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Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*

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Abstract

The aim of this study was to determine the toxicity of the aqueous extract of neem leaves, a product extensively used in fish-farms as alternative for the control of fish parasites and fish fry predators, for the neotropical fish *Prochilodus lineatus*. The 24 h LC_{50} of neem leaf extract for juveniles *P. lineatus* was estimated as 4.8 g L^{-1}; the fish were then exposed for 24 h to 2.5, 5.0 and 7.5 g L^{-1} or only clean water (control). Plasma glucose levels were higher in fish exposed to 2.5 g L^{-1} and 5.0 g L^{-1} neem extract, relative to control, indicating a typical stress response. Neem extract did not interfere with the osmoregulating capacity of the fish, as their plasma sodium, chloride, total protein and osmolarity did not change. The presence of the biopesticide interfered with the antioxidant defense system of *P. lineatus*, as there was a decrease in liver catalase activity at all neem concentrations and the detoxifying enzyme glutathione-*S*-transferase was activated in fish exposed to 5.0 g L^{-1}. Fish exposed to all neem extract concentrations exhibited damaged gill and kidney tissue. These results indicate that although neem extract is less toxic to *P. lineatus* than other synthetic insecticides used in fish-farming it does cause functional and morphological changes in this fish species.

Keywords: Acute toxicity; *Azadirachta indica*; Biomarkers; Characidae

1. Introduction

In Brazil, apart from parasites, which are the major cause of lost production in fish-farming (Ranzani-Paiva et al., 1997), the predation of the fry by dragonfly larvae also incurs great losses in productivity (Gáradi et al., 1988). Both fish parasites and fish predators are mainly controlled with toxic chemicals, mostly applied indiscriminately and without adequate training (Senhorini, 1991; Rodrigues et al., 1997). These chemical pesticides are well recognized as an economic approach to controlling pests, however, at the same time, they are highly toxic to other species in the environment, particularly fish (Rao, 2006). Thus the use of pesticides in aquaculture systems to control fish diseases, parasites and other pests not only leads to high levels of residues in the animals but also may interfere with the maintenance of their homeostasis and thus affect their performance (Barton and Iwama, 1991; Wendelaar Bonga, 1997).

In view of the environmental problems caused by the use of synthetic chemicals and the growing need for alternative methods of pest control that minimize this damage, there has been extensive research on pest control by substances from plants (Wan et al., 1996). One of the most promising natural compounds is azadirachtin (AZA), an active compound extracted from the neem tree (*Azadirachta indica*), whose antiviral, antibacterial and antifungal properties have been known for 2000 years (Isman et al., 1990; Harikrishnan et al., 2003). The chemistry and biological activity of both neem extracts and purified AZA have been investigated in various countries (Biswas et al., 2002). Experiments in the field have successfully demonstrated that neem extract has a potential as a pest-control agent (Martinez, 2002; Kreutzweiser et al., 2004). Because of that neem has been used successfully in aquaculture systems to control fish predators (Dunkel and Ricilards, 1998) and pathogenic bacteria such as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Escherichia*

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coli and *Myxobacteria* spp (Das et al., 2002). Kreutzweiser et al. (1999) performed tests on Neemix® 4.5, a product made from neem, and showed that at high concentrations of azadirachtin, neem products can cause mortality among aquatic insects, including the dragonfly *Ophiogomphus* sp. In Brazil the 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 (Martinez, 2002), 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 fish (Rábago-Castro et al., 2006).

Geographically, the south of Brazil is a region of widely differing microclimates, permitting diverse types of aquaculture, including twenty six species of farmed fish. From these 65.4% are common species native to the region (Poli et al., 1999), among which the bottom-feeding *Prochilodus lineatus* (Valenciennes, 1847). This neotropical fish species is one of the most important medium size fish for subsistence and commercial fishing (Jensch-Junior et al., 2005) and is a potential species for fish culture considering its high tolerance to changes in water pH (Takasusuki et al., 2004) and temperature (Barrinuevo and Fernandes, 1995) and its high productivity in fish-farms.

In light of the scarcity of data on the toxic effects of herbal medicines on neotropical fish, the aim of this study was to determine the lethal concentration of the aqueous extract of neem leaves for *P. lineatus* and to verify possible alterations in some biochemical, physiological and histopathological parameters in response to an acute exposure to different concentrations of this extract.

