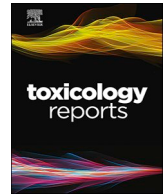




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Chitosan from crustacean shell waste and its protective role against lead toxicity in *Oreochromis mossambicus*

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ABSTRACT

In the current study heavy metal removing capability and antioxidant properties of chitosan supplemented diet tested in lead poisoning induced *Oreochromis mossambicus* in comparison with and standard fish diet. *O. mossambicus* fishes weighed (20 ± 2 gm) were purchased from a local commercial fish pond and acclimated to the laboratory conditions for 10 days. After that fish were divided into four groups, each group received respective feed throughout the experimental period. The fish fed with standard diet exhibited drastic weakening of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GR), glutathione peroxidase (GPx), ascorbic acid, α -tocopherol and β -carotene and also displayed abnormal histological changes in gills, liver, and intestine. The fish fed with a chitosan supplemented diet for 10 days showed substantial enhancements in antioxidant levels and also normal histological structures of organs in the fish.

1. Introduction

Lead (Pb) is a significant intimidation in an amphibious ecosystem because of its harmful nature, aggregation and bio-magnification in the food chain [1]. The primary sources of Pb are construction works, batteries, firearms, industrial wastes, vehicle pollutant emission, mining, herbicides, household wastes and agricultural wastes [2–7]. Pb concentrates in fish through water or feeds and at high concentrations, it turns into toxic.

Chitosan is a derivative of deacetylated chitin [8,9]. Chitosan is nontoxic, biodegradable and bioactive [10]. It is employed in food production because of its antimicrobial and antioxidant actions and film-forming capability [11]. Chitosan has a good effect on fish growth, immunostimulatory and antioxidants [12].

Although heavy metal removing capability of a chitosan is known, Sun and Wang [13] studied the absorption properties of N-succinyl chitosan with Pb(II) ions. There is no precise information available on the effect of an isolated chitosan from crustacean shell wastes on removal of lead toxicity from live fish model. Based on the above literature, in the current study, chitosan feed are subjected to analysis of heavy metal removal and health benefits of chitosan feed. The aqueous solution of Pb salts were subjected to heavy metal removal in fish intestine via live fish *O. mossambicus*.

The design of experiments was conducted with heavy metal removal efficiency and health benefits of chitosan feed. The analysis of variance

(ANOVA) was carried out to validate the heavy metal removal efficiency and health benefits of chitosan feed. Inductively Coupled Plasma Optical Emission Spectrometer, Superoxide Dismutase, Catalase, Reduced Glutathione, Glutathione Peroxidase, Ascorbic acid, α -Tocopherol, β -Carotene and histopathological analysis were used to characterize the properties of chitosan feed.

2. Materials and methods

2.1. Sample collection

We gathered crustacean shells from resident landing centre, Parangipettai, Tamil Nadu, India. Shells washed with tap water and dehydrated in a hot air oven at 100 °C and all the shell wastes were crumbling as thin particles and saved in the refrigerator (4 °C) until further work.

2.2. Fish and experimental conditions

O. mossambicus fishes weighed (20 ± 2 gm) were purchased from local commercial fish pond from Kurinjipadi (11.5642 °N, 79.5960 °E), Tamil Nadu, India. Fishes were acclimatized in 250 L aquarium tanks in water supplied from the UV sterilized and de-chlorinated water reservoir (temperature of 26 ± 2 °C, pH 7.6 ± 0.5 , dissolved oxygen 7.0 mg/L). During acclimatization, fish were fed with standard fish feed [14].

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2.3. Chitosan preparation

We prepared chitosan from 2gm of fine crustacean shell powder following the protocols of Mohanasrinivasan et al. [15]. The powder was demineralized by treating with 100 ml of 4 M hydrochloric acid (HCl) for 1 h at 75 °C. After the incubation, samples were washed with double-distilled water for several times to ensure the removal of minerals. In the next step, we subjected samples to deproteinization, by exposing the sample to 50 ml 2 M sodium hydroxide (NaOH) at 100 °C for 18 h. After the process, all the samples were washed with distilled water. Further, the samples were decolorized with the mixture of chloroform, methanol and distilled water in a 1:2:4 ratio and again the samples were washed with distilled water, dried in the oven at 60 °C for 24 h. For chitosan preparation, the sample was treated with 1:1 (W/W) NaOH at 80 °C for 2 h. After the NaOH was drained off and washed with distilled water, the prepared chitosan was dried at room temperature and stored in the refrigerator until further use.

2.4. Chitosan feed preparation

The chitosan supply was prepared according to Zaki et al. [16]. The prepared chitosan feed contained 51.7 % crude protein, 8.8 % crude lipid, 7.2 % crude ash, and 14.1 % crude carbohydrate.

