The Protective Role of Brown Alga *(Sargassum Crassifolia)* Against the Degenerative Toxic Effects Induced by Nimbecidin in Muscles of Nile Tilapia, *Oreochromis Niloticus* L.

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Abstract: This study was undertaken to evaluate the potential effect of the brown alga *Sargassum crassifolia* against biochemical and histological alterations in the muscles of fresh water fish, *Oreochromis niloticus,* exposed to 1/10 LC_{50} (0.03 ppm) of Nimbecidin (azadirachtin). *S. crassifolia* was added to the basal diet of fish in different proportions (0, 10 and 25%) and the experiment lasted for 4 weeks. Exposure of fish to Nimbecidin induced significant decrease in the activity of reduced glutathione, catalase and in the total protein content of fish muscles. Also many histopathological changes including degeneration and necrosis, disorganized myofibers with chromophobic cytoplasm, haemocytic infiltration, oedema and inflammations were noticed in Nimbecidine - treated fish. Exposing fish to Nimbicidin and *Sargassum crassifolia* (10% and 25%) led to marked improvement in the examined biochemical parameters together with the histological structure of muscles . This improvement was more obvious at high concentration of *Sargassum crassifolia.* In conclusion , the results of the present work indicated that the brown alga *Sargassum crassifolia* had ameliorative effect against muscle damage induced by Nimbicidin and this may be mediated by its potent antioxidant activities.

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Key words: *Sargassum crassifolia*, *Oreochromis niloticus*, antioxidant, neem, Nimbecidin, muscles, Glutathione, Catalase, Total protein.

1. Introduction

Marine algae are rich sources of structurally new and biologically active metabolites. In recent years, there have been many reports of macroalgae derived compounds that have a broad range of biological activities, such as antiviral (Ehresmann *et al*., 1977), antimicrobial (Devi *et al.,* 2008), anti-inflammatory (Dar *et al.*, 2007; Kang *et al.*, 2008 and Ananthi *et al*., 2010), anticarcinogenic and antioxidant activities (Kim *et al.*, 2005; Athukorala *et al*., 2006; Kukovinets and Kislitsyn, 2006; Devi *et al.,* 2008; Iwai, 2008; and Ananthi *et al*., 2010).

Free radicals scavengers and antioxidants can reduce lipid peroxidation and the generation of ROS. The importance of antioxidants in human health has become increasingly clear due to spectacular advance in understanding the mechanisms of their reaction oxidants. Furthermore, interest in employing antioxidants from natural sources to increase the shelf-life of foods is considerably enhanced by consumer preference for natural ingredients and concerns about toxic effects of synthetic antioxidants(Heo, *et al*., 2005). During the last years , several authors reported that certain species of brown and red algae contained multitude of bioactive compounds that have antioxidant properties (Matanjun *et al*., 2008, Plaza *et al.,* 2008). Recently, the antioxidant activity of the brown algae *Sargassum crassifolia* inhibiting Jeddah corniche , Saudi Arabia, was reported (Al-Amoudi *et al*., 2009).

Neem; *Azadirachta indica* (*A. indica*), is one of the most promising medicinal plants, having a wide spectrum of biological activity , well known for its insecticidal properties. Neem leaf extract and neem-based products were successfully implied in fish farming as antipararasitic and antibacterial agent (Harikrishnan *et al.,* 2003 and Mousa *et al*., 2008) and as control of fish fry predators (Dunkel and Ricilards, 1998). Martinez and Souza (2002) reported that aqueous extract of neem leaves and other neem-based products have been extensively used in fish-farms as alternative for the control of fish parasites and fish fry predators such as dragonfly larvae. Although neem extract is considered of low toxicity towards non-target aquatic life, water extracts of the bark of the neem plant caused respiratory problems in *Tilapia zilli* **(**Omoregie and Okpanachi, 1997), while long exposure to low concentrations of the crude extract of *A. indica* delayed the growth of this cichlid fish (Omoregie and Okpanachi, 1992). Such results indicate that neem extracts added to water may cause disturbances on fish. Consequently it is important to recognize the effects that the employ of these products used to

prevent the appearance of diseases may have in different parameters of aquatic life (Rábago-Castro *et al.,* 2006).