2. Materials and methods

2.1. Fish

Juvenile *P. lineatus* (Characiformes: Prochilodontidae) (8.99±1.84 g; 9.37±0.67 cm; $n=60$), obtained from the Universidade Estadual de Londrina hatchery station, were acclimated for 7 days in a 600 L aquarium filled with well water (temperature 20–21 °C, pH 7.5–7.7 and hardness 80–90 mg L^{-1} $CaCO_3$) with constant aeration and a 12 h:12 h dark: light cycle. During this time, the fish were fed with commercial pellet food each 48 h, except during and the day preceding the experiments.

2.2. Preparation of aqueous neem leaf extract

A. indica leaves, dried and finely chopped, were obtained from Sementes Aliança (Jaboticabal, SP, Brazil). To prepare the aqueous extract the leaves were dissolved in well water, at a concentration of 25 g of dried leaves per liter of water, for 24 h at room temperature (as described by Cruz et al., 2004). The mixture was filtered and the extract (25 g L^{-1}) was used immediately in the experiments, in different dilutions.

2.3. Determination of 24 h LC_{50}

Short-term (24 h) static toxicity tests were run to determine the median lethal concentration (LC_{50}) of neem leaf extract to *P. lineatus*. Tests were conducted in 100 L glass aquaria, 6 fish per aquarium, containing neem leaf extract diluted in well water to the following concentrations: 0 (control group), 2.5, 5.0, 7.5, 10.0 and 12.5 g L^{-1}. Photoperiod, water temperature, pH and hardness were maintained as described for the acclimation period. Deaths and abnormal behavior of fish were recorded every 6 h for 24 h. The value of 24 h LC_{50} and the related confidence interval were estimated by the Spearman–Karber method (Hamilton et al., 1978) with Toxstat® software (Ver. 3.5, Western Eco-Systems Technology, USA).

2.4. Acute toxicity tests

To evaluate neem acute effects, fish were distributed in four groups of six fish each, comprising three experimental groups and one control. Each group was placed into 100 L glass aquaria, not exceeding 1 g of fish per liter. Experimental groups were exposed for 24 h to three concentrations of neem extract corresponding to a sublethal (50% LC_{50}), a lethal (LC_{50}) and an over lethal (150% LC_{50}) concentration. The control group was simultaneously exposed to clean water alone. The experiments were carried out in static systems. Temperature, pH, dissolved O_2 and conductivity were monitored continuously. The tests were conducted in duplicates. At the end of 24 h of exposure, at least 6 fish for each of the four groups were anaesthetized with benzocaine (0.1 g L^{-1}), and blood was collected from the caudal vein, using heparin-coated syringes. Immediately after blood sampling fish were sacrificed by cervical section and gills, liver and the posterior kidney were removed. Liver samples were stored frozen at −80 °C and gills and kidney samples were fixed in Bouin's fluid.

2.5. Blood analysis

To determine the hematocrit, blood was centrifuged for 5 min at 5000 *g* in glass capillaries in a microhematocrit centrifuge (LC5, Luguimac SRL, Argentina). The remaining blood was centrifuged for 5 min at 5000 *g* (Fanem Centrimicro, Brazil) and the plasma stored at −20 °C. Plasma concentration of Na^+ was measured by flame photometry (Analyser 900, Brazil) and of Cl^- by spectrophotometry (1203 UV, Shimadzu, Japan), with the aid of a commercial kit (Analisa, Brazil). Osmolarity was measured in a freezing micro-osmometer (Osmomat 030, Gonotec, Germany). Glucose concentration was assayed enzymatically with glucose peroxidase (Diagnostica, Brazil) and total plasma protein was measured in a spectrophotometer by the method of Lowry et al. (1951).

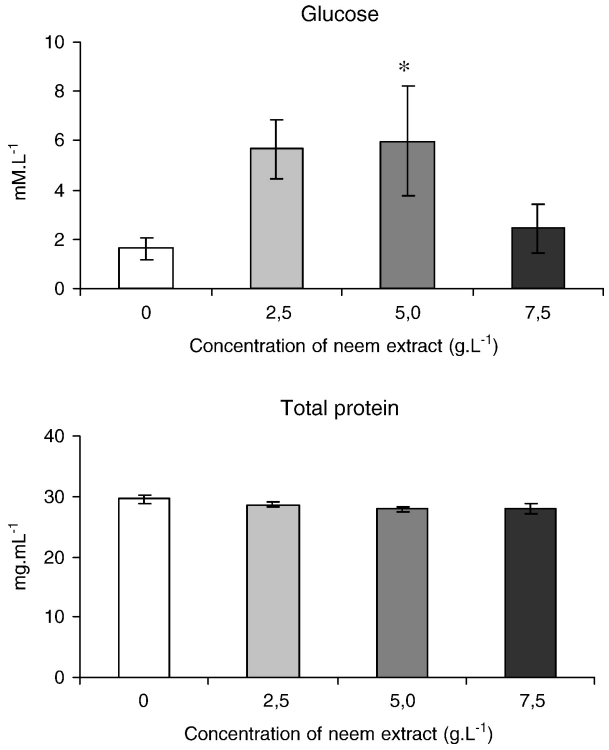

Fig. 1. Plasma concentrations of glucose and total protein of *P. lineatus* exposed for 24 h to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}). Concentration 0 corresponds to control group (CTR). Bars represent means and vertical lines the SE (n=5–6). *Indicates a significant difference from CTR ($P \leq 0.05$).

