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AMELIORATORY EFFECT OF METHANOLIC LEAF EXTRACT OF *MORINGA OLEIFERA* ON SOME LIVER FUNCTION AND OXIDATIVE STRESS MARKERS IN SODIUM FLUORIDE TOXICITY IN SEA BREAM *SPARUS AURATUS L.*

Rayed H. Khalil[1]*, Amany M. Diab[2], Medhat S. Shakweer[3], Hanan A. Ghetas[4] and Khallaf M.[5]

[1]Department of Poultry and Fish, Faculty of Veterinary Medicine, Alexandria University, Egypt.
[2]Aquatic Microbiology, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt.
[3]Fish Diseases and Management, Department of Internal Medicine, Infectious and Fish Diseases, Faculty of Veterinary Medicine, Mansoura University.
[4,5]Lecturer of Aquatic Animal Medicine and Management Department Faculty of veterinary medicine- Sadat City University.

***Corresponding Author: Dr. Rayed H. Khalil**

Department of Poultry and Fish, Faculty of Veterinary Medicine, Alexandria University, Egypt.

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ABSTRACT

Background: Because of the potential antioxidant, anti-inflammatory, and immune-modulating properties of Moringa (Moringa oleifera), there is an increased interest in its potential role in the prevention and control of sodium fluoride toxicity in cultured sea bream. **Objective:** This study investigated the effects of sodium fluoride on sea bream Sparus auratus L. and protection effect of Moringa oleifera by exposed of sea bream to 1/10 dose of sodium fluoride 96hr-LC_{50} (6.1 mg/L) and study the changes in antioxidant enzyme activities of gills tissues. **Material and methods:** Two hundred and sixty sea bream was used for determination of LC_{50} and chronic toxicity the fish divided into four groups of fifty fishes each. Control group, received no any treatment; 1/10 dose of sodium fluoride LC_{50} (6.1 mg/L), sodium fluoride plus Moringa oleifera extract and Moringa oleifera extract 1% of diet. **Results:** The results showed that the sodium fluoride at dose of 6.1 mg/L the level of SOD; CAT in gills and total protein in the serum of sea bream were decreased. On the other side the level of AST; ALT and creatinine were increased in the serum of sea bream during sodium fluoride toxicity. The SOD; CAT in gills at the sodium fluoride exposed group was found lower than the M. oleifera supplemented groups. Exposure of sea bream to sodium fluoride for 8th weeks resulted in significant changes in mRNA abundance for a limited subset of the analyzed of hepatic sea bream genes. GSR mRNA levels decrease significantly upon incubation with sodium fluoride, in liver, irrespectively to the length of the treatment, whereas the corresponding levels of sea bream feed with 1% M. oleifera were indistinguishable from controls. **Discussion and conclusion:** On the basis of present findings it could be concluded that increased sodium fluoride content in water causes adverse effect on fish. The changes of plasma biomarkers as antioxidant enzymes were the physiological responses of sea bream to the stress of sodium fluoride exposure. Moringa oleifera tree can be grown to produce more natural products and environmentally friendly materials.

KEYWORDS: Moringa oleifera, sodium fluoride toxicity, sea bream.

INTRODUCTION

Fluoride has been known as strong, hard anion and cumulative toxic agent (Guney *et al.*, 2007) occurs naturally mostly distributed in the rivers, lake and seas around the world (Azmat, 2009). Fluoride is a cumulative toxin and the most damaging environmental pollutant, has affinity to accumulate in the tissues of organisms, making adverse effects to aquatic life at very low levels of exposure (Azmat *et al.*, 2007; Masoud *et al.*, 2006; Aziz, 2012). Fluoride may be considered as a xenobiotic to the biological ecosystem at elevated level disturb the normal metabolic pathways of an organism. Fish are the main aquatic food chain organisms may often accumulate large amounts of certain metals (Trivedi and Goel, 1986). Fish are commonly used to assess the quality and health condition of aquatic ecosystems and as such can serve as bio indicators of environmental pollution (Dautremepuits *et al.*, 2004). (JIANJIE *et al.*, 2013) stated that the fluoride is ubiquitously distributed in natural waters. Elevated fluoride may cause histopathological changes and induce oxidative stress in the gills of the common carp (*Cyprinus carpio*).

Recently, an increasing number of studies on fish have shown that a high concentration of F may have negative

effects, such as slower growth and development, increased mortality, metabolic disorders, pathological changes in tissues, F accumulation in and deformity of bone tissue, and ecological pressure on fish (Chen *et al.*, 2013; Chen *et al.*, 2014).

This study investigated the ameliorative effects of *Moringa Olvera* against toxicity of sodium fluoride on sea bream; by determination of 96hr-LC_{50} for sodium fluoride on sea bream *Sparus auratus L.* Chronic toxicity by exposed of sea bass to 1/10 dose of sodium fluoride 96hr-LC_{50} values. Changes in antioxidant enzyme activities of sea bream gills as activities of superoxide dismutase (SOD) and catalase (CAT); changes in enzyme activities of serum as aspartate amino transferase; alanine amino transferase were studied. Amelioration of sodium fluoride toxicity by addition of *Moringa oleifera* to the ration of exposed sea bream by the rate of 1%. Detection of gen expressions of glutathion-s- transferase in liver of toxicated sea bream after 8th weeks of toxicity were carried out.

MATERIAL AND METHODS

Chemicals

Sodium fluoride was purchased from Lab Service Co. Egypt and dissolved in water. Kits for determination of liver functions test (Aspartate amino transferase; Alanine amino transferase) and antioxidant parameter (catalase and Superoxide Dismutase) were purchased from the Biodiagnostic Company, Cairo, Egypt. All chemicals used were of analytical grade.

Moringa leaves preparation: Moringa leaves were obtained by personal communication and added to the ration by 1% in ration. The Moringa leaves were harvested and air-dried under a shed until they were crispy to touch while retaining their greenish coloration. The leaves were milled to obtain a powder.

Gas chromatography–mass spectrometry (GC-MS) analysis: Trace GC Ultra-ISQ mass spectrometer with a direct capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm) was injected by 10 µl of MOL methanolic extract. The column oven temperature was started 60 °C and then increased by 5 °C/min. till reach 280 °C. The injector and detector (MS transfer line) temperatures were kept at 250 °C. Helium flow rate of 1 ml/min. was used as carrier gas for 37.83 minutes. The solvent delay was 2 min. and diluted samples of 1 µl were injected automatically using auto-sampler AS3000 coupled with GC in the splitless mode. The ion source and quadrupole temperatures were set at 200 and 150°C, respectively. The mass spectra of the identified components were determined by comparison to NIST 11 mass spectral database.

Fish for experimental work

A total of 260 apparently healthy sea bream *(Sparus auratus L)* were collected from private fish farms at Borg-El Arab, Alexandria Governorate and previously acclimated in full glass aquaria measuring (40×30×40 cm) and maintained in aerated marine water at 25 ± 2^{0}C for 14 days. They seemed healthy and had a uniform size and weight with average body weight 40 ± 3 grams.

Experimental design of LC_{50} of Sodium fluoride in sea bream

A total number of 60 apparently health sea bream, weighting 30 ± 2 grams were selected after the period of acclimation about two weeks and then divided into six equal groups; each group contained of 10 fish. The first five groups were consistently exposed to 0, 20; 40; 60; 80 and 100 mg L^{-1} of sodium fluoride while the control group (group 6) was act as a control group and the determine the LC_{50} were carried out according to (Klassen, 1991) (Table 3). The dead fish were removed immediately. Behavioral changes, clinical toxic signs and postmortem lesions of tested fish were closely followed up and recorded daily. The lethal concentration of sodium fluoride after 96 hour (96-h LC_{50}) of exposure was calculated according to (Behrens and Karber, 1953).

Experimental design of chronic experiment

Four aquaria were used for experimented sea bream with an average body weight of 40 ± 3 g and divided to four equal groups (50 fish per each). Fifty fish were served as a control negative group. The groups were arranged as the following; G (2) exposed to sodium fluoride (1/10 LC_{50}, 6.0 mg. /L.); G (3) sodium fluoride (6.0 mg. /L.) plus *M. oleifera* extract by 1% in ration and G (4) supplemented by *M. oleifera* extract by 1% in ration only. The experiment was extended to 8 weeks where fish samples were taken every 14 days from all aquaria for analyses (Table 4). Settled fish wastes were cleaned daily by siphoned with three quarters of the aquarium's water, which was replaced by aerated water from the water storage tank. Water temperature was kept at 25 ± 1 °C and 35‰ salinity.

Fish diets: Fish were fed on a commercial fish diet containing 45% crude protein. The diet was daily provided at a fixed feeding ratio of 3 % of body weight of fish as described by (Eurell *et al.*, 1978) (Table 1).

Sample collection and preparation

At 2nd, 4th, 6th and 8th weeks during the experimental period, blood samples were collected from different groups via the caudal vessels from 3 fish using disposable syringe (Hawak *et al.*, 1965). The serum was collected with a micropipette and then was stored in sterile Eppendorf tubes at -20°C until used for assay (Lied *et al.*, 1975). At same time of blood sampling the specimen of gills were collected at 2nd, 4th, 6th and 8th weeks during the experimental period for measurement of different antioxidant parameters from different groups.

Clinico-biochemical analysis: Determination of serum aspartate amino transferase (S.AST) and serum alanine amino transferase (S.ALT) were estimated according to (Reitman and Frankle, 1957).

Methods for determination of antioxidants enzyme assay in gills tissue

The gills were washed in an iced cold 1.15% KCl solution, blotted and weighed. They were then homogenized with 0.1 M phosphate buffer (pH 7.2), before putting the organs each into the mortar; laboratory sand was added to it (acid washed sand) and it was blended together in the mortar with pestle. The resulting homogenate was centrifuged at a speed of 2500 rpm for 15 mins after which it was removed from the centrifuge. The supernatant was decanted and stored at -21°C until spectrophotometric determination of antioxidant enzymes activity using UV-VISIBLE spectrophotometer (Habbu *et al.*, 2008). Catalase (CAT) activity was determined by measuring the decrease of H_2O_2 concentration at 410 nm according to (Koroliuk *et al.*, 1988). Superoxide dismutase (SOD) activity in supernatant was determined according to (Kostiuk *et al.*, 1990).

Transcript expression analysis of gen expressions of glutathion-s- transferase in liver of toxicated sea bream after 8th weeks in liver of different treatments

At the end of the experiment (8th week) another liver samples were collected from different treatments for Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA expression of antioxidant related gene (glutathion-s- transferase) was performed (Table 2). The total mRNA was extracted from tissue samples from the control and treated groups (n=6 per group) using an mRNA extraction kit according to the manufacturer's instructions. The quality of the extracted RNA was confirmed with 2 % agarose electrophoresis following the manufacturer's protocol (Abd El-Rahim *et al.*, 2010; Puerto *et al.*, 2011).

