Total hardness (mg/L)	166.3 ± 1.74
PH	7.3 ± 0.03
Total dissolved solid (mg/L)	181.4 ± 2.13
Sodium (mg/L)	12.2 ± 0.24
Dissolved oxygen (mg/L)	8.35 ± 0.06
Chlorides (mg/L)	8.93 ± 0.31
Sulphates (mg/L)	38.4 ± 0.21
Potassium (mg/L)	1.6 ± 0.52
Calcium (mg/L)	37.9 ± 0.4
Water temperature (°C)	27.4 ± 0.32
Magnesium (mg/L)	17.8 ± 0.24
Nitrates (mg/L)	0.8 ± 0.01

Analysis of oxidative stress and antioxidant enzymes: Fish were retained and dissected for enzymatic examination at days 14, 21 and 28th of the research trial. Visceral organs (brain and kidneys) were removed, washed, preserved in chilled solution and finally processed for estimation of ROS (Hayashi and Su, 2007), TBARS (Iqbal *et al*., 1996) and GSH (Jollow *et al*., 1974). The levels of some antioxidant enzymes including POD (Chance and Maehly, 1955), SOD (Kakkar *et al*., 1984), and CAT (Chance and Maehly, 1955) were evaluated in brain and kidneys of fish.

Histopathological examination: For histopathological observations, brain and kidneys were removed from the fish at 14, 21 and 28th days of research trial and were immediately fixed in formaldehyde solution. According to earlier research, each experimental fish was anaesthetized individually with clove oil (5.0 mg/L) before sample collection and dissection (Islam *et al*., 2019). Absolute and relative weight of brain and kidneys was computed. The brain and kidneys were immediately collected, washed and fixed in 15% formaldehyde solution. After 3 days of fixation, the collected tissues were dehydrated in ascending grades of alcohol, cleared in xylene, sectioned with automated microtome and stained with Hematoxylin and Eosin (Ghaffar *et al*., 2021). Finally, the prepared sectioned were observed with the help of light microscope (Nikon Eclipse 80i, Nikon Co., and Tokyo, Japan).

Statistical analyses: Data acquired from the brain and kidneys tissues for enzymatic oxidative stress, absolute and relative weight of the brain and kidneys, and antioxidant activity of dried broccoli was analyzed by using the IBM statistical software (SPSS). Tukey's test was used to compare all collected information at a significance level of $p<0.05$.

RESULTS

Absolute and relative weight of tissues: In current study absolute and relative weight of brain was measured in fish (*Tilapia niloticus*) subjected to ZnO NPs and dried broccoli powder (Fig. 1). Data indicated that with the application of ZnO NPs absolute and relative weight of brain significantly ($p<0.05$.) increased with increasing zinc oxide NPs concentrations from 150mg/kg to 450mg/kg in diet while the addition of dried broccoli in diet at low concentrations of ZnO NPs, absolute and relative weight of brain was measured non significantly ($p<0.05$.) as compared to control fish. The absolute and relative weight of kidneys (Fig. 2) significantly ($p<0.05$.) increased in fish received higher concentrations of zinc oxide NPs of groups T2 and T3. The results unveiled that the addition of broccoli showed a non-significantly ($p<0.05$.) alterations in weight of kidneys compared to control.

