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Effects of Seed Extract of *Croton officinalis* (Alston) on the Antioxidant Status of *Oreochromis mossambicus* (Peters)

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Abstract

A number of plant products have been used as fish poisons from time immemorial since they are readily available and economical. Freshwater fish, *Oreochromis mossambicus*, were exposed to different concentrations of the fish poison, seeds of *Croton officinalis*, for 96 hours. The LC_{50} value for 96-hour exposure to toxin was found to be 1.18 ppm. A comparative investigation of the effect of toxin at different sub-lethal concentrations on activities of antioxidant enzymes Catalase, Superoxide dismutase, Glutathione peroxidase, Glutathione-S-Transferase, and on lipid peroxidation in gills, liver, heart, kidney, and muscle tissues were carried out. The antioxidant enzymes significantly increased at lower doses, 0.13 ppm and 0.25 ppm and thereby enabled the organism to overcome the oxidative stress induced by toxin. On exposure to 0.42 ppm of toxin, the levels of malondialdehyde, conjugated dienes, and hydroperoxides significantly increased with the corresponding decrease of antioxidant enzymes suggesting that a severe antioxidant stress was experienced by fish exposed to higher concentration of toxin.

Introduction

Oxidative stress potentially is experienced daily by all aerobic life when antioxidant defenses are overcome by pro-oxidant forces and is the basis of many physiological aberrations. Among the most used biomarkers of oxidative stress are antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST). SOD lowers the steady state levels of O_2, CAT is mainly located in the peroxisomes and is responsible for the reduction of H_2O_2 produced from the metabolism of long chain fatty acids in peroxisomes. GST catalyzes the conjugation of xenobiotics with glutathione, whereas GPX catalyses the reduction of both H_2O_2 and lipid peroxides and is considered as an efficient protective enzyme against lipid peroxidation (LPO) (Winston & Di Giulio 1991). LPO is a molecular mechanism of cell injury leading to generation of peroxides and lipid hydroperoxides,

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which can decompose to yield a wide range of cytotoxic products, most of which are aldehydes like malondialdehyde (MDA), 4-hydroxynonenal (HNE) etc.

Naturally occurring fish poisons are widely used from time immemorial for easy harvesting and for the elimination of unwanted fishes in culture ponds. The present study focuses on a biochemical investigation of the effects of naturally occurring fish poison *Croton officinalis* (Alston) on the activities of antioxidant enzymes and on LPO in different tissues of a tropical teleost *Oreochromis mossambicus* (*O. mossambicus)* (Tilapia) adapted to freshwater.

Materials and Methods

Experimental design

The freshwater *O. mossambicus* (15 ± 3g) were collected from the culture ponds of Rice Research Institute, Vytilla, Kerala and were acclimated to laboratory conditions for a month. The characteristics of the water in tank is as follows: dissolved oxygen content was 7.8 ppm, hardness was below detectable amounts, pH 7.0 ± 0.37, temperature 26 ± 3°C, and salinity 0 ppt. Aqueous extract of seeds of *Croton officinalis* (collected from Pathanamthitta District, Kerala, India) was used as toxin for the experiment. For conducting the biochemical study, fishes were divided into four groups and were taken in four separate tanks with the first group serving as the control and the other three as toxin-treated groups that received a sub-lethal concentrations of toxin, 0.13 ppm (i.e. 1/10 of LC_{50}/96 h), 0.25 ppm (i.e. 1/5 of LC_{50}/96 h), and 0.42 ppm (i.e. 1/5 of LC_{50}/96 h). The experimental animals were dosed for 7 days.

Biochemical studies

The marker enzymes in oxidative stress i.e. the antioxidant enzymes were assayed and the products of LPO were estimated in the gill, liver, heart, kidney, and muscle of both control and toxin-treated groups.