Quantitative Real Time-PCR: The first strand cDNA from different samples was used as templates for RT-PCR with a pair of specific. The sequences of specific primer and product sizes are listed in Table 1. β- actin was used as a housekeeping gene for normalizing mRNA levels of the target genes (FAROUK *et al.*, 2015). The relative quantification of the target to the reference was determined by using the 2−ΔΔCT method if Ef for the target (GST) and the reference primers (β-Actin) as follows: The relative expression was calculated by using the $E^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The equation: $E^{-\Delta\Delta Ct} = E^{(\mathbf{A}\ \mathbf{Ct}\ \text{gene of interest} - \mathbf{Ct}\ \text{reference gene}) - (\mathbf{B}\ \mathbf{Ct}\ \text{gene of interest} - \mathbf{Ct}\ \text{reference gene})}$ Where *A* represents exposed fish and *B* control fish. The method gives a fold change value compared to the control with adjustment to a reference gene. The equation relies on Ct (cycle threshold) values which are the numbers of polymerase chain reactions it takes for the amplification curve to reach a manually set threshold in the exponential phase.

Statistical analysis: All data are expressed as the mean ± standard deviations (SDs), and the levels of significance are cited. SPSS statistical package version 17.0 for Windows (IBM, Armonk, NY, USA) was used for all data analysis. Differences in values were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. Differences were deemed significant when $p<0.05$.

RESULTS

The data illustrated in Table (5) and presented in Figure (1) and revealed the chemical composition of *Moringa oleifera* that analyzed by GC–MS led to identification of some antioxidant agents as; eugenol (45.23 %), caryophyllene (11.35%), hexadecanoic acid (7.23%), phenol (6.14%), octadecenoc acid (3.29%), heptadecyne (3.18 %), cyclopropanoctonic (4.17%), heptatriacotanole (1.26%) and quercetin (0.89%), respectively.

Results of determination of LC_{50} sodium fluoride in sea bream: A result of LC50 of sodium fluoride in sea bream was summarized in Table (6) and Figure (2). The obtained results showed that the lethal concentration $_{50}$ (LC_{50}) of sodium fluoride in sea bream was 61 mg/L; so the 1/10 dose of LC_{50} of sodium fluoride in sea bream to induce chronic toxicity was 6.1 mg/L.

Results of clinical manifestation during toxicity of 1/10 dose of LC_{50} of sodium fluoride in sea bream (6.1 mg/L): The clinical signs during the 1/10 dose of LC_{50} of sodium fluoride in sea bream (6.1 mg/L) it started to appear at the 1st week post addition of sodium fluoride and continued until the end of the experimental period (8th week). The clinical signs of the experimentally intoxicated sea bass were showed marked loss of orientation and equilibrium, and increased mucus secretion and finally gasping for breath, reduction of swimming ability, darkening of color, and then death after long term of exposure. Other behavioral observations revealed fish suffering from anorexia lose weight, go through a period of violent movement which degrades into aimless wandering, and finally lose their equilibrium. Consequently, fish moved to the corners of the test aquaria, which can be regarded as an adaptive/ avoidance behavior of the fish to toxicant. A significant morphological change in gills, a major respiratory organ and kidney as well as intestine in fish, associated with sodium fluoride toxicity.

Effect of sodium fluoride and/or *M. oleifera* on Aspartate aminotransferase in serum of sea bream

Our results indicated that there were statistically significant increases in the activities of AST in the serum of sea bream exposed to sodium fluoride. But levels of the activities of AST in the serum of *M. oleifera* supplemented group decreased significantly in comparison to the control group. In relation to control group, sodium fluoride significantly ($p<0.05$) increased the serum levels of AST at 6th and 8th weeks of exposure period of 6.1mg /L. of sodium fluoride which indicate hepatotoxicity (Table 7). *M. oleifera* alone has no effect on the measured of levels of AST, fortunately it returned the increased levels of AST to their normal values in sodium fluoride + *M. oleifera* treated fish.

Effect of sodium fluoride and/or *M. oleifera* on Alanine aminotransferase in serum of sea bream

The present results indicated that there were statistically significant increases in the activities of ALT in the serum of sea bream exposed to sodium fluoride Table (8). But levels of the activities of ALT in the serum of *M. oleifera* supplemented group decreased significantly in comparison to the control group. In relation to control group, Sodium fluoride significantly ($p<0.05$) increased the serum levels of ALT at 6^{th} and 8^{th} weeks of exposure period of 6.1mg. /L. of sodium fluoride. *M. oleifera* alone has no effect on the measured of levels of ALT, fortunately it returned the increased levels of ALT to their normal values in Sodium fluoride + *M. oleifera* treated fish.

Effect of sodium fluoride and/or *M. oleifera* on catalase activity in gill tissue of sea bream

The results revealed that the fish exposed to 6.1mg. /L of sodium fluoride for 8^{th} weeks the catalase activities of gills tissue of sea bream were increased (Table 9). In group feed on *M. oleifera* and at the same times exposed to 6.1mg. /L of sodium fluoride for 8^{th} weeks the catalase activity was gradually decreased compared to the control and treated groups. However, CAT activity significantly ($p<0.05$) increased in sodium fluoride exposed fish at 8^{th} weeks days of exposure period. And in gills, there were no significant ($p>0.05$) changes in CAT activity at all experimental period.