2.5. Determination of lethal concentration (LC₅₀) of Pb

The fish was exposed for 96 h at different concentrations of Pb, maintaining water quality at temperature 26 ± 2 °C, dissolved oxygen (DO) 7.25–0.4 mg/L and pH 7.65 ± 0.5 in the experimental vessels with triplicates. Fish mortality was observed daily. Six fish from each experimental tank were sampled at 24, 48, 72, and 96 h of disclosure. LC₅₀ value for 96 h was discovered at 12.08 mg/L [17].

2.6. Experimental setup

Fish were turned over to a glass tank of 50 L capacity equipped with good aeration. Fish were subdivided into four groups (each group consists of 20 fishes). Group-1 served as control, Group-2 where provided with chitin supplementary diet (CSD), Group 3 exposed to lead and fed with CSD, and Group 4 exposed with lead and fed with standard fish diet (SPF). For the initial four days of the experiment, all the fishes were fed with standard fish diet, after four days fishes were fed with experimental diets. Pb(NO₃)₂ with 99.5 % purity was dissolved in double-distilled water. The test concentration was replaced with fresh solution for every 24-hrs interval until 96 h of exposure to sustain proper toxicity [2].

2.7. Heavy metal accumulation analysis

Gills, liver, intestine and muscle tissues collected from fish after the experiment were dried at 60 °C, and determined for Pb concentration.

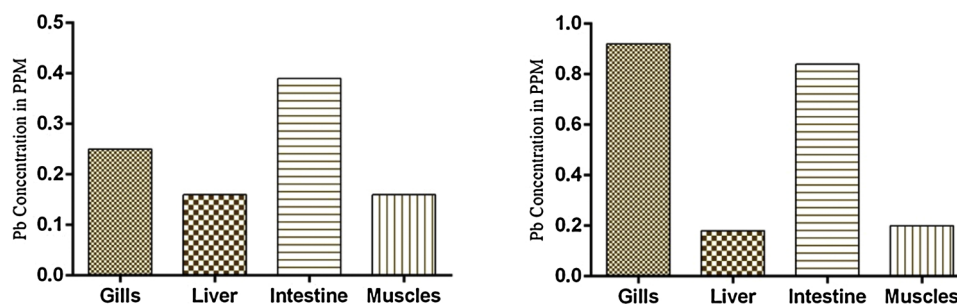


Fig. 1. Graphical representation of Pb accumulation in gills, liver, muscles and intestine of lead toxicity induced fishes and chitosan treated group and lead toxicity induced group chitosan non-treated fishes.

Samples were digested with concentrated nitric acid (HNO₃) and HCl (4:1) for 24 h. The samples were subjected to complete dryness by placing them on a hotplate. The ash obtained was made up to a 20 ml solution using 20 % HNO₃. The sample mixture was filtered through Whatman filter paper and again the Pb concentration was determined by using Inductively Coupled Plasma Optical Emission Spectrometer (ICP–OES) (Software–winlab 32; Perkin Elmer, Optima 2100DV) at the wavelength of 220.353.

2.8. Antioxidant analysis

All the experimental fish organs such as gills, liver, intestine and muscles were analyzed for antioxidants such as superoxide dismutase [18], catalase [19], reduced glutathione [20], glutathione peroxidase [21], ascorbic acid [22], α -tocopherol [23] and β -carotene [24] following the test protocols.

2.9. Histopathological study

Histological evaluation was conducted on all four groups; the fish were anaesthetized in ice-cold water and sacrificed. Tissues like gills, muscles, liver and kidney were fixed in 10 % buffered formalin. After 24 h of fixation, samples were dehydrated in a series of ethanol, cleared in xylene, and finally embedded in paraffin wax. Block of tissue has been cut into 5 μ m thick sections and stained with hematoxylin and eosin (H and E). Stained tissues were mounted with DPX and allowed it for air drying. Later tissues were examined with a light microscope for changes due to metal exposure [25].

2.10. Statistical evaluation

The data were subjected to statistical analysis using SPSS-15 the values are given as mean ± SD and significance (*P < 0.05, **P < 0.01, ***P < 0.001) between the groups was analyzed by one way ANOVA.

3. Results

3.1. Lead accumulation in *O. mossambicus*

Accumulation of Pb in gills, liver, intestine and muscle tissues of *O. mossambicus* is shown in the Fig. 1. Although the Pb accumulation was recorded in all the tissues of fish induced with Pb toxicity, the accumulation in excess was noted in fish fed with standard diet (Fig. 1A). However, the Pb accumulation was low in the fish fed with a chitosan supplemented diet (Fig. 1B). The order of Pb accumulation in different tissues in groups 3 and 4 experimental fish was gills > intestine > liver > muscles. Among the tissues, gills seemed to be efficient in accumulation.

