Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(5): 1103 – 1118 (2022)
www.ejabf.journals.ekb.eg

The prevention impact of the green algal extract against genetic toxicity and antioxidant enzyme alteration in the Mozambique tilapia

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ARTICLE INFO

Article History:
Received: Sep. 13, 2022
Accepted: Oct. 3, 2022
Online: Oct. 21, 2022

Keywords:
Oreochromis mossambicus,
Dioxin,
ROS generation,
DNA damage,
Gene expression

ABSTRACT

Algal studies are primary for ecological risk assessment and toxicology by evaluating lethal and sub-lethal toxic impacts of potential toxicants on inhabitants of numerous ecosystems. *Dunaliella salina*, a green marine alga, is characterized by its carotenoid accumulation and is widely used in many health and nutritional products. Our experiment was designed to evaluate algal extract's ability to inhibit genetic alterations induced by mutagen agents such as dioxin in the Mozambique tilapia. The expression of three stress genes was examined: heat shock protein 90 (Hsp90), CYP1A1 as one of the main cytochrome P450 enzymes, and metallothionein (MT). The study exhibited a characteristic sensitivity to metal treatments. Liver samples were collected from all fish to analyze bio-indicators, including superoxide dismutase (SOD), malondialdehyde (MDA), and reactive oxygen species (ROS). While gills samples were used for DNA fragmentation assay. Results showed that oxidative stress in the dioxin group's liver significantly changed indicators. However, the dioxin group significantly increased the SOD, MDA enzyme activities, and ROS formation. Interestingly, the genes Hsp90, CYP1A1, and MT expression were significantly down-regulated in *Dunaliella salina* groups. Nevertheless, DNA fragmentation in gill organs was affected by exposure to dioxin in fish. Thus, it was concluded that the methanolic extract of an isolated strain *Dunaliella salina* is effective against mutagen agent dioxin by inhibiting genetic alterations in fish organs with an antioxidant defense system to conquer oxidative damage.

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INTRODUCTION

Extensive research has identified deterioration in aquatic ecosystems over the past era **(Ameur *et al*., 2012)**. Fish Biomarkers can be utilized as early warning signs in marine environments. Pollution may permit environmental management to alleviate potential adverse impacts at higher degrees of biological organization **(Hassanin & Kaminishi, 2019; Abdel-Gawad *et al*., 2020)**.

The discharge of chemical pollutants into the aquatic environment has toxic effects on marine organisms. It has been postulated that humans consume organochlorinated chemicals mostly through fish diet **(Abdel-Gawad *et al.*, 2018; Sciuto *et al*., 2018)**. Fortunately, an enormous emphasis on risk assessments of chemicals linked to hormone system disruption in living organisms, particularly wildlife and humans started only a few decades. Cytochromes, particularly CYP1A1 is the main oxidative enzyme affected by anthropogenic chemicals. Most heat shock proteins (HSPs) respond rapidly to stress, thus can be used as biomarkers of exposure to different compounds like organic compounds in fish **(Faheem *et al*., 2020)**.

Dioxins represent a group of organic micropollutants classified as persistent organic pollutants. They are very poisonous to humans and persist in the lipid component of cells and tissues, allowing them to infiltrate the human food chain. These compounds cause developmental and reproductive damage in fish, birds, and mammals exposed to them in the environment **(Sciuto *et al*., 2018)**. Dioxin can induce oxidative stress, leading to impaired DNA fragmentation in fish **(Canesi & Fabbri, 2015)**. Oxidative stress is an essential factor in induced toxicity. Antioxidant enzyme activities such as superoxide dismutase (SOD) and lipid peroxidation levels malondialdehyde (MDA) are used as oxidative stress biomarkers indicating an early warning of environmental pollution **(Nel *et al*., 2006; Rezayian *et al*., 2019)**.

Given that microalgae are in direct contact and interact with pollutants in the marine systems, monitoring the effects of microalgae on toxic chemicals, such as endocrine disruptors is of considerable importance **(Ebenezer *et al*., 2013; Rasheed *et al.*, 2019)**. Microalgae represent an important source of biologically active compounds. On the other hand, their large-scale commercial production began barely a few decades ago **(Becker, 2004; Christaki *et al.*, 2011)**. *Dunaliella salina* has been utilized as a model organism in the molecular mechanism studies for over than 100 years. *Dunaliella* has attracted great attention for promoting health-modulating impacts and participating in decreasing the risk of developing various metabolic diseases **(El-Baz *et al*., (2019)**.

Natural bioactive chemicals found in microalgae are beneficial to human health. Among these, strong antioxidants such as carotenoids and polyphenols were highlighted. These chemicals control the gene expression of enzymes that defends against oxidative

stress in addition to scavenging reactive oxygen and nitrogen radicals **(Zanella & Vianello, 2020)**. Therefore, the objective of this study was to assess the effect of marine microalgae *Dunaliella salina* in reducing fish genetic alterations and overcome oxidative damage associated with water pollutants.

MATERIALS AND METHODS

Materials

Reagents for water quality analysis, determination of total protein, biochemical and molecular analyses were purchased from Sigma Aldrich and Invitrogen (Germany). All of the chemicals and reagents used were of the highest purity.

Extraction of *Dunaliella salina*

D. salina was cultivated, harvested, and methanolic extracted at National Research Centre (NRC) according to the method of **Bassem *et al*. (2020)**.