Effect of sodium fluoride and/or *M. oleifera* on super oxide dismutase activity in gill tissue of sea bream

Levels of SOD activity in the gills tissue of sea bream after chronic exposure to 6.1 mg /L for 8^{th} weeks to sodium fluoride and/or *M. oleifera* are summarized in (Table 10). The levels of SOD activity in the gills, tissue of sea bream after chronic exposure to sodium fluoride group were little higher, but not significantly compared to the control group. There was significant difference in SOD activity in the tissue of sea bream after chronic exposure to 6.1 mg /L for 8^{th} weeks to sodium fluoride and/or *M. oleifera* groups. However, in general, the activities of SOD enzymes in the *M. oleifera* treated groups were higher than those in the toxicated and controls groups, but the SOD activities and the extent of changes were not more obvious than those in the sodium fluoride exposed group. There were significant ($p>0.05$) changes in the activity of gills tissue of sea bream SOD enzyme in *M. oleifera* and chronic exposure to 6.1 mg /L for 8^{th} weeks to sodium fluoride + *M. oleifera* treated groups compared with the control, however it was significantly ($p< 0.05$) increased in Sodium fluoride exposed fish at 2^{nd}, 4^{th}, 6^{th} and 8^{th} weeks in a time-dependent manner.

Quantitative analysis of mRNA abundance of stress genes in liver tissues of sea bream

Exposure of sea bream to sodium fluoride (6.1mg / L) for 8^{th} weeks resulted in significant changes in mRNA abundance for a limited subset of the analyzed of hepatic sea bass genes (Figure 3). GSR mRNA levels decrease significantly upon incubation with sodium fluoride, in liver, irrespectively to the length of the treatment, whereas the corresponding levels of fish treated with *M. oleifera* were indistinguishable from controls. These data indicate that sodium fluoride seems to be a poor GSR inducer in toxicated sea bass, whereas liver seem to respond better at the long term. Changes in GSR mRNA levels were in general mild and restricted to particular subsets of samples. GSR mRNA levels significantly decreased at the end of toxicity with sodium sulphate. Bivariate correlation analysis of mRNA abundance levels of different genes in liver tissues defined different clusters of co-regulated genes. Levels of mRNA from oxidative metabolism-related genes (GST) showed a very high correlation in.

Table. 1: The ingredient composition (%) of the basal diet (without supplementation of *Moringa oleifera* extract.

Ingredients	**%**
Fish meal (65%)	35
Barley	30
Ash	10.5
Soybean meal	23
Crude fiber (CF)	12.5
Wheat bran	14.5
Ether extract (EE)	2.46
Limestone	1.8
N-free extract (NFE)	56.04
Bone meal	1.0
Digestible energy, Kcal/g (DE)	2.63
Salt	0.2
Calcium	1.10
Premix	0.3
Phosphorus	0.59
DL – methionine	0.2
Lysine	0.97
Methionine	0.57

Each 3 Kg vitamin and mineral mixture provides: Vitamin A: 12000000 IU, Vit.D3: 2200000 IU, Vit. E: 10000 mg, Vit. K, :2000 mg, Vit.B:11000mg, Vit.B2 :4000mg, Vit.B6 :1500mg, Vit.B12 :10mg, Pantothenic Acid : 10000mg, Niacin :20000mg, Biotin :50 mg, Folic Acid : 1000mg, Choline chloride : 500gm, Selenium: 100mg, Manganese : 55000mg, Zinc : 50000mg, Iodine : 1000 mg and carrier CaCo3, to 3000 gm.

Table. 2: Primer sequences used for RT-PCR.

Gene name	Forward primer	Reverse primer
β-actin[a]	CCTCACCCTCAAGTACCCCAT	TTGGCCTTTGGGTTGAGTG
GST	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTATCCGC

[a] Housekeeping gene

Table. 3: Experimental design for determination of LC_{50} of Sodium fluoride in sea bream.

Group Number	Concentration of Sodium fluoride by mg L-1	Number of *O. niloticus*
1	0 (control)	10
2	20	10
3	40	10
4	60	10
5	80	10
6	100	10

The data were also assessed according to Behrens–Karber's method using the following formula (Klassen, 1991):-

$LC_{50} = LC_{100} \sum A \times B / N$ as mg/L;

- Where LC_{50} and LC_{100} indicate the lethal doses for 50% and 100% of the tested fish.
- Value ''A" gives the differences between the two consecutive doses,
- ''B" the arithmetic mean of the mortality caused by two consecutive doses and
- ''N" the number of tested fish in each group.

Table. 4: Design of the chronic experiment.

Groups	Treatments	Number of sea bream	Dose of Sodium fluoride and other additives	Reference
G (1)	Control (without treatment)	50	0	
G (2)	Sodium fluoride only	50	6.0 ppm	1/10dose of LC_{50}
G (3)	Sodium fluoride plus *Moringa oleifera* extract	50	6.0 ppm+ *Moringa oleifera* extract by 1% in ration	
G (4)	*Moringa oleifera* extract	50	*Moringa oleifera* extract by 1% in ration	