Catalase (E.C.1.11.1.6) was assayed using the method of Maehly and Chance (1955). SOD (E.C.1.15.1.1) was assayed using the method of Kakkar et al. (1984). The assay of GPX (E.C. 1.11.1.9) was carried out by the method of Rotruck et al. (1973). GST (E.C.2.5.1.18) was assayed by the method of Beutler (1986). MDA was estimated by the method of Niehaus and Samuelson (1968). Conjugated diene (CD) was estimated by the method of Retnagol and Ghoshal (1966). Hydroperoxide (HP) was estimated by the method of Mair and Hall (1977). Protein was estimated by the method of Lowry et al. (1951).

Statistical analysis

Testing of statistical difference between test and control groups were carried out by two-way ANOVA (Tukey) using the software SPSS 10.0 package. p value < 0.05 was considered as significant.

Results

The results are presented in Table 1

Table 1. Effects of seeds of *Croton officinalis* on different tissues of *Oreochromis mossambicus*
[Values are mean ± SD from fish in each group]

Tissue	Enzyme	Control	0.13ppm	0.25ppm	0.42ppm
Gills	CAT*	$^{A}12.02 \pm 0.416^{d}$	$^{D}96.89 \pm 3.248^{d}$	$^{C}10.28 \pm 0.206^{d}$	$^{B}7.24 \pm 0.246^{d}$
	SOD**	$^{A}11.99 \pm 0.436^{e}$	$^{B}24.94 \pm 0.93^{e}$	$^{C}16.79 \pm 0.612^{e}$	$^{D}10.342 \pm 0.377^{e}$
	GPX#	$^{B}12.37 \pm 0.418^{c}$	$^{D}27.81 \pm 1^{c}$	$^{C}22.08 \pm 0.81^{c}$	$^{A}14.26 \pm 0.52^{c}$
	GST##	$^{A}27.94 \pm 1.07^{c}$	$^{D}191.46 \pm 7.036^{c}$	$^{C}79.64 \pm 2.77^{d}$	$^{B}58.38 \pm 2.236^{d}$
	MDA$	$^{A}0.018 \pm 0.004^{c}$	$^{B}0.091 \pm 0.001^{c}$	$^{C}0.035 \pm 0.003^{c}$	$^{D}0.095 \pm 0.005^{c}$
	CD$	$^{A}30.95 \pm 1.132^{c}$	$^{B}35.11 \pm 1.289^{c}$	$^{C}45.37 \pm 1.543^{c}$	$^{D}49.53 \pm 1.721^{c}$
	HP$	$^{A}14.83 \pm 0.505^{d}$	$^{B}17.29 \pm 0.599^{d}$	$^{C}25.84 \pm 0.868^{d}$	$^{D}35.35 \pm 1.262^{d}$
Liver	CAT*	$^{A}11.68 \pm 0.373^{c}$	$^{D}26.62 \pm 0.93^{c}$	$^{C}8.62 \pm 0.3^{c}$	$^{B}4.09 \pm 0.142^{c}$
	SOD**	$^{A}10.026 \pm 4.362^{d}$	$^{B}18.41 \pm 0.661^{d}$	$^{C}14.34 \pm 0.523^{d}$	$^{D}11.01 \pm 0.402^{d}$
	GPX#	$^{B}8.51 \pm 0.315^{a}$	$^{D}17.23 \pm 0.631^{a}$	$^{C}6.88 \pm 0.251^{a}$	$^{A}4.05 \pm 0.148^{a}$
	GST##	$^{A}35.08 \pm 1.291^{c}$	$^{D}88.39 \pm 3.01^{c}$	$^{C}83.96 \pm 3.063^{c}$	$^{B}56.58 \pm 2.066^{c}$
	MDA$	$^{D}0.304 \pm 0.006^{d}$	$^{A}0.062 \pm 0.007^{d}$	$^{B}0.146 \pm 0.003^{d}$	$^{C}0.109 \pm 0.005^{d}$
	CD$	$^{C}47.25 \pm 1.724^{d}$	$^{D}69.01 \pm 2.478^{d}$	$^{A}30.84 \pm 1.074^{d}$	$^{B}35.07 \pm 1.259^{d}$