Fish experiment

Fish under study (Mozambique tilapia, *Oreochromis mossambicus*) were obtained from the National Research Centre farm (Nubaria, Egypt). Fish were transported to the Biotechnology and Biodiversity Conservation Laboratory in big plastic water containers with de-chlorinated tap water (24.52.1°C and pH 7.2-8.2) and battery aerators as a source of oxygen.

Experimental design

After the adaptation period, Mozambique tilapia fish were divided into the following groups: The 1st group contained fish with only standard fish diet; the 2nd to the 4th group, fish were fed different concentrations of marine algae (*Dunaliella salina*) in addition to standard fish feed as follows; 5%, 10% and 20%. Whereas, fish groups from 5-7 were introduced fish diets with 5%, 10% and 20% of *Dunaliella salina* microalgae meal in addition to dioxin (1/10 of the LD50~22 mg/kg), respectively. The 8th group was fed only on a diet with dioxin, with concentration of 1/10 of the LD50~22 mg/kg. The experiment lasted for one month. On the other hand, one group of fish (the control)was sacrificed at the beginning of the investigation to determine the toxicity in the DNA from the polluted environment by dioxin and to be compared later with other groups after feeding.

Bacteriological quality

Total coliform was determined in water samples throughout the experiment by MPN/100 method, the most probable number techniques (MPN) according to **APHA (2012)** and **Sabry *et al*. (2018)**.

Enzyme activity analysis

The activities of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured in liver samples following the procedures of **Ma'rifah *et al*. (2019)**. Fish were sacrificed on the final day of the experiment; the liver was carefully extracted, weighed and diced into minute portions, washed in ice-cold phosphate-buffered saline (PBS, 0.01 M, pH = 7.4) to eliminate excess blood, and kept at 20°C until further analysis (5 days). Within 10 minutes of administering the stop solution, the optical density was measured at

450nm. Using the relevant standard curves, the concentration of MDA and the activity of SOD were measured, and the results were represented as nmol/mg for MDA and U/mg for SOD. All data were normalized to the liver's weight.

DNA fragmentation analysis

Quantitative analysis of DNA fragmentation

Genomic DNA fragmentation was determined in fish tissues according to **Lu *et al*. (2002)**. For laddering nuclear DNA, the phenol/chloroform method was used to isolate the genomic DNA. Afterwards, the DNA was electrophoresed on an agarose gel and stained in Tris-acetate/EDTA with ethidium bromide. By subjecting the gels to UV transillumination, the DNA fragments were visualised and photographed. To measure the rate of DNA fragmentation, the diphenylamine reaction method **(Khalil *et al*., 2011)** was carried out. The tissues were homogenized and lysed in lysis buffer and then centrifuged at 4°C at 12 000rpm. Afterwards, the obtained pellets were re-suspended in lysis buffer. Thus, the supernatants (S) and cell pellets (P) were incubated at 4°C overnight with tri-chloroacetic acid (TCA). After centrifugation, the pellets were re-suspended in TCA and incubated for half an hour at 80°C. The samples were incubated overnight with DPA solution at room temperature. The rate of DNA fragmentation at 600nm was measured and calculated using the following formula: %Fragmented DNA= [OD(S)/ OD(S) + OD (P)] X 100.

Qualitative analysis of DNA fragmentation

Fish gills tissues were used to determine apoptotic DNA fragmentation using the nuclear DNA laddering pattern, following the method of **Lu *et al.* (2002)**. The tissues were homogenized and lysed overnight at 37°C in 0.5mL of DNA lysis buffer, containing 50 mM Tris-HCl, 10 mM EDTA, 0.5 percent triton, and 100 g/mL of proteinase K (pH 8.0). The lysate was then incubated for two hours at 37°C with 100g/ mL of DNase-free RNase, followed by three extractions of equal amounts of phenol/chloroform (1:1 v/v), and a re-extraction with chloroform by centrifuging at 15,000 rpm for 5 minutes at 4°C. At -20°C, the isolated DNA was precipitated in two volumes of ice-cold 100% ethanol with a 1/10 volume of 3 M sodium acetate, and a pH value of 5.2 at -20°C for one hour **(Khalil *et al*., 2011)**. This step was followed by a 4°C centrifuge for 15 minutes at 15,000 rpm. The DNA pellet was air-dried and dissolved in 10 mM TrisHCl/1 mM EDTA, pH 8.0 after being washed with 70% ethanol. In a Tris/acetate/EDTA (TAE) solution, the DNA was electrophoresed on a 1.5 percent agarose gel and stained with ethidium bromide (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). As a molecular size marker, a 100-bp DNA ladder (Invitrogen, USA) was utilized, and DNA fragments were imaged using UV transillumination.

Assessment of reactive oxygen species (ROS) formation

A flow cytometer assessed the generation of intracellular ROS molecules in fish tissues according to **Elateek *et al*. (2021)**. The liver samples were homogenized, single-

cell suspensions were formed, and a fluorescent probe as an oxidation-sensitive DCFH-DA probe was utilized. During the reaction, fish cells were easily taken up the DCFH-DA into cells. Hence, through the action of cellular oxidants, oxidation of the DCFH compound into fluorescent dichlorofluorescein (DCF) was performed. The flow cytometer measured the fluorescence emission (with excitation 488 nm and emission 525 nm). The approximate number of cells counted was 1×10^5 cells for each treatment, each of which was performed in triplicate.

