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Assessment of certain heavy metals (mercury and cadmium) and hormonal (methyltestosterone and trenbolone acetate) residues in farmed Oreochromis niloticus and mitigation of heavy metal residues using acetic acid immersion

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ABSTRACT

Tilapia nilotica is regarded as a crucial component in Egyptian fish aquaculture. Heavy metals impair fish health and ecosystems, and also pose a significant risk to human health, whenever consumed with contaminated fish. The unconscious use of hormones in aquaculture production has sparked great debate among researchers due to the potential hazards to human health. The present investigation objective was to assess the frequency of mercury (Hg), cadmium (Cd), Methyltestosterone (MT) and Trenbolone acetate residues in farmed Nile tilapia fish (Oreochromis niloticus) in Kafr El Sheikh, Egypt, and judge their acceptability compared to the tolerable daily intake and maximum residue limits (MRLs) of the National Egyptian Food Safety Organization (NFSA), CX/MRL2-2018.Then mitigation of heavy metals through immersion in acetic acid solution (5%). Sixty farm fish samples of O. niloticus collected from three separate fish farms, A, B, and C (20 of each) at Kafr Elsheikh governorate. In farms A, B, and C, the average Hg value was 0.87±0.01 mg/kg, 0.61±0.01 mg/kg, and 0.43±0.01 mg/kg, respectively. Meanwhile the average Cd value was 0.32±0.01 mg/kg, 0.17±0.01 mg/kg, and 0.09±0.01 mg/kg, respectively. The average values of MT and trenbolone acetate were 1.13 ± 0.01, 0.89 ± 0.01and 0.41 ± 0.02 µg/Kg and 0.36 ± 0.01, 0.21 ± 0.01 and 0.10 ± 0.01 µg/Kg, respectively. According to MRLs of Hg 33.3% (20/60) of total examined fish samples were unsuitable for human consumption, 23.3% (14/60) exceeded the cadmium MRL recorded by EOS [29], while comparing the evaluated O. niloticus to the MRLs for Trenbolone acetate—2 µg/kg—all of the samples were determined to be safe for human consumption. However, 5% (3/60) of the evaluated samples exceeded the acceptable daily intake of MT (2µg/kg). Contamination results with these residues suggested that further governmental action is needed to locate and eradicate contamination sources and mitigate potential public health hazards.

1.INTRODUCTION:

Fish are the major product of aquaculture. Numerous vital nutrients, including proteins, vitamins, minerals and polyunsaturated omega-3 fatty acids, are found in fish [1]. Fish flesh is an ideal food item that meets all national nutritional standards and is rich in protein, minerals, and vitamins. It is also easily digested [2].The necessity for land-based fish farming has increased due to the decrease in marine fish supply and the rise in seafood consumption [3].

The most significant species in private fish farms, Tilapia nilotica, is the main product of Egypt's farmed fish value chain [4]. Approximately 6 million tons of tilapia were produced worldwide in 2020 [5]. Because of its numerous advantageous qualities, such as its excellent resistance to disease, high survival rate, remarkable capacity to survive in difficult environments, and rapid growth, tilapia is a common culture species [6, 7].

The vast majority of fish farms in Egypt, especially the unofficial and private ones, used drainage waters, which comprised sewage and sanitation for the villages these drains served as well as industrial and agricultural discharges. So, the water in these fish farms is heavily contaminated with heavy metals and various chemical pollutants. [8]. The main sources of heavy metal pollution in water are thought to be industrial and agricultural outputs like pesticides, phosphate fertilizers, coal and oil combustion, plastics, and fertilizers. Because heavy metals take a long time to leave the body of a consumer whether human or animal. They are categorized as hazardous substances [9]. Tilapia Fish can absorb heavy metals to levels higher than those found in the environment, accumulating them in a variety of organs including the gill surface, kidneys, liver, and gastrointestinal tract wall [10]. Mercury and cadmium are heavy metals that pose a significant risk to human health [11]. They are among the most dangerous heavy metals because when consumed in excess or over an extended period of time, they have a toxic effect or be extremely hazardous even at low concentrations [12]. One list of possible human carcinogens includes cadmium. Chronic exposure to cadmium has been linked to a number of illnesses, such as liver and kidney ailments, cardiac problems, and weakened immune systems. It also played a major part in the development of chronic renal failure [13]. Mercury compound is readily absorbed, fat soluble, and accumulates in erythrocytes and the central nervous system, it is particularly hazardous to health [14]. In general, children, newborns, and fetuses are more vulnerable than adults to the neurological effects of methyl mercury [15].