	HP$	$^{B}28.96 \pm 1^{e}$	$^{C}34.52 \pm 1.177^{e}$	$^{D}52.06 \pm 1.809^{e}$	$^{A}27.26 \pm 1^{e}$
Heart	CAT*	$^{A}11.42 \pm 0.377^{e}$	$^{D}48.33 \pm 1.679^{e}$	$^{C}40.31 \pm 1.399^{e}$	$^{B}30.52 \pm 1.049^{e}$
	SOD**	$^{A}11.28 \pm 0.411^{c}$	$^{B}15.09 \pm 0.515^{c}$	$^{C}13.35 \pm 0.4^{c}$	$^{D}11.69 \pm 0.427^{c}$
	GPX#	$^{B}11.44 \pm 0.384^{b}$	$^{D}21.92 \pm 0.764^{b}$	$^{C}16.51 \pm 0.604^{b}$	$^{A}4.43 \pm 0.159^{b}$
	GST##	$^{A}19.38 \pm 0.742^{b}$	$^{D}45.6 \pm 1.641^{b}$	$^{C}35.21 \pm 1.292^{b}$	$^{B}21.87 \pm 0.804^{b}$
	MDA$	$^{A}0.021 \pm 0.005^{d}$	$^{A}0.113 \pm .0.002^{d}$	$^{C}0.028 \pm 0.01^{d}$	$^{D}0.127 \pm 0.015^{d}$
	CD$	$^{A}40.22 \pm 1.538^{d}$	$^{B}43.05 \pm 1.574^{d}$	$^{C}47.58 \pm 1.626^{d}$	$^{D}51.04 \pm 1.742^{d}$
	HP$	$^{A}7.9 \pm 0.164^{c}$	$^{B}9.54 \pm 0.323^{c}$	$^{C}12.36 \pm 0.424^{c}$	$^{D}15.7 \pm 0.538^{c}$
Kidney	CAT*	$^{A}4.14 \pm 0.15^{b}$	$^{D}14.12 \pm 0.48^{b}$	$^{C}6.34 \pm 0.216^{b}$	$^{B}2.57 \pm 0.087^{b}$
	SOD**	$^{A}10.58 \pm 0.386^{b}$	$^{B}12.13 \pm 0.44^{b}$	$^{C}11.62 \pm 0.403^{b}$	$^{D}9.54 \pm 0.348^{b}$
	GPX#	$^{B}12.58 \pm 0.45^{d}$	$^{D}67.02 \pm 2.328^{d}$	$^{C}18.37 \pm 0.626^{d}$	$^{A}9.74 \pm 0.391^{d}$
	GST##	$^{A}6.66 \pm 0.228^{a}$	$^{D}22.46 \pm 0.827^{a}$	$^{C}15.48 \pm 0.542^{a}$	$^{B}10.02 \pm 0.394^{a}$
	MDA$	$^{A}0.011 \pm 0.002^{a}$	$^{B}0.03 \pm 0.001^{a}$	$^{B}0.024 \pm 0.003^{a}$	$^{B}0.032 \pm 0.001^{a}$
	CD$	$^{A}8.8 \pm 0.305^{a}$	$^{B}12.07 \pm 0.418^{a}$	$^{C}17.67 \pm 0.646^{a}$	$^{D}34.15 \pm 1.184^{a}$
	HP$	$^{A}13.77 \pm 0.501^{b}$	$^{B}15.21 \pm 0.522^{b}$	$^{C}21.31 \pm 0.742^{b}$	$^{D}28.68 \pm 0.97^{b}$
Muscle	CAT*	$^{A}3.51 \pm 0.12^{a}$	$^{D}5.25 \pm 0.179^{a}$	$^{C}4.66 \pm 0.159^{a}$	$^{B}3.15 \pm 0.121^{as}$
	SOD**	$^{A}8.05 \pm 0.324^{a}$	$^{B}8.94 \pm 0.342^{a}$	$^{C}8.43 \pm 0.302^{a}$	$^{D}8.17 \pm 0.3^{a}$
	GPX#	$^{B}5.77 \pm 0.202^{b}$	$^{D}27.46 \pm 1^{b}$	$^{C}15.27 \pm 0.548^{b}$	$^{A}5.97 \pm 0.218^{b}$
	GST##	$^{A}7.26 \pm 0.252^{a}$	$^{D}28.75 \pm 1.052^{a}$	$^{C}12 \pm 0.438^{a}$	$^{B}9.71 \pm 0.357^{a}$
	MDA$	$^{A}0.062 \pm 0.004^{b}$	$^{B}0.055 \pm 0.004^{a}$	$^{C}0.025 \pm 0.005^{a}$	$^{D}0.141 \pm 0.007^{b}$
	CD$	$^{A}19.85 \pm 0.727^{b}$	$^{B}21.05 \pm 0.735^{b}$	$^{C}27.88 \pm 0.95^{b}$	$^{D}39.92 \pm 1.36^{b}$
	HP$	$^{A}5.67 \pm 0.197^{a}$	$^{B}7.05 \pm 0.244^{a}$	$^{C}10.59 \pm 0.357^{a}$	$^{D}13.11 \pm 0.459^{a}$

