EFFECT OF SUPPLEMENTAL DIETARY L-ASCORBIC ACID (VITAMIN C) ON MERCURY DETOXICATION, PHYSIOLOGICAL ASPECTS AND GROWTH PERFORMANCE OF NILE TILAPIA (*Oreochromis niloticus* L.)

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Abstract

The effect of supplemental dietary L-ascorbic acid (A.A.) on mercury intoxication, some biochemical parameters, growth performance and survival rate of Nile tilapia (*Oreochromis niloticus* L.) was studied. Nile tilapia (25-35 g/fish) was assigned to five treatments, with three replicates each. The first treatment was left as control, the second and the third ones were exposed to ¼ and ½ LC_{50} of mercuric chloride, respectively. The fourth and the fifth ones were exposed to ¼ and ½ LC_{50} of mercuric chloride, respectively, while fish was fed 500 mg L-ascorbic acid/kg diet. Fish were fed frequently a diet of 32% CP at a rate of 3% of live body weight twice daily for 60 days. The obtained results showed significant reduction of total protein in muscle while hepatic total protein was significantly increased in fish exposed to ¼ and ½ LC_{50} of Hg. The activity of aspartate amninotransferase (AST) was significantly increased in muscles of fish exposed to low and high doses of Hg only whereas AST activity in fish exposed to low and high doses of Hg + AA became similar to that of control fish. The activity of alanine aminotransferase (ALT) in fish muscle was the highest in fish exposed to high doses of Hg, while the lowest values were observed in muscle of control fish. AST and ALT activities in liver decreased in fish exposed to sublethal doses of mercury only and recovered with dietary ascorbic acid at low and high doses of Hg. Fish exposed to high dose of Hg produced the lowest final weight. Vitamin C enhanced the growth of fish exposed to low and high dose of Hg to be similar to that of control and that exposed to low dose, respectively. Subsequently, vitamin C enhanced weight gain, SGR and survival rate, while they were decreased significantly with high dose of Hg in comparison to control. Feed intake linearly decreased in fish exposed to high dose of Hg. Contrarily, FCR was linearly increased with fish exposed to high dose of Hg. The best FCR and PER were obtained with control fish and low dose +AA with non-significantly difference (2.9 and 3.0, respectively).

Introduction

The increase in fish culture during the last years has been causing the increase of risk factors concerning fish health. One of these risks is heavy metal pollution. Mercury is one of the high toxic metals and it grouped under European Economic Community's black list pollutants because of its high toxicity, persistence and bioaccumulation in the ecosystem (Moore and Ramamoorth, 1984). Mercurials have found widespread use in many industries as insecticides, fungicides, bactericides and pharmaceuticals. The estimated global mercury discharge is 1300 metric tons each year to water from natural resources (US National Academy of Science, 1977) and Watson (1979) predicted the total discharges of mercury on land through human activity to be 6609 metric tons in the year 2000. Most of the increases in mercury discharge into the environment occur in the less developed regions of the world.

Thus, some researchers have been given emphasis to investigations with data for treatment, which is not only curative but also prophylactic (Verlhac and Gabaudan, 1994), such as the case of use of vitamins on the supplementation of commercial diets. Among these investigations, the use of vitamins as A, D, C and E is paramount, because they are closely related to the performance of fish immune system. The need for these vitamins differ according to the species, age and raising period, and the research in this field is still much limited. Ascorbic acid (vitamin C) is an essential nutrient in aqua-feeds, and is an indispensable nutrient required to maintain the physiological processes of different animals including fishes (Tolbert, 1979). Most of fish, including tilapia are not capable of vitamin C biosynthes is (Chatterjee, 1973) due to the absence of the enzyme L-gulonolactone oxidase, which is responsible for synthesis of ascorbic acid (Wilson, 1973). However, fish depend upon exogenous source of vitamin C. At their natural environment, vitamin C would be present in natural food, but in intensive fish culture its supplementation becoming necessary. Small amount of this vitamin is sufficient to prevent and cure scurvy, however, larger amount may be essential to maintain good health during environmental adversities, situation of physiological stress and conditions of infectious and parasitic diseases (McDowell, 1989; Lim, 1996).