Anabolic steroids are widely used for meat production in various regions of the world, despite their restriction as a livestock supplement in some areas (such as the European Union) [16]. The synthetic hormone 17α-methyltestosterone is extensively employed in male monosexual development of Nile tilapia [17]. The usage of hormone-based steroids in fish farming is raising concerns all around the world. In fish farming, steroid hormones can be used to promote growth when unisexual species grows larger and more quickly than the other [18]. For greater financial gain, it is utilized to improve the fish's size, weight, and growth [19]. A concern over hormone residues in fish that could harm humans, fish, and the environment led to prohibition of anabolic steroids, including 17α-MT, by numerous governments and food regulatory agencies [20]. For decades, livestock producers have utilized trenbolone acetate (TRB), a synthetic anabolic-androgenic steroid, as an anabolic stimulant to enhance muscle development and feed conversion. Several metabolites of TRB acetate, such as βTRB and 17α-trenbolone (αTRB), and trendione, have occasionally been found at physiologically significant amounts in water samples, primarily connected to livestock operations [21, 22]. A worrying global trend is the unreported use of hormones in fish aquaculture. The possible concerns to human health, such as endocrine disorders and cancer, as well as environmental hazards, the use of hormones in aquaculture production has generated a lot of discussion among researchers [23]. Steroid hormone has an adverse effect on customers in several ways. It results in early puberty and increased risk of cancer in youngest [24]. In addition, it decreased fertility in females [19]. Particularly, trenbolone has been connected to severe violent episodes [25, 26] as well as cardiac problems [27]. Increased aggressiveness and blood pressure are among the short-term side effects of trenbolone that have been reported [28].

The levels of these serious residues were compared with the standard limits of the Egyptian Organization for Standardization "EOS" [29] the maximal residual limits "MRL" of mercury is 0.5 mg/kg, 0.05 mg/kg for cadmium, metheyletestosterone (2 μg/kg) and Trenbolone acetate 2 μg/kg for evaluation of their acceptability for human consumption. Additionally, the application of certain trials to control such toxic heavy metals residues in fish was carried out by using acetic acid.

2-MATERIAL AND METHODS

2.1.Selecting the study area

The Kafr Elsheikh governorate, the study location, was chosen because it well-known for producing fish and has several fish farms. The primary fish-producing region in Egypt is Kafr Elsheikh governorate, which produces 324,479 tons of fish (55% of the country's farmed fish output) and 259,583 tons of tilapia (44% of the country's farmed tilapia production) [30].

2.2.Samples collection:

A total of sixty randomly selected Oreochromis niloticus farm fish samples were taken from three distinct fish farms in Kafr Elsheikh governorate of Egypt, A, B, and C (20 of each). After being collected, the samples were placed in an airtight ice box and taken to a lab to be examined for any potentially dangerous residues.

2.3. Determination of mercury and cadmium [31]

In order to detect cadmium residues, 1 g of each sample was really macerated with a sharp scalpel and digested with 10 ml of a digestion mixture (60 ml of 65% nitric acid and 40 ml of 70% perchloric acid) in a screw-capped tube. In order to digest 0.5 g of the macerated sample for mercury, 10 ml of a concentrated H_2SO_4/HNO_3 solution (1:1) was used.

The amounts of mercury and cadmium were then measured using an Atomic Absorption Spectrophotometer (VARIAN, Australia, model AA240 FS, Australia) after the digest, blanks, and standard solutions were aspirated.