* one IU = Change in absorbance at 230 nm/min, expressed/mg protein
** Units/mg protein
\# µg of GSH/min/mg protein
\#\# nmoles of CDNB complexed/min/mg protein
$ mmol/100 g wet tissue

Control compared with toxin-treated groups are represented as values with lower case varies significantly ($P < 0.05$) between tissues and values with upper case varies significantly ($P < 0.05$) between concentrations.

Two-way ANOVA (Tukeys test) showed that there is significant difference ($p < 0.05$) between control and toxin-treated groups with respect to all the parameters tested.

Catalase activity in different concentrations of toxin-treated groups varied significantly compared to control. Among the tissues, CAT activity in gills ($p < 0.05$) was highest. Between different concentrations of toxin-treated groups, tissues at 0.13 ppm showed highest activity. ANOVA showed an overall significant change ($p < 0.05$) in SOD activity in gills, liver, heart, kidney, and muscle tissues. SOD activity was highly elevated in 0.13 ppm treated groups. All tissues at 0.42 ppm showed least activity. GPX activity was found to be highest in kidney at 0.13 ppm ($p < 0.05$) compared to control. Activity of GPX at 0.25 ppm and 0.42 ppm also showed significant increase ($p < 0.05$) compared to control. Among the tissues, all tissues at 0.13 ppm showed an increased GPX activity. Fishes exposed to 0.13 ppm of toxin showed highest GST activity ($p < 0.05$) compared to control. Comparison between tissues showed that gills at 0.13 ppm showed highest GST activity.

ANOVA showed an overall significant change ($p < 0.05$) in the level of MDA, CD, and HP compared to control. Comparison between different concentrations showed that tissues at 0.25 ppm showed highest level of MDA. Among the toxin-treated groups, CD and HP were found to be highest in 0.42 ppm treated group. No significant variation in the levels of CD and HP in muscle at 0.13 ppm was observed compared to control.

Discussion

All organisms posses effective mechanism to prevent and neutralize the free radical–induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, GSH, GPX, and GST. When the balance between reactive oxygen species (ROS) production and antioxidant defence is lost, oxidative stress results, which through a series of events deregulates the cellular functions leading to various pathological conditions. Any compound natural or synthetic with antioxidant properties might contribute towards the partial or total alleviation of damage.

Toxicity of oxygen is due to the production of oxygen-derived freeradicals, the most common ones being superoxide (O_2^-), hydroxyl free radical (OH^-), and the singlet oxygen. Under normal conditions also, free radicals are produced during several physiological processes. During mitochondrial respiration, 1%–5% free radicals are produced (Yau-Huei Wei 1998), and immune response by activated phagocytes (Babior et al. 1973) also produces free radicals. These normal levels of free radicals are scavenged

by the normal amounts of antioxidant enzymes. However, the substantial increase in the levels of these highly reactive radicals occurs when the animal is subjected to stress conditions like environmental chemicals or pollutants (Pedragas et al. 1993). This is reflected in the increased production of the antioxidant enzymes.