Ascorbic acid (vitamin C) is essential for producing collagen and bone minerals, assists in metabolizing iron and helps in activation of vitamin D. It also assists in reducing the harmful effects of hormones produced by the adrenal gland during prolonged periods of stress (Lovell, 1989; Navarre and Halver, 1989). Also, it has an important role in a great number of biochemical processes such as synthesis of collagen which is an intercellular protein and principal constituent of skin, scales, mucosa, cartilaginous tissues, bones and conjunctive tissue formation, which involves all the organs of the body (McDowell, 1989). Agrawal *et al.* (1978) reported that high levels of ascorbic acid are efficient to enhance tolerance to environmental stressors e. g. aldrin toxicity. Several functions especially immunoactivity and resistance to toxicants and stress are affected, in aquaculture species, by dietary ascorbate deficiency and result in increased fish mortality (Marchie *et al*., 1996). Therefore, the present study was carried out to investigate the effect of high level of dietary ascorbic acid (vitamin C) on intoxication of inorganic mercury and its impact on some biochemical parameters, growth performance and survival rate of Nile tilapia, *O. niloticus* L.

Materials and methods

Fish and culture technique

Healthy fish of Nile tilapia (*Oreochromis niloticus*) weighing 25-35 g/fish were collected from the nursery ponds of Central Laboratory for Aquaculture Research at Abbassa, Abo-Hammad, Sharkia, Egypt. Fish were acclimated in indoor tank for 2 weeks to laboratory conditions. Ten fish were frozen at -20^{o}C for chemical analysis. The fish were distributed randomly in glass aquaria of 120-liter capacity each at a rate of 10 fish/aquarium that containing aerated water. Each aquarium was supplied with compressed air via air-stones from air pumps. Well-aerated water supply was provided from a storage fiberglass tank. The temperature was adjusted to 27^{o}C by using thermostats.

Chemicals used were mercuric chloride ($HgCl_2$) produced by El-Nasr Co. for Chemicals, Egypt, as sources for Hg^{2+} and L-ascorbic acid equivalents in the form of ascorbyl monophosphate magnesium salt (1 mg of ascorbic acid is equivalent to approximately 2.4 mg of ascorbyl monophosphate magnesium salt (Sigma, St. Louis, Mo, USA). Dietary ascorbic acid level throughout this study are given as added level only i.e. 500 mg/kg diet.

The 96-hr LC_{50} for mercury chloride was determined by Behreus and Karber (1953) and it was 0.5 mg/l. Fish was exposed to ¼ and ½ LC_{50} of $HgCl_2$ (0.125 and 0.250 mg/l, respecti) for 60 days. The fish were assigned to five treatments with three replicates each. The first treatment was left as control, the second and the third ones were exposed to ¼ and ½ LC_{50} of Hg Cl_2, respectively. The fourth and the fifth ones were exposed to ¼ and ½ LC_{50} of Hg Cl_2, res pectively, while fish was feeding 500mg L-ascorbic acid/kg diet. Fish were fed frequently a diet containing 32% CP at a rate of 3% of live body weight twice daily for 60 days. After application of the toxicant, siphoning a portion of water from each aquarium was doneevery 3 days for excreremoval and an equal volume of water containing the same concentrations of toxicants replaced it. Fish in each aquarium was biweekly weighed and subsequently the amount of given feed was calculated. Dead fish were removed and recorded daily.

Proximate analysis of diet and fish

The basal diet and samples of 12 fish from each treatment were analyzed using standard methods of the Association of Official Analytical Chemists (AOAC, 1990) for determination of moisture, crude protein, total lipids and ash.

Growth parameters

Growth performance was determined and feed utilization was calculated as follows:

$$\text{Specific growth rate (SGR)} = 100\ (\ln W_2 - \ln W_1)\ T^{-1}$$

where W_1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period.

$$\text{Feed conversion ratio (FCR)} = FI\ (B_2 + B_{dead} - B_1)^{-1}$$

where FI, B_1 and B_2 are the feed intake, the biomass at the start and end, respectively, and B

$_{dead}$ is the biomass of the dead fish.

$$\text{Protein efficiency ratio (PER)} = (B_2 - B_1)\ PI^{-1}$$

where B_1 and B_2 are the biomass at the start and the end of the experiment, and PI is the protein intake.