Expression analysis of stress related genes

RNA isolation

To separate total genomic RNA from the liver tissues of all treated fish, TRIzol® extraction chemical (Invitrogen) was used. The RNA pellet was stored in DEPC treated water once the isolation operations were completed. The pellet of extracted RNA was processed with an RNAse-free DNAse kit to digest any potential DNA remnants (Invitrogen, Germany). RNA aliquots were kept at -20°C or used for reverse transcription right away **(Salem *et al*., 2018)**.

Reverse transcription reaction

First, the cDNA copy was synthesised from liver tissues using the Strand cDNA Synthesis Kit (RevertAidTM, MBI Fermentas) via reverse transcription reaction (RT). The cDNA copy of the liver genome was obtained using an RT reaction program including 10 minutes at 25°C, one hour at 42°C, and 5 minutes at 95°C. Finally, tubes containing cDNA copy were placed on ice and used for cDNA amplification **(Khalil *et al*., 2018)**.

Quantitative real time-PCR

The qRT-PCR analyses were performed with the SYBR® Premix Ex TaqTM kit (TaKaRa, Biotech. Co. Ltd.), utilizing the synthesized cDNA copies from liver tissues. A melting curve profile was conducted for each reaction. The expression of a housekeeping gene was used to standardize the quantitative values of the target genes (Table 1). The quantitative values of specific genes to the GAPDH gene were determined using the $2^{-\Delta\Delta CT}$ method.

Table 1. Primers Sequences Used for PCR

Gene	Primer sequence (5´ –3´) a	Accession No.
HSP 90	F: CAACGCAGACTCACTTCACC R: GCGACTCAGAGCTGTAGACT	GR675649.1
CYP1A1	F: GTTTCTGACTCCTCCCTCCC R: AGAATAGATGACACCCCGGC	GR703248.1
MT	F: CAAGACTGGAACCTGCAACT R: CTGGTGTCGCATGTCTTTCC	S75042.1
GAPDH	F: TGTTCGTCATGGGTGTCAAC R: CGTCAACCGTCTTCTGTGTG	AY140649.1

* F: forward primer; R: a reverse primer.

Statistical analysis

The data obtained from enzyme activity analysis, and the DNA fragmentation analysis and assessment of ROS formation were examined using the General Linear Models (GLM) procedure of the Statistical Analysis System **(SAS, 1982),** followed by Scheffé-test to detect the significant differences among fish groups. The values were expressed as mean±SEM. The statements of significance were based on the probability of $P < 0.05$.

RESULTS

Total coliform count

The results reported in Fig. (1) indicate that the total coliform counts were not detected in the control and microalgae 20 % group water samples. While, they were 5.0 (log MPN/100 ml) at dioxin group. Non-significant differences were detected among water samples in algae plus dioxin group (10% and 20%).

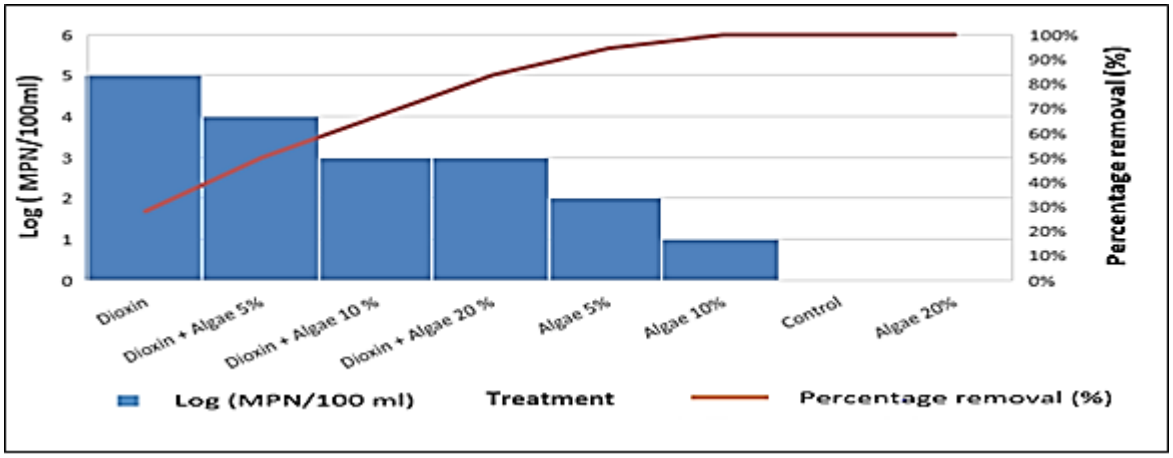

Fig. 1. Average count of total coliform (log MPN/100ml) and total coliform (%) removal upon the addition of algae to fish diet

Enzyme activity

The results of Mozambique tilapia liver are presented in Fig. (2A, B). Oxidative stress indicators included antioxidant enzymes (SOD) as well as non-enzyme antioxidant (MDA). Enzymes activities of SOD and MDA were higher in fish liver samples fed diet mixed with dioxin. The increased liver SOD activity is due to advises of a higher formation level of intracellular H_2O_2. The SOD activity of dioxin fish was significantly elevated, compared to the other fish groups. MDA production was significantly reduced in the liver samples of those fed on algae in comparison to fish group treated with algae and dioxin. Decreased MDA rates in aquatic species exposed to pollutants is often related to increase levels of antioxidant activity.