2.4. Control of heavy metals using acetic acid

The effect of 5% acetic acid used by dipping on the concentration of experimentally contaminated fish fillet with cadmium and mercury was studied. In this trial, 18 pieces of fish fillet each weighed 50 g were divided into 3 groups. The first were individually inoculated with 40 mg/Kg ionic mercury and 10 mg/Kg ionic cadmium and left without any further treatment and set as a control group. The 2nd group was inoculated with 40 mg/Kg ionic mercury standard solution and soaked for 30, 60, and 90 minutes in 5% acetic acid. The 3rd group was inoculated with 10 mg/Kg ionic cadmium soaked in 5% acetic acid for 30, 60 and 90 minutes. This trial was repeated 3 times at different days and the reduction percentage of each heavy metal were recorded.

2.5. Determination of hormonal residues [32]

Fish muscle was stripped and 10 grams of ground muscle were homogenized with 10 ml of 67 ml of PBS buffer using a mixer for five minutes. Two grams of the homogenized sample were then combined with 5 ml of tertiary butyl methyl ether (TBME) in a centrifugal screw-capped vial, which was shaken vigorously for thirty to sixty minutes. Furthermore, after saving the supernatant and centrifuging the contents for ten minutes at 3000 rpm, the TBME extraction was done once more. Moreover, 1 mL of 80% methanol was used to dissolve the dried extract after the supernatants were mixed and evaporated using a N_2 evaporator. After diluting the methanolic solution with 2 mL of 20 mM PBS-buffer, the mixture was filtered via a RIDA C18 column (a solid phase extraction column equipped with a C18 end-capped sorbent that has an average particle size of 50 μm). The filtered filtrate was then utilized in an ELISA kit.

2.6.ELISA procedures [32]

The kits utilized were Ridascreen ELISA (RBio-pharm GmbH, Germany). The manufacturer's instructions for the ELISA test kit provided methyl testosterone standard solutions at different concentrations of 4.5, 1.5, 0.5, 0.25, 0.125, and 0 ppb in aqueous solution, which were utilized for the calibration curve. Both the standard and the samples undergo double analysis. Actually, 20 μl of standards or samples were added along with 50 μl of the diluted enzyme conjugate (peroxidase conjugated testosterone). After adding 50 μl of the diluted antibody solution and gently shaking the plate, the mixture was incubated for two hours at room temperature. Each well had 250μl of distilled water, and the liquid spilled out of them all. The washing process was performed twice after discarding the water after rinsing. Moreover, 50 microliters of urea peroxide, the substrate, and 50 microliters of tetramethylbenzidine, the chromogen, were added. Following a comprehensive mixing process and a 30-minute dark incubation period at room temperature, 100μl of the stop solution (1M sulfuric acid) was added. After mixing, the color remained steady for 60 minutes and absorbance was measured at 450 nm.

The testosterone calibration curve was used to determine the methyl testosterone concentrations (ppb) in the samples. The absorbance value of the zero standard was divided by the mean of the absorbance values obtained for the standards, and the result was multiplied by 100 (the percentage maximum absorbance) to produce the calibration curve.

The relationship between absorbance and testosterone is reverse.

O.D. standard (or sample) / O.D. zero standard x 100 = % maximal absorbance.

The estimated results (% maximum absorbance) for the standards were plotted (on the Y-axis) against the testosterone equivalent concentration (ppb) on the logarithmic X-axis. The calibration curve was nearly linear in the 0 to 4.5 ppb range.

3.RESULTS:

Table (1) revealed the concentration of compares the mercury and cadmium contamination in Oreochromis niloticus collected from three distinct fish farms, namely A, B, and C (20 of each) in Kafr Elsheikh governorate, Egypt and detection of its acceptability compared to the maximum residual limits of EOS. Farm A had the greatest amounts of cadmium and mercury pollution, while C had the lowest. According to Egyptian Organization for Standardization "EOS" [29] the maximum residual limits ("MRL") for mercury are 0.5 mg/kg and 0.05 mg/kg for cadmium, so the examined samples exceed the MRL presented by Egyptian authority and have a public health hazard effect, so the unaccepted farm fish samples according to their mercury limits were 8(40%), 7(35%) and 5(25%), respectively,. So, accepted samples from all examined samples was, 40(66.7%) did not exceed the MRLs and were approved for human consumption, while unaccepted samples with total 20 (33.3%). According to cadmium residual limits were 6(30%), 5(25%) and 3(15%), respectively, with a total 14(23.3%), unaccepted samples lead to a public hazard for human consumption, however 14(70%), 15(75%) and 17(85%), respectively, with total 46(76.7%) samples did not exceed the MRLs and were authorized for ingestion by humans