Biochemical analyses

At the end of experiment, 3 fish were sacrificed and samples of liver and muscles were taken and frozen for further biochemical analysis. Protein contents in muscle and liver were determined colorimetrically according to Henry (1964). Activities of aspartate amninotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957). Kits reagents used for determination of protein, AST and ALT were supplied by Egyptian American Co. for Laboratory Services, Egypt.

Statistical analysis

The obtained data were subjected to analysis of variance according to Snedecor and Cochran (1982). Differences between means were at the 5% probability level using Duncan's new multiple range test (Duncan, 1955).

Results

Physiological parameters

A significant reduction in RBCs, Hb and Ht in fish blood was observed in fish exposed to sublethal doses (¼ and ½ LC_{50}) of inorganic mercury. However, all these blood parameters were increased at high dose of Hg to be similar to those exposed to low dose of Hg (Table 1). As shown in Table 1, glucose level was significantly increased in fish blood exposed to low and high doses of Hg only (110.11 and 124.8 mg/L, respectively; $P<0.05$), however, glucose level in fish blood exposed to low and high doses of Hg + AA became similar to that of control and fish exposed to low dose (84.78 and 107.92 mg/L, respectively; $P<0.05$).

A significant reduction of total protein in fish muscles was observed in fish exposed to sublethal doses (¼ and ½ LC_{50}) of mercury (7.45 and 6.03 mg/g fresh wt, respectively). Ascorbic acid (vitamin C) increased total protein content in fish muscle at high dose of Hg to be similar to those exposed to low dose of Hg (7.64 mg/g fresh wt; Table 2). As shown in Table 2, AST activity was significantly increased in muscles of fish exposed to low and high doses of Hg only (287.5 and 295.5 U/g fresh wt, respectively; $P<0.05$). AST activities in fish exposed to low and high doses of Hg+AA became similar to that of control fish. Concerning the activity of ALT in fish muscle, it is noticed that the highest ALT activities were the highest in fish exposed to high doses of Hg (19.7 U/g fresh wt.). The lowest value was observed in muscles of control fish.

Total protein content in fish liver was significantly increased in fish exposed to low and high doses of Hg (21.9 and 25.5 mg/g fresh wt., respectively; Table 3). On the other

hand, vitamin C significantly lowered total protein in fish exposed to low dose of Hg to be similar to that of control fish (19.2 mg/g fresh wt; $P<0.05$). Also, it enhanced values of total protein in fish exposed to high dose of Hg+AA (Table 3). Hepatic AST and ALT activities were decreased at high dose of Hg without dietary ascorbic acid. Vitamin C enhanced AST activity at low and high doses (173.3 and 167.3 U/g fresh wt., respectively). ALT activity in liver of fish fed dietary vitamin C and exposed to low and high doses of Hg (Table 3) exhibited recovery trend.

Growth performance

Results in Table 4 and Figure 1 showed that the final weight of Nile tilapia was significantly decreased ($P<0.05$), while mercury dose increased. The least final weight was obtained with fish exposed to high dose of Hg (31.9 g/fish). Dietary vitamin C increased the final weight of fish exposed to high dose of Hg to be similar to that exposed to low dose and completely reduced the toxicity of low dose of Hg to be similar to control fish. There is no significant difference between control and treated low dose +AA (41.9 and 41.8 g/fish, respectively; $P<0.05$). Also, fish exposed to Hg low dose was approximately similar to high dose +AA. Similarly, weight gain and SGR were decreased significantly with high dose of Hg, while low dose +AA did not significantly differ as compared to control group.

Regarding the survival rate, it increased with increasing Hg toxicity where the least survival rate was obtained with high dose of Hg (58.8%). Ascorbic acid (vitamin C) enhanced the survival rate of fish exposed to low and high doses (84.3% and 79.7%, respectively; Table 5).

Results in Table 5 showed that feed intake of fish exposed to high dose of Hg was the lowest (36.1 g feed/fish). Contrarily, feed conversion rate (FCR) of fish exposed to high dose of Hg was higher (11.28), while the best FCR was obtained with control fish (2.9). Results in Table 5 indicated that protein efficiency ratio (PER) was higher with control fish and low dose +AA (1.133 and 1.150, respectively), while the least one was obtained with fish exposed to high dose of Hg.