2.6. Biochemical assays

All enzymes activities were measured spectrophotometrically (1203 UV, Shimadzu, Japan) at 25 °C. Samples of frozen liver were quickly weighed and then homogenized in 0.1 M potassium phosphate buffer (1:10 w/v) and centrifuged (14,700 *g*) for 20 min at 4 °C in a refrigerated centrifuge (Joan BR-4i, France). The supernatant was separated for catalase (CAT) and glutathione-*S*-transferase (GST) assays.

CAT activity was determined by measuring the rate of decomposition of hydrogen peroxide at 240 nm, as described by Beutler (1975). GST activity was measured by enzymatic conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, according to Keen et al. (1976). Total protein in the homogenized liver supernatant was estimated by the method of Lowry et al. (1951). CAT and GST activities were expressed in U (μmol min^{-1} mg^{-1} protein) and mU (nmol min^{-1} mg^{-1} protein), respectively.

2.7. Histological procedure

The second gill arch from the right side of each fish and the posterior kidney were fixed in Bouin's fluid for 8 and 12 h, respectively, and embedded in paraffin wax. Serial sections (5 μm) were stained with hematoxilin–eosin (HE) and 10 sections for each animal were examined under the light microscope (Olympus CH30, Japan).

The occurrence of histological alterations in gills and kidney was evaluated semi-quantitatively by the Degree of Tissue Change (DTC), which is based on the severity of the lesions. For DTC calculation (modified from Poleksiæ and Mitroviæ-Tutundžiæ, 1994) the alterations in each organ were classified into three progressive stages: stage I — alterations which do not alter the normal functioning of the tissue; stage II — which are more severe and affect the associated tissue function; stage III — which are very severe and cause irreparable damage. A value of DTC was calculated for each animal by the formula: DTC = 1 Σa + 10 Σb + 100 Σc; where a, b and c correspond to the number of alterations of stages I, II and III, respectively. The DTC value obtained for each fish was used to calculate the mean index for control and neem-exposed groups. Mean DTC values between 0 and 10 indicate functionally normal organ; 11 and 20: slightly damaged tissue; 21 and 50: moderately damaged tissue; 51 and 100: severely damaged tissue; and >100: irreparable damaged tissue.

2.8. Statistical analysis

For each parameter analysed differences among groups exposed to all concentrations of neem leaves extract, including the control group, were tested for significance by one-way parametric ANOVA or the Kruskal–Wallis test, where appropriate, and the Student–Newman–Keuls (SNK) multiple-range test was used to identify the differences. $P \leq 0.05$ was taken as significant.

3. Results

The 24 h LC_{50} of neem leaf extract for *P. lineatus* was estimated as 4.8 g L^{-1}, with confidence interval ranging from 3.7 to 6.2 g L^{-1}. The concentrations of neem extract tested on the acute experiments were 2.5, 5.0 and 7.5 g L^{-1}, corresponding to 50%, 100% and 150% of the 24 h LC_{50}, respectively.

During the toxicity tests dissolved oxygen, pH and temperature remained at 5.4±0.46 mg O_2 L^{-1}, 7.5±0.12 and 20.3±0.32 °C respectively, but the conductivity increased with rising concentration of neem in the water (Table 1).

The plasma glucose level was significantly higher (F [3,18] = 3.46; P=0.04) in fish exposed to 2.5 g L^{-1} neem than in the controls. Fish exposed to 5.0 g L^{-1} neem also exhibited raised glucose levels, but this group was not significantly different from the control group (Fig. 1). The concentrations in the

Table 1
Values of dissolved oxygen (DO), pH, temperature and conductivity of the water, at the beginning and at the end of the toxicity tests with different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1})

[Neem] (g L^{-1})	DO (mg L^{-1})	pH	Temperature (°C)	Conductivity (μS cm^{-1})
0 (CTR)	6.4–5.9	7.2–7.3	20.2–20.5	162.0–162.0
2.5	5.1–5.9	7.6–7.3	20.5–21.3	424.0–426.0
5.0	4.6–7.2	7.6–8.0	18.5–18.7	726.0–761.0
7.5	5.6–5.9	7.2–8.4	20.7–21.3	1226–861.0

Initial (t=0) and final (t=24 h) measurements are shown for each group.

