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Study on the toxicity of neem (*Azadirachta indica* A. Juss) leaf extracts as phytopiscicide on three life stages of Mozambique tilapia (*Oreochromis mossambicus* Peters*)* with special reference to their ethological responses

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Abstract

Acute toxicity of leaf extracts of neem (*Azadirachta indica* A. Juss) on three different stages of fresh water weed fish *Oreochromis mossambicus* was investigated in the present study. The 24, 48, 72 and 96 h LC_{50} values for *O. mossambicus* fry were 3.29, 2.62, 2.19 and 1.67 g/l respectively. These values were 4.96, 3.56, 2.74, 2.27 g/l and 7.58, 7.00, 6.28, 5.83 g/l for juvenile and adult fish respectively. Toxicity factor values of the toxicant to the fish irrespective of stage were increased with the time of exposure. During the study, ethological changes in the adult fish exposed to two sub-lethal concentrations of the toxicant (10 and 20% of 96h LC_{50}) were also observed. It showed an alteration in behaviour pattern with the increasing concentration of the neem leaf extracts and time of exposure.

Keywords: Acute toxicity, ethological responses, neem, *Oreochromis mossambicus.*

Introduction

Recently water soluble neem (*Azadirachta indica* A. Juss) extracts extensively used in fish farms to control fish pathogens (Das et al., 2002) and fish fry predator insects (Martinez, 2002). Besides, many studies illustrated on its piscicidal potentiality to control unwanted predator and weed fish during pond preparation before fish seed stocking (Fafioye, 2012). In India, Mozambique tilapia was introduced into pond and reservoir ecosystem in 1952, with a view to filling up unoccupied niches and spread all across the country within a few years due to its prolific breeding capacity and adaptability to wide range of environmental condition. But, its performance in open water bodies of the country has been discouraging over the last many years due to its uncontrolled breeding capacity, which led to excessive recruitment, stunting growth and a low percentage of marketable-sized fish, dampened the initial enthusiasm for tilapia as a food fish and thus considered as a weed fish (Jhingran, 1991). Mousa et al., (2008) and Fafioye (2012) assessed the acute toxicity of

neem leaf extracts on Nile tilapia and its effects on their health status. The extracts of neem bark may cause respