EFFECT OF NEEM LEAF EXTRACT ON FRESHWATER FISHES AND ZOOPLANKTON COMMUNITY

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Abstract

Neem (*Azadirachta indica*) is a medicinal plant of containing diverse chemical active substances of several biological properties. So, the aim of the current investigation was to assess the effects of water leaf extract of neem plant on the survival and healthy status of Nile tilapia (*Oreochromis niloticus*), African cat fish (*Clarias gariepinus*) and zooplankton community. The laboratory determinations of lethal concentrations (LC_{100} and LC_{50}) through a static bioassay test were performed. The 24 h LC_{100} of neem leaf extract was estimated as 4 and 11 g/l, for juvenile's *O. niloticus* and *C. gariepinus*, respectively, while, the 96-h LC_{50} was 1.8 and 4 g/l, respectively. On the other hand, the 24 h LC_{100} for cladocera and copepoda were 0.25 and 0.45 g/l, respectively, while, the 96-h LC_{50} was 0.1 and 0.2 g/l, respectively. At the highest test concentrations, adverse effects were obvious with significant reductions in several cladoceran and copepod species. Some alterations in glucose levels, total protein, albumin, globulin as well as AST and ALT in plasma of treated *O. niloticus* and *C. gariepinus* with $^1/_2$ and $^1/_{10}$ LC_{50} of neem leaf water extract compared with non-treated one after 2 and 7 days of exposure were recorded and discussed.

It could be concluded that the application of neem leaf extract can be used to control unwanted organisms in ponds as environment friendly material instead of deleterious pesticides. Also, extensive investigations should be established for the suitable methods of application in aquatic animal production facilities to be fully explored in future.

Key words: Fish, Zooplanktons, Neem, *Azadirachta indica*

INTRODUCTION

Recently the application of medicinal plants from different families in the management of aquaculture ponds is gaining momentum because they are safe, effective, widely available and inexpensive. Also, to produce fish free from any chemicals of public health hazards.

Neem; *Azadirachta indica* (*A. indica*), is one of the most promising medicinal plant, having a wide spectrum of biological activity, well known for its insecticidal properties (ICAR, 1993). Every part of neem tree have been known to possess a wide range of pharmacological properties, especially as antibacterial, antifungal, antiulcer, antifeedant, repellent, pesticidal, molluscicidal, ecdysone inhibitor and sterilant and is

thus commercially exploitable (Biswas *et al.*, 2002; Das *et al.*, 2002), and hence, traditionally used to treat large number of diseases (Van Der Nat *et al.,* 1991). This eco-friendly native tree of India is perhaps most researched tree in the world. Water soluble extract of *A. indica* leaves was found to possess significant hypoglycemic, hypolipidemic, hepatoprotective, anti-fertility and hypotensive activities.

Both fish parasites and fish predators which cause great economic losses in productivity are mainly controlled with toxic chemicals, mostly applied indiscriminately and without adequate training (Senhorini, 1991; Rodrigues *et al.*, 1997). 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 several 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).

Neem has been used successfully in aquaculture systems to control fish predators (Dunkel and Ricilards, 1998). Martinez (2002) stated 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 dragon-fly 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 zillii* (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).

Zooplanktons are the natural food items of many marine and freshwater fishes and crustaceans. They have been used extensively to rear larvae and fry (De Pauw *et al.* 1981; Tay *et al*. 1991). Studies have shown that fry performed better when fed zooplankton than when fed with artificial dry diets (Dabrowski 1984; Dave 1989). Such results indicated that neem extracts added to water have been expressed toxicity in the natural feed (zooplankton). Consequently it is important to recognize these disturbances.

In the present study, we determine the toxicity of the aqueous extract of neem leaves on the survival and healthy status of Nile tilapia (*Oreochromis niloticus*), African cat fish (*Clarias gariepinus*) and its ecological impacts on zooplankton community.

MATERIALS AND METHODS

Preparation of aqueous neem leaf extract

Azadirachta indica (*A. indica*) leaves were obtained from the surrounding area of Abbassa laboratory, dried and finely chopped, then dissolved in tap water, at a concentration of 500 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 (500 g/l) was used immediately in the experiments, in different dilutions.

Experimental Fish and zooplanktons

Apparently healthy Nile tilapia; *Oreochromis niloticus* and African cat fish; *Clarias gariepinus* weighed 50.2 ± 2.5 and 100 ± 4.5 g, respectively were collected from the Fish Farm of Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad, Sharkia, Egypt and acclimated in indoor tanks supplied with dechlorinated tap-water and continuous aeration for 2 weeks. In addition, zooplankton samples were collected from the same site. Total volumes of the composite samples were approximately 30 L. Water was filtered through a 40 umm mesh collection net, sub samples were removed from this volume during mixing with a large-bore, Hensen–Stemple pipette. Sub-sample volumes ranged from 1 to 10 ml and enumerated at 25x magnification.

Determination of 24-h LC_{100} and 96-h LC_{50}

Static toxicity tests were run to determine lethal and sublethal concentrations (24-h LC_{100} and 96-h LC_{50}) of neem leaf extract to *Oreochromis niloticus* and *Clarias gariepinus* as well as cladocera and copepods. For fish, tests were conducted in 30 L glass aquaria, 6 fish per aquarium, containing neem leaf extract diluted in tap water to the following concentrations: 0 (control group), 1, 5, 10, 15, 20, 25 and 30 g /l. For zooplankton, tests were conducted in 1 L glass beakers, with abundance of 20 individual of cladocera (*Daphnia sp.*) or 20 individual of copepods (*Cyclops sp.*) per beaker, containing neem leaf extract diluted in tap water to the following concentrations, 0 (control group), 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 and 0.45 g /l. Each treatment had 3 replicates. The test containers were examined and counted every 3 days. Once the test individuals began to reproduce, the neonates were discarded. All laboratory conditions were maintained constant. Deaths and abnormal behavior fish as well as cladocera and copepoda (*Daphnia sp.* & *Cyclops sp.*) were recorded every 2 h for the 1st day, then every day for other 3 days. The value of 24 h-LC_{100} and the 96-h LC_{50} were estimated. The survival rate for cladocera and

copepoda (*Daphnia sp.* & *Cyclops sp.*) was estimated for 21 days of exposure to the mentioned concentrations.

Physiological and biochemical assays for fish

To evaluate acute effects of neem leaf extract, fish were distributed in three groups of eight fish each, comprising two experimental groups and one control. Each group was placed into 80 L glass aquaria. Experimental groups were exposed for 2 and 7 days to two concentrations of neem leaf extract corresponding to a sublethal (10% & 50% LC_{50}). The control group was simultaneously exposed to dechlorinated tap water. The experiments were carried out in static systems. Temperature, pH, and dissolved O_2 were monitored continuously. The tests were conducted in duplicates. At the end of the 2^{nd} and 7^{th} day of exposure, at least 6 fish for each of the three groups were removed and blood was collected from the caudal vein, using heparin-coated syringes, then centrifuged at 3000 rpm for 10 minutes at low temperature; plasma was collected and stored at –20 ^{o}C for biochemical assays.

Plasma aspartate amino-transferase (AST) and alanine amino-transferase (ALT) were determined according to Reitman and Frankel (1957). Total plasma protein was determined according to Henry (1964). Albumin was determined colorimetrically according to Wotton and Freeman (1982). Globulin was obtained by the subtraction of albumin from total protein. Plasma glucose was determined colorimetrically using glucose kit according to Trinder (1969). All kits used were produced by Egyptian American Co. for Laboratory Services, Egypt.

Statistical Analysis

The data were statistically analyzed using Duncan's multiple range test to determine differences in means (Duncan, 1955).

RESULTS AND DISCUSSIONS

Medicinal plants are environment friendly containing diverse biologically active principles. 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, its place of origin or even the individual tree (Luo *et al.*, 1999 and Winkler *et al.*, 2007). The 24-h LC_{100} of neem leaf extract for Nile tilapia and cat fish were estimated as 4 and 11 g/l, respectively. While the 96-h LC_{50} were 1.8 and 4 g/l, respectively. Also, the 24-h LC_{100} of neem leaf extract for cladocera and copepoda was 0.25 and 0.45 g/l, respectively, while, the 96-h LC_{50} was 0.1 and 0.2 g/l, respectively (Table 1). Compared to other synthetic pesticides used in fish farming, such as carbamates and organophosphates, neem based products are certainly less toxic to fish (Wan *et al.*, 1996). Results indicated that tilapia is more sensitive to neem

leaf water extract than cat fish. The neem leaf extract applications appeared to affect the abundance of the major crustacean zooplankton groups at all test concentrations. By the end of the sampling period, the abundance of copepods was low overall, but was lower than controls at all other treatment levels (Tables, 2 & 3). Significant treatment effects on zooplankton communities were detected at all test concentrations. Among adult and juvenile copepods, negative effects were evident at the lowest test concentration.