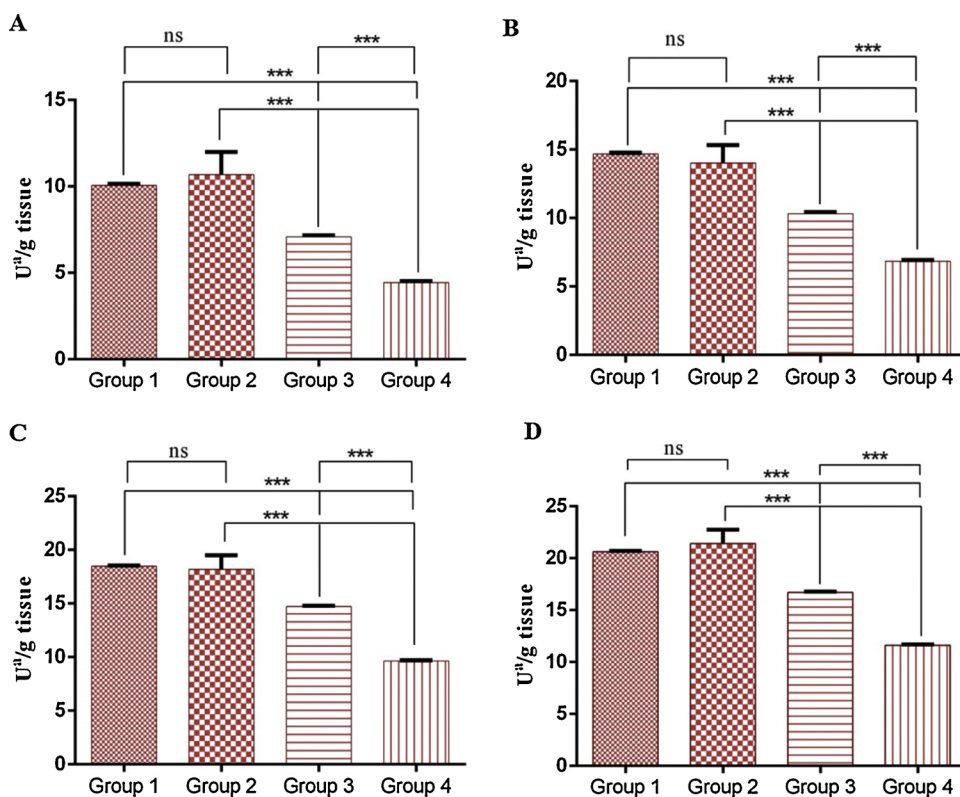


Fig. 2. Chitosan shows a protective role by modifying lead toxicity induced oxidative stress in *O. mossambicus*. Antioxidant activity of chitosan on lead toxicity induced oxidative stress was assessed by antioxidants levels of (A) Superoxide dismutase (SOD) one unit of enzyme activity which results in 50 % inhibition of nitro blue tetrazolium reduction/minute of SOD activity in gills, liver, intestine and muscles of control and experimental groups. Fishes were exposed to lead toxicity (25 % of LC_{50}) for 4 days and 1 % chitosan supplemented feed treated and non-treated effect on these parameters were analyzed. Values were represented as mean \pm SEM. Statistical significance was performed by one way ANOVA followed by turkeys multiple comparison method. Values are statistically significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns.

3.2. Oxidative stress

3.2.1. Superoxide dismutase (SOD)

SOD is the group of enzymes playing an important role in defense against ROS production, and it is the first level of defense mechanism against ROS as well is an important biomarker for toxic chemicals and heavy metals. In the present study, we assessed the effect of chitosan on Pb toxicity induced in *O. mossambicus*. The enzymatic activity of SOD significantly reduced (** $P > 0.001$) in the fish fed with standard diet (Fig. 2D) when compared with other experimental groups 1, 2 and 3. However, there was an increase in the level of SOD in the fish fed with chitin diet (Fig. 2C), similar to the fish non-exposed to Pb toxicity and fed with chitosan diet (Fig. 2B).

3.2.2. Catalase (CAT)

CAT is an important antioxidant scavenging H_2O_2 . The reduced activity CAT leads to hyper production of hydroxide free radicals. Although there was no change in CAT in control (Fig. 3A), the fish exposed with lead for 96 h and fed with standard diet showed a significant level of reduction in CAT levels (Fig. 3D) when compared with control and chitosan control group (Fig. 3A and B). However, CAT level was significantly increased (** $P > 0.001$) in the fish fed with the chitosan diet (Fig. 3C). However, there was no significant change in CAT in the chitosan alone treated group (Fig. 3B).

3.2.3. Reduced glutathione (GR) and Glutathione peroxidase (GPx)

After 96 h of exposure, GR and GPx enzymatic activity was responded just like as other antioxidants, fish induced with Pb toxicity and fed with standard diet exhibited significantly (** $P > 0.001$) reduced activities of GR and GPx when compared with the experimental groups 1 and 2 (Figs. 4 and 5 D). However, there was no significant change in the activities of GR and GPx in chitosan alone treated group (Figs. 4 and 5B). Interestingly the activities of GR and GPx increased in the fish exposed to Pb and fed with chitosan diet (Figs. 4 and 5C).

3.2.4. Vitamin A, C, and E

Levels of Vitamin A, C and E in gills, liver, intestine and muscles were significantly lower (** $P > 0.001$) in the fish exposed to Pb and fed with standard diet (Figs. 6–8 D). In contrast, the levels of vitamins were higher in all the tissues of the fish fed with chitosan (Figs. 6–8C). There was no significant change in the control and chitosan supplement diet alone feed fish (Figs. 6–8A and B).

3.3. Histological changes

3.3.1. Gills

Light microscopic (10X and 40X) analysis of gills showed the arrangements of primary and secondary lamellar structures. Gill tissues from the control and chitosan control exhibited normal architecture. Hypertrophy of mucous cells and hyperplasia of the epithelial cells (Fig. 9 D and H) were observed in the gills tissues of fish that were exposed to Pb and fed with standard diet for 10 days. Pb exposed fish gills were found to have severe lamellar fusions, shortening and curling of secondary lamellae. Primary lamellas were severely damaged by the presence of aneurysms (Fig. 9H). Many lamellae were severely damaged by the presence of oedema. Interestingly chitosan fed fish after Pb exposure showed a reduction in the number of hypertrophy of mucous cells and hyperplasia characters (Fig. 9C and G). Severe damages like aneurysms and oedema were not found in the fish gill, fed alone with chitosan diet, which was similar to control group.

3.3.2. Liver

Liver tissue was observed normal in the control and chitosan control groups of fish. The fish exposed to Pb and fed with standard diet showed several changes in liver, specifically hepatocellular degenerations, vacuolated areas, necrosis (Fig. 10 D and H), hemorrhage and increased sinusoidal space. But the fishes exposed to Pb and treated with chitosan diet minimise the effects of Pb toxicity and improved the cellular changes like hypertrophy, vacuole formations and cellular degenerations (Fig. 10C and G).

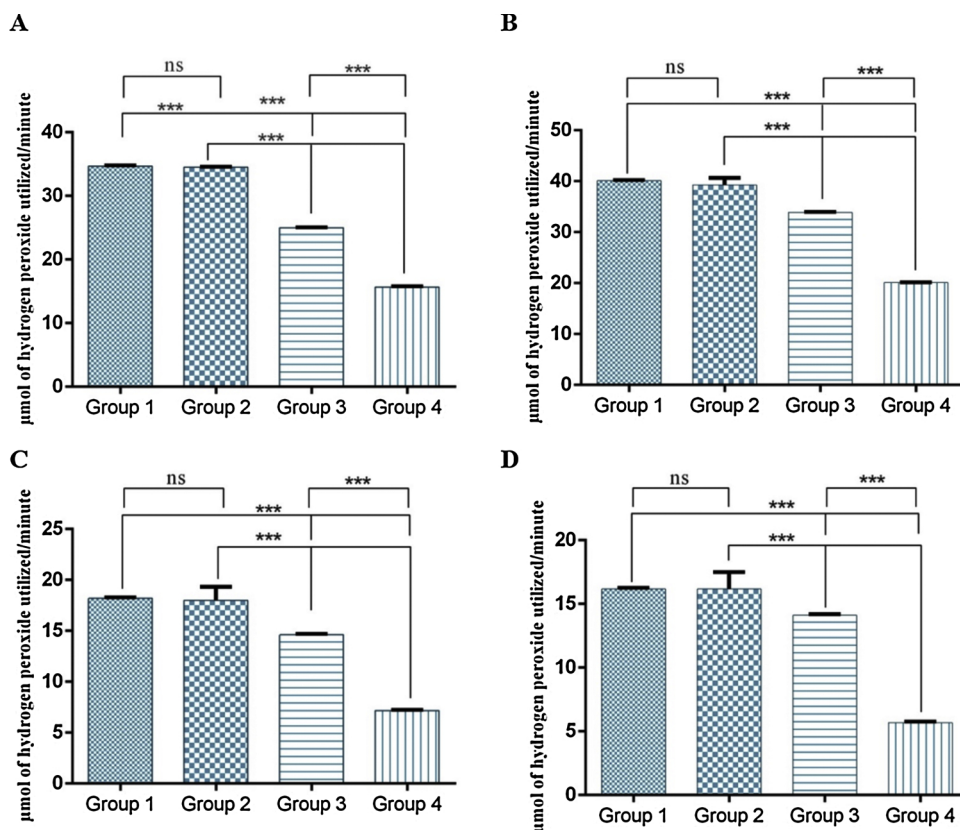


Fig. 3. Protective role of a chitosan against lead induced antioxidant stress was analyzed in gills, liver, intestine and muscles of the control and experimental groups. Catalases (CAT) were assessed as μmol of hydrogen peroxide utilized/minute of catalase activity in gills, liver, intestine and muscles. Statistical significance was performed by one way ANOVA followed by turkeys multiple comparison method and values are statistically significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns.

3.3.3. Intestine

The fish exposed to 25 % of 96 h LC_{50} concentration of Pb showed damage to intestinal tissues. The fish exposed to Pb and fed with a standard diet exhibited a higher number of degenerated nucleus and apoptotic cells, swelling, and crack appearances in the tissues (Fig. 11 D and H). However, the fish fed with a chitosan diet displayed reduction in vacuolated cells and swelling. On the other hand, there was no change in chitosan alone fed fish and control fish.

3.3.4. Muscles

Light microscopic examination of the fish showed a well-organized muscle bundle, and Pb did not exhibit any disarrangement in muscle fibers.

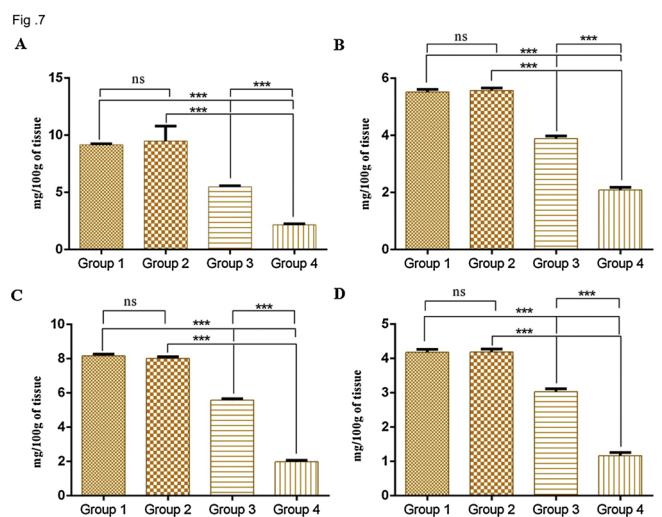
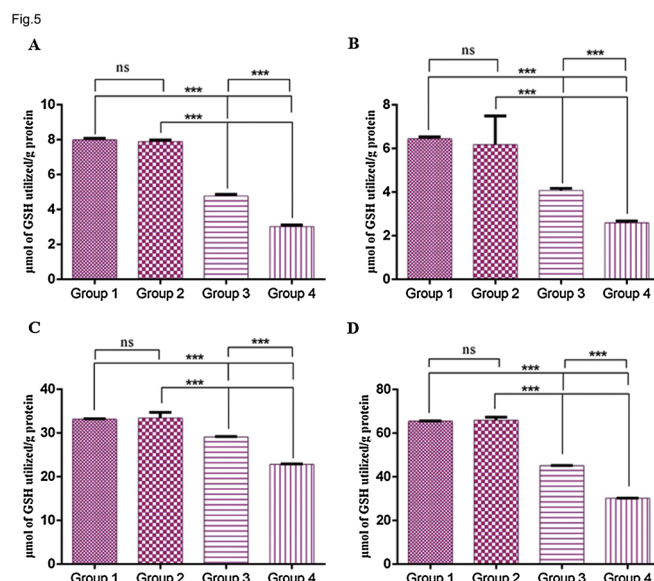
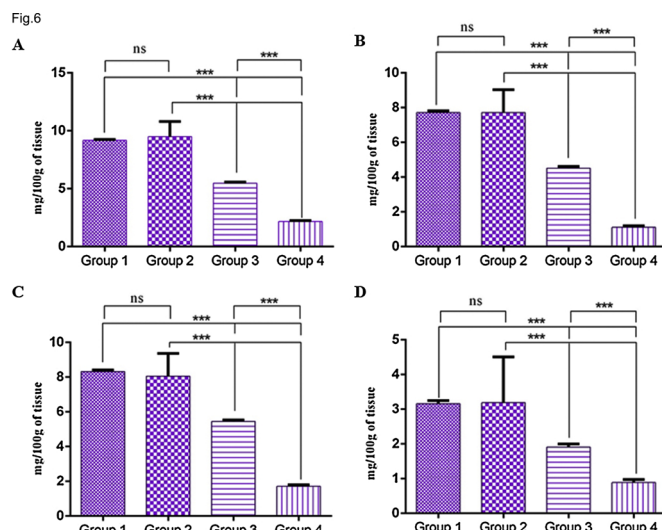
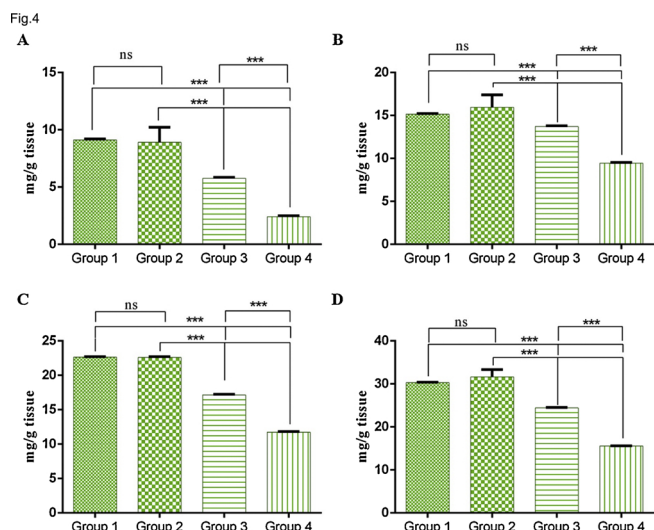
4. Discussion

Industrialization and urbanization have released heavy metals in high concentrations to an aquatic environments. Among the heavy metals, Pb is one of the important metals poses a serious problem of toxicity in higher concentrations. For the past two decades accumulation of Pb in the aquatic environment is getting higher [26]. Exposure of Pb to the fish results in accumulation of Pb in various organs such as gills, liver, intestine and muscles [27]. Accumulation triggers free radicals, specifically ROS. These highly reactive molecules can damage biological macromolecules and induce morphological changes as well as loss of antioxidants in fish [28]. Chitosan is the second most abundant biopolymer in nature, and it is a common component in crustaceans, insects and some microorganisms. Previous studies have reported that a chitosan supplemented diet increases many biological activities such as macrophage activity, antioxidant, cellular responses [29]. The main functional groups of chitosan which are which are potential points for adsorption of metal ions are $-\text{OH}$ and $\text{N}-\text{H}_2$. These

influences the process forming of complex between heavy metal and functional group [30].

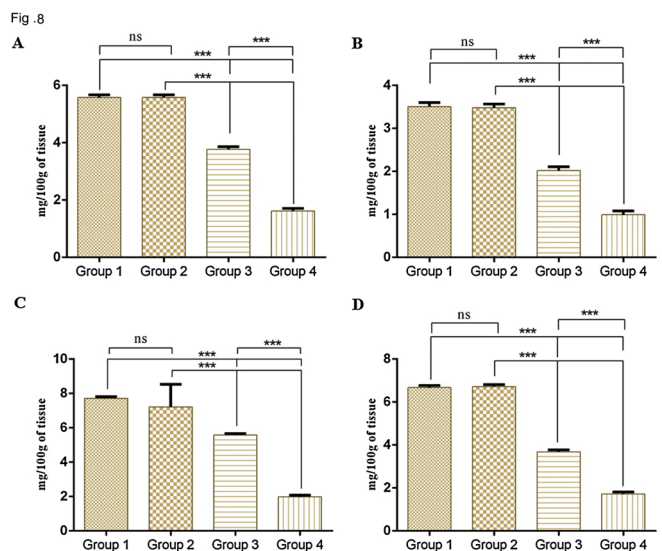
Numerous studies have demonstrated that antioxidant activity of fish plays a vital role in the protection of the organism against free radicals formed by the natural metabolic process and external chemical stimulus [31]. Degeneration of the antioxidant system might contribute to protein oxidation, lipid peroxidation and nucleic acid oxidation and ultimately lead to cell death [32,33]. Usually, heavy metals such as Pb, Hg and Cd have had a high affinity for the sulfhydryl ($-\text{SH}$) groups presents in the enzyme complexes [33,34]. Additionally, vitamins are the important organic compound necessary for normal growth and healthy immune functions, Studies have found that vitamins in the fish diet help to increase the immune responses [13]. Vitamins help to improve the antioxidant system in the fish system to prevent the damages caused by ROS and also to prevent damages to vital biological molecules in the cells [11]. The present study proved that fish antioxidants were severely spoiled due to exposure of Pb, a decrease in levels of antioxidants and histological changes in gills, liver, and intestine, but not in muscles due to low accumulation of Pb in muscle tissues.

The control fish in the present study exhibited normal ranges of antioxidants in all tissues. Pb has a high affinity with antioxidants, and hence there was a reduction of antioxidants and correlated with the degeneration of tissues exposed to Pb. Light microscopic examination of the tissues revealed an extensive cell change due to Pb toxicity in the fish Pb causes morphological changes in the way of dilation of blood vessels, necrosis, aneurysms and detachment in the fish [17]. Fishes treated with Pb displayed a significant level of a decrease in the levels of SOD, CAT, GR and GPx and Vit A, C and E in the gills, liver, and intestine. Pb intoxicated fish fed with a chitosan diet significantly protected gills, liver, and intestine. There were no significant changes in morphological and antioxidants in chitosan alone fed fish, which



Figs. 4 and 5. Effect of chitosan supplemented feed on heavy metal toxicity induced stress in *O. mossambicus*. Chitosan shows an improving role in lead toxicity induced fishes. Enzymatic antioxidant activity was assessed by (A) Glutathione Reductase (GR) (mg/g tissue) and (B) Glutathione peroxidase (GPx) (μmol of GSH utilized/g protein) in gills, liver, intestine and muscles. Statistical significance was performed by one way ANOVA by turkey's method, values were significant at *P < 0.05; **P < 0.01; ***P < 0.001; ns.

indicated a nontoxic nature of the chitosan for fish [35]. This was also evident by non-significant level of changes in SOD, CAT, GR, GPx and vitamins A, C and E. The activation of SOD, CAT, GR and GPx is the first defense mechanism through apoptosis against ROS produced by Pb exposure. The fish exposed to Pb and fed with chitosan exhibit increased levels of antioxidants and prevent gill, liver and intestine damage from apoptosis. Chitin and chitosan are the natural polymers [14] capable of nullifying the toxic effect of Pb, but the exact mechanism is not clearly understood. It may be due to the formation of a complex of a chitosan with Pb. The chitosan forms a complex and eliminates cross linkage between OH⁻ or Amino chains combined with metals and forms water-insoluble linkages. Another possibility is that chitosan acts as an insoluble support for the metals and forms a hybrid or the composite materials [36]. According to Ngo and Kim [37], chitosan has an antioxidant activity but less than ascorbic acid, and it increases antioxidant activity such as SOD, CAT, and GPx. Previous studies have also reported that water-soluble chitosan derivatives have a free radical



Figs. 6–8. Effect of chitosan on vitamins in heavy metal toxicity induced fishes. Effect of lead heavy metal stress on Vit-A, C and E and protective role chitosan were analyzed on *O. mossambicus* was analyzed in gills, liver, intestine and muscles. (A) Vit-A, (C) Vit - E (mg/100 g of tissue) was analyzed. Statistical significance was performed by one way ANOVA by turkey's method, values were significant at *P < 0.05; **P < 0.01; ***P < 0.001; ns.

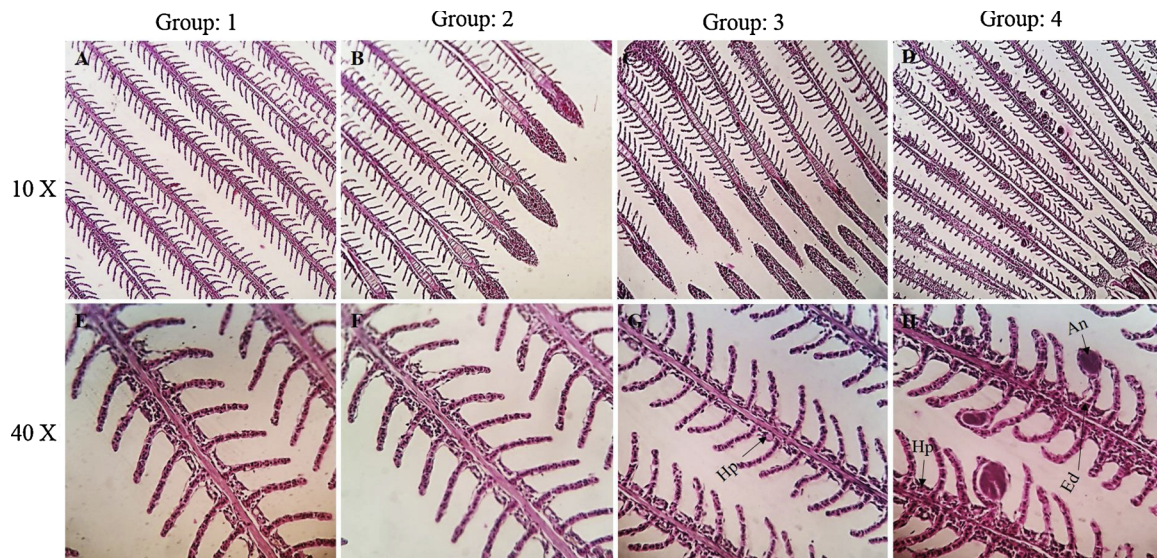


Fig. 9. Photograph of H and E stained (10 and 40 X) gills of *O. mossambicus* (H). Hypertrophy of mucous cells, hyperplasia of the epithelial cells and aneurysms. (C and G) Reduced number of hypertrophy of mucous cells and hyperplasia.