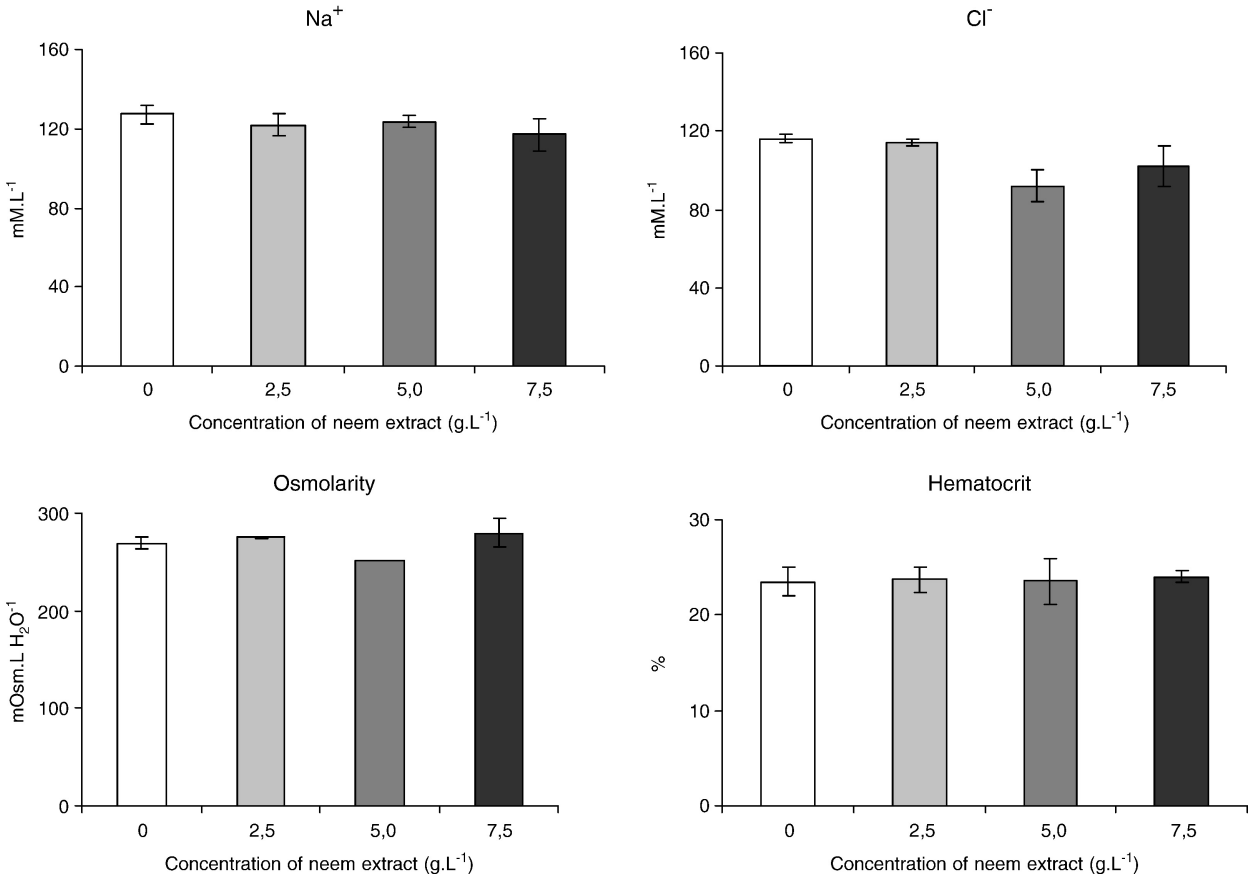

Fig. 2. Plasma concentrations of sodium (Na^+) and chloride (Cl^-), osmolarity and hematocrit of *P. lineatus* exposed for 24 h to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}). Concentration 0 corresponds to control group (CTR). Bars represent means and vertical lines the SE (n=4–7).

plasma of total protein, Na^+ and Cl^- and the osmolarity and hematocrit did not vary significantly among any of the groups exposed to neem or the control group (Figs. 1 and 2).

In all groups exposed to neem the hepatic CAT activity was significantly lower (F [3,22]=16.86; P<0.001) than in the control group (Fig. 3). GST activity was significantly higher (F [3,21]=3.17; P=0.05) in fish exposed to the intermediate neem concentration than in the control group (Fig. 3).