Results in Table 6 showed that moisture content was significantly higher in fish exposed to high dose of Hg (81.7%) whereas it ranged from 79.1 to 80.2% without significant difference among other treatments. On contrary, values of protein content and total lipids in fish fillet were the lowest in fish exposed to high dose of Hg (15.43 and 1.19 %, respectively). On the other hand, ash content in fish fillet among the other treatments did not exhibit significant variations.

Discussion

The metabolic pathways of fish can be severely altered by a variety of biological, chemical and physiological factors, which could be assessed throughout several biochemical procedures. The influence of toxicant on total protein content in liver and muscle of fish has also been taken into account in evaluating response of the fish against stressors and was studied by many workers. Verma and Tonk (1983) observed a decrease in protein content of

muscle in mercury exposed fish, *Notopterus notopterus*. The reduction in muscle total protein may be attributed to the great energy demands and cellular damage that occurred in the tissue of toxicated fish (Haggag *et al*., 1993; Shalaby, 1997). Moreover, the obtained results also indicated that hepatic total protein increased following the exposure to sublethal concentrations of inorganic mercury. Similar results were obtained by Shalaby (1997) who recorded a significant increase in liver total protein in common carp, *Cyprinus carpio* exposed to metals Cu, Cd or Zn. He also reported that, it could be suggested that fish exposed to toxicant might compensate any possible protein loss by increasing its protein synthesis.

The decrease in hepatic AST enzyme activity is similar to the obtained results by Gill *et al*. (1990) who found a marked reduction in hepatic, branchial and renal AST and ALT in rosy barb (*Puntius conchonius*) after toxication with mercuric chloride. They mentioned that, the reduced levels of aminotransferase in various organs may result from tissue damage and consequently the reduction of enzyme turnover causally related to the presence of toxic mercury. Also, Abu El-Ella (1996) and Shalaby (2000) found similar results with grass carp, *Ctenopharyngodon idella* and common carp, *Cyprinus carpio* when exposed to Cd, respectively. These results may be attributed to liver necrosis (because of toxicant) that led to leakage from liver into the blood and/or tactual inhibition of liver enzymes.

On the other hand, Moore and Ramamoorth (1984) reported that chronic/sublethal poisoning of mercury may show several clinical symptoms including (1) inhibition of enzymes and protein synthesis in liver, kidney and brain, (2) structural alterations of fish epidermal mucus, (3) reduction in sperm viability, embryogenesis, and survival of second generation fry, (4) reduction in olfactory response, vision, and respiration, (5) reduction in fin generation time in fish, and (6) decability to osmoregulation. It is appeared from these symptoms that sublethal poisoning may lead to decrease in the ability of fish survive, grow and reproduce in nature.

The obtained results revealed that toxicated fish were recovered when fed dietary ascorbic acid (vitamin C). However, fish growth is highly flexible and is one of the complex activities where it represents the net outcome of a series of environmental and physiological factors that begin with food intake, digestion, absorption, assimilation and other metabolic activities. All these processes may affect the final fish product (Brett, 1979; Bugaev *et al.,* 1994; McDowall, 1994). Therefore, it could be concluded that dietary ascorbic acid (vitamin C) is efficient for reduction of Hg toxicity. These results are much expected because ascorbic acid (vitamin C) is closely related to the immunological system performance, and has antioxidant properties, favors integrity and fluidity of membranes (Brake, 1997) controlling the oxidizing reactions of fatty acids, thus keeping cellular respiration and avoiding cell death (Verlhac and Gabaudan, 1994). This antioxidant activity of ascorbic acid (vitamin C) makes it a hunter of free radicals, thus preventing the autointoxication of immunological cells such as macrophages which are the first processors of the information about the alien bodies and maximizing the defense of fish (Brake, 1997).

Beyond phagocytosis mechanism, ascorbic acid (vitamin C) is involved in leukocyte migration and retarded hypersensibility (Thomas and Holf, 1978, cited by Soliman *et al*., 1994). It also participates in the mutagenic proliferation of lymphocytes, in the increase of

the level of serum complements and in the production of interferon; therefore it is considered as anti-infection vitamin (Soliman *et al*., 1994). On the other hand, ascorbic acid (vitamin C) has been shown to enhance also the urinary elimination of metal to reduce hepatic and renal burden of metal (Dhawan *et al.*, 1988; Ghazaly, 1994).

