Evaluation of the Anti-Genotoxicity and Growth Performance Impacts of Green Algae on *Mugil cephalus*

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Abstract: Fish meal has traditionally been used as a major ingredient in commercial aquatic feeds as the most important source of protein. However, fish meal is an expensive feed ingredient and the supplies often vary unpredictably because of overfishing or large-scale transient oceanic changes. Algae have received attention as suitable alternative protein sources for farmed fish since their protein content and production rate are high. On the other hand, pollution of the aquatic environment has become a major concern of society. Perhaps one of the more serious concerns is the potential for exposure to substances that are genotoxic. In the present study *Mugil cephalus* were fed fish diet contains 10%, 20% and 30% of *Ulva lactuca* or *Caulerpa prolifera* for 8 weeks. The results revealed that addition of small amounts of *Caulerpa prolifera* meal to fish diets resulted in considerable effects on growth, body composition, stress responses, liver function and DNA protection. The results of the current study demonstrate that *Caulerpa prolifera* could be as fish nutrient when be added to the fish diet for several reasons: (a) it improves the fish diet; and (b) it enhances the DNA repairing of fish and therefore inhibit fish diseases.
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Key words: *Mugil cephalus*, *Caulerpa prolifera*, *Ulva lactuca*, Fish meal replacement, Growth performance, DNA damage; Gene Expression.

1. Introduction

Although no other food producing sector is growing as fast as aqua-culture, improvement of marine fish production is important for increasing the aquatic food production, which is essential to overcome the deficiency of animal protein in Arabian countries. In the years 2004–2009 worldwide Nile tilapia, *Oreochromis niloticus* (L.), production increased from about 1.46 million metric tons (mt) to 2.54 mt; an increase of 74% in only 5 years (FAO 2011).

Fish meal has traditionally been used as a major ingredient in commercial aquatic feeds as the most important source of protein. However, fish meal abundance and price are factors strongly influencing costs of compound feeds and therefore of intensive and semi- intensive aquaculture operations. Over the past decade, fish meal production has at best kept stable but the price has increased (New and Wijkström 2002; Naylor *et al*. 2009). As protein is the most expensive component in aquaculture feeds, the need to find alternative plant protein sources to replace fish meal in aquaculture diets has been a research challenge for the last 30 years (Viola *et al*. 1982). Several plants are commonly used or are under evaluation as alternative protein sources in fish feeds, for example, soybeans (Webster *et al*. 1992), rapeseed/canola (Mwachireya *et al*. 1999), *Jatropha sp*. (Kumar *et al*. 2010), peas (Borgeson *et al*. 2006), duckweed (Fasakin *et al*. 1999), lupins (Chien and Chiu 2003) and flax (Drew *et al*. 2007).

Algae have received attention as suitable alternative protein sources for farmed fish since their protein content and production rate are high. Macro- and microalgae, such as *Ulva*, *Ascophyllum*, *Laminaria*, *Undaria*, *Porphyra*, *Spirulina*, and *Chlorella*, have been evaluated as feed additives in earlier studies. The addition of small amounts of algae meal to fish diets resulted in considerable effects on growth (Hashim and Maat-Saat 1992; Wassef *et al*. 2001; Azaza *et al*. 2008), feed utilization (Nakagawa *et al*., 1984), lipid metabolism (Nakagawa *et al*. 1984; Nakagawa *et al*. 1987), body composition (Nakagawa *et al*. 1986; Nakagawa *et al*. 1987), stress responses (Nakagawa *et al*. 1984), liver function, disease resistance (Nakagawa *et al*. 1997), and carcass quality (Hashim and Maat-Saat 1992). Algae are receiving increasing attention as a possible protein source for fish diets, particularly in tropical developing countries where algal production rates are high.

Pollution of the aquatic environment has become a major concern of society. The Red Sea is a deep semi-enclosed and narrow basin connected to the Indian Ocean by a narrow sill in the south and to the Suez Canal in the north. Oil industries in the Gulf of Suez, phosphate ore mining activities in Safaga-Quseir region and intensified navigation activities are pollution sources that could have serious genotoxicity

impacts on the marine environment and the coastal ecosystems of the Red Sea (El Mamoney and Khater 2004).

So the main objectives of the present study are: examine of the effect of feed supplementation with green algae on histopathological features and genotoxicity in *Mullets sp.* collected from polluted area in Red Sea; evaluation the effect of different dietary quantities of the green algae on the growth of *Mullets sp.* and determine the gene expression of growth hormone and Insulin-like growth factor-1 genes in relation to green algae supplementation using Real-time PCR.