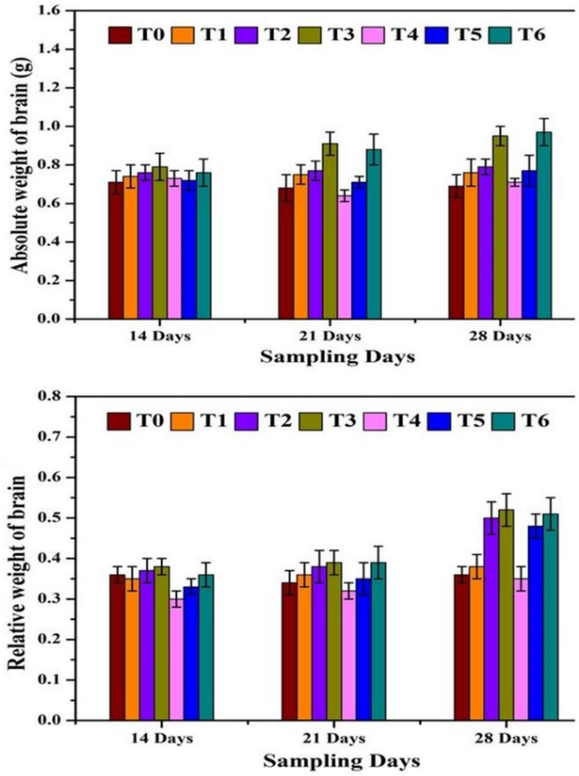

Fig. 1: Photograph unveiling comparison of variation of absolute and relative weight of brain of fish treated with different doses of nanoparticles and broccoli.

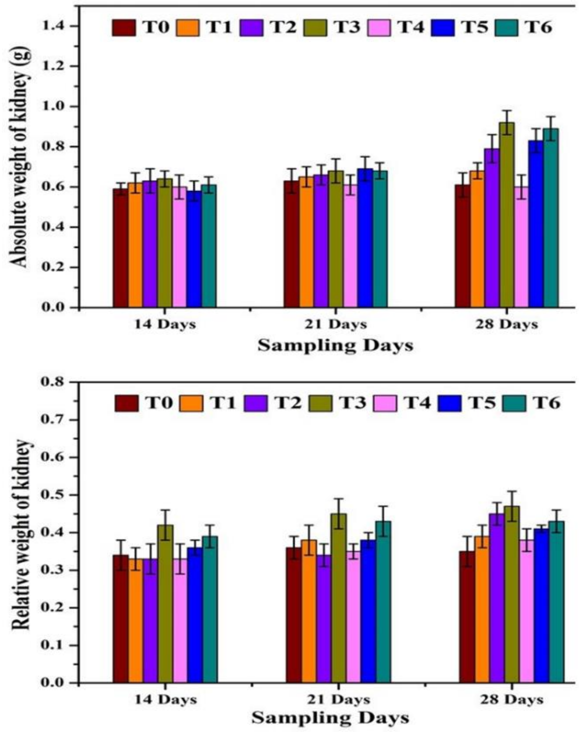

Fig. 2: Photograph unveiling comparison of variation of absolute and relative weight of kidneys of fish treated with different doses of nanoparticles and broccoli.

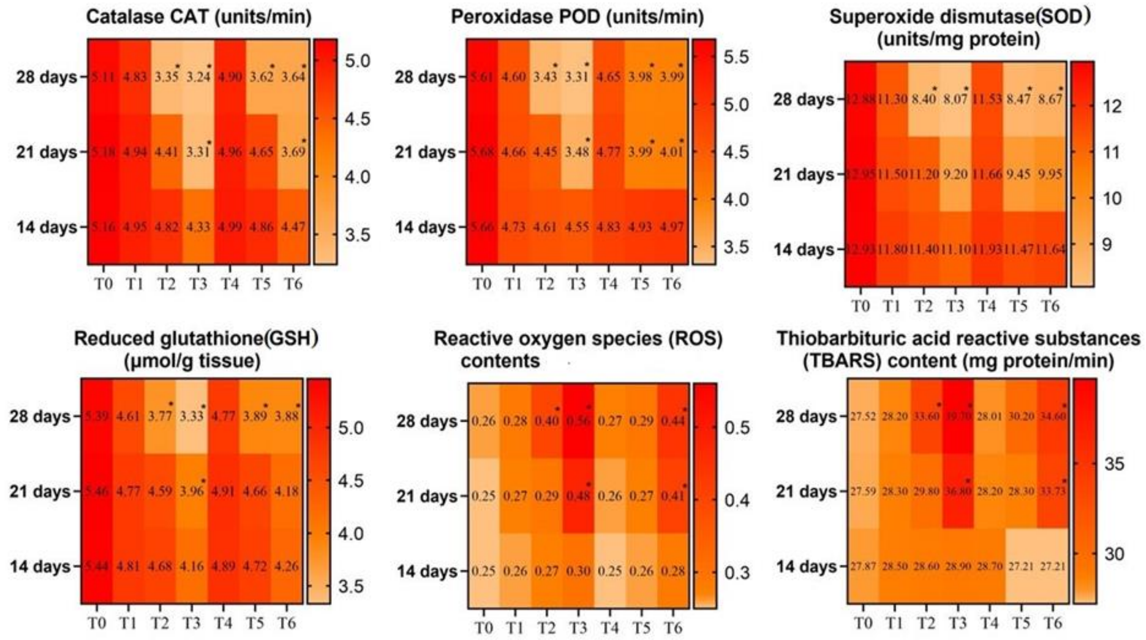

Fig. 3: Photograph unveiling comparison of variation in antioxidant enzymes and oxidative stress profile in kidneys of fish at variable concentrations of nanoparticles and broccoli.

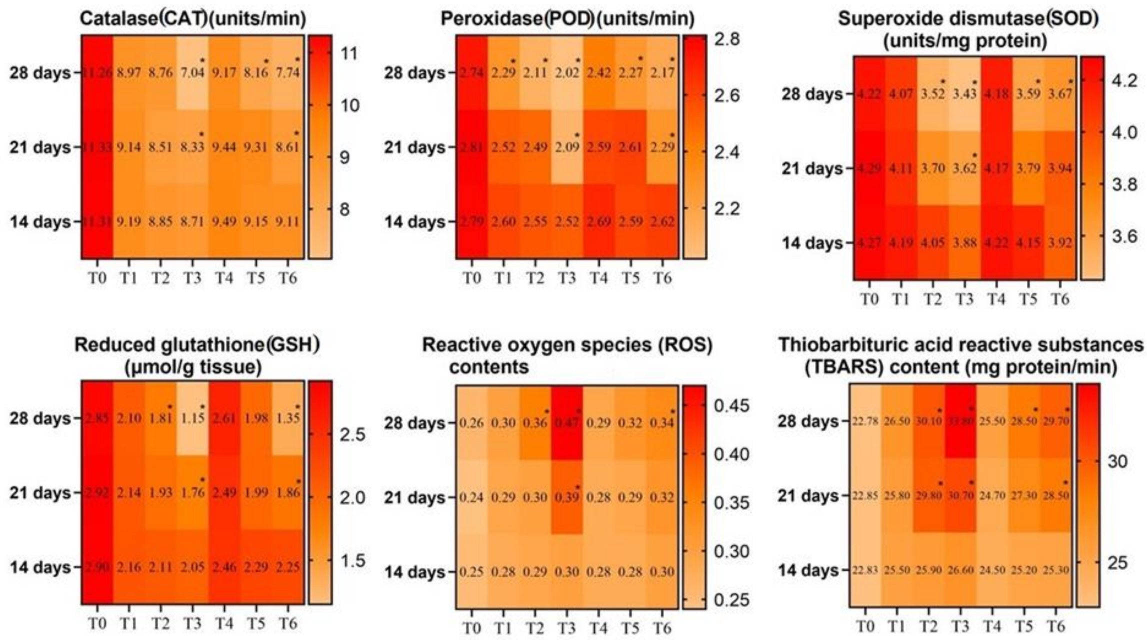

Fig. 4: Photograph unveiling comparison of variation in antioxidant enzymes and oxidative stress in brain of fish at variable concentrations of nanoparticles.

Enzymatic oxidative stress parameters analysis: The results on the quantity of antioxidant enzymes in kidneys and brain of fish (*Tilapia niloticus)* treated with ZnO NPs (150 mg/kg to 450 mg/kg) as well as broccoli (dry powder) revealed that the levels of ROS and TBARS increased significantly while SOD, POD, CAT and reduced glutathione (GSH) in kidneys reduced significantly ($p<0.05$) when compared to control fish. At day 14, the quantity of oxidative stress (ROS and TBARS) and antioxidant enzymes (SOD, POD, CAT and GSH) in fish treated with zinc oxide nanoparticles alone and zinc oxide nanoparticles in diet containing broccoli at 14 days (Fig. 3) was non significantly different from each other and compared to control group. The quantity of ROS and TBARS was significantly escalated in fish of group T3 at day 21th of trial (Fig. 3) and in groups T2-T3 at day 28th (Fig. 3) as compared to control group (T0). The results revealed that the quantity of these biomarkers substantially reduced in fish exposed to diet containing broccoli at days 21 and 28th in a time-dependent manner. Peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in the kidneys and brain (Fig. 4) of fish (*Tilapia niloticus*) subjected to ZnO NP alone was significantly reduced compared to control group (T0). The supplementation of diet containing broccoli considerably reversed the quantity of these biomarkers in fish of groups T4-T6 compared to fish received nanoparticles alone.

Histopathological analysis: Results on severity of histopathological lesions in brain of different fish treated with nanoparticles and broccoli are presented in Table 2. Different mild to severe microscopic lesions in brain (Fig. 5) like inflammatory reactions, edema, atrophy of neuron, microgliosis, vacuolation of neuron, neuronal degeneration, congestion, necrosis of neuron, hypertrophy of nuclei of neuron, hypertrophy of cytoplasm of neuron and atrophied nuclei of neuron were observed at days 21 and 28^{th} of trial. Microscopically, results on severity of histopathological lesions in kidneys of different fish treated with nanoparticles and broccoli are presented in Table 2. Different mild to severe microscopic lesions like atrophy and obliteration of renal tubules, nuclear hypertrophy, edema, ceroid formation, deterioration of glomerulus, widening of Bowman's space, necrosis of tubular cells, melanomacrophage aggregates, and congestion at day 21^{th} and 28^{th} of trial were observed.

Table 2: Histopathological changes in kidneys and brain tissues of treated fish (*Tilapia niloticus*) exposed to variable concentrations of ZnO NPs and dried broccoli.

Histopathological ailments	Treatment groups					
	TI	T2	T3	T4	T5	T6
Brain						
Inflammatory reactions	+	++	+++	+	+	+++
Edema	+	++	+++	+	++	+++
Atrophy of neuron	++	++	+++	++	++	++
Microgliosis	+	++	+++	+	++	+++
Vacuolation of neuron	++	+++	++++	++	+++	+++
Neuronal degeneration	++	++	+++	++	++	++
Congestion	+	++	+++	+	-	++
Necrosis of neuron	+	++	+++	+	-	++
Hypertrophy of nuclei of neuron	++	+++	+++	++	++	++
Hypertrophy of cytoplasm of neuron	++	+++	+++	++	++	++
Atrophid nuclei of neuron	++	+++	++++	++	++	++
Kidneys						
Atrophy and obliteration of renal tubule	+	++	+++	+	++	++
Nuclear hypertrophy	+	++	+++	+	+	++
Edema	++	++	++	+	++	++
Ceroid formation	+	++	+++	+	-	++
Deterioration of glomerulus	++	+	++	+	++	++
Widening of Bowman's space	++	+	++	+	+	++
Necrosis of tubular cells	+	+	+++	+	+	++
Melanomacrophage aggregates	+	++	+++	+	+	++
Congestion	++	++	+++	+	+	+++