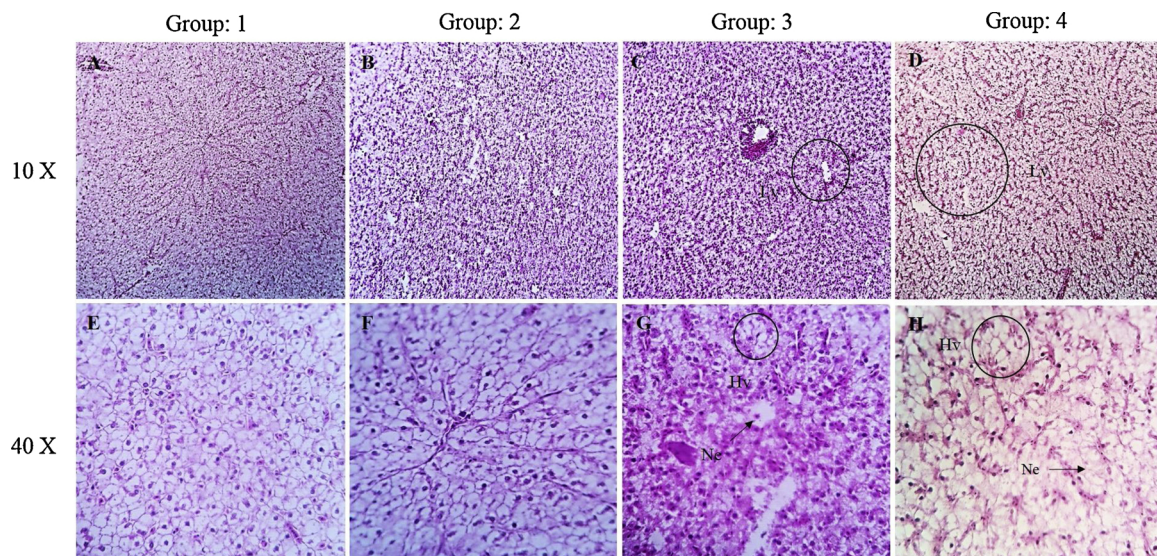


Fig. 10. Photograph of H and E stained (10X and 40X) liver of *O. mossambicus* (D and H) hepatocellular degenerations, vacuolated areas, necrosis. (C and G) Reduced level of hepatocellular degenerations, vacuolated areas and necrosis.

scavenging activity, by converting unstable amino groups into stable forms [38]. The present study found an increased level of antioxidant activities in the fish fed chitosan alone. This indicated that alterations caused by the Pb were connected with suppression of antioxidants and tissue damages due to free radical generation protected the fish against Pb intoxication by increasing the levels of antioxidants.

5. Conclusion

Based on their proved properties, chitosan feed can be a very promising material for heavy metal removal and also improves health of the fish. Aiming to improve their heavy removal and health benefits, fish feed can be modified by cross linking for forming composites. However, the main limitation of the current study is their low solubility

in aqueous medium. The cationic nature of chitosan influences the absorption mechanism of chitosan feed. Although there is a wide range of chitosan derivatives with absorption properties and health benefits, choosing the most suitable type is still in research. This research has a great area for improvement, and based on a large quantity of promising results, it is hops that chitosan feed can be applied commercially instead only at laboratory conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

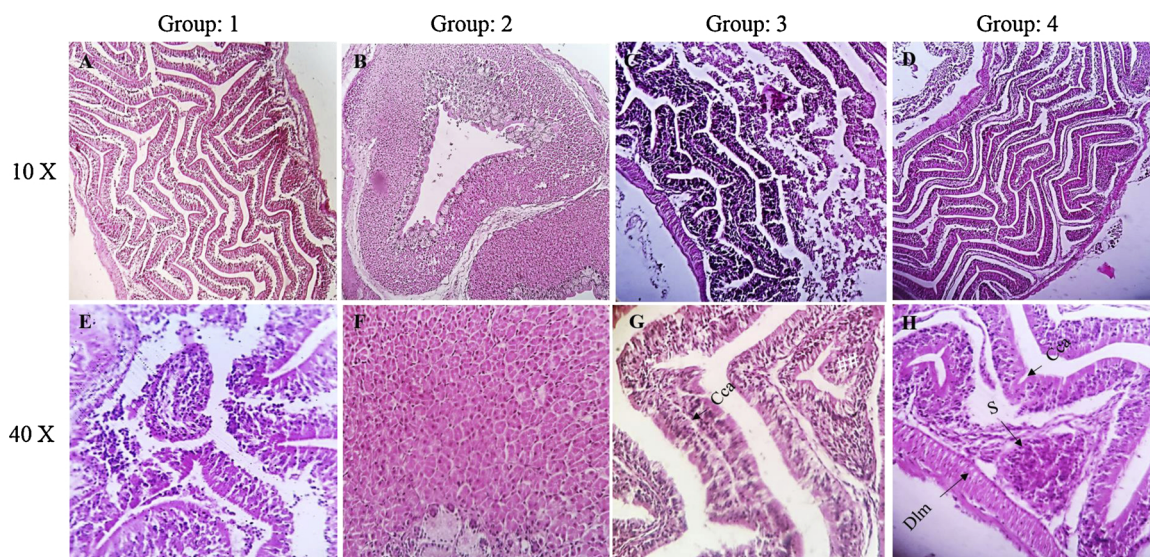


Fig. 11. Photograph of H and E stained (10X and 40 X) intestine of *O. mossambicus* (D and H) degenerated nucleus and apoptotic cells, swelling, crack appearances in intestine.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2020.02.006>.

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