2. Materials and Methods

2.1. Materials and Chemicals

Reagents for DNA Fragmentation, Micronucleus Test, Real Time-PCR kits and primers were purchased from Sigma-Aldrich and Invitrogen (Germany). All reagents and chemicals were of the highest purity available.

2.2. Experimental Animals

Eighty *Mugil cephalus* fish were collected from Red Sea in the port of oil production Company (Floating port, Gulf of Suez, Red Sea). The fish were transported in large plastic water containers supplied with battery aerators as a source of oxygen and containing de-chlorinated tap water (24.5±2.1°C and pH 7.2-8.2). Fish were maintained on *ad libitum* standard fish food. After an acclimation period of 1 week, 10 fish were sacrificed and stored under -80 ºC for molecular analysis, while other fish (n=70) were divided separately into seven experimental groups (10 fish/ group) and were placed into fish aquariums.

2.3. Experimental Design

The *Mugil cephalus* collected from several places in the port of oil production Company, Gulf of Suez, Red Sea were allocated after adaptation time in several groups as follows: First group contained fish fed standard fish diet; second to fourth groups contained fish fed fish diet contains 10%, 20% and 30% of Ulva algae meal, respectively; fifth to seventh groups contained fish fed fish diet contains 10%, 20% and 30% of Caulerpa algae meal, respectively. The feeding period was 2 months. On the other hand, one group of fish was sacrificed in the beginning of the experiment to determine the toxicity on the DNA resulted from the polluted environment and to be compared with other groups after feeding.

2.4. Fish Diets

Algae meal with a ratio of 10, 20 and 30 % was added to fish diets. All ingredients were grounded, mixed (in a horizontal helix ribbon mixer) and dry pelleted through a 2.4-mm die at 50 °C (CPM, C-300 model). Diets were subsequently stored at 5 °C. The diets contained local marine fishmeal (FM), soybean meal (SBM), maize meal (MM) and *Ulva lactuca* meal (UM) as well as *Caulerpa prolifera* meal (CM). The latter was collected from the Red Sea. This material was washed with fresh water then sun dried for 2 days. All ingredients were finely ground to a fine powder using a laboratory hammer mill and sieved to pass through a 0.25 mM sieve. FM, SBM and MM were obtained from commercial suppliers. Locally available commercial fishmeal is prepared by drying and powdering low quality fish; the resulting fishmeal is of poor quality as it contains low amounts of protein as well as high levels of ash. Prior to feed formulation, proximate compositions of algae ingredients were determined (Table 1).

2.5. DNA Fragmentation

2.5.1 DNA Fragmentation Analysis Using Diphenylamine Reaction Procedure

Liver tissues of *Mugil cephalus* were used to determine the quantitative profile of the DNA fragmentation. Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml of lysis buffer containing, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.2% triton X-100, centrifuged at 10 000 rpm (Eppendorf) for 20 min at 4°C. The pellets were re-suspended in 0.5 ml of lysis buffer. To the pellets (P) and the supernatants (S), 0.5 ml of 25% tri-chloroacetic acid (TCA) was added and incubated at 4°C for 24 h. The samples were then centrifuged for 20 min at 10 000 rpm (Eppendorf) at 4°C and the pellets were suspended in 80 ml of 5% TCA, followed by incubation at 83°C for 20 min. Subsequently, to each sample 160 ml of DPA solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg:ml)] was added and incubated at room temperature for 24h (Burton 1956). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm wavelength using the formula:

$$\% \text{ Fragmented DNA} = [OD(S) / OD(S) + OD(P)] X100$$

2.5.2 DNA Fragmentation Analysis Using DNA Gel Electrophoresis Laddering Assay

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described according to Lu *et al*. (2002). Briefly, liver tissues were homogenized**,** washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris–HCl, 10 mM EDTA. 0.5% Triton, and 100 µg/ml proteinase K, pH 8.0) for overnight at 37 °C. The lysate was then incubated with 100 µg/ml DNase-free RNase for 2h at 37°C, followed by three extractions of an equal volume of phenol/chloroform (1:1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 °C. The extracted DNA was precipitated in two volume of ice-cold 100% ethanol with 1/10 volume of

3 M sodium acetate, pH 5.2 at −20 °C for 1h, followed by centrifuging at 15,000 rpm for 15 min at 4 °C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris–HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris–acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