Table 2
Histological alterations found in the gills of *P. lineatus* following acute exposure to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}) and their respective stages of damage to the tissue and frequency of occurrence

Gill lesions	Stage	Neem leaf extract concentrations (g L^{-1})			
		0	2.5	5.0	7.5
Hyperplasia of gill filament epithelium	I	0	+0	++	+++
Hyperplasia of the lamellar epithelium	I	+0	+	+++	+++
Decrease of interlamellar space	I	0	+0	+++	+++
Epithelial lifting of the lamella	I	0	0	+++	+++
Incomplete fusion of several lamellae	I	0	0	0	+0
Complete fusion of several lamellae	I	0	0	+0	0
Lamellar disorganization	I	+	++	++	+++
Lamellar blood sinus dilates	I	0	+0	+	++
Lamellar blood sinus constricts	I	+	+	+++	+++
Lamellar aneurism	II	0	+	+	+++
Rupture of the lamellar epithelium	II	0	0	++	++

Note. 0 = absent; 0+ = rarely present; + = fairly frequent; ++ = frequent; +++ = very frequent.

The histological alterations found in the gills of fish exposed to neem extract are detailed in Table 2. The more frequent branchial changes were considered to be at stage I in severity and were found mainly in fish exposed to the two highest neem concentrations. Lamellar aneurism and rupture of the lamellar epithelium were the only lesions of stage II and they were found frequent only in fish exposed to 5.0 g L^{-1} and 7.5 g L^{-1} neem. Very severe stage III lesions were not found in the gill of the animals studied. Fish exposed to all neem leaf extract concentrations presented values of DTC in the gills significantly higher (F [3,21]=152.02; P<0.001) than control (Fig. 4). Animals exposed to lethal and over lethal concentrations presented DTC values between 10 and 20, indicating slightly damaged gill tissue. In contrast, fish exposed to the sublethal neem concentration, presented mean DTC value of 4.17, which was significantly lower than the other experimental groups and indicated functionally normal gill tissue.

The alterations found in the posterior kidney of the fish exposed to neem and their frequencies of occurrence are shown in Table 3. The most important change found in the renal corpuscle of *P. lineatus* was glomerular expansion, resulting in reduction of Bowman's space. In the tubules, the most frequent alterations were: cytoplasmic vacuolation, granular degeneration and narrowing of tubular lumen. Very severe stage III

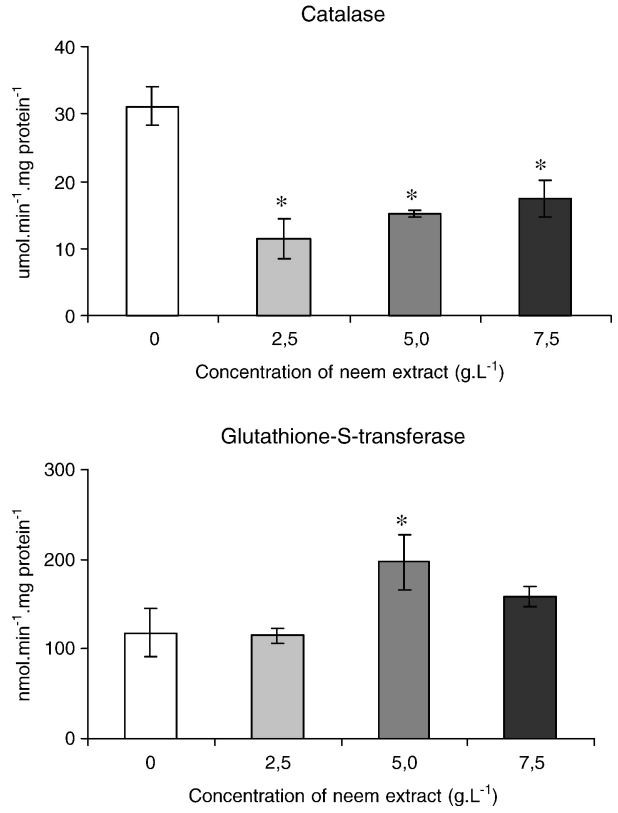

Fig. 3. Catalase and glutathione-*S*-transferase liver activities of *P. lineatus* exposed for 24 h to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}). Concentration 0 corresponds to control group (CTR). Bars represent means and vertical lines the SE (n=5–6). *Indicates a significant difference from CTR ($P \leq 0.05$).

lesions were not observed. Mean kidney DTC values calculated for all experimental groups were between 34 and 42.67, indicating moderately damaged kidney tissue, and the values for fish exposed to the two highest neem leaf extract concentrations were significant higher (F [3,20]=9.82; P<0.001) than control (Fig. 4).