Fish exposed to higher concentration of the plant extract exhibited respiratory distress, erratic swimming, off feed and nervous manifestations. Winkaler *et al.*, (2007) noticed that fish exposed to all neem extract concentrations exhibited damaged gill and kidney tissue.

Table 1. Lethal and sublethal concentrations of neem leaf aqueous extract for Nile tilapia, African cat fish and zooplankton community (cladocera and copepoda)

Fish and zooplanktons	24-h LC_{100} (g/l)	96-h LC_{50} (g/l)	$^1/_2$ LC_{50} (g/l)	$^1/_{10}$ LC_{50} (g/l)
Nile tilapia	4	1.8	0.9	0.18
African cat fish	11	4	2.0	0.4
Cladocera	0.25	0.1	-	-
Copepoda	0.45	0.2	-	-

Table 2. Effect of different concentrations of neem leaf aqueous extract on the survival rate of mature (organism/l) cladocera (*Daphnia sp.*) throughout different periods.

Days / Treat.	Zero time	3 days	6 days	9 days	12 days	15 days	18 days	21 days
Cont	100	98.3	98.3	98.3	98.3	98.3	98.3	98.3
0.05	100	80	70	56.6	46.6	41.6	35	28.3
0.10	100	51.6	43.3	38.3	30	23.3	15	8.3
0.15	100	18.3	1.6	0	0	0	0	0
0.20	100	2.2	0.1	0	0	0	0	0
0.25	100	0	0	0	0	0	0	0
0.30	100	0	0	0	0	0	0	0
0.35	100	0	0	0	0	0	0	0
0.40	100	0	0	0	0	0	0	0
0.45	100	0	0	0	0	0	0	0

Table 3. The effect of different concentrations of neem leaf aqueous extract on the survival rate (organism/l) of mature copepoda (*Cyclops sp.*) throughout different periods.

Days / Treat.	Zero time	3 days	6 days	9 days	12 days	15 days	18 days	21 days
Cont	100	100	98.3	98.3	98.3	96.6	96.3	96.3
0.05	100	91.6	90	86.6	86.6	78.3	73.3	68.3
0.10	100	86.6	80	75	71.6	66.6	56.6	48.3
0.15	100	80	76.6	71.6	50	63.3	55	45
0.20	100	70	63.3	56.6	49.4	45	38.3	30
0.25	100	55	46.6	38.3	31.6	26.6	21.6	13.3
0.30	100	20.1	1.8	0.5	0	0	0	0
0.35	100	8.1	3.5	0	0	0	0	0
0.40	100	0	0	0	0	0	0	0
0.45	100	0	0	0	0	0	0	0

The plasma glucose level was significantly higher in Nile tilapia exposed to 0.9 or 0.18 g/l of neem leaf extract after the 2nd and 7th day of exposure and the increase was dose-dependent. On the other hand, plasma glucose didn't exhibit any significant changes in cat fish exposed to 2 or 0.4 g/l of neem leaf extract after the 2nd or 7th day of exposure (Table 4). The increase in blood glucose can be viewed as part of a stress response triggered by the presence of neem leaf extract in water (Winkaler *et al.*, 2007). The increase in blood glucose in might be resulted from an increase in plasma catecholamine and corticosteroid hormones (Pickering, 1981). Moreover, Gupta (1974) mentioned that the hyperglycemia induced by any toxicant might be explained by the inhibition of the neuro-effector sites in the adrenal medulla leading to hyper secretion of adrenaline, which stimulates the breakdown of glycogen to glucose.

The quantitative determination of total plasma protein reflects the liver capacity of protein synthesis and denotes the osmolarity of the blood and the renal impairments. So, it is of valuable effect in the diagnosis of the toxicity of the fish. Martinez *et al.* (2004) mentioned that fish under stress may mobilize protein to meet energy requirements needed to sustain increased physiological activity. In the present study, *O. niloticus* showed significant increases in serum total protein, albumin, and globulin. Thus, it may be inferred that the observed hyperglycemia during acute exposure to sublethal concentrations of neem leaf extract, is sufficient satisfy the raised energy demands arising from the chemical stress and don't use the excess of protein as previously mentioned by Winkaler *et al.* (2007). In case of cat fish, the results showed slight changes in these parameters indicating to its more tolerance to the neem leaf water extract than in tilapia (Tables 4 and 5).