2.6. Micronucleus Test by Acridine Orange Fluorescent Staining

Acridine orange staining of erythrocytes was performed according to Ueda *et al*. (1992). To assess this assay, ten *Mugil cephalus* from each location were sacrificed after exposure period. The gills cells were collected from all fish and re-suspended in a small volume of fetal calf serum (FBS; Sigma) on a 0.003% acridine orange-coated glass slide. The slide was then covered with a cover glass to prepare gills specimens. Slides were dried overnight and fixed with methanol for 10 min. Gills specimens were examined in a blinded manner using fluorescence microscopy at 600X or higher magnification with a blue excitation wavelength (e.g. 488 nm) and yellow to orange barrier filter (e.g., 515 nm long pass). Two slides per one fish were labeled to get blinded micronuclei scoring. To avoid obtaining unbiased results, the slides were observed once by one observer who has sufficient experience of micronucleus test. The number of micronucleated polychromatic erythrocytes (%MnPCEs) was measured at a rate of 3000 polychromatic erythrocytes (PCEs) per one fish

2.7. Histopathological Examination

Tissue specimens from fish of several experimental groups were fixed in neutral buffered formalin 10 % and processed by conventional method, embedded in paraffin, sectioned at 4-5 um and stained by Haematoxylin and Eosin (Bancroft *et al*., 1996).

2.8. Gene Expression Analysis:

2.8.1 RNA Extraction

TRIzol® Reagent (cat#15596-026, Invitrogen, Germany) was used to extract total RNA from brain and liver tissues of *Mugil cephalus* according to the manufacturer's instructions with minor modifications. Briefly, tissue samples (50 mg) were homogenized in 1 ml of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then, the samples were vortexed vigorously for 15 seconds and incubated at room temperature for 3 min. The samples were centrifuged for no more than 12 000 g for 15 min at 4 °C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 °C for 10 min and centrifuged at not more than 12,000 x g for 10 min at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7 500 g for 5 min at 4 °C. The supernatant was removed and RNA pellet was air-dried for 10 min. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Total RNA was treated with 1 unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

2.8.2 Reverse Transcription (RT) Reaction

The complete Poly(A)$^+$ RNA isolated from brain and liver tissues of *Mugil cephalus* was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

2.8.3 Semi-Quantitative Real Time-Polymerase Chain Reaction (sqRT-PCR)

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. An iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine the *Mugil cephalus* cDNA copy number. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers (Kumar *et al*. 2013). Each experiment included a distilled water control.

Primer design program (Primer3, version 0.4.0) was used to build the primers for *Mugil cephalus* GH and IGF-1 genes. The semi quantitative values of RT-PCR (sqRT-PCR) of GH (GH-F: 5′- CATG CAC AAG GTG AGG AAG A -3′, GH-R: 5′- AGG TCT CAA CCT GCA AAC ATC-3′, GenBank: AF134605.1) and IGF-1 (IGF1-F: 5′- TCT TCA AGA GTG CGA TGT GC-3′, IGF1-R: 5′- ACA GCT TTG GAA GCA GCA CT -3′, GenBank: AY427954.1) genes were normalized on the bases of β-actin (β-actin-F: 5′-CAC ACT GTG CCC ATC TAC GA-3′, β-actin-R: 5′-TCC TTC TGC ATC CTG TCA GC-3′, Wong *et al*. 2001) expression. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

2.8.4 Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (Bio-Rad 2006):

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the

ΔC_T method if Ef for the target (GH&IGF-1) and the reference primers (β-Actin) are the same (Bio-Rad 2006):

$Ratio_{(reference/\ target\ gene)} = Ef^{C_T(reference)\ \_\ C_T(target)}$

2.9. Statistical Analysis

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS, Version 9.1, Statsoft Inc., Tulsa, USA) followed by Scheffé-test to assess significant differences between groups. The values were expressed as mean ± SEM. All statements of significant were based on probability of $P \leq 0.05$.

3. Results

DNA Fragmentation

Quantitative DNA fragmentation was determined in *Mugil cephalus* collected from several places in Floating port. The DNA damage was examined in liver tissues of collected fish using gel electrophoresis laddering assay and diphenylamine reaction procedure (Figs. 1 and 2, respectively). The results of gel electrophoresis laddering assay revealed that fish collected from Floating port and killed immediately after collection showed more bands (as an evidence of DNA damage) due to oil pollution compared with the fish collected and then fed UM or CM for 2 months (Fig. 1). On the other hand, fish collected and then fed standard diet for 2 months revealed also more bands than those fed UM or CM for 2 months (Fig. 1).

The results of diphenylamine reaction revealed that, the DNA damage increased significantly in fish killed immediately after collection compared with fish fed UM or CM for 2 months (Fig. 2). Furthermore, the DNA damage in fish fed standard diet for 2 months increased significantly compared with fish fed UM or CM for 2 months (Fig. 2).