Table (1): Mean values of mercury and cadmium residues (mg/kg) and their acceptability in comparison to the maximum residual limits of EOS in the investigated samples of farmed Tilapia. (n=20).

Fish farm	Mercury	Cadmium				
	Mean value	Accepted	Unaccepted	Mean value	Accepted	Unaccepted
Farm A	0.87 ± 0.01 A	12(60%)	8(40%)	0.32 ± 0.01A	14(70%)	6(30%)
Farm B	0.61 ± 0.01 B	13(65%)	7(35%)	0.17 ± 0.01B	15(75%)	5(25%)
Farm C	0.43 ± 0.01 C	15(75%)	5(25%)	0.09 ± 0.01C	17(85%)	3(15%)
Total (60)	---	40(66.7%)	20(33.3%)	---	46(76.7%)	14(23.3%)

**Means with different capital letters in the same columns are significantly different due to fish farms (P<0.05).

Maximum permissible limits of Mercury 0.5 mg/kg, cadmium0.05 mg/kg according to EOS [29].

Table (2) was investigated the effect of acetic acid (5%) on heavy metal contamination in the fish fillets, when experimentally dipped of fish fillet inoculated with 40 mg/kg mercury in a 5% acetic acid solution for 30, 60, and 90 minutes, the levels of mercury was lowered by 32.3%, 53.8% and 69% respectively, while the reduction level of cadmium inoculated into fish fillet (10 mg/kg) after dipping in 5% acetic acid solution for 30, 60, and 90 minutes were 36%, 57% and 69%, respectively. The decrease level increase with longer acetic acid dipping times.

Table (2): Effect of acetic acid (5%) on the concentration of mercury experimentally inoculated into to fish fillets.

Storage time	Mercury	Cadmium				
	Control	Acetic acid treated group	Reduction	Control	Acetic acid treated group	Reduction
	mg/kg	mg/kg	%	mg/kg	mg/kg	%
Zero time	40	40	---	10	10	---
30 minutes	40	27.1	32.3	10	6.4	36
60 minutes	40	19.5	53.8	10	4.3	57
90 minutes	40	12.4	69	10	3.1	69

Moreover, Table (3) investigated the levels of 17α-Methyltestosterone contamination in Oreochromis niloticus. Farm A exhibited the highest maximum and average 17α-Methyltestosterone concentrations (2.62 and 1.13 µg/kg, respectively), followed by farms B and C. The Trenbolone acetate contamination levels compared to farms B and C, Farm A had the highest maximum and average concentrations of Trenbolone acetate (0.70 and 0.36 µg/Kg, respectively).

Table (3): 17α-Methyltestosterone and trenbolone acetate contamination levels in Oreochromis niloticus samples collected from three different fish farms in Kafr Elsheikh governorate Egypt (20 of each).

Fish farm	17α-Methyltestosterone	Trenbolone acetate				
	Minimum	Maximum	Mean±SE	Minimum	Maximum	Mean±SE
Farm A	ND	2.62	1.13 ± 0.01	ND	0.70	0.36 ± 0.01
Farm B	ND	2.27	0.89 ± 0.01	ND	0.49	0.21 ± 0.01
Farm C	ND	1.53	0.41 ± 0.02	ND	0.33	0.10 ± 0.01

Data are expressed as mean ±SE

ND not detected

Table (4) presents a safety evaluation of Oreochromis niloticus consumption in relation to the recommended maximum doses of 17α-Methyltestosterone and trenbolone acetate by the National Egyptian Food Safety Organization (NFSA). Five percent (3/60) of the evaluated Oreochromis niloticus surpassed the acceptable daily intake of testosterone (2 µg/kg) and all examined Oreochromis niloticus samples were deemed safe for human consumption when compared to the maximum permissible residue for Trenbolone acetate, which is set at 2 µg/kg by the National Egyptian Food Safety Organization (NFSA).