4. Discussion

Comparisons of the sensitivity of different fish species to neem are questionable, since the amount of active compounds in a given weight of neem varies widely with the part of the plant (Luo et al., 1999), its place of origin or even the individual tree (Isman et al., 1990; NRC, 1992). Cruz et al. (2004) have made a quantitative HPLC analysis of the azadirachtin content in the aqueous extract of neem leaves which was prepared by the same method and with the same stock of neem leaves as in the present work. The content of azadirachtin in this aqueous extract was found to be 11.8 mg of azadirachtin per liter of water extract. According to these authors this concentration is similar to that found by Govindachari et al. (1999) in the aqueous extract of neem kernel. Thus, these results indicate that the aqueous extract of neem leaves used in the present research does contain the bioactive compound azadirachtin, the most active one for insect control.

The 24 h LC_{50} of neem leaves extract found here for *P. lineatus* was 4.8 g L^{-1} which corresponds to 5.7 mg of azadirachtin L^{-1} (Cruz et al. 2004). If a simple comparison is made between the 24 h lethal concentration of azadirachtin found here for *P. lineatus* and the 96 h LC_{50} for the Pacific silver salmon, *Onchorhynchus kisutch*, (>4 mg L^{-1}) (Wan et al., 1996), it appears that both fish species present similar sensibility to azadirachtin. However, interpretation and comparisons of LC_{50} values need to be done with caution, considering that the values obtained by toxicity tests are very dependent upon the conditions under which tests were performed, and given the small number of studies on the toxicology of *A. indica* related to fish, the lack of detailed information on the methods used in published research makes these comparisons even more dubious.

Compared to other synthetic insecticides used in fish-farming, such as carbamates and organophosphates, neem-based products are certainly less toxic to fish. Thus, the 96 h LC_{50} of the synthetic carbofuran and malathion for the silver salmon were, respectively, 0.5 and 0.2 mg L^{-1}, whereas the 96 h LC_{50} of the neem products Margosan-O® and Pherotech, and neem extract for the same species were 38, 81 and 13 mg L^{-1} respectively (Wan et al., 1996). Neem was also shown to be less toxic to *P. lineatus* than the herbicide Trifluralin (24 h LC_{50}=0.25 mg L^{-1}) and the insecticide Azodrin (96 h LC_{50}=28.28 mg L^{-1}) reported by Martinez and Cólus (2002).

Exposure to various types of stressors, including those related to current aquaculture practices, may induce changes in some of the hematological variables of fish (Heath, 1995) which are frequently used to assess fish health (Martinez and Souza, 2002). In this study, neem extract in the water did not lead to significant changes in the hematocrit of *P. lineatus*, in contrast to the situation described by Harikrishnan et al. (2003), who

Table 3
Histological alterations found in the posterior kidney of *P. lineatus* following acute exposure to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}) and their respective stages of damage to the tissue and frequency of occurrence

Kidney lesions	Stage	Neem leaf extract concentrations (g L^{-1})			
		0	2.5	5.0	7.5
Changes in corpuscle					
Enlargement of glomerulus	I	0+	+	++	++
Reduction of Bowman's space	II	0	++	+++	+++
Blood cells in Bowman's space	II	0	0	+	+
Changes in tubules					
Nuclear hypertrophy	I	0	+	+	+
Cytoplasmic vacuolation	I	+	++	+++	+++
Granular degeneration	I	+	++	+++	+++
Narrowing of tubular lumen	I	+	+++	++	++
Tubular regeneration	I	0	0	++	0
Hyaline droplet degeneration	II	0	0	+	++
Nuclear degeneration	II	0	+	++	++
Tubular degeneration	II	0	0+	++	++
Cellular rupture	II	0	0+	+	+

Note. 0 = absent; 0+ = rarely present; + = fairly frequent; ++ = frequent; +++ = very frequent.