Micronucleus Assay

Effect of oil pollution on MnPCEs formation in the erythrocytes of gills tissues of *Mugil cephalus* is summarized in Fig 3. The results showed that incidence of MnPCEs increased significantly in fish killed immediately after collection compared with those collected and fed UM or CM diets. In addition, fish collected and fed standard diet revealed significantly higher incidence of MnPCEs compared with those fed UM or CM (Fig. 3).

Histopathological Analysis

Comparison between fish fed UM or CM and fish collected immediately from the Red Sea is summarized in Fig. 4. Only the liver tissues revealed alterations between fish fed UM or CM and fish collected immediately from the Red Sea. Cytopathological evaluations of hematoxylin and eosin stained hepatocytes of *Mugil cephalus* inhabiting fish fed UM and CM for 8 weeks (Figs. 4a and 4b) and control fish collected from the polluted area in the Red Sea (Figs. 4c and 4d). The results demonstrated a normal liver parenchymal architecture characterized by the appearances of hepatic lobules (HL) (Fig. 4a). Moreover, arrangement of hepatocytes in the form of plates radiating outward from a central vein (CV), sinusoids (S), the vascular channels (Fig. 4b) were showed. Figs 4c and 4d demonstrated the presence of hepatic lobules (HL), sinusoids (S) and portal canals (Fig. 4c). Furthermore, certain cytological alterations like hepatocyte vacuolation, corresponding to a higher glycogen (G) and/or lipid (L) content (Panel d), cellular swelling, hepatocellular membrane

disintegration and blood congestion in the sinusoids (Fig. 4d) are also observed.

Feed Intake and Growth Performance

Acceptability and palatability of the experimental diets were good based on visual observation during the feeding. There was no mortality during the entire experimental period. Results of body mass of fish fed several diets are summarized in Fig. 5. The results of the present study revealed that feeding of *Mugil cephalus* on CM diet as a ratio of 10% and 20% replacement with soybean meal increased the body mass rate more than in 30% of same diet. In addition, the body mass rate of fish fed 10% and 20% of CM diets was significantly higher than in those fed UM diets (10, 20 and 30%). However, the body mass rate of fish fed 10 or 20% CM diets was relatively similar to that in fish fed standard diet (control group).

Expression of GH and IGF-1 Genes

Results of expression of GH and IGF-1 genes in brain and liver of fish are shown in Figs 6 and 7, respectively. IGF-1 gene expression was directly proportional to body mass gain of fish. Highest levels of GH and IGF-1 expression in brain and liver were observed in control group. The same trend was observed in fish fed 10% and 20% of CM. However, the fish fed 30% of CM exhibited decrease in the expression of GH and IGF-1 gene compared to those fed standard diet or 10% or 20% of CM. On the other hand, the expression of GH and IGF-1 in fish fed control died or CM was significantly higher than those fed UM expecially at the highest dose of UM.

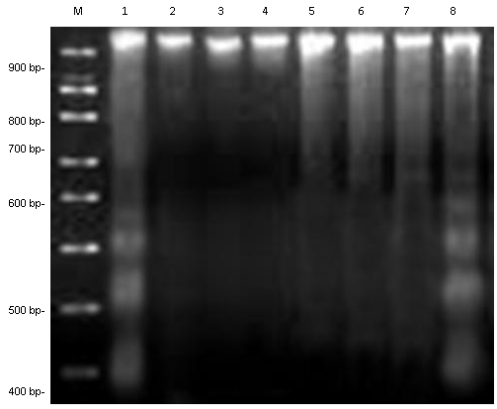


Fig. (1): DNA fragmentation detected with agarose gel of DNA extracted from liver tissues of *Mugil cephalus* collected from Red Sea analyzed by DNA gel electrophoresis laddering assay. M represents DNA marker. Lane 1 represents DNA from fish fed standard diet. Lanes 2–4 represent liver tissues of fish fed Ulva diet. Lanes 5–7 represent liver tissues of fish fed *Caulerpa* diet. Lane 8 represents DNA from fish collected before the feeding experiment.