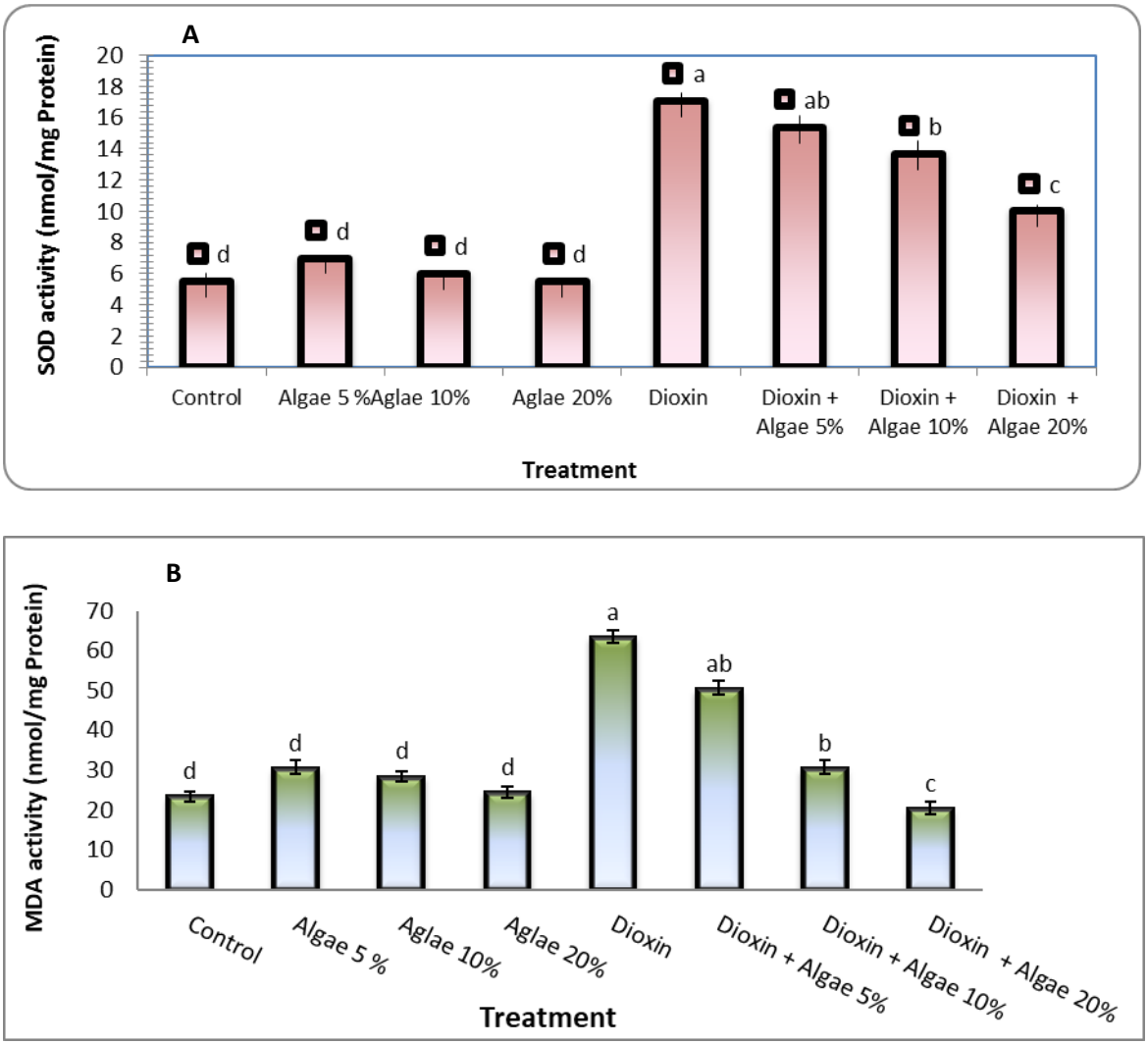

Fig. 2. Enzyme activities analysis of SOD (A) and MDA (B) in fish fed on algae and/or dioxin Data are presented as mean ± SEM. [a,b,c,d:] Mean values of tissue with unlike superscript letters were significantly different ($P< 0.05$).

DNA fragmentation in fish

Fig. (3A, B) show that *Oreochromis mossambicus* treated with microalgae and the control group exhibited the smallest percentage of DNA fragmentation. Whereas, the combined diet of *Dunaliella salina* and dioxin induced DNA fragmentation elevation with respect to the microalgae group. The gill is a metabolically active organ in fish and can accumulate more polluted compounds than other organs. Our results revealed a relationship of dioxin concentration in gills tissues and DNA fragmentation of fish.