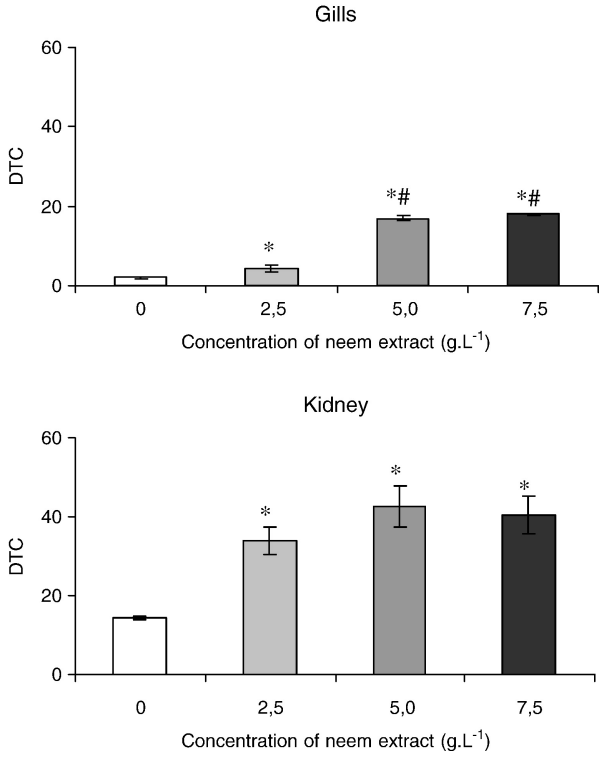

Fig. 4. Degree of tissue change (DTC) calculated for gills and posterior kidney of *P. lineatus* exposed for 24 h to different neem leaf extract concentrations (0, 2.5, 5.0 and 7.5 g L^{-1}). Concentration 0 corresponds to control group (CTR). Bars represent means and vertical lines the SE ($n=5$–6). *Indicates a significant difference from CTR; #Indicates a significant difference from 2.5 g L^{-1} ($P \leq 0.05$).

observed increases in the hematocrit, hemoglobin (Hb) content and number of erythrocytes in carp infected with *A. hydrophila* and treated for 20 or 30 days with neem extract (1 g L^{-1}). Other toxic agents used in aquaculture may promote hematological changes; one such case is copper sulphate, which increased the hematocrit, Hb and the red blood cell count of *P. lineatus* after 96 h of exposure at 20 °C (Carvalho and Fernandes, 2006).

A variety of toxicants can interfere in fish osmoregulation and the usual method that has been used to detect the effects of compounds on osmotic and ionic regulation has been to measure the concentrations of individual ions and total osmolarity in fish plasma (Heath, 1995; Barcarolli and Martinez, 2004). In our study, blood concentrations of Na^+ and Cl^- and blood osmolarity of *P. lineatus* exposed to neem, like the hematocrit, did not differ significantly from the values in the control group. Thus, the exposure to these levels of neem in the water, during 24 h, did not interfere in the osmoregulation processes of *P. lineatus*, despite the excessive conductivity recorded in the water of the neem-treated fish.

In the present study, the fish exposed to 2.5 g L^{-1} neem exhibited a significant rise in blood glucose. A slight hyperglycemic response was also observed in *P. lineatus* exposed to 5.0 g L^{-1} neem, but this glucose rise was not significant, probably because of the large deviations of the results in this group. This increase in blood glucose can be viewed as part of a stress response triggered by the presence of neem leaf extract in water. Hyperglycemia has also been found in *P. lineatus* acutely exposed to lead for 6, 12 and 24 h (Martinez et al., 2004). Conversely, in fish exposed to the highest concentration of neem (7.5 g L^{-1}) no significant alteration in blood glucose was observed. According to Heath (1995) high doses of some pesticides can cause an immediate hyperglycemia, followed by hypoglycemia before death. Thus, the absence of a hyperglycemic response might indicate that this concentration, which is above the 24 h LC_{50} (confidence interval 3.73–6.15 g L^{-1}), represents a situation of exhaustion, where there was a depletion of liver glycogen and a host of other changes, making the organism incapable to respond and consequently less able to survive (Wendelaar Bonga, 1997; Martinez et al., 2004).

Fish under stress may also mobilize protein to meet energy requirements needed to sustain increased physiological activity (Martinez et al., 2004). 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. Hussein et al. (1996) also observed a significant fall in the total plasma protein level in *Oreochromis niloticus* after continuous exposure to the herbicide atrazine for 14 and 28 days. In contrast, in the present study the fish showed no variation in total plasma protein levels in any group. Thus, it may be inferred that during acute exposure to sublethal concentrations of neem leaf extract, the observed hyperglycemia is sufficient to satisfy the raised energy demands arising from the chemical stress.

Many environmental pollutants, including pesticides, are capable of inducing oxidative stress in fish (Gabryelak and Klekot, 1985; Hincal et al., 1995; Dorval et al., 2003; Pandey et al., 2003; Sayeed et al., 2003; Monteiro et al., 2006). This event results in the formation of highly reactive compounds such as free radicals or oxyradicals (O_2^-, H_2O_2 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).