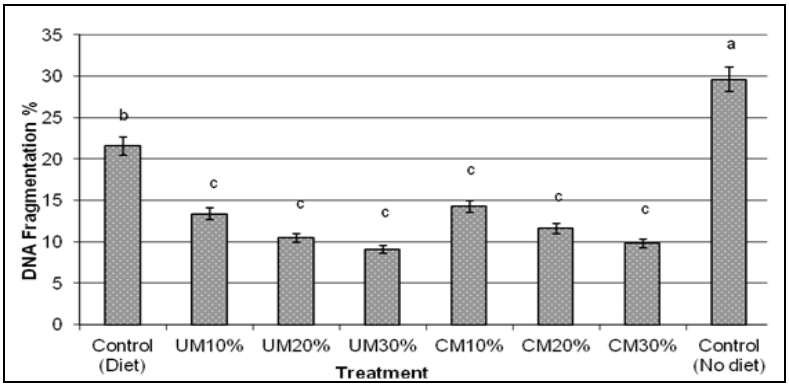


Fig. (2): DNA fragmentation in liver tissues of *Mugil cephalus* collected from Red Sea analyzed by diphenylamine reaction procedure. Results are expressed as mean±SEM of data from at least ten samples. [a,b] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$, Scheffé-Test).

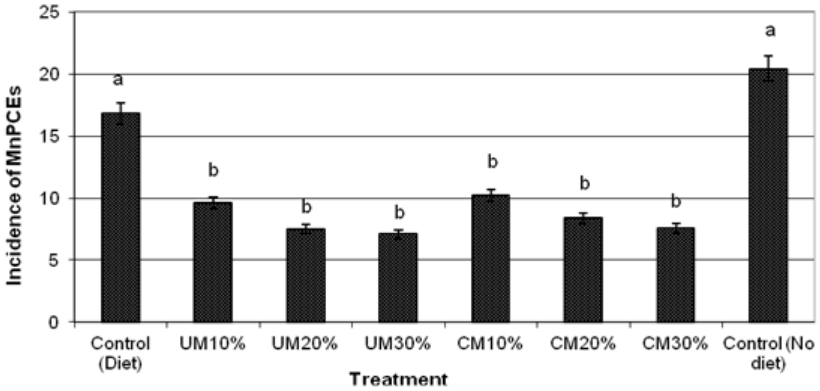


Fig. (3): Micronucleated polychromatic erythrocyte MnPCEs in gills cells of *Mugil cephalus* collected from Red Sea. Results are expressed as mean±SEM of data from at least ten samples. [a,b] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$, Scheffé-Test).

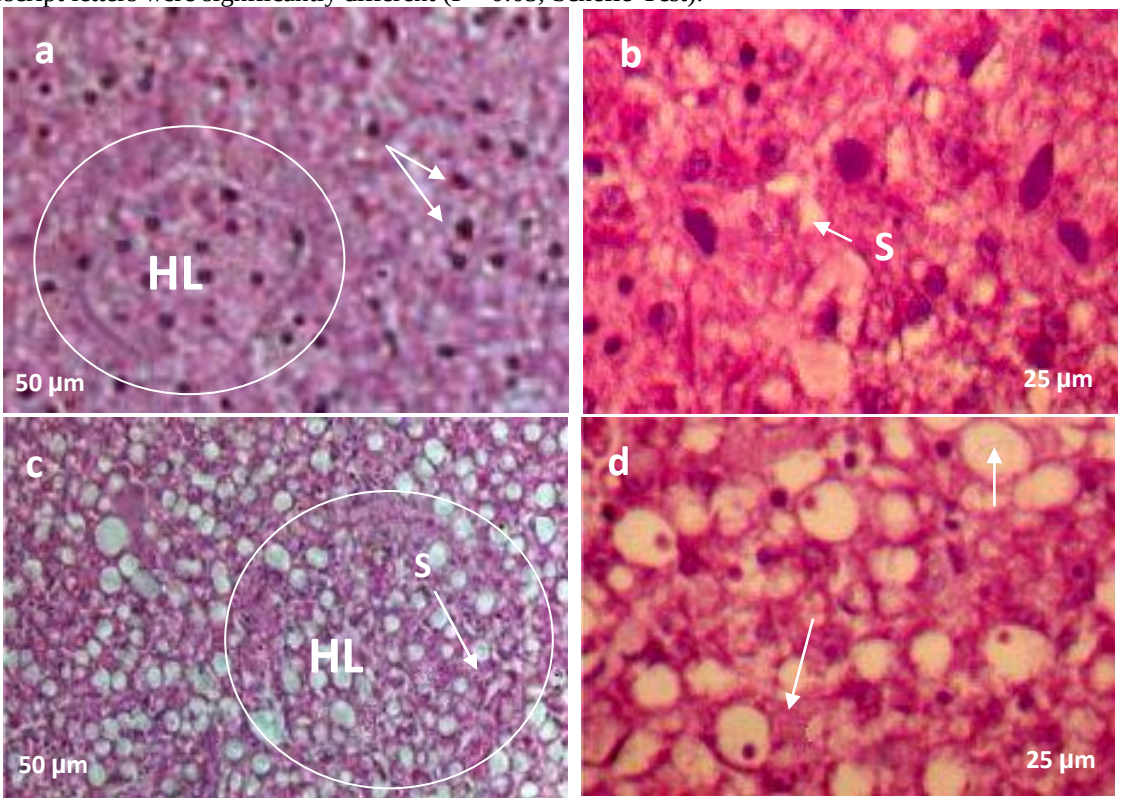
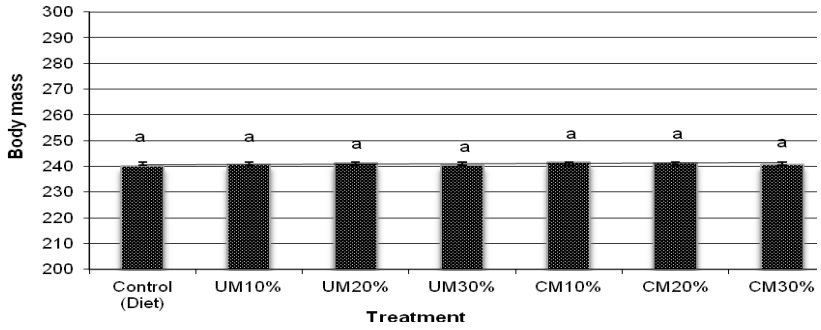