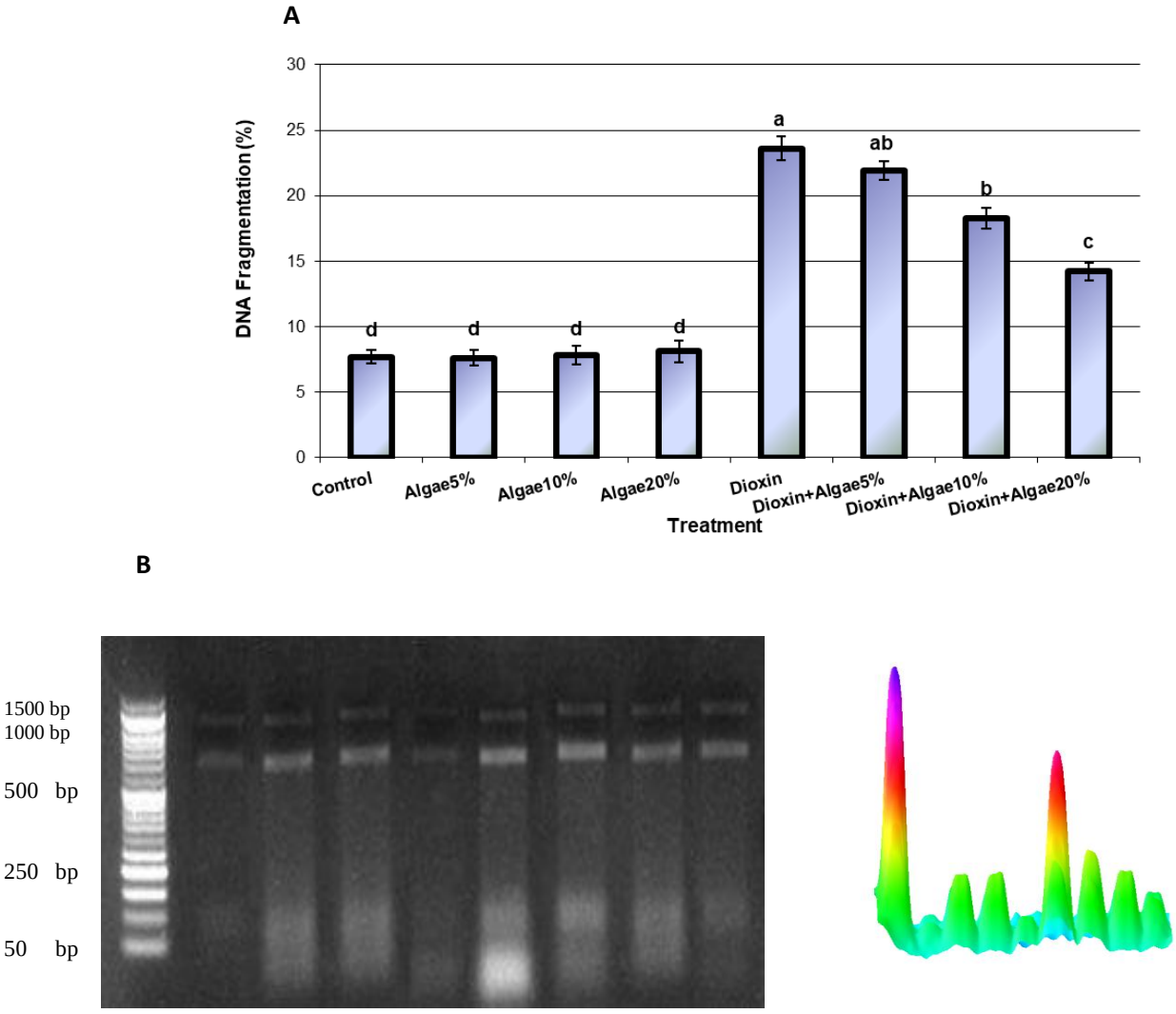

Fig. 3. (A) DNA fragmentation detected by diphenylamine reaction method **(B)** Agarose gel in fish fed on algae and/or dioxin.

M: DNA marker, Lane1: control fish, Lanes 2-4: fish fed 5%, 10% and 20% algae, respectively, Lane5: fish exposed to dioxin, Lanes 6-8: fish exposed to dioxin plus 5%, 10% and 20% algae, respectively. Data are presented as mean ± SEM. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$).

ROS formation assay

The actual levels of ROS determined in fish sample are presented in Fig. (4). The results indicate a significant difference between fish fed on algae group (5%, 10% and 20%) and the algae plus dioxin group (5%, 10% and 20%). High generation of ROS leads to exceeding cells' antioxidant defence known as "oxidative stress". Cells under oxidative stress exhibited various dysfunctions owing to damage caused by ROS to lipids, proteins in addition to DNA. Hence, the experiment showed that the activity of ROS in the fish fed on algae plus dioxin was significantly higher than that in the algae group.

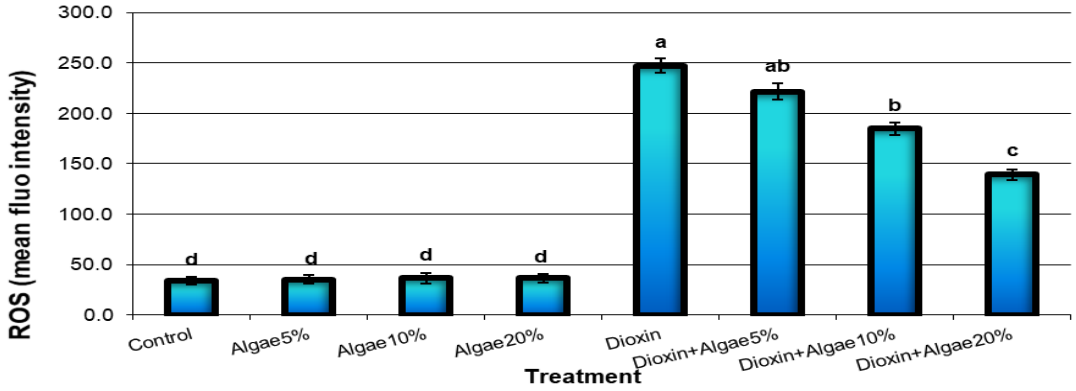

Fig. 4. Intracellular ROS levels in tissues of fish fed on algae and/or dioxin

Data are presented as mean ± SEM. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$).