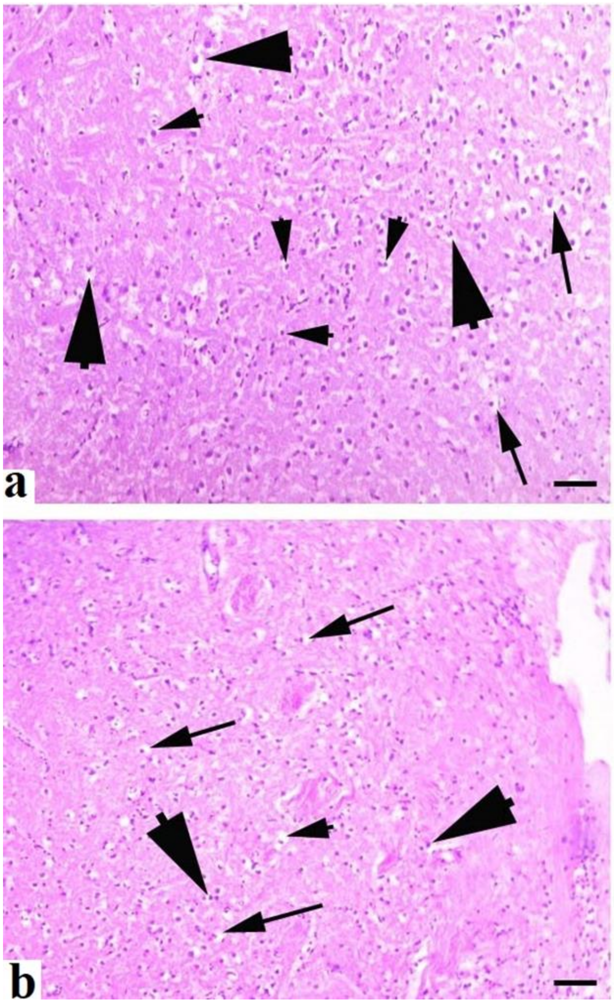

Fig. 5: Photomicrograph of brain of fish treated with higher doses (300 and 450 mg) showing different microscopic lesions a) inflammatory reactions, edema, atrophy of neuron/neuronal degeneration, necrosis of neuron (thin arrow heads), hypertrophy of cytoplasm and eccentric nuclei of neuron (thick arrow heads) and atrophid nuclei (arrows) and b) eccentric nuclei and hypertrophy of cytoplasm (arrows), atrophid nuclei (thin arrow head) and necrosis of neurons (thick arrow heads) at days 28^{th} of trial. H &E stain; 400X.

DISCUSSION

Different reports have indicated that aquaculture is extensively developing and expanding in different parts of world. However, the presence of various xenobiotics in seas, rivers and lakes induce undesirable effects on aquatic animals resulting in damages to multiple tissues including gills (Naz *et al.,* 2023), liver (Naz *et al.,* 2021), kidneys (Mahmood *et al.,* 2021; Naseem *et al.,* 2022), growth retardation (Akram *et al.,* 2022), morphological and nuclear changes in red blood cells (Akram *et al.,* 2021), DNA damage (Akram *et al.,* 2021; Raza *et al.,* 2022) in fresh water fish. Therefore, monitoring of toxic effects of different xenobiotics in aquatic animals is imperative to mitigate the toxicity of such chemicals in target and non-target animals. Therefore, this experimental research describes the physiochemical examination of dried broccoli and its potential of lowering of oxidative stress induced by zinc oxide nanoparticles. The results on physiochemical analysis showed that dried broccoli contains proteins, dietary sugars, carbohydrates antioxidants which are considered as a good reducing agent (Atoui *et al.*, 2005). Antioxidants in broccoli were measured by using the ferric reducing antioxidant power (FRAP) test, the 2, 2- diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, and 3-ethylebenzo thiazoline-6-sulphonic acid (ABTS) radical cation decolonization assays suggested that it contains FRAP, DPPH and ABTS. Similar to our physiochemical analysis, earlier studies have also reported presence of similar bioactive chemicals as well as other compounds including glycosylates, glucoraphanin and polyphenolic (Thomas *et al.*, 2018; Drabińska *et al.*, 2018). It is recorded that broccoli contains DPPH radical scavenging activity in its floret (Guo *et al.*, 2001) and is used to control the electron transfer ability in an acidic environment (Wojdyło *et al.*, 2007). In this research, absolute and relative weight of brain and kidneys were significantly ($p<0.05.$) high in fish received higher doses of zinc oxide NPs in diet alone while addition of dried broccoli in diet at low concentrations of ZnO NPs considerably reduced the absolute and relative weight of brain as compared to fish received nanoparticles alone in fish (*Tilapia niloticus*) at