The present work was aimed to study the effect of *Sargassum crassifolia,* a brown alga from Saudi Arabia coasts, against the toxicity induced by Nimbecidin , natural plant insecticide derived from neem plant, in the muscles of the fresh water tilapia, *O. niloticus*.

2- Materials and methods

2.1. Nimbecidin

Nimbecidin®, a commercial natural pesticide derived from neem tree, *Azadirachta indica*. The active ingredient of nimbecidin is azadirachtin (Aza) which makes about 0.03% of its chemical composition (90.57%), Hydroxye1 (5.00%), Epichlorohydrate (0.50%), Aromax (5.90%).

2.2. Algal materials

Sargassum crassifolia (brown alga) was collected along Jeddah corniche, about 22 km through the longitudinal direction of N-W. The collected samples were carefully cleaned from epiphytes and washed several times with tap and distilled water then air-dried, chopped into small pieces and powdered and preserved in a dissector for the determination of protein, fat and ash contents.

2.2.1. Crude protein content

Crude protein was determined by measuring the total nitrogen content of the sample multiplied by the empirical factor 6.25. This method accomplished briefly in three stages: digestion by sulfuric acid, distillation and titration (Hepher *et al.,* 1983).

2.2.2. Crude lipid content (ether extract)

Crude lipid content was determined by weighing the filter paper containing the dried algal sample and then transferred to Soxhlet apparatus using petroleum ether at 60-80 °C for 12 hrs. The sample with filter paper was dried and reweighed, the difference between sample weights indicates the total lipid content in the sample (AOAC, 1995).

2.2.3. Ash content

Ash content was determined by weighing the crossable containing the dried sample and then transferred to a muffle furnace at 550 °C for 8 hrs. The crossable containing the sample was reweighed, the difference between sample weights indicates the ash content (AOAC, 1995).

2.3. Experimental animals

Nile tilapia(*Oreochromis niloticus*) with an average weight 14± 1.2g were obtained from a fish hatchery in Abbassa, Abo-Hammad, Sharkia, Egypt and transferred immeddiately to the laboratory in sacs filled with fresh water and supplied with continuous system of aeration. In the laboratory , fish were kept in 3.3 m^3 aquaria filled with dechlorinated tap water and supplied with continuous system of filtration and aeration. Temperature was maintained at 21 ± 2 °C and dissolved oxygen values at 7.0 ± 0.5 mg/L. Fish were fed with wheat bran(3% of total fish weight/day) and acclimatized for 15 days before the beginning of the experiments.

2.4. Toxicity test of Nimbecidin pesticide

Following acclimatization, ten groups of ten fishes were placed in 100 L aerated aquaria and exposed to a series of Nimbecidin concentrations that would permit the computation of the 96 hrs LC_{50} value and mortalities of fish were recorded daily.

2.5. Experimental Design:

In a four week experiment, the fish were assigned to 6 groups of 50 fish each:

Group 1 (G1): Animals of this group were used as controls and fed on basal diet without *S. crassifolia.*

Group 2 (G2): The diet of this group consisted of the basal diet (90%) mixed with S. *crassifolia (*10%).

Group 3 (G3): Animals of this group were fed on basal diet (75%) mixed with S. *crassifolia (* 25%).

Group 4 (G4): Animals of this group were exposed to 0.03 ppm (1/10 LC_{50}) of Nimbecidin and fed on normal basal diet.

Group 5 (G5): Animals of this group were exposed to 0.03 ppm (1/10 LC_{50}) of Nimbecidin and fed on basal diet (90%) mixed with S. *crassifolia (*10%).

Group 6 (G6): Animals of this group were exposed to 0.03 ppm (1/10 LC_{50}) of Nimbecidin and fed on basal diet (75%) mixed with S. *crassifolia (*25%).

At termination of 2nd and 4th experimental weeks, samples of fishes from all groups were dissected and muscle samples were removed.

2.6. Biochemical analysis

For biochemical study, muscle samples from all fish groups were washed in saline solution and homogenized (10% w/v) in ice-cold normal saline. The homogenate was centrifuged at 10 000xg for 20 min at 4ºC and the resultant supernatant was used for biochemical assay according to the method described by Chitra *et al.* (1999).

2.6.1. Antioxidants: Reduced glutathione (GSH) and catalase

The biochemical assays of reduced glutathione (GSH) and catalase were determined according to the methods described by Ellman (1959), Aebi et al. (1974) and Okhawa *et al.* (1979) respectively. The activity of glutathione was expressed as micromoles per wet gram of tissue and catalase was expressed as IU per mg protein.

2.6. 2. Total protein content

The total protein content of muscles was estimated by the method of Bradford (1976) and expressed as mg per gram of tissue.

2.7. Histological study

For histological study, samples of trunk muscles from control and treated fish were removed, rapidly fixed in Bouin's fluid and routinely processed for paraffin embedding. Sections were cut at 5 µm and stained with haematoxylin and eosin.

2.8. Statistical analysis

Biochemical results were subjected to one-way analysis of variance (ANOVA) and represented as mean ± SD of eight fishes per group. Differences in mean values between groups were assessed by Tukey's test and were considered statistically different at $p < 0.05$.

3- Results

3.1. Effect of Nimbecidin on the mortality of *Oreochromis niloticus*

The toxicity of Nimbecidin against *O. niloticus* was evaluated to determine the median lethal and sublethal concentrations.

Observation of the mortality rate over a period of 96 hours of *O. niloticus* exposed to various concentrations of Nimbecidin showed that the percentage of mortality of fishes depended on the concenterations of Nimbecidin. The LC50 of Nimbecidin for *O. niloticus* was found to be 0.33 ppm.

3.2. Analysis of algal material.

Results of analysis of the chemical composition of S. *crass folia* were 15.31 ± 0.36 mg/g dry weight (for crude protein), 1.86 ± 1.5 mg/g dry weight(for crude lipid) and 35.34 ± 1.04 mg/g dry weight(for ash).

3.3. Biochemical analysis

3.3.1. Antioxidants

3.3.1.1. Reduced glutathione (GSH) :

Data in Table (1) shows that there was a significant reduction ($p<0.05$) in the activity of the mitochondrial antioxidant reduced glutathione (GSH) in the muscles of fish treated with Nimbecidin compared with that of control.

Treating animals with Nimbecidin together with S. *crassifolia* (10% and 25%) for 2 & 4 weeks induced significant increase ($p<0.05$) in glutathione (GSH) when compared with control animals (group 1) or animals treated with Nimbecidin alone (group 4).

3.3. 1.2. Catalase :

Significant decrease ($p<0.05$) in catalase was recognized in fish exposed to Nimbecidin (groups 4) compared to that in control animals (group 1) or in animals fed with 10% and 25% of S. *crassifolia* (group 3 & 4 respectively). When animals treated with both Nimbecidin plus 10% S. *crassifolia* for 2 weeks, no *significant* change was recorded in catalase activity compared with that in fish exposed to Nimbecidin alone. Significant increase in the activity of catalase was recorded in animals treated with Nimbecidin plus 25% S. *crassifolia* for 2 weeks and in animals treated with Nimbecidin together with S. *crassifolia* (10% and 25%) for 4 weeks (Table 2) .

3.3.2. Total protein:

Results exist in (Table 3) reveled that there was a significant ($p<0.05$) decrease in the total protein content in the muscles of animals treated with Nimbecidin as compared with control (group 1). Animals given Nimbecidin and S. *crassifolia* (10% and 25%) for 2 & 4 weeks showed significant increase in the total protein compared with Nimbecidin treated group.

3.4. Histological results:

Control fish (Fig. 4a) and fish fed on 10% and 25% S. *crassifolia* supplementations (Figs 4b & c respectively) had normal muscle structure with no apparent tissue lesions.

In the muscles of animals treated with Nimbecidin for two weeks (Fig. 4d) degeneration and necrosis processes, disorganized myofibers with chromophobic cytoplasm, haemocytic infiltration, oedema and inflammatory changes were noticed. Less proliferated muscle lesions were recorded in animals exposed to Nimbecidin and fed on diet containing 10% and 25% S. *crassifolia* (Figs. 4e &f respectively).

Exposure of fish to Nimbecidin for 4 weeks (Fig. 5a) induced sever histopathological changes including necrosis and inflammatory changes, oedema and appearance of microhemorrhagic zones .These pathological changes were less pronounced in fishes fed diet supplied with 10% and 25% *S. crassifolia* (Figs 5b &c respectively).

Table 1: Effect of *S. crassifolia* and / or Nimbecidin on the activity of reduced glutathione (µmol/wet g tissue) in the muscles of fish at two time periods

Groups	Time periods(weeks)	
	2weeks	4weeks
Group 1	171.18±6.07[a]	171.18±6.07[a]
Group 2	172.86±9. 1[a]	176.91±11.01[ac]
Group 3	176.82±11.81[a]	187.67±10.09[c]
Group 4	164.18±6.27 [b]	163.26±8.43[b]
Group 5	210.36±11.26[c]	204.47±14.66[d]
Group 6	240.19±13.39[d]	212.89±13.11[e]

Data are represented as mean ± SD of eight specimens.
Data with different superscript letters in a column are significant to each other at $p<0.05$

Table 2: Effect of *S. crassifolia* and / or Nimbecidin on the activity of catalase (µ/mg protein) in the muscles of fish at two time periods

Groups	Time periods(weeks)	
	2weeks	4weeks
Group 1	3.77±0.61[a]	3.71±0.38[a]
Group 2	3.98±0.88[a]	3.87±0.56[a]
Group 3	3.99±o.78[a]	3.99±o.52[a]
Group 4	2.84±0.16[b]	3.06±0.01[b]
Group 5	2.86±0.23 [b c]	3.29±0.08[a]
Group 6	3.06±0.19[c]	3.77 ± 0.08 [c]

Data are represented as mean ± SD of eight specimens.
Data with different superscript letters in a column are significant to each other at $p<0.05$

Table 3: Effect of *S. crassifolia* and / or Nimbecidin on the total protein content (mg/g tissue) in the muscles of fish at two time periods

Groups	Time periods(weeks)	
	2weeks	4weeks
Group 1	0.64±0.03[a]	0.65±0.04[a]
Group 2	0.66±0.06[ac]	0.67±0.06[ac]
Group 3	0.7±0..06[c]	0.71±0.06[c]
Group 4	0.61±0.02[b]	0.60±0.03[b]
Group 5	0.65±0.02[a]	0.64±0.03[a]
Group 6	0.85±0.02[d]	0.73±0.04[d]

Data are represented as mean ± SD of eight specimens.
Data with different superscript letters in a column are significant to each other at $p<0.05$