Table 4. Effect of sublethal concentrations of neem leaf aqueous extract on plasma glucose (mg/dl) and total protein (g/dl) of Nile tilapia and African cat fish for different periods.

Fish		Nile tilapia		African cat fish	
Concentrations	Parameters / Periods	Glucose	T. protein	Glucose	T. protein
control		41.56 ± 1.51^{C}	1.60 ± 0.05^{C}	35.98 ± 1.12^{A}	2.03 ± 0.11^{A}
$^{1}/_{2}$ 96 h LC_{50} (g/l)	2 days	108.49 ± 2.01^{A}	3.62 ± 0.12^{A}	36.22 ± 1.32^{A}	2.09 ± 0.02^{A}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		69.23 ± 2.11^{B}	2.13 ± 0.06^{B}	35.85 ± 2.14^{A}	1.99 ± 0.05^{A}
control		59.38 ± 1.35^{C}	1.65 ± 0.10^{B}	32.48 ± 1.52^{A}	2.11 ± 0.22^{A}
$^{1}/_{2}$ 96 h LC_{50} (g/l)	7 days	123.77 ± 1.44^{A}	1.93 ± 0.13^{A}	33.21 ± 1.42^{A}	2.24 ± 0.14^{A}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		118.52 ± 2.13^{B}	1.82 ± 0.08^{A}	32.50 ± 1.95^{A}	2.15 ± 0.23^{A}

Means with the same letter in the same square are not significantly different at P<0.05

Table 5. Effect of sublethal concentrations of neem leaf aqueous extract on plasma albumin and globulin g/dl of Nile tilapia and African cat fish for different periods.

Fish		Nile tilapia		African cat fish	
Concentrations	Parameters / Periods	Albumin	Globulin	Albumin	Globulin
control		1.20 ± 0.02^{B}	0.38 ± 0.02^{C}	0.99 ± 0.13^{A}	1.02 ± 0.07^{A}
$^{1}/_{2}$ 96 h LC_{50} (g/l)	2 days	2.03 ± 0.01^{A}	1.45 ± 0.21^{A}	1.01 ± 0.18^{A}	1.02 ± 0.08^{A}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		1.31 ± 0.11^{B}	0.84 ± 0.14^{B}	0.99 ± 0.14^{A}	0.99 ± 0.05^{B}
control		1.18 ± 0.12^{A}	0.48 ± 0.17^{C}	0.96 ± 0.11^{B}	1.06 ± 0.10^{A}
$^{1}/_{2}$ 96 h LC_{50} (g/l)	7 days	1.23 ± 0.14^{A}	0.69 ± 0.11^{B}	1.22 ± 0.14^{A}	1.01 ± 0.02^{A}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		0.73 ± 0.02^{B}	1.05 ± 0.21^{A}	1.18 ± 0.11^{A}	0.84 ± 0.04^{B}

Means with the same letter in the same square are not significantly different at P<0.05

Results in table (6) showed significant increase in plasma AST and ALT activities on the 2nd day of exposure to $^{1}/_{10}$ LC_{50} and significant decrease in case of exposure to $^{1}/_{2}$ LC_{50} in both fish species. On the other hand, it showed significant decrease in both fish species during the 7th day of exposure to the two concentrations in comparison with the control groups. The increase in plasma AST and ALT were attributed to the hepatocellular damage as a result of toxic effect of low concentrations of neem leaf water extract. The same results were previously recorded by Daabees *et al.* (1992); Nesckovic *et al.* (1996) and Mousa (1999) in case of exposure to other toxic agents. The decreases occurred in case of exposure to the high concentrations were attributed to the inhibition of enzymes synthesis as a result of toxic effect of the neem leaf water extract as previously mentioned by Mousa (2004) and Shalaby *et al.* (2007) in other toxicological studies. Many environmental pollutants,

including pesticides, are capable of inducing oxidative stress in fish (Sayeed *et al*., 2003 and Monteiro *et al*., 2006). This event results in the formation of highly reactive compounds such as free radicals or oxy-radicals 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). Impairment in anti-oxidative 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).

Table 6. Effect of sublethal concentrations of neem leaf aqueous extract on plasma AST and ALT (u/l) of Nile tilapia and African cat fish for different periods.