In the same regard, Ghazaly (1994) studied the effect of different levels of ascorbic acid on experimental copper intoxication in *Tilapia zillii*. He found that ascorbic acid has been shown to have protective and therapeutic effects against copper intoxication, however it reduced fish mortality and poisoning signs, lowered metal content of tissues and prevented the inhibition of GOT and LDH activities. He also found that ascorbic acid enhanced transport of copper to kidney. Moreover, many investigators used ascorbic acid (vitamin C) for disease preventing. Eya (1996) found that a concentration of 46 mg ascorbic acid/kg of diet would prevent broken-skull disease in African catfish anomaly and allow optimum synthesis of vertebral collagen. Leonardo *et al*. (2000) studied the effect of different levels of dietary ascorbic acid (vitamin C) on the occurrence of ectoparasites of Nile tilapia larvae and found that the optimum level of ascorbic acid (1000 mg/kg diet) was efficient for reduction of ectoparasites occurrence.

However, it could be recommended that high levels of ascorbic acid (vitamin C) (>500 mg/kg diet) be used to be efficient in toxicity reduction, preventing disease and enhancing fish tolerance to environmental stress.

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Table 1. Changes in red blood corpuscles count (RBCs; C/mm^3), hemoglobin content (g/100 ml), Hematocrit value (%) and glucose level (mg/L) in blood of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
RBCs count	1.74 a ± 0.017	1.33 b ± 0.090	1.28 c ± 0.001	1.69 a ± 0.015	1.34 b ± 0.088
Hemoglobin content	4.32 a ± 0.073	3.92 a ± 0.094	2.80 b ± 0.264	4.26 a ± 0.068	3.86 a ± 0.087
Hematocrite value	12.86 a ± 0.736	13.60 a ± 0.686	10.20 b ± 0.249	12.62 a ± 0.536	12.93 a ± 0.856
Glucose	86.71 b ± 5.95	110.11 b ± 3.45	124.8 a ± 8.10	84.78 b ± 6.25	107.97 b ± 4.65

Means followed by the same letter in a row are not significantly different at P<0.05.

Table 2. Changes in total protein content (mg/g fresh wt.), AST (U/g fresh wt.) and ALT (U/g fresh wt.) in muscle of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
Total protein	9.97 a ± 1.06	7.45 b ± 0.63	6.03 c ± 0.34	7.89 b ± 0.34	7.64 b ± 0.57
AST	256.3 b ± 7.4	287.5 a ± 6.8	295.5 a ± 7.4	263.1 b ± 6.1	262.8 b ± 6.6
ALT	15.1 c ± 2.3	17.4 b ± 2.8	19.7 a ± 2.5	17.9 b ± 2.4	17.8 b ± 0.6

Data are represented as means of 9 samples.
Means followed by the same letter in a row are not significantly different at P<0.05.

Table 3. Changes in total protein content (mg/g fresh wt.), AST (U/g fresh wt.) and ALT (U/g fresh wt.) in liver of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
Total protein	17.4 c ± 1.6	21.9 b ± 3.3	25.5 a ± 1.1	19.2 bc ± 1.7	22.0 b ± 3.0
AST	173.4 a ± 7.4	153.3 b ± 3.5	142.3 c ± 5.7	173.3 a ± 7.9	167.3 ab ± 6.1
ALT	28.9 a ± 2.6	27.4 ab ± 3.2	22.9 d ± 0.7	27.7 ab ± 2.3	25.3 b ± 2.3

Data are represented as means of 9 samples.
Means followed by the same letter in a row are not significantly different at P<0.05.

Table 4. Growth performance of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
Initial weight (g/fish)	28.4 ± 0.07	28.7 ± 0.04	28.7 ± 0.04	28.6 ± 0.04	28.7 ± 0.04
Final weight (g/fish)	41.9 a ±1.4	36.7 b ±0.26	31.9 c ±0.23	41.8 a ±0.54	36.3 b ±0.17
Weight gain (g/fish)	13.5 a ±1.6	8.0 b ±0.03	3.2 c ±0.08	13.2 a ±0.66	7.6 b ±0.057
S G R (%/d)	0.648 a ±0.028	0.410 b ±0.016	0.178 c ±0.013	0.637 a ±0.024	0.391 b ±0.014
Survival (%)	95.3 a ±2.3	82.0 b ±2.0	58.8 c ±4.8	84.3 b ±4.3	79.7 b ±3.6

Means followed by the same letter in a row are not significantly different at P<0.05.