Effect of dioxin on the induction of stress-related genes

Gene expression changes in Hsp90, Cyp1a1 and MT were evaluated in livers treated with dioxin only and *Dunaliella salina* plus dioxin as displayed in Figs. (5- 7). Hsp90 levels were determined by real-time PCR. As shown in Fig. (5), Hsp90 decreased significantly in control but increased significantly in the groups treated with dioxin alone and in case of dioxin with a low dose of *Dunaliella salina*. These were due to heat shock proteins generally rising in response to stress. Fig. (6) shows that cytochrome P450 results indicate upregulation of Cyp1a1 expression in the liver of fishes exposed to dioxin alone and in case of dioxin with a low dose of *Dunaliella salina.* In addition, metallothoine in levels expression were downregulated in all other groups treated with/ or without *Dunaliella salina* (Fig. 7).

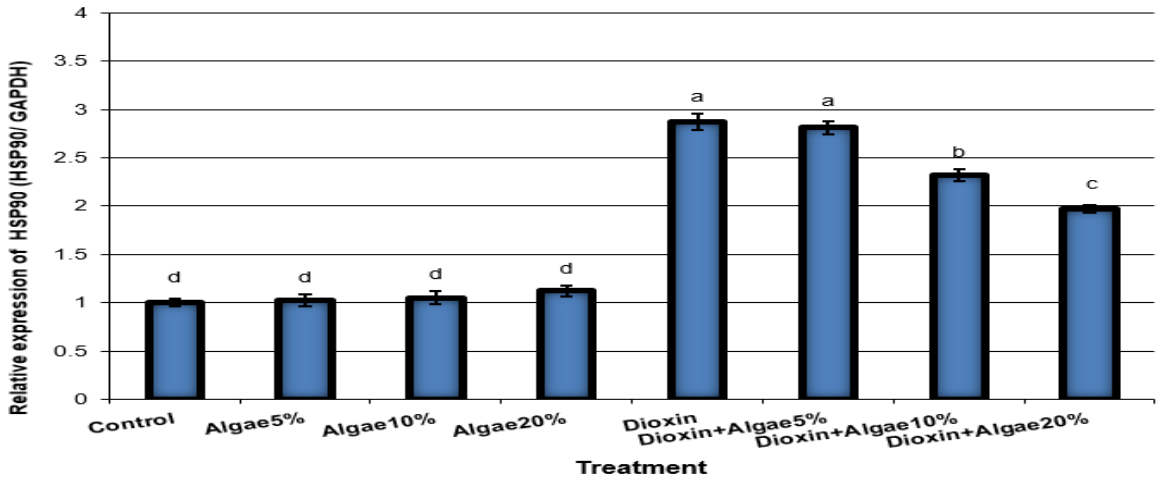

Fig. 5. The expression alterations of HSP90 gene in liver tissues of fish fed algae and/or dioxin

Data are presented as mean ± SEM. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$).