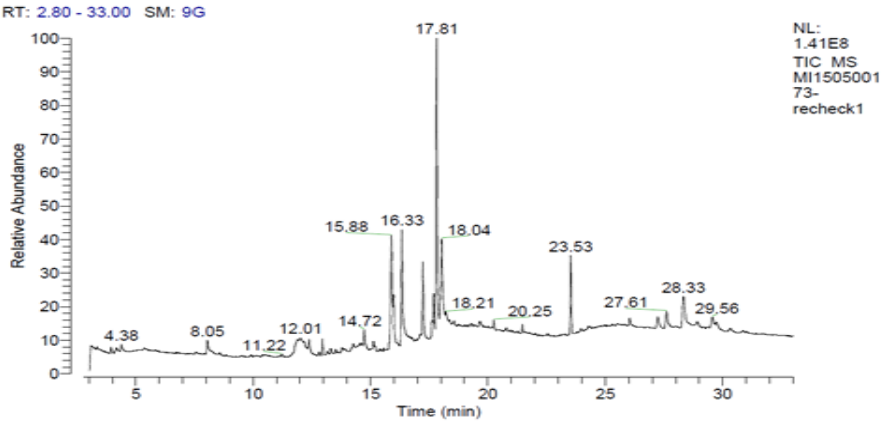

Fig. 1: GC-MS chromatogram of *Moringa oleifera* methanolic extract.

Table. 5: GC-MS analysis of Moringa leaves methanolic extract.

No.	Compound Name	RT (Minutes)	Area %	Molecular Formula
1	Eugenol	17.81	45.23	C10H12O2
2	Caryophyllene	15.88	11.35	C15H24
3	Hexadecanoic acid	28.33	7.23	C38H68O8
4	Phenol	27.61	6.14	C12H14O3
5	Octadecenoic acid	29.56	3.29	C19H36O2
6	Heptadecyne	20.25	3.18	C17H32O
7	Cyclopropanoctonic	31.24	4.17	C22H38O2
8	Heptatriacotanole	28.42	1.26	C37H76O
9	Quercetin	31.19	0.89	C18H16O7

Table. 6: Results of determination of LC_{50} sodium fluoride in sea bream.

Sodium fluoride dose (mg/L)	No. of exposed fish	No of dead fish				Overall deaths within 96 h	A	B	AB
		D1	D2	D3	D4				
0 (control)	10	0	0	0	0	0	0	0	0
20	10	0	0	0	0	0	20	0	0
40	10	0	0	1	1	2	20	1.0	20
60	10	1	1	1	2	5	20	3.5	70
80	10	1	2	2	2	7	20	6.0	120
100	10	2	2	3	3	10	20	8.5	170
									∑AB =390

Where

✓ A = differences between the two consecutive doses and

✓ B = arithmetic mean of the mortality caused by two consecutive doses.

96 h LC_{50} = LC_{100} - ∑ (A x B)/N = 100 – 390/10 = 61.0 ppm. Or (mg/L)

- So lethal concentration 50 (LC_{50}) of Sodium fluoride in sea bream = 61 mg/L.
- So 1/10 dose of LC_{50} of Sodium fluoride in sea bream to induce chronic toxicity was = 6.1 mg/L.

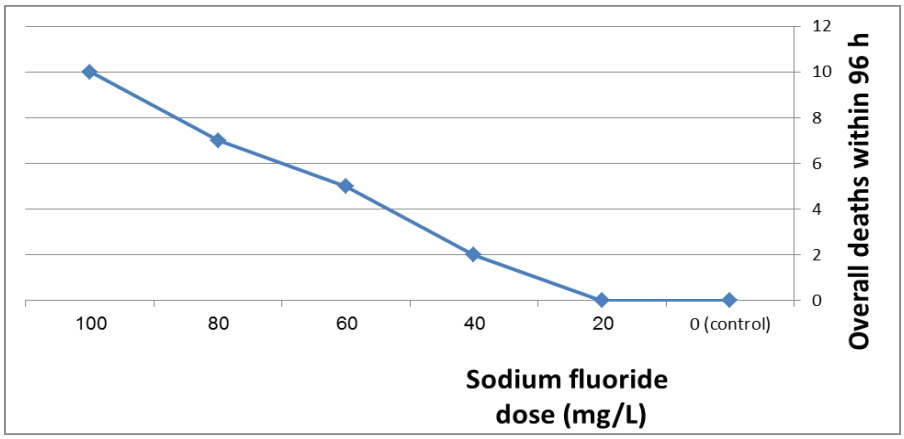

Fig. 2: LC_{50} of Sodium fluoride in sea bream.

Table. 7: Effect of sodium fluoride and / or *M. oleifera* on S.AST (IU/L) in serum of sea bream.

Groups	Period of exposure			
	2nd week	4th week	6th week	8th week
CTR	48.13± 0.24 $^{A\,b}$	47.01± 1.28 $^{A\,c}$	46.95± 0.43 $^{A\,c}$	46.84± 0.78 $^{A\,c}$
SF	53.84± 0.16 $^{D\,a}$	58.79± 0.22 $^{C\,a}$	68.43± 0.71 $^{B\,a}$	75.29± 1.16 $^{A\,a}$
FM	49.65± 0.61 $^{D\,b}$	54.79± 0.81 $^{C\,b}$	64.94± 0.77 $^{B\,b}$	72.19± 1.61 $^{A\,b}$
ME	41.80± 1.00 $^{A\,c}$	40.70± 0.34 $^{A\,d}$	39.35± 0.58 $^{A,B\,d}$	36.64± 0.45 $^{B\,d}$

Different superscript small letters within the same column indicate significantly different mean values ($p< 0.05$) between different groups. Different superscript capital letters within the same raw indicate significantly different mean values ($p< 0.05$) between different periods of exposure. CTR, Control; SF, Sodium fluoride (6.1 mg/L.); FM: Sodium fluoride (6.1 mg/L.) + *Moringa oleifera* extract (1% in ration); ME, *M. oleifera* extract (1% in ration).