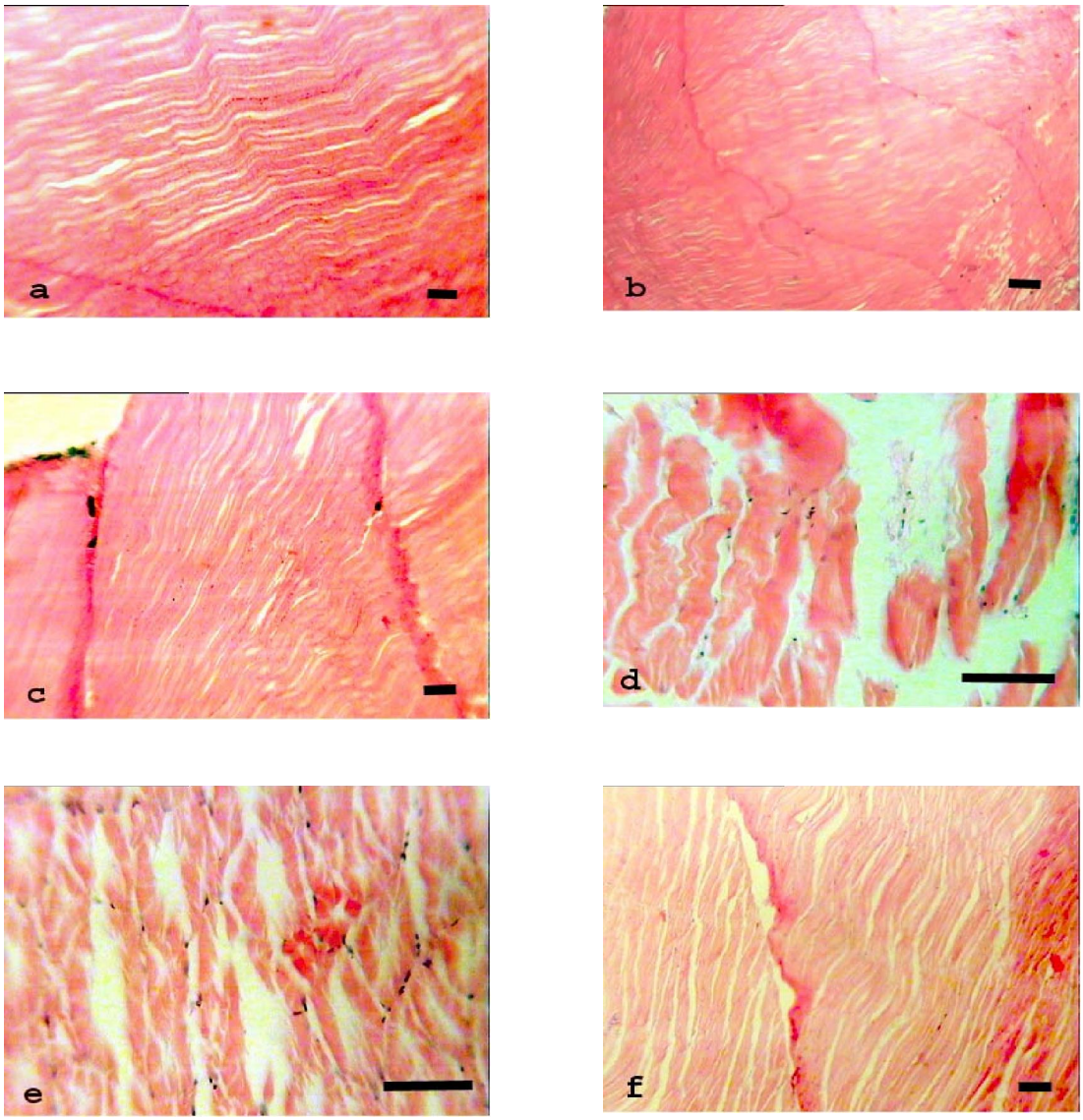

Fig. 4: Sections in muscles of control Nile tilapia, *O. niloticus* and fish fed on ascending doses (0%, 10% and 25%) of *S. crassifolia* supplementations and subjected to 0.03 ppm Nimbecidin, or without it, for 2 weeks duration. (a) Control fish (Group 1), (b) Fish fed on supplementary 10% *S. crassifolia* (Group 2). (c) Fish fed on supplementary 25% *S. crassifolia* (Group 3) (d) Fish subjected to Nimbecidin (Group 4) showing degeneration and necrosis processes, disorganized myofibers with chromophobic cytoplasm, haemocytic infiltration, oedema and inflammatory changes. (e) Fish subjected to Nimbecidin+ supplementary 10% *S. crassifolia* (Group 5). Muscle lesions appeared less proliferated. (f) Fish subjected to Nimbecidin+ supplementary 25% *S. crassifolia* (Group 6). Muscle lesions appeared less proliferated. H&E-stained muscle sections. Bar= 100 µm.