Table (4): Safety evaluation of Oreochromis niloticus consumption's in relation to the recommended daily testosterone and trenbolone acetate dosages by the National Egyptian Food Safety Organization (NFSA) (n=20).

Fish farm	17α-Methyltestosterone	Trenbolone acetate						
	Accepted	Unaccepted	Accepted	Unaccepted				
	NO	%	NO	%	NO	%	NO	%
Farm A	18	90	2	10	20	100	Zero	Zero
Farm B	19	95	1	5	20	100	Zero	Zero
Farm C	20	100	Zero	Zero	20	100	Zero	Zero
Total(60)	57	95	3	5	60	100	Zero	Zero

This according to Joint FAO/WHO Expert Committee on Food Additives "JECFA" [33].

4. DISCUSSION:

The purpose of the current study was to evaluate the frequency of mercury and cadmium residue contamination in Oreochromis niloticus and the mitigation of such contamination by immersion in acetic acid. Also estimating the levels of Trenbolone acetate and Methyltestosterone (MT) in farm fish samples of (Oreochromis niloticus) and estimate the consumption acceptability with reference to maximum residue limits (MRLs) authorized by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and its public health risk. As one of the most important aquaculture species, Nile tilapia is within the reach of consumers worldwide in terms of price and nutritional value, thus further research is needed to enhance the current production methods [34]. Earlier assessment study in Menoufia governorate, Egypt by Atomic Absorption Spectrophotometer revealed that 32.5% (13/40) and 40% (16/40) of fish samples were positive for mercury (mean value 0.46±0.03mg/kg) and cadmium (mean value 0.10±0.01 mg/kg), respectively [35].

In 25 fish samples (Tilapia nilotica) from Kafr El Sheikh governorate, Egypt, mercury was found in 11/25(44%) with mean value 0.89±0.01 [36]. 28% (7/25) of analyzed tilapia samples. collected from Kafr El Sheikh governorate, Egypt, were exceed the MRL of cadmium having an average of 0.01±0.17 mg/kg [37]. The current findings revealed that mercury is found in 20 samples with mean value 0.87 ± 0.01, 0.61 ± 0.01 and 0.43 ± 0.01mg/kg in farm A, B and C, respectively which was much higher than these obtained by Ragab [38] (0.050 ± 0.010 mg/kg), Mohamed [39] (0.13±0.02 mg/kg) in Oreochromis niloticus and according to El- said [40] study the average mercury concentration in Oreochromis niloticus was 0.037±0.115 mg/kg. While lower than Falandysz and Piotrowska [41] (4.6 ±2.4 mg/kg) wet weight and nearly similar to Sohsah [42] (0.81±0.05 mg/kg).

Considering table (1) it is obvious that the mean value of cadmium were 0.32 ± 0.01, 0.17 ± 0.01 and 0.09 ± 0.01 mg/kg in farm A, B and C, respectively. The recorded results were lower than that obtained by Kaoud and El-Dahshan [43] (1.21±0.05 mg/kg), Baidoo [44] (1.56±0.17 mg/kg) and Abo- Zahra [45] who found that the concentration of cadmium in three different farms in Kafr El- Sheikh governorate were (4, 3.6 and 3 mg/kg) in first, second and third farm, respectively. However, the cadmium levels recorded in this study were higher than those reported by Ayeloja [46] who discovered that the cadmium content was (0.073±0.02 mg/kg).

According to the WHO, consuming foods is the main way that people are exposed to heavy metals. Certain heavy metals, like cadmium and mercury, can cause major illnesses in humans even at low concentrations [47]. Methyl Mercury is more concentrated in the fetus's brain than in the mother's, and it can pass through both the placenta and blood brain barriers [48].