Table. 8: Effect of sodium fluoride and / or *M. oleifera* on S.ALT (IU/L) in serum of sea bream.

Groups	Period of exposure			
	2nd week	4th week	6th week	8th week
CTR	53.17 ± 0.31 $^{A\,b}$	52.64 ± 0.94 $^{A\,b}$	51.10 ±1.11 $^{A\,c}$	50.75 ± 1.41 $^{A\,c}$
SF	55.78 ± 0.24 $^{C\,a}$	58.46 ± 0.49 $^{C\,a}$	64.47 ± 0.60 $^{B\,a}$	68.63 ± 0.60 $^{A\,a}$
FM	53.49 ± 0.36 $^{D\,a,b}$	56.73 ± 0.34 $^{C\,a}$	61.22 ± 0.93 $^{B\,b}$	64.53 ± 0.49 $^{A\,b}$
ME	48.42 ± 0.65 $^{A\,c}$	45.99 ± 0.28 $^{A,B\,c}$	43.54 ± 0.60 $^{B,C\,d}$	40.87 ± 0.38 $^{C\,d}$

Different superscript small letters within the same column indicate significantly different mean values ($p< 0.05$) between different groups. Different superscript capital letters within the same raw indicate significantly different mean values ($p< 0.05$) between different periods of exposure. CTR, Control; SF, Sodium fluoride (6.1 mg/L.); FM: Sodium fluoride (6.1 mg/L.) + *Moringa oleifera* extract (1% in ration); ME, *M. oleifera* extract (1% in ration).

Table. 9: Effect of sodium fluoride and / or *M. oleifera* on CAT activity (µmol O2/min/mg protein /ml.) in gills tissue of sea bream.

Groups	Period of exposure			
	2nd week	4th week	6th week	8th week
CTR	2.61 ± 0.006 $^{B\,a}$	2.61 ± 0.069 $^{B\,a,b}$	2.74 ± 0.035 $^{A,B\,a}$	2.86 ± 0.048 $^{A\,a}$
SF	2.55 ± 0.008 $^{A\,a}$	2.46 ± 0.031 $^{A,B\,b}$	2.38 ± 0.017 $^{A,B\,b}$	2.28 ± 0.023 $^{B\,b}$
FM	2.58 ± 0.012 $^{A\,a}$	2.53 ± 0.048 $^{A,B\,b}$	2.62 ± 0.024 $^{A\,b}$	2.39 ± 0.011 $^{B\,b}$
ME	2.68 ± 0.026 $^{C\,a}$	2.79 ± 0.011 $^{B,C\,a}$	2.89 ± 0.124 $^{A,B\,b}$	3.00 ± 0.148 $^{A\,a}$

Different superscript small letters within the same column indicate significantly different mean values ($p< 0.05$) between different groups.

Different superscript capital letters within the same raw indicate significantly different mean values ($p< 0.05$) between different periods of exposure. CTR, Control; SF, Sodium fluoride (6.1 mg/L.); FM: Sodium fluoride (6.1 mg/L.) + *M. oleifera* extract (1% in ration); ME, *M. oleifera* extract (1% in ration)

Table. 10: Effect of sodium fluoride and / or *M. oleifera* on SOD activity (U/min/mg protein /ml.) in gills tissue of sea bream.

Groups	Period of exposure			
	2nd week	4th week	6th week	8th week
CTR	0.45± 0.0024 $^{A,B\,a,b}$	0.46± 0.0043 $^{A\,a}$	0.45± 0.0017 $^{B\,b}$	0.45± 0.0018 $^{B\,b}$
SF	0.44± 0.0005 $^{A\,b}$	0.43± 0.0018 $^{A,B\,c}$	0.42± 0.0011 $^{B,C\,c}$	0.42± 0.0032 $^{C\,c}$
FM	0.45± 0.0029 $^{A\,a,b}$	0.45± 0.0020 $^{A\,b}$	0.42± 0.0012 $^{B\,c}$	0.42± 0.0036 $^{B\,c}$
ME	0.46± 0.0046 $^{A\,a}$	0.46± 0.0028 $^{A\,a}$	0.46± 0.0039 $^{A\,a}$	0.46± 0.0064 $^{A\,a}$

Different superscript small letters within the same column indicate significantly different mean values ($p< 0.05$) between different groups.

Different superscript capital letters within the same raw indicate significantly different mean values ($p< 0.05$) between different periods of exposure. CTR, Control; SF, Sodium fluoride (6.1 mg/L.); FM: Sodium fluoride (6.1 mg/L.) + *Moringa oleifera* extract (1% in ration); ME, *Moringa oleifera* extract (1% in ration)