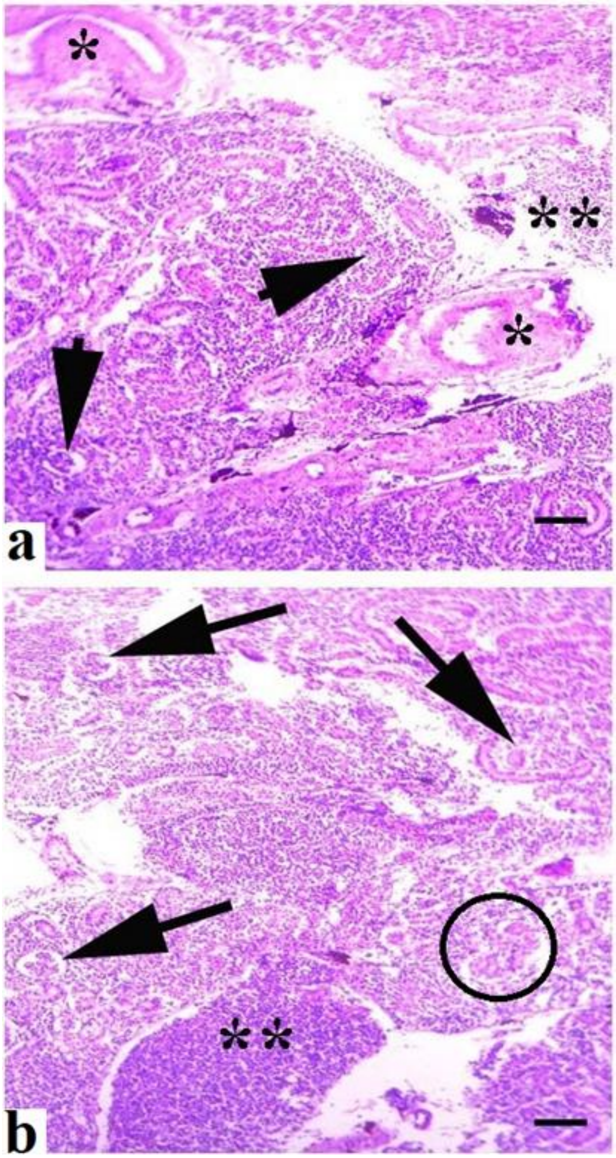

Fig. 6: Photomicrograph of kidneys of fish treated with higher doses (300 and 450 mg) of nanoparticles showing severe microscopic lesions a) edema (*), inflammatory cells (**), atrophy and obliteration of renal tubule (arrow heads) and b), inflammatory exudate (**), widening of Bowman's space (arrows), necrosis of tubular cells and deterioration (circle) and disorganization of renal tubules at day 28 of trial. H & E stain; 400X.

14, 21 and 28th days of research trial. The accumulation of inflammatory exudates as a result of injury due to oxidative stress caused by ZnO NPs could be the possible reason of increase in both the relative and absolute weight of various visceral tissues. Previously, increased relative and absolute weight of visceral organs in fish and rats exposed to several toxins causing inflammation (Cervantes-Camacho *et al.,* 2020; Namratha *et al.,* 2022) has been recorded.