Fig. (4). Photomicrographs of liver tissues of *Mugil cephalus*. Comparison between fish fed UM and CM (a and b) for 8 weeks and control (c and d) fish collected from Red Sea. Cytopathological evaluations of hematoxylin and eosin stained hepatocytes were used. In this Fig., panels a and b demonstrate a normal liver parenchymal architecture revealed by the appearances of hepatic lobules (HL) (a, bar = 50 µm). Moreover, panel b (bar = 25 µm) showed arrangement of hepatocytes in the form of plates radiating outward from a central vein (CV), sinusoids (S), the vascular channels. In contrary, several lesions were observed in Panels c and d which they demonstrate the presence of hepatic lobules (HL), sinusoids (S) and portal canals (c, bar = 50 µm). In addition, certain cytological alterations like hepatocyte vacuolation, corresponding to a higher glycogen (G) and/or lipid (L) content (d, bar = 25 µm).

A



B

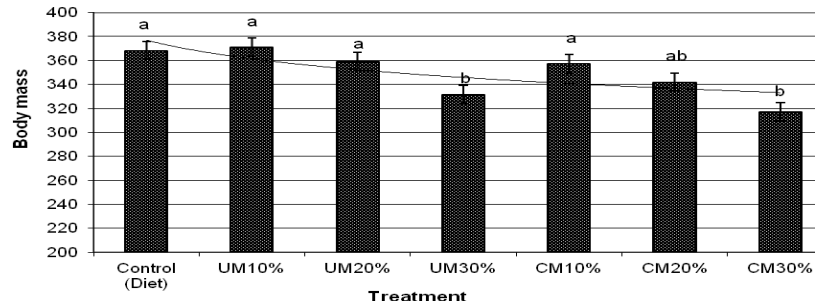


Fig. (5): Body mass *of Mugil cephalus* before (a) and after (b) feeding on several diets. Results are expressed as mean±SEM of data from at least ten samples. [a,b] Mean values within tissue with unlike superscript letters were significantly different ($P< 0.05$, Scheffé-Test).

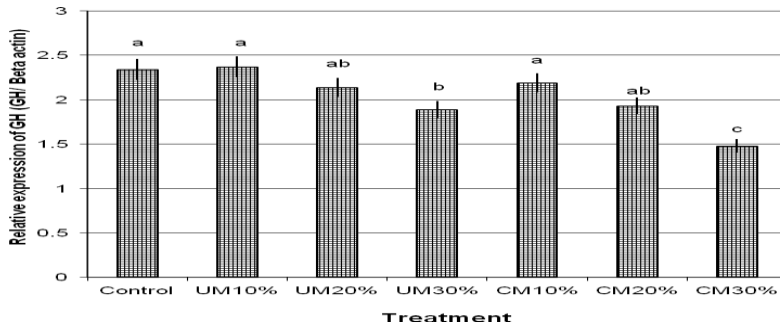


Fig. (6). Semi-quantitative Real Time-PCR analysis of GH in brain tissues of *Mugil cephalus* fed several fish diets. Means with different letters, within tissue, differ significantly ($P \leq 0.05$).