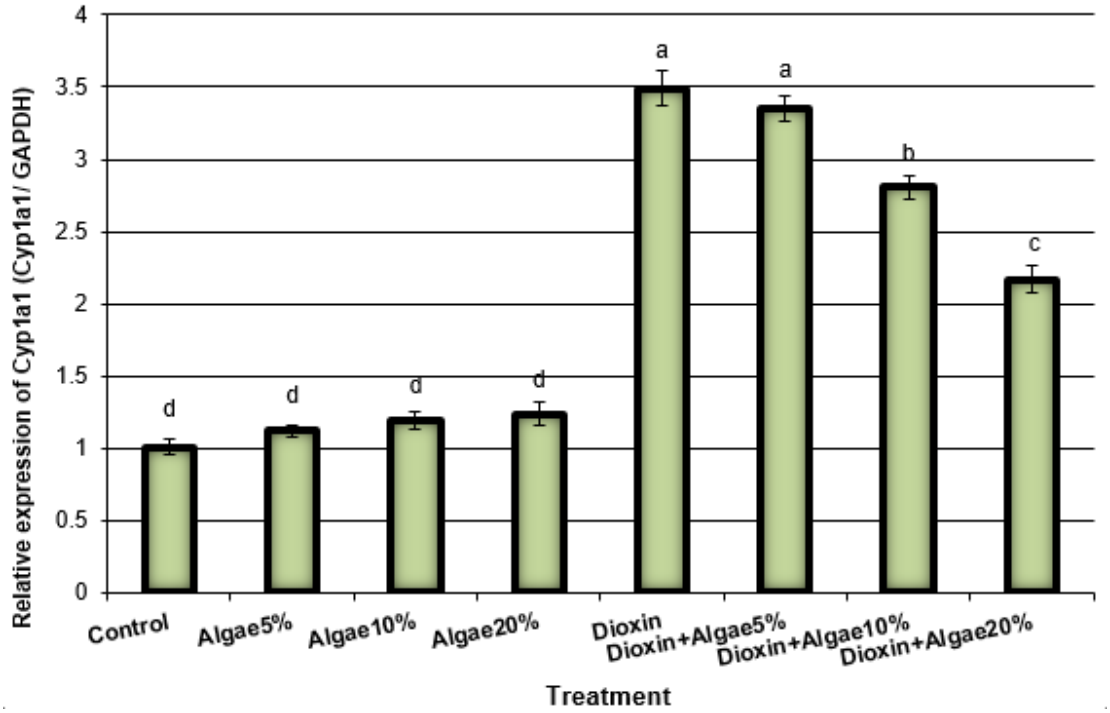

Fig. 6. The expression alterations of Cyp1a1 gene in liver tissues of fish fed algae and/or dioxin Data are presented as mean ± SEM. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$).

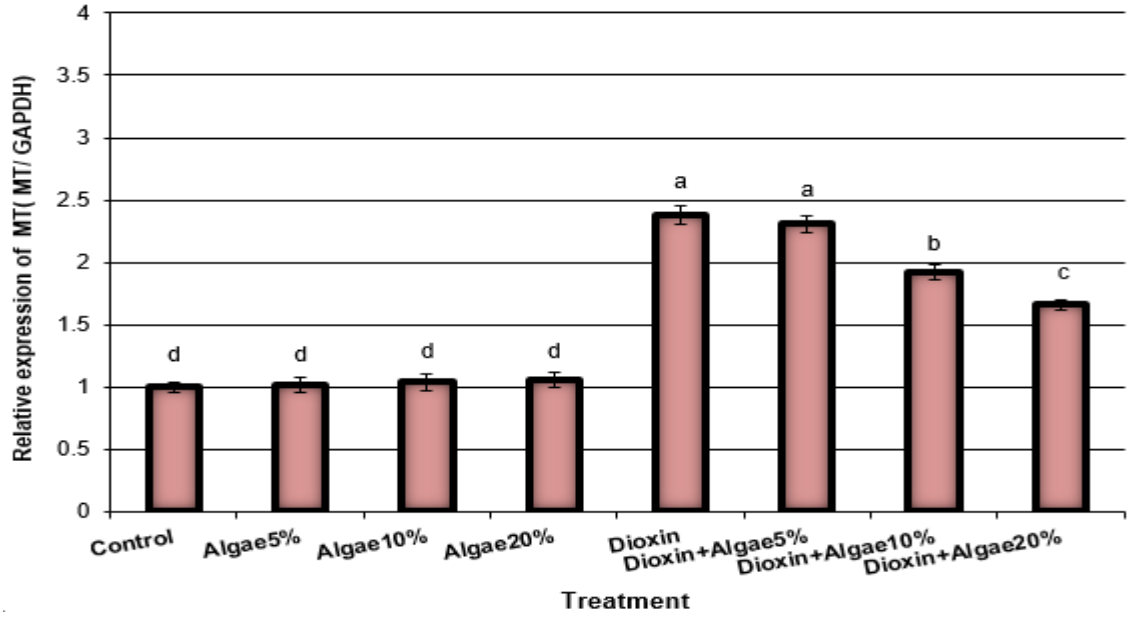

Fig. 7. The expression alterations of MT gene in liver tissues of fish fed algae and/or dioxin

Data are presented as mean ± SEM. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$).

DISCUSSION

Global demand for algal diets is growing. Marine microalgae are progressively consumed for the functional benefits of health and nutrition. Algal-derived food products have a lot of evidence of their health advantages. It has been documented that organic pollutants such as dioxin-like compounds could be released into the aquatic environment, accumulate in fish, and subsequently induce potential risks on fish gene expression and enzyme activity **(Caballero *et al.*, 2016)**. In line with the data obtained from this study, dioxin could induce oxidative damage to the liver of Mozambique tilapia. However, mutagenic chemicals produce reactive oxygen species, which can react with essential macromolecules, viz. lipids, proteins, and nucleic acids, both naturally and during metabolism. A balance between production and the removal of oxygen free radicals is always maintained in natural conditions. While, the imbalance in this mechanism would result in oxidative stress and pathogenic alterations in various cells. The main cause of brain and other organ damage caused by mutagenic chemicals is lipid peroxidation of the cell membrane **(Mehrepak *et al.*, 2016)**. Several studies reported that common carp (*C. Carpio*) exposed to a toxic condition induced DNA oxidation and oxidative damage, as well as gene expression alterations in various tissues and organs **(Mustafa *et al.*, 2011, 2012)**.