The results are nearly the same to the results obtained by Mita [49] who recorded that the decline in mercury levels up to 64.70 and 88.71% when soaked in acetic acid for 30 minutes. Acetic acid is a weak organic acid that is colorless, soluble in water/alcohol/glycerol, and can damage the intricacy of the binding between metal and protein, resulting in the dissolution of metal to a reduced amount. Atta [50] who reported that the immersion of fish in acetic acid solution (5%) have a favorable impact on lowering the level of cadmium in fish fillets. Immersion of fish in acetic acid solution reduced Cd concentration in fish fillet from 0.36 mg/kg significantly to 0.34 mg/kg after 1 minute immerging in 1% acetic acid. The author claimed that this impact is more prominent when the acid content is increased and the length of immersion is extended. After three minutes submerged in 5% acetic acid, fish with the lowest quantity of lead were detected. The author further suggested that the interaction between metals in fish bodies and acetic acid may be the cause of the decline in Cd concentration, which results in the formation of a water-soluble salt that is released in the immersion solution.

Fish fillets containing higher concentrations of acetic acid and longer soaking times showed lower levels of heavy metals, since acetic acid was able to chelate mercury and cadmium [51].

In order to increase production yield, MT is frequently used as an anabolic steroid to cause Nile Tilapia fry to become male [52]. For many years, livestock producers have utilized trenbolone acetate as an anabolic steroid to enhance feed conversion and boost muscle growth [53].

Previous research found that the hormonal residues of progesterone and testosterone were present in all three fish species in both farm and market samples. The levels of these hormones ranged (above ADI) from 2.1 to 16.96 μg/kg and 31.47–731.57 μg/kg ($p < 0.05$), respectively. Only market samples of rui and catla from Rajshahi district contained residues of the estrogen hormone; no residue was found in tilapia fish, and the hormone levels (above ADI) varied from 8.23 to 40.13 μg/kg [23]. The ELISA method can be used as a fast screening method in approved control laboratories to determine whether fish contain 17α-Methyltestosterone and Trenbolone acetate [32].

According to results recorded in table (3) these results were agree with that obtained by Aabu-Taleb [54] who found that the fish sample mean value of MT was 0.81±0.03 μg/kg , Ibrahim [55] recorded 0.227±0.007ppb of trenbolone and Abd El Maksod et al. [56] who found MT in all examined 25 (100%) samples of farmed Tilapia with mean value 1.1±0.12ppb and trenbolone acetate found in 1 (4%) with level 0.28 ppb in some farmed fish species marketed in Sharkia governorate. Higher results obtained by Hamada [57] who recognized that the concentration's mean value of MT hormone in farmed Nile tilapia was 3.48+0.27ppb. Reduced results have been detected by Marzouk [58] who observed that the mean value of Mt was 0.535±0.03ppb in Tilapia fish.

Raw fish, which is vital to human nutrition, should be safe and devoid of any substances that could harm people's health. However, the anabolic hormones used in animal husbandry for a variety of objectives often leave behind residues, which pose a risk to the public health [59] such as elevated blood pressure, digestive issues, and benign and malignant liver tumors [60].

5. CONCLUSION.

The study's findings indicate that Farm A has the highest levels of mercury contamination, exceeding 0.5 mg/kg, cadmium contamination above 0.05 mg/kg and the highest average and maximum levels of 17α-Methyltestosterone and trenbolone acetate. Levels of mercury, cadmium and 17α-Methyltestosterone pose a significant public health hazard, as they are above the safe permissible limits. These residue concentrations can be harmful to human health. All of the examined Oreochromis niloticus samples were determined to be safe for ingestion by comparing them to the MRLs for Trenbolone acetate, which are 2 μg/kg. Despite the low hormone prevalence and residue levels at current levels, regular human exposure—whether daily or frequent—along with the residues' cumulative potency could have serious negative effects on health. To ensure food safety and public health, these harmful residues must be continuously monitored.

The study also found that the use of acetic acid was highly effective in reducing and controlling the level of heavy metal pollutants, such as mercury and cadmium. This suggests that acetic acid could be a valuable tool in mitigation the contamination of heavy metals and reducing the public health risks.

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