As reported by Kappus (1986) and Di Giulio et al. (1989), antioxidant defence consists of enzymes including SOD, CAT, GPX, and GST. One of the important features of these enzymes is their inducibility under conditions of oxidative stress (Akcha et al. 2000).

The results obtained in the present study reveal that SOD and CAT appeared to be significantly elevated in *O. mossambicus* exposed to 0.13 ppm concentration of toxin (aqueous extract of seeds of *Croton officinalis*) for 7 days. SOD and CAT are included in the primary antioxidant enzymes, which help in the detoxification of ROS formed from the toxin by decreasing the peroxide levels or by maintaining a steady supply of metabolic intermediates like glutathione (GSH) and NADPH (Kappus 1985). The tissue specific increase in CAT activity showed the following trend: Gills > Heart > Liver > Kidney > Muscle. The tissue specific increase in SOD activity showed the following trend: Gills > Liver > Heart> Kidney > Muscle. The increased activities of SOD and CAT in gills may be related to their physiological role in respiration. In fact, in fish, the extraction of oxygen from water occurs primarily at the gill surface and therefore the gills posses a more rapid and efficient enzymatic mechanism against increased levels of oxygen radicals (Afonso et al. 1996). The increase in tissue SOD activity suggests an increased generation of intracellular hydrogen peroxide that could be adequately detoxified by CAT activity, which was also significantly higher in gills, liver, heart, kidney, and muscle tissues of *O. mossambicus* exposed to 0.13 ppm of toxin for 7 days. Reduction of superoxide anion radicals by CAT prevents the formation of free radical intermediates from the toxin by oxygen reduction mechanisms (Reddy 1997).

GPX is characterized by its ability to reduce hydrogen peroxide and a large number of organic hydroperoxides. In the present study, GPX activity in different tissues showed the following trend: Kidney > Gills > Muscle > Heart > Liver. GPX and other glutathione metabolizing enzyme activities are strongly dependent on tissues, species, and developmental stage (Aceto et al. 1994). Glutathione was found in higher concentration in the kidney and has been directly or indirectly implicated in the maintenance of normal kidney function (Colowick et al. 1954), which may have resulted in increased GPX activity.

When compared to control, GST activity significantly increased in 0.13 ppm, 0.25 ppm, and 0.42 ppm. The maximum increase was seen in gills followed by liver, heart, muscle, and kidney. In the primary metabolism of xenobiotics, electrophilic reactive intermediates can be generated (Miller & Miller 1979; Selkisk et al. 1980). GST

constitutes a versatile mechanism against chemically induced damage. Gadagbui et al. (1996) also support the view that *O. mossambicus* is more likely to excrete xenobiotics as glutathione conjugates or mercapturic acids because of its high GST activity.

LPO, as measured by the concentration of MDA, did not significantly increase in tissues of fish exposed to 0.13 ppm and 0.42 ppm of toxin when compared to control group. This could be due to the increase of nonenzymatic antioxidants, as well as by the increased activity of GST, which can prevent the formation of MDA (Christophersen 1986).

On the other hand, activities of CAT, SOD, GPX, and GST were significantly inhibited in fish exposed to 0.42 ppm of toxin for 7 days. The decreased SOD and CAT activity observed in 0.42 ppm in toxin-treated experimental animal may be related to the increase in O_2^- production. CAT inhibition by O_2^- was previously described by Kono and Fridovich (1982). This shows that in experimental animal tissue dosed in 0.42 ppm, SOD, CAT, and GPX adaptive response were not enough to protect cells against the damage by oxyradicals generated from the toxin, whereas the levels of CD, HP, and MDA significantly increased in fish exposed to 0.42 ppm of toxin when compared to control group, the maximum increase was observed in liver followed by other tissues.

The foregoing results show that at lower doses, toxin-induced oxidative stress in *O. mossambicus* was overcome to a large extent by its antioxidant defence mechanism. In contrast, fishes treated at higher doses experienced LPO to a large extent, which is indicated by its high level of MDA, CD, and HP.

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