Catalase is the primary cellular enzymatic defense against H_2O_2, converting it into H_2O and O_2, and is critical for the process of scavenging free radicals (Dorval et al., 2003). In the current study, fish exposed to any of the concentrations of neem showed significant reduction in hepatic CAT activity, which is likely to affect the capacity of liver cells to defend themselves and respond to contaminant-induced oxidative stress. Impairment in antioxidative enzymes will produce an imbalance between pro- and antioxidant system causing the formation of toxic hydroxyl radicals with direct consequences on cell integrity and cell function itself (Winston and DiGiulio, 1991). A lower level of CAT activity might be due to an increased production of the superoxide radical (O_2^-), as an excess of this anion is known to inhibit CAT activity (Bainy et al., 1996). This mechanism may well explain the present results, indicating that neem extract may induce oxidative stress in *P. lineatus*. Reduced hepatic CAT activity was also found in carp (*Cyprinus carpio*) exposed for 4 days to 300 mg L^{-1} of chitosan, a natural polymer extracted from the exoskeleton of

crustaceans that has insecticidal properties (Dautremepuits et al., 2004).

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). It has been demonstrated that the activity of these enzymes may be enhanced in fish exposed to polycyclic and polychlorinated hydrocarbons (Zhang et al., 1990) and organophosphorus insecticides (Monteiro et al., 2006). Even low level organic contamination can lead to increased hepatic GST activity in fish (Machala et al., 1997). Almeida et al. (2005) registered higher liver glutathione-*S*-transferase activity in *P. lineatus* exposed to sediment potentially polluted with organic contaminants from agricultural sources and municipal landfill. Thus, the enhanced hepatic GST activity following the exposure to 5.0 g L^{-1} of neem extract should be related to the metabolism of organic compounds in the extract. The lack of an increased GST activity at 7.5 g L^{-1} neem may reflect impairment in the detoxifying capacity of the fish at levels above the LC_{50}.

Gills are extremely important in respiration, osmoregulation, acid–base balance and excretion of nitrogenous wastes in fish and they represent the greatest area of the animal in contact with external environment (Heath, 1995). Therefore, their morphology can be very useful as a parameter in environmental monitoring (Schwaiger et al., 1997). In the present study, the gills of *P. lineatus* exposed to neem extract showed the occurrence of histological alterations. Some of these alterations such as epithelial lifting and hyperplasia of the epithelial cells can be considered adaptive, since they increase the distance between the external environment and the blood, thus serving as a barrier to the entrance of contaminants (Mallat, 1985; Hinton et al., 1992; Poleksiæ and Mitroviæ-Tutundžiæ, 1994; Fernandes and Mazon, 2003). Lamellar aneurism, on the other hand, represents a lesion that can result from the rupture of pillar cells, and thus corresponds to the deleterious effect of xenobiotics on branchial tissue (Martinez et al., 2004). These lesions may result in decreased oxygen-uptake capacity of the gill making the fish exposed to neem less able to get adequate oxygen for its total metabolic activity. In this case a subsequent internal hypoxia would be expected to cause an increase in hematocrit due to swelling of the erythrocytes which occurs whenever fish blood cells are exposed to hypoxia. However, as it was already reported, no significant change in the hematocrit of *P. lineatus* exposed to neem was observed after 24 h. Alterations in hematological variables would possibly be observed at longer times of exposure, such as 96 h, as a consequence of gill damage.

Fish kidney receives the major part of the blood coming from the gill and is one of the first organ to be affected by contaminants in the water (Tophon et al., 2003). As for the gills, the same alterations have been described in the kidney of fish exposed to various contaminants, suggesting that histopathological changes in the kidney are also not specific to the stressors. Usually, the alterations in the posterior kidney of fish exposed to water contamination correspond to changes in tubules, such as granular and hyaline degeneration and changes in the corpuscle, such as dilatation of capillaries in the glomerulus and reduction of Bowman's space (Takashima and Hibiya, 1995). These alterations are among the ones observed in the posterior kidney of *P. lineatus* exposed to neem extract. The presence of degenerating cells in the tubules, together with the absence of necrosis in the kidneys studied here indicates that this tissue suffered damage after exposure to neem extract, but the short period of exposition may have prevented the establishment of necrosis in this organ. Even so, according to the DTC values the lesions observed after 24 h exposure to neem extract were severe enough to interfere on some important kidney functions of *P. lineatus*.

Overall, these results indicate that it is important to assess the functional and morphological responses in fish exposed to sublethal concentrations of plant extracts used in aquaculture. The aqueous extract of neem, although much less toxic to *P. lineatus* than other pesticides, does promote important functional and morphological changes in this fish and thus further work should be carried out in order to establish clear criteria for its safe use in fish-farming.

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