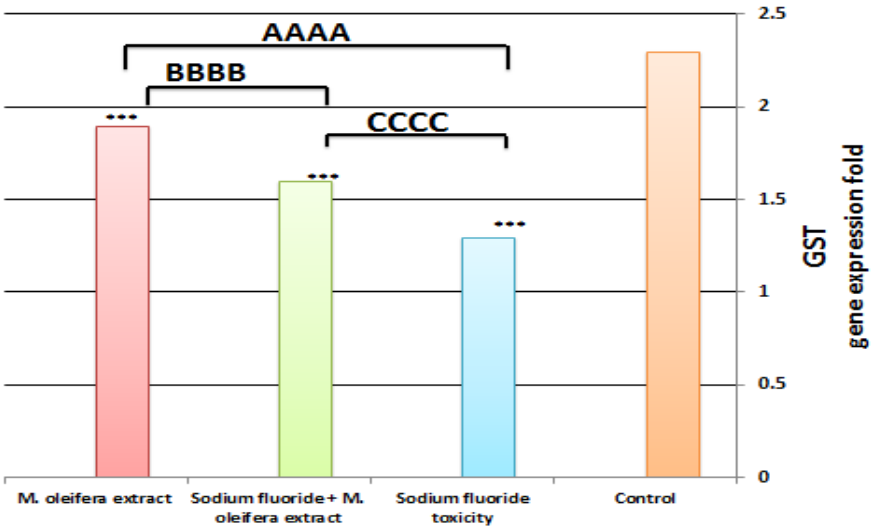

Fig. 3: Effect of Moringa leaves on glutathione S-transferase mRNA expression in sea bream. ***p < 0.001 vs control ^{AAAA} p < 0.001 vs. Sodium fluoride toxicity ^{BBBB} p < 0.001 vs. Sodium fluoride (6.1 mg/L.) + *M. oleifera* extract (1% in ration) ^{CCCC} p < 0.001. vs. *M. oleifera* extract (1% in ration). Triplicate samples were analyzed to obtain an average concentration for each treatment.

DISCUSSION

Fluorine belongs to the VIIA group of the periodic table and is widely distributed in the environment, mainly as fluorite, a calcium salt of the ionic form fluoride (Nielsen, 2009). High fluoride concentrations can induce detrimental effects on cells and organisms as they can act as general inhibitors of various enzymes (Barbier *et al.*, 2010). As regards the aquatic environment, fluoride is present in unpolluted waters at concentrations ranging from 0.01 to 0.3 mg/l; however its level can increase more than 100 times as a consequence of human activities (Camargo, 2003). Fish can accumulate fluoride through the food chain, but few reports have determined the toxicity of dietary fluoride to fish (Yoshitomi *et al.*, 2007). As for the toxic effects, fluoride can lead to reduced male and female fecundity, growth impairment and mortality in aquatic organisms (Casellato *et al.*, 2013; Ballarin *et al.*, 2014). The obtained results showed

that the lethal concentration 50 (LC_{50}) of sodium fluoride in sea bream was 61 mg/L; so the 1/10 dose of LC_{50} of sodium fluoride to induce chronic toxicity was 6.1 mg/L. Due to its high biological activity and small ionic radius, it penetrates easily into the organisms and tissues. It has adverse chronic effects on different tissues (Devi and Piska, 2006 a, b). The clinical sea bream (6.1 mg/L) it started to appear at the 1st week post addition of sodium fluoride and continued until the end of the experimental period (8^{th} week). The clinical signs of the experimentally intoxicated sea bream were showed marked loss of orientation and equilibrium, and increased mucus secretion and finally gasping for breath, reduction of swimming ability, darkening of color, and then death after long term of exposure. Similar results were reported by (Farha *et al.*, 2014) who found that the *T. mossambica* exposed to sub lethal concentration of sodium fluoride (1.5 g /70L and 3.0 g/70L NaF) after 7 and 14 days showed marked apathy then loss of orientation and equilibrium and increased mucus secretion and finally gasping for breath, reduction of swimming ability, darkening of color and then death after long term of exposure.

Many metals are known to be powerful oxidants. Redox active metals such as Cr, Cu and Fluorine deplete major antioxidants in the cell, especially thiol containing antioxidants and enzymes (Pinto *et al.*, 2003).

These enzymatic responses are associated with increased ROS production leading to "oxidative stress" occurring when the ROS generation rate exceeds that of their removal (Ercal *et al.*, 2001; Pinto *et al.*, 2003; Martinez-Alvarez *et al.*, 2005). The leaves of *Moringa oleifera* rich in starch, mineral, iron, vitamin A, B and C, calcium, protein and including the essential sulfur amino acid, methionine and cysteine (Foidl *et al.*, 2001). Chemically Modified *Moringa oleifera* leaves powder was used by (Reddy *et al.* 2012) for optimization of Cd, Cu and Ni biosorption. Removing of Cd from waste water was achieved using fresh leaves as biosorbents (Eman, 2014). Without any exception from the previous mentioned results of fish exposed to 6.1mg. /L of sodium fluoride for 8^{th} weeks the catalase and super oxide dismutase activity of serum and gills tissue of sea bream were decreased. This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation; therefore liver tissue was thought to be the best to present the response of CAT activity to metal exposure (Gül *et al.*, 2004; Avci *et al.*, 2005).

Sensitivity of SOD and CAT activities to metal exposures were also supported with our previous results (Atli and Canli 2008, 2010). They concluded that toxicants may induce different antioxidant/prooxidant responses depending on their ability to produce ROS. The response of the antioxidant system could differ when organisms exposed to metals and some other factors. For instance, (Garcia Sampaio *et al.*, 2008) showed that single-factor sodium fluoride exposure was found to be insufficient to decrease the SOD activity in fish whereas under hypoxia and combined-factors of hypoxia Cu led a significant decrease in its activity. The SOD-CAT system, the first line of defense system against oxidants varied according to the response of fish antioxidant system to counteract with the toxicity of hardness and metal exposures (Garcia Sampaio *et al.*, 2008; Atli and Canli, 2010). Onah *et al.* 2016 reported that *Moringa oleifera* extracts supplementation was associated with significant decreases in the levels of both liver and kidney GST, SOD and CAT.