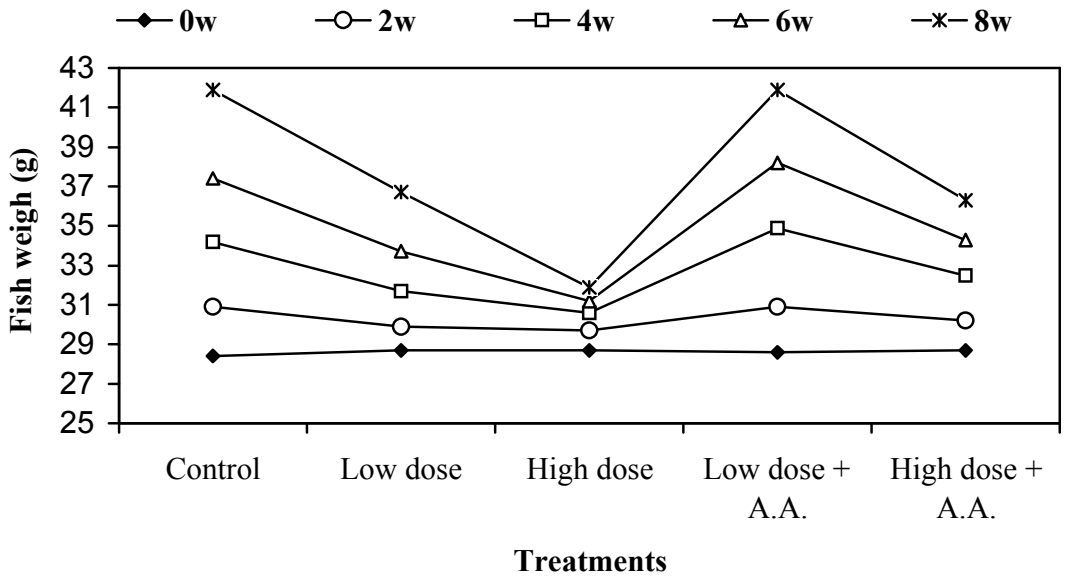

Fig. 1. Changes in body weight (g/fish) of Nile tilapia (*O. niloticus*) fed L-ascorbic acid (A.A.) and exposed to low and high doses of mercury.

Table 5. Feed intake, food conversion ratio (FCR) and protein efficiency ratio (PER) of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	rch Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
Feed intake (g feed/fish)	39.2 a ± 0.23	37.2 b ± 0.31	36.1 c ± 0.17	39.7 a ± 0.07	37.7 b ± 0.18
FCR	2.90 a ± 0.16	4.65 ab ± 0.21	11.28 c ± 1.20	3.01 a ± 0.15	4.96 b ± 0.24
PER	1.133 a ± 0.106	0.738 b ± 0.041	0.307 c ± 0.029	1.150 a ± 0.058	0.693 b ± 0.034

Means followed by the same letter in a row are not significantly different at $P<0.05$.

Table 6. Proximate chemical analysis (%; on wet weight basis) in muscle of Nile tilapia (*O. niloticus*) fed dietary L-ascorbic acid (A.A.) and exposed to low or high doses of mercury.

Items	Treatments				
	Control	Low dose	High dose	Low dose + A.A.	High dose + A.A.
Moisture	79.8 b ± 0.48	80.2 ab ± 0.37	81.7 a ± 0.62	79.8 b ± 0.69	79.1 b ± 0.48
Crude Protein	16.22 ab ± 0.55	15.94 ab ± 0.46	15.43 b ± 0.44	16.40 ab ± 0.48	17.18 a ± 0.60
Total Lipids	2.12 a ± 0.07	2.02 ab ± 0.06	1.19 d ± 0.05	1.92 bc ± 0.06	1.80 c ± 0.06
Ash	1.86 a ± 0.05	1.84 a ± 0.05	1.68 a ± 0.06	1.88 a ± 0.37	1.92 a ± 0.05

Means followed by the same letter in a row are not significantly different at $P<0.05$.