In our investigation, the results revealed significantly increased quantity of ROS and TBARS in treated fish exposed to higher concentrations of ZnO NPs. The escalated values of ROS and TBARS in different visceral tissues might be due to induction of oxidative stress via higher generation of free radicals. Moreover, the supplementation of broccoli in diet noticeably reduced the oxidative stress biomarkers and increased the level of different antioxidant enzymes suggesting presence of antioxidant potentials. ROS and TBARS are well known and reliable indicators of inflammatory response and are frequently used to determine the extent of tissue damage (Akram *et al*., 2021). The results of this study indicated noticeably reversal of oxidative stress biomarkers when broccoli was included in diet of fish. The possible cause of reversal of these parameters could be due to presence of carotenoids (lutein and beta carotene) antioxidants in broccoli which protect cells from injury caused by free radicals (Hwang and Lim, 2014). In current investigation, the brain and kidneys of fish exposed to ZnO NPs showed noticeably higher levels of enzymatic antioxidant parameters in fish treated with zinc oxide nanoparticles along with broccoli. The quantity of CAT, SOD, and POD were substantially improved in fish treated with broccoli in kidneys and brain. The possibility these changes might be related to presence of DPPH radical scavenging activity in broccoli (Guo *et al*., 2001). It is recorded that GSH plays a critical part in the non-enzymatic antioxidant system by acting as a reductant during the conjugation of xenobiotics (Bhutta *et al.,* 2022). The induction of oxidative stress and depletion of antioxidant enzymes in brain and kidneys of fish in current study could be related to physicochemical and structural features of zin oxide NP (Makhdoumi *et al*., 2020). Earlier studies have highlighted that the increased oxidative stress, depletion of antioxidant enzymes and histopathological ailments in treated animals mainly occurred due to increased generation of free radicals induced by NPs (Manke *et al*., 2013). Furthermore, several acellular factors like composition of NPs, size, particle surface, and metals and cellular factors including mechanism of mitochondrial respiration, interaction of nanoparticles with cells, immune response and activation of associated molecular events and cell signaling pathways (Manke *et al*., 2013; Makhdoumi *et al*., 2020).

Different histological changes like edema, atrophy of the neuron, micro-gliosis, vacuolation of the neuron, neuronal degeneration, congestion, necrosis of the neuron, hypertrophy of the nuclei of the neuron, hypertrophy of the cytoplasm of the neuron, and atrophied nuclei of the neuron. Numerous other studies have also observed similar histopathological changes in brain of *Catla. catla* (Bose *et al.,* 2013), *Oreochromus niloticus* (Ayoola and Ajani 2008), *Oreochromus mossambicus* (Gobi *et al.,* 2018; Murali *et al.,* 2018) and other fishes caused by various harmful contaminants (Gobi *et al.,* 2018; Murali *et al.,* 2018). Various mild to severe microscopic lesions such as nuclear hypertrophy, edema, ceroid formation, deterioration of the glomerulus, widening of Bowman's space, necrosis of tubular cells, and congestion in the kidney tissues might be due to induction of process of lipid peroxidation leading to generation of ROS and TBARS (Ghazanfar *et al.,* 2018; Kiran *et al.,* 2022) causing abnormalities in cellular membranes. Previously, severe microscopic abnormalities in tilapia, deterioration of the glomerulus, atrophy and obliteration of the renal tubule in *C. catla* (Faheem *et al*., 2019) and H. fossilis (Pal and Reddy 2018; Vasu *et al.,* 2019) have been observed. The severity of histopathological abnormalities was remarkably reduced in brain and kidneys of fish given broccoli. The decrease in histopathological changes in brain and kidneys of fish might be due to reversal of antioxidants and increased cytochrome P450 (beneficial for detoxification) in treated fish (Villa-Cruz *et al*., 2009).

Conclusions: On the basis of phytochemical examination, it is suggested that broccoli contains important contents including carbohydrates, minerals and antioxidants compounds. Therefore, it is recommended that broccoli should be used in aquaculture as a natural antioxidant to mitigate the toxic effects such as induction of oxidative stress by zinc oxide nanoparticles.

Authors contribution: RI planed the experiment. SRK and KM executed the research. SRK, RI and RH collected the data and were involved in analysis. RH and MA involved in interpretation. SRK, RH, NN and SSN prepared the final manuscript.

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