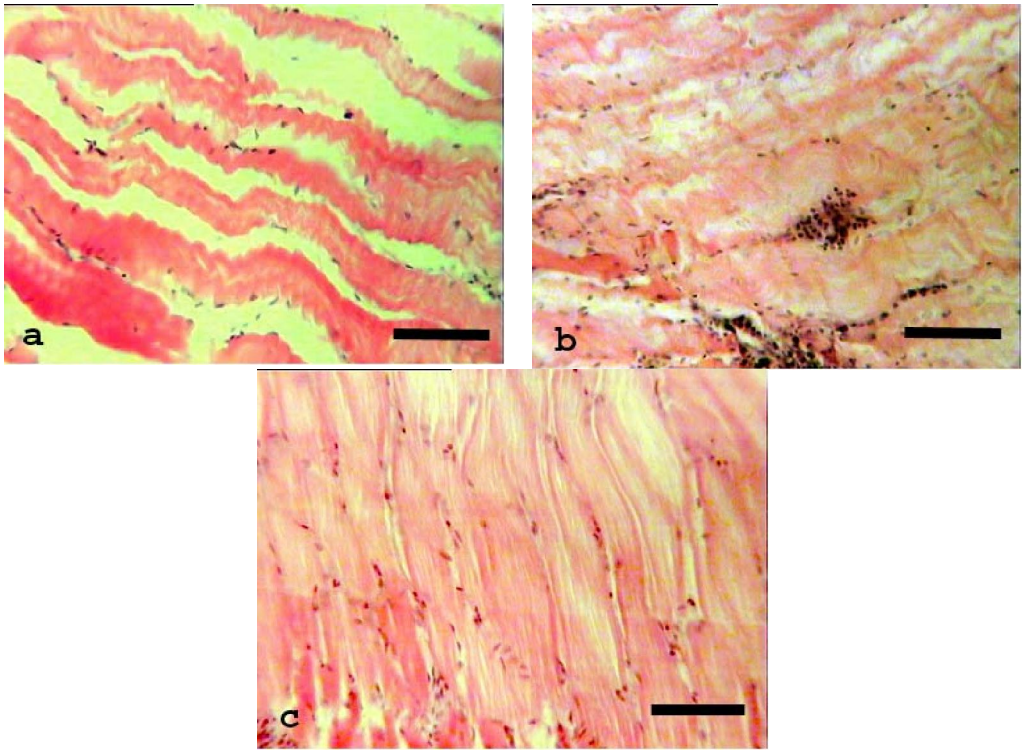

Fig. 5: Sections in muscles of Nile tilapia, *O. niloticus*, treated with 0.03 ppm Nimbecidin and ascending doses (0%, 10% and 25%) of *S. crassifolia* supplementation for 4 weeks duration. (a) Nimbecidin-subjected fish (Group 4) showing sever necrotic and inflammatory changes, oedema microhemorrhagic zones were observed. (b) Fish subjected to Nimbecidin+ supplementary 10% *S. crassifolia* (Group 5). Muscle lesions appeared less proliferated. (c) Fish subjected to Nimbecidin+ supplementary 25% *S. crassifolia* (Group 6) showing myofibrils with central nucleus and partially uniform cytoplasm and some degree of fat infiltration in the interstitial tissue. H&E-stained muscle sections. Bar= 100 µm.