Alterations in enzymes profile are important pollution indices. Alteration in enzymes activity may be related with the muscles morphology and physiology. Cell membrane permeability lets the enzymes leaching or decrease membrane permeability allows it to accumulate in cell. Cellular damage is another major cause of decrease or increase in enzyme activity result in the inhibition of carbohydrate protein metabolism (FARHA, 2012). Fluoride is an oxidizing agent and a well-known reversible enzymatic inhibitor that inhibits the enzyme activity of at least 80 proteins. Fluoride ions act as enzymatic poisons, disturbing enzyme activity and, finally, interfering metabolic processes such as glycolysis and synthesis of proteins (Camargo, 2003). The elevation of Aspartate amino transferase (AST) and Alanine aminotransferase (ALT) is due to altered liver function due to fluoride toxicity and utilization of amino acids (Devi and Piska, 2006b). *M. oleifera* improved the serum (ALT), (AST), creatinine (SOD) and (CAT) during Lead acetate administration in fish (Christian *et al.* 2016). (Fakurazi *et al.* 2012; Uma *et al.*, 2010) showed that *M. oleifera* leaves protected against acetaminophen-induced liver damage by decreasing liver enzymes and hepatic lipid peroxidation as well as increasing antioxidant enzymes levels. (Sharifudin *et al.*, 2013) also showed that *M. oleifera* leaves and flowers at 200 and 400 mg/kg prevented acetaminophen-induced hepatotoxicity. (Ouedraogo *et al.*, 2013) reported that *M. oleifera* at doses of 150 and 300 mg/kg body weight prevent gentamicin induced nephrotoxicity in rabbits by significantly decreasing the markers of kidney damage including lipid peroxidation, serum creatinine and urea as well as histological changes. (Onah *et al.*, 2016) reported that *M. oleifera* extracts supplementation was associated with significant decreases in the levels of ALP, ALT, and AST, GGT, bilirubin, urea, creatinine and uric acid. Results showed decrease in total protein levels in serum of sea bream exposed to 6.1 mg. /L sodium fluoride for 8th weeks**.** Furthermore, there were statistically significant changes in levels of total protein in serum of groups exposed to sodium fluoride and/or *M. oleifera* groups. On the same manner, (Kumar *et al.*, 2007 ; Bajpai and Tripathi, 2010) found that the toxicity of fresh water catfish exposure to two sub-lethal doses of fluoride NaF (35 mg F ion/L and 70 mg F ion/L) for 90 days after their a significant decrease of total protein occurred. The (Devi and Piska, 2006a) found that the

fluoride decrease of the tissue proteins of fresh water cat fish. The decreased levels of protein in the serum, liver, kidney, muscle, and brain of NaF exposed fish were found by (Kumar *et al.,* 2007; Bajpai and Tripathi, 2010). Results indicated that there were statistically significant increases in the levels of albumin in the serum of sodium fluoride + *M. oleifera* group. But levels of albumin in the serum of sodium fluoride group decreased significantly in comparison to the control group. On the same manner, the (Fırat *et al.*, 2011; JIANJIE *et al.*, 2013) reported that the elevated sodium fluoride cause decrease of albumin and globulin in fish.

A significant increase of creatinine in serum of sea bream occurred at sodium fluoride exposure group to 6.1mg. /L. for 8th weeks. Moreover, an increase in the level of creatinine in serum of sea bream exposed to sodium fluoride + *M. oleifera*, but it was significantly higher ($P<0.05$) only at sodium fluoride exposure of sea bream to 6.1mg. /L. for 8^{th} weeks. Fluoride ions have been reported to act as enzymatic poisons, inhibiting enzyme activities and ultimately, interrupting metabolic process (FCDSW, 1984). (Onah *et al.*, 2016) reported that *M. oleifera* extracts supplementation was associated with significant decreases in the levels of bilirubin, urea, creatinine and uric acid. This study showed lead induced changes in some liver and kidney function parameters as well as some oxidative markers of these organs and also revealed possible amelioratory effects to these changes after *Moringa oleifera* extracts supplementation.

A large cluster of oxidative metabolism genes (GST) showed a tight correlation in their mRNA levels in our analysis, particularly in the liver. These genes are under the control of AREs in many systems (Jones *et al.*, 2007), and their coordinate expression may reflect variations in the redox status of the fish. In this study, a decreased in GST activity of *sea bream* was recorded after 7th day sodium fluoride exposure in contrast to *M. oleifera* + sodium fluoride exposure. Decreased GST activity could also be due to compensate the ROS impact on antioxidant system with other antioxidant enzymes. (Dautremepuits *et al.*, 2004) observed a decrease in antioxidant enzyme activity in the liver of *Cyprinus carpio* exposed to Cu and they indicated that excess Cu causes a rapid GSH oxidation even at low non-toxic Cu concentrations in hepatocytes followed by GST depletion. However, it was also interesting to see that total GSH level was not altered in where alterations in GST activity occurred. *M. oleifera* is an important dietary antioxidant and significantly decreases the adverse effect of reactive species such as reactive oxygen that can cause oxidative damage to macromolecules such as lipids, DNA and proteins which are associated in several diseases (You *et al.*, 2000).

CONCLUSION

On the basis of present findings it could be concluded that increased sodium fluoride content in water causes adverse effect on fish. The changes of plasma biomarkers as antioxidant enzymes; aspartate amino transferase; alanine amino transferase and creatinine were the physiological responses of sea bream to the stress of sodium fluoride exposure. *Moringa oleifera* tree can be grown to produce more natural products and environmentally friendly materials.

CONFLICT OF INTEREST STATEMENT

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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