The assessment of contaminants' impact on organisms considering the alterations of significant enzymes in specific processes is commonly used to study the state of oxidative stress **(Huang *et al.*, 2020)**. SOD is considered as the first line of defence against oxidative stress. Compared to the control group, SOD activity increased significantly in dioxin groups in the current experiment. Excessive ROS formulation in fish after being fed on dioxin plus marine algae, which exceeds the ability of SOD to eliminate ROS, explains the results. According to **Li *et al.* (2016)**, SOD activity in the *Carassius auratus* liver was inhibited after exposure to high concentration of fluorinated PFDDs. Moreover, MDA is an essential degradation product of lipid hydroxides often used as an effective bio-indicator for evaluating lipid hydroxides under oxidative stress of aquatic species. There is a correlation between the level of MDA and free radicals in body cells **(Dong *et al.*, 2018)**. In the present study, a significant increase was detected in the MDA contents of algae and dioxin groups at different concentrations. This increase in fish liver may indicate a significant time-dependent effect. Therefore, it can be inferred that exposure to mutant chemicals at high concentrations caused excessive production of ROS in a short time. However, the antioxidant system's ability to remove ROS was restricted, and the residual oxygen free radicals attacked the biofilm's polyunsaturated fatty acids, causing lipid peroxides and an increased MDA content **(Huang *et al.*, 2020)**. In this experiment, the high MDA content in dioxin-treated groups was evident, indicating that dioxin was more likely to induce the generation of ROS in fish's liver, causing damage to the cell membrane.

Surprisingly, the increase in DNA strand breaks due to oxidative stress could be linked to ROS generation in the cells exposed to mutagenic agents. The antioxidant defence status as determined by the levels of antioxidant enzyme activities (such as SOD in the liver tissues) plays a vital role against toxicity-induced genetic changes. The above suggested that it is either that the level of oxidative stress was insufficient to induce a defence response, or the timing of antioxidant defence response differed from the interval between stress application and subsequent tissue/cell sampling **(Aniagu *et al*., 2006)**. Despite slight increases in DNA oxidation in the current study, the expression of genes involved in DNA repair remained high in the liver cells of fish fed natural products such marine algae. Several research on animals and humans have proved the health benefits of omega-3 fatty acids **(Simopoulos, 2008; Rasmy *et al*., 2011)**. These studies proposed that omega-3 fatty acids improve serum lipid levels and are beneficial against diseases associated with oxidative stress, such as cancer and obesity. Notably, omega-3 fatty acids differ from other fatty acids in relation to their storage as triglycerides in mature adipocytes. Omega-3 fatty acids can diminish adipose tissue lipid substances and suppress adipocyte growth **(Ruzickova *et al*., 2004)**. Although marine fish is the most common commercial source of omega-3 fatty acids, the marine microalga *Dunaliella salina* was used as an alternative source of omega-3 fatty acids in this study. It was found that fish with dioxin & *Dunaliella salina* extract decreased the mRNA expression levels of Hsp90, Cyp1a1 and MT significantly, reducing the antioxidant enzymes activities and the DNA damage. In this context, **Yook *et al*. (2015)** found that dietary omega-3 fatty acids were suppressors of anabolic lipid genes and promoters of catabolic lipid genes, which is consistent with the present finding.

Moreover, *Dunaliella salina* extract reduced the rate of DNA damage in fish in the current study. These findings support previous research suggestig an extract rich in omega-3 fatty acids and carotenoids, which are important scavengers against the production of numerous free radicals including the reactive oxygen species (ROS) **(Zhu & Jiang, 2008)**. The unicellular green microalgal may be beneficial in the production of bioactive chemicals such as n-6/n-3 PUFA and carotenoids. Omega-3 fatty acids and 9-cis-carotene content, which have been reported to limit DNA damage and genetic alterations, might therefore be linked to the protective action of the present *Dunaliella salina* algal extract **(Xue *et al*., 2008)**. *D. salina* contains chlorophyll-α, violaxanthin, and veucherxanthin, which could enhance fish immune system and used to prevent viral nervous necrosis (VNN) infection in cantang grouper. Upon the addition of algal extract to fish feed, a decline was detected in the proportion of Nf-kB expression in the brain of a VNN-infected cantang grouper **(Khumaidi *et al*., 2015; Yuwanita *et al*., 2020)**.

CONCLUSION

In conclusion, the current study reveals that dioxin can produce considerable alterations in SOD and MDA in the liver of fish after a month of exposure. These findings suggest that dioxin exposure may enhance ROS generation, causing oxidative damage in fish liver. Furthermore, growing outspread exposure to various polluted chemicals causes DNA damage in fish. We suggest that Mozambique tilapia can be utilized as a model to predict the risks that exposure to dioxin-like compounds poses to feral fish populations. The results of the current study may propose that *Dunaliella salina* was able to prevent the genetic alterations induced by dioxin in Mozambique tilapia organs.

Additionally, due to the high levels of omega-3 fatty acids and carotenoids in *Dunaliella salina* extract, it was able to reduce the rate of DNA damage in fish. Carotenoids are regarded a key scavenger against generations of many free radicals, including reactive oxygen species. The data obtained in this study may be used as a base for further evaluation of *Dunaliella salina* against other organic micropollutants compounds. Unfortunately, a critical number of these compounds are a part of the environmental chain pollution, making it difficult to evaluate their potentially harmful effects. Therefore, we should emphasize the inhibition of organic aquatic pollution.

Acknowledgements

This work was financially supported by the Biotechnology and Genetic Conservation group laboratory, Water Pollution Research Centre of Excellence for Advanced Sciences (CEAS), National Research Centre, Egypt.

Author contributions

All authors contributed to the conception and design of the experiments. All the authors gave their approval for the final version of the manuscript.

Compliance with ethical standards

The research described herein was performed on Mozambique tilapia (*Oreochromis mossambicus*). This study was conducted in strict accordance with the guidelines of the Ethical Committee, National Research Centre, Egypt, on the care and use of animals for scientific purposes.

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