4- Discussion

The nutritional value of algal species depends particularly on its biochemical composition. In the presen study total protein given by *S. crassifolia* was 15.31mg/g dry weight and it is comparable to that reported by Al-Amoudi *et al*. (2009) for such species (16.7mg/g). Burtin (2003) reported that the protein content in brown seaweeds are generally ranging from 5 to 15% of dry weight, compared to red and green seaweeds (ranging from 10 to 30%). Chemical analysis of the seaweeds collected from the Persian Gulf of Iran showed that the brown seaweed *Gracilaria corticata* had the highest protein content (19.3%, dry weight basis) and *S. ilicifolium* had the lowest (8.9%) (Ghadikolaei *et al.*, 2011). The differences in chemical composition between seaweeds are taxonomically and environmentally related (Herbreteau *et al.,* 1997 and Lourenço *et al.,* 2002).

In the present study, lipid content of *S. crassifolia* was found to be 1.86 mg/g dry weight and it is within the range for most seaweeds (1–3%) (; Sánchez-Machado *et al*., 2004 Matanjun *et al*., 2009 and Ghadikolaei *et al.*, 2011). Matanjun *et al.* (2009) found that the total lipid content in *E. cottonii*, *C. lentillifera* and *S. polycystum* were 1.10%, 1.11% and 0.29%, respectively.

Ash was the most abundant component of dried seaweeds. The level of ash recorded currently for *S. crassifolia* was 35.34 mg/g dry weight and it is comparable to those previously reported by Ortega-Calvo et al., (1993), Rupérez,(2002) and Matanjun *et al*., (2009).

Chemical and some plant pesticides may induce oxidative stress leading to generation of free radicals and alteration in antioxidant or oxygen free-radical scavenging enzyme system and disturb membrane structure (El-Shenawy *et al*., 2010).

In fish, many environmental pollutants, including pesticides, are capable of inducing oxidative stress (Hincal *et al*., 1995; Dorval *et al*., 2003; Pandey *et al.,* 2003; Sayeed *et al.,* 2003; Monteiro *et al.,* 2006). The authors mentioned that this event results in the formation of highly reactive compounds such as free radicals or oxyradicals (O2−, H2O2 and .OH) that frequently react with cellular macromolecules, leading potentially to enzyme inactivation, lipid peroxidation, DNA damage and

even cell death (Van der Oost *et al.,* 2003).

Glutathione-S-transferases (GST) are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites, and are involved in the detoxification of both reactive intermediates and oxygen radicals (Van der Oost *et al.,* 2003). In the present study exposure of Nile tilapia (*Oreochromis niloticus*) to sublethal dose of Nimbecidin induced significant reduction in the activity of glutathione (GSH). Similar findings were observed by Winkaler *et al.* (2007) who observed enhanced hepatic GST activity in freshwater fish *Prochilodus lineatus* following exposure to 5.0 g /L of neem extract and attributed it to the metabolism of organic compounds in the extract. Deplition of GSH level was also recorded in gills, muscles and intestine of *O. mossambicus* exposed to sub-lethal concentration (30 µg/L) of profenofos for 28 days (Kavitha and Rao, 2009) and in the freshwater fish, *Channa punctata*, in response to heavy metals (Pandey *et al.*, 2008).

Administration of S. *crassifolia* in combination with Nimbecidin induced significant elevation in GSH activity in comparison to that in animals exposed to Nimbicidin alone.

Elevation of GSH activity could be attributed to the antioxidant activity of *S. crassifolia* (Al-Amoudi *et al*., 2009).

Catalase is one of the free radical scavenging enzymes which form the first line of defence against oxidative injury converting H2O2 into H2O and O2 (Dorval *et al.* 2003).