Fish		Nile tilapia		African cat fish	
Concentrations	Parameters / Periods	AST	ALT	AST	ALT
control	2 days	18 ± 1.21^{B}	23 ± 1.02^{B}	20 ± 0.52^{B}	16 ± 2.01^{B}
$^{1}/_{2}$ 96 h LC_{50} (g/l)		11 ± 1.04^{C}	11 ± 1.41^{C}	13 ± 1.00^{C}	10 ± 0.98^{C}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		32 ± 1.65^{A}	41 ± 0.54^{A}	34 ± 1.34^{A}	48 ± 2.41^{A}
control	7 days	16 ± 0.68^{A}	20 ± 1.01^{A}	22 ± 0.69^{A}	18 ± 0.57^{A}
$^{1}/_{2}$ 96 h LC_{50} (g/l)		10 ± 1.01^{C}	9 ± 1.20^{B}	11 ± 1.21^{C}	10 ± 0.64^{B}
$^{1}/_{10}$ 96 h LC_{50} (g/l)		13 ± 0.88^{B}	10 ± 1.10^{B}	17 ± 1.14^{B}	19 ± 1.22^{A}

Means with the same letter in the same square are not significantly different at $P<0.05$

From the present study, it could be concluded that the application of neem leaf extract can be used to control unwanted organisms in ponds as environment friendly material instead of deleterious pesticides. Also, extensive investigations should be established to provide information for the suitable methods of application in aquatic animal production facilities to be fully explored in future for its safe use in aquaculture.

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تأثير المستخلص المائي لورق أشجار النيم على أسماك المياه العذبة والهائمات الحيوانية

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تعتبر أشجار النيم من الأشجار ذات الفوائد الطبية المتعددة لإحتوائها على العديد من الخواص البيولوجية والمركبات الطبيعية حيث يحتوي كل جزء من هذه الشجرة على خاصية بيولوجية مستقلة أو مركب طبيعي مختلف عن الأجزاء الأخرى ، لذلك أجريت هذه الدراسة لمعرفة تأثير المستخلص المائي لأوراق هذه الشجرة على معدل الإعاشة وبعض النواحي البيوكيميائية في سمكة البلطي النيلي وسمكة القرموط الأفريقي ، وكذلك معرفة الجرعات المميتة من هذا المستخلص لبعض الهائمات الحيوانية شائعة الإستخدام في تغذية صغار هذه الأسماك. وقد أوضحت النتائج المعملية أن الجرعات المميتة خلال ٢٤ ساعة لسمكة البلطي والقرموط كانت ٤ ، ١١ جرام/لتر على التوالي وكانت ٠,٢٥ ، ٠,٤٥ جرام/لتر للهائمات الحيوانية (الكلادوسيرا والكوبيبودا) على التوالي. أما الجرعات المميتة للنصف خلال ٩٦ ساعة فكانت ١,٨ ، ٤ جرام/لتر لسمكة البلطي والقرموط على التوالي وكانت ٠,١ ، ٠,٢ للكلادوسيرا والكوبيبودا جرام/لتر على التوالي. وعند تعرض الأسماك محل الدراسة لنصف الجرعة المميتة للنصف وكذلك عشرها تبين أن هذه التركيزات قد أحدثت اضطرابات في بعض القياسات البيوكيميائية في البلازما بعد يومين وسبعة أيام من التعرض مقارنة بالمجموعات الضابطة ، غير أن هذه التغيرات كانت أكثر وضوحا في سمكة البلطي مقارنة بالقرموط الأفريقي دلالة على أن سمكة البلطي أكثر حساسية من سمكة القرموط لهذا المستخلص المائي من أوراق النيم. وتشير النتائج أيضا إلى أن الهائمات الحيوانية تتأثر بتركيزات منخفضة كثيرا عن تلك التي تتأثر بها الأسماك محل الدراسة.

ويمكن القول بأن التركيزات العالية من مستخلص أوراق النيم لها سمية واضحة لأسماك البلطى بينما أسماك القرموط تقاوم هذة التاثيرات ، لذلك يجب الحذر عند استخدام هذا المستخلص للمعالجة أو القضاء على الكائنات الغير مرغوب فيها في أحواض التربية بحيث يكون بتركيزات منخفضة لتفادى آثاره الضارة على الأسماك المذكورة وكذلك للحفاظ على الغذاء الطبيعي لها (الهائمات الحيوانية). كما يجب اجراء دراسات مكثفة لإستخدام هذا النبات بالطرق الآمنة للوصول للغرض المنشود من استخدامه.