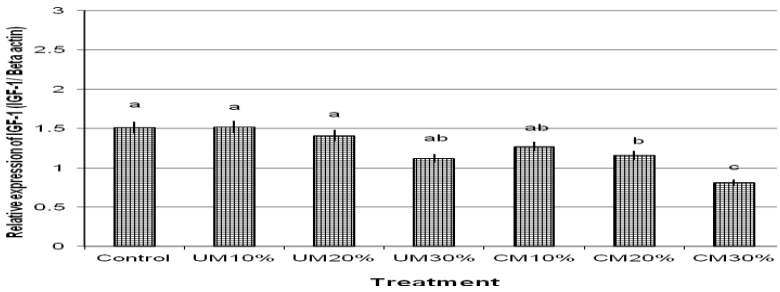


Fig. (7). Semi-quantitative Real Time-PCR analysis of IGF-1 in liver tissues of *Mugil cephalus* fed several fish diets. Means with different letters, within tissue, differ significantly ($P \leq 0.05$).

Table (1): Proximate composition (expressed as % dry matter) profile of different kinds of algae

Algae	Nutrient contents (% DM)				
	CP	CL	CF	CA	GE
Ulva lactuca	14.2	1.4	2.1	47.9	7.7
Caulerpa prolifera	29.6	4.7	2.2	19.0	15.8

CP: Crude protein; CL: Crude lipid; CF: Crude fibre; CA: Crude ash; DM: Dry matter

4. Discussion

Exposure of organisms to a genotoxic chemicals may result in the formation of a covalently attached intermediate to the organism's DNA (adduct). Thus, detection of adducts provides a way of documenting exposure. This approach was used in the current study to examine the effect of oil pollution on the DNA damage, MnPCEs formation and histopathological alterations in *Mugil cephalus* collected from the oil polluted location (Floating port) and the potential anti-genotoxic effect of UM and CM against oil pollution. The results revealed that fish collected from Floating port resulted in increase the DNA fragmentation and micronuclei formation.

In agreement with the present results, Tuvikene *et al*. (1999) reported that oil processing area in northeast Estonia is heavily polluted with polycylic aromatic hydrocarbon (PAHs) and also with other compounds. Studies with freshwater species exposed to the PHAs, polychlorinated biphenyls (PCBs) or petroleum byproducts, as reported by Winter *et al*. (2004), who also confirm the genotoxicity of these pollutants in freshwater organisms. The effects of oil pollution to *Mugil cephalus* observed in the present study could be resulted in different forms of biological damage.

The process of PAHs biotransformation in fish, in contrast to that which occurs with the most of chemical compounds, frequently converts these xenobiotics into intermediary highly toxic reactive substances (Maria *et al*., 2002) that may cause oxidative damage to DNA. The genetic effects, such as DNA strand breakage and micronuclei presence may cause mutagenicity, carcinogenicity, teratogenicity and subsequent population alterations (Van Der Oost *et al*., 2003).

The action mechanism of PAHS to induce toxicity in marine fish of Red Sea has been not studied yet. However, it is known that PAHs can generate ROS via production of PAH metabolites that include redox-cycling quinones and radical cations, the latter being associated with DNA damage (Miranda *et al*., 1995). Also, PAHs can damage mitochondrial DNA and perturb mitochondrial function, alter gene expression which may enhance oxidative stress in these organelles (Li *et al*., 2008). Induction of stress related proteins including CYP450 via the AHR by compounds such as chlorinated dioxins and PCBs can lead to ROS generation and oxidative damage (Nebert *et al*., 2000), associated with the recalcitrance of these compounds to metabolism. Numerous PAHs also induce CYP450s via the AHR, but this induction has not been thought to generate ROS per se, due to the suitability of PAHs as CYP substrates. However, ROS generation may be enhanced in co-exposures to PAHs which include both AHR agonists and CYP inhibitors, exposures which give rise to synergistic cell toxicity (Billiard *et al*., 2006).

Up to date there is no data regarding the role of green algae Ulva or Caulerpa in inhibition the genetic toxicity resulted from oil pollution in *Mugil cephalus.* However, some reports on the green algae in deceasing the genotoxic effect was demonstrated. Bhagavathy and Sumathi (2012) demonstrated that green algae were able to decrease the genotoxic effect of carcinogenic agents in human lymphocytes. This effect was due to the carotenoids existed in the green algae.

This study reports the first use of ulva meal (UM) and caulerpa meal (CM) in *Mugil cephalus* diets as a partial substitution of soybean meal (SBM), in which ulva and caulerpa meal can constitute up to 20% of the *Mugil cephalus* diet without any depressive effect on fish growth and feed utilization efficiency. Beyond this level (20%), fish demonstrated decreasing growth. These findings are in agreement with Azaza *et al*. (2008), who found that ulva meal increased the growth rate of Nile tilapia when product was included in fish diet by up to 20% in practical diets with no detrimental effects. In addition, our recent work (Stadtlander *et al*., 2013) reported that low replacement level (15%) of fish meal with red algae (Nori) improves growth and feed utilization of Nile tilapia.

In comparison with terrestrial plants like soybean or cotton- seed *caulerpa spp*. have a considerable advantage as sources of plant derived protein or fish meal replacements because they contain, except for non-starch polysaccharides (NSP), no or very low levels of anti-nutrient factors (ANF) such as trypsin inhibitor, phytic acid and tannins. Only saponins have been found in consider able amounts. Although saponins are generally considered to be anti-nutrients, studies in common carp (*Cyprinus carpio*) and Nile tilapia showed that low levels of saponins can have a

positive effect on growth and metabolism (Francis *et al*. 2001, 2002a,b).

The growth-promoting effect might be related to the presence of saponins in the caulerpa 10% and 20% diets, while in caulerpa 30% the amount of saponins might already be too high and became detrimental for the fish. The same trend was showed in ulva died. Furthermore, ulva meal has more phytic acid (0.55 mg/g) and trypsin inhibitor (0.14) compared with those in caulerpa meal (0.21 mg/g and 0.00, respectively).

In contrast to the anti-nutritional properties described for NSP, an extremely wide range of potentially beneficial biological activities like anti-allergic, anti-cancer, anti-oxidant, anti-bacterial, anti-viral, hypoglycaemic, immunostimulant and prebiotic were reported for algae (Bhatia *et al*. 2008). Although it has not been tested, porphyran might have acted as immunostimulant in the ulva and caulerpa. This mechanism explained why both ulva and caulerpa algae have protective activity against DNA damage compared to fish not fed algae diets.

The GH–IGF-1 network plays an integral role in mammalian growth (Jones and Clemmons, 1995). Cao *et al*. (2009) have suggested that IGF-1 is an important hormone involved in growth of carp. To the best of our knowledge, this is the first study to demonstrate a correlation with GH and IGF-1 gene expression with growth performance when *Mugil cephalus* fed CM and UM a protein source.

The present study was aimed to measure the levels of GH and IGF-I mRNA in the brain (including pituitary) and liver, respectively, to search for the underlying mechanism how regulate the green algae gene expression in relation of growth performance.

The GH-IGF-I axis provides an integrated signal for growth and nutrient partitioning (Beckman and Dickhoff 1998; Mingarro *et al*. 2002). In the present study for all diet groups, there was positive correlation between expression of GH gene and growth performance. Highest growth performance and GH expression were observed for the CM10% and CM20% groups, which were statistically similar ($P > 0.05$) to that for control group and significantly ($P < 0.05$) higher than for all other groups.

Our results are in agreement with Aksnes *et al*. (2006), wherein they observed that ≥75% FM protein replaced by a mixture of plant protein sources (SBM, soy protein concentrate, corn gluten meal, wheat gluten, extruded peas, rapeseed meal) balanced with indispensable amino acids in rainbow trout and gilthead sea bream leads to increased in GH concentration in plasma and liver GH gene expression. They concluded that growth performance was inversely related to GH gene expression, similar trend has been observed in our study.

Mounting evidence suggests that IGF-1 plays a similar role in the growth of fish (Duan 1998; Moriyama *et al*. 2000). Perez- Sanchez and Le Bail (1999) first proposed that the GH–IGF-1 network could be used as a marker of growth performance and nutritional status in cultured fish. The current study indicated IGF-1 gene expression in liver of *Mugil cephalus* differs significantly depending on dose of algae used. Relative expression of IGF-1 gene in liver was highest for the CM10 and 20% groups, which were statistically not different to that for control group and significantly ($P < 0.05$) higher than for all other groups which was parallel to growth performance. Our results are in agreement with Kumar *et al*. (2013), Gómez-Requeni *et al*. (2004), Dyer *et al*. (2004), Aksnes *et al*. (2006), Li *et al*. (2006), wherein they have observed that IGF-1 was in parallel to growth rates in carp, trout, sea bream, channel cat fish, barramundi and Atlantic salmon. Results of the present study and the study of Dyer *et al*. (2004) suggest that mRNA expression and circulating plasma concentrations of IGF-1 is a useful tool to predict growth rates of fish.

In Conclusions: the results of the current study demonstrate that *Caulerpa prolifera* could be as fish nutrient when be added to the fish die for several reasons: (a) it improves the fish diet which it increases the body mass and up-regulates the expression of growth genes such as GH and IGF-1; and (b) it enhances the immunity of fish which it inhibit the DNA and therefore inhibit fish diseases.

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