In the present study depletion in catalase values was found in muscles of fish treated with Nimbicidin. Bainy *et al.* (1996) declared that the depletion in level of catalase activity might be due to an increased production of the superoxide radical (O2−), as an excess of this anion is known to inhibit catalase activity. Similarly, Winkaler *et al.* (2007) found that fish exposed to the sublethal concentrations of neem extract showed significant reduction in hepatic catalase activity which is likely to affect the capacity of liver cells to defend themselves and respond to contaminant-induced oxidative stress. In tilapia, Avci *et al.* (2005) reported significant reduction in CAT activity in the muscle of *O. niloticus* obtained downstream of Kizilirmak River, Turkey, due to exposure to petrochemical industry contamination. Depletion was also recorded in various organs in fish in response to different stressors (Kavitha and Rao, 2009).

CAT activity in the muscles of *O. niloticus* was found to be ameliorated after treating with 10% and 25% of *S. crassifolia*. Atencio *et al.* (2009) demonstrated that low dose of Se supplementation has shown to protect CAT activity in the liver ande kidney of cyanobacterial cells-exposed *O. niloticus*. Also, Karthikeyan *et al.* (2010& 2011) reported that the diminishing in activity antioxidant enzymes including CAT in liver, blood and kidney in rats was restored significantly after an ameliorating period using the brown alga *P. boergesenii* at a concentration of 150 mg/kg body weight.

In the current study, administration of Nimbicidin induced reduction in the total protein content in the muscles of treated fish. Also, Hussein *et al.* (1996) observed a significant fall in the total plasma protein level in *O. niloticus* after exposure to the herbicide atrazine for 14 and 28 days. Jee *et al.* (2005) reported decreased blood proteins and albumin levels in *Sebastes schlegeli,* the Korean rockfish, exposed to a sublethal concentration of cypermethrin (pyrethroid insecticide) for 8 weeks. On the other hand, Winkaler *et al.* (2007) reported that the fish showed no variation in total plasma protein levels after exposure to sublethal concentrations of neem leaf extract. Administration of *S. crassifoliahas has* the ability to increase the total protein after 2 and 4 weeks of treatment, In tilapia, the defensive role of vitamin E on protein oxidation in liver and kidney of microcystins-exposed tilapias has been demonstrated (Prieto et al., 2006).

Results obtained in the current work revealed that Nimbicidin caused many histological lesions which are progressively altered with time. These lesions included degenerative, oedemic disorganized myofibers with chromophobic cytoplasm, haemocytic infiltration, vacuolization, necrotic effects, and presence of microhemorrhagic zones. Similar damaging effects were observed in many target organs in fish after administration of toxic substances such as aluminum (Poleo *et al.,* 1994, lead (Yousif, 1998), cadmium (Hellstron et *al.,* 2001), copper (Mourad and Wahby, 1999, Takasuki *et al.,* 2004 and Sukaty, 2010), phenol (Mohammed, 1995), hexavalant chromium (Mishra, 2008) and after exposure to radiation (Ergenjeva and Shagava, 2006).

Treatment with S. *crassifolia* attenuated Nimbicidin-induced histopathology in fish muscles. The pathological alterations were less proliferated in muscles of fish treated for 4 weeks with Nimbecidin combined with 25% supplementary S. *crassifolia*. This protective effect of S. *crassifolia* could be attributed to its antioxidant activity which is time and dose dependant. Atencio *et al.* (2009) investigated the antioxidant role of selenium (Se) and its capability to repair tissue alterations induced by exposure to cyanobacterial cells in the liver, kidney, heart and gastrointestinal tract in Nile tilapia, *O.*

niloticus.. Other studies were carried out on the protective effect of some antioxidants against toxins in fish such as vitamin E (Prieto *et al.,* 2008), and calcium (Abdel-Tawab, 2005 and Sukaty, 2010) . Yen and Duh (1994) showed that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical which is responsible for damaging of the membranes of cell organelles.

Conclusion

The results of the present work indicated that the brown alga *Sargassum crassifolia* had ameliorative effect against muscle damage induced by Nimbicidin and this may be mediated by its potent antioxidant activities which had the ability to restore the membranal structure and function of the cell organelles in the muscles of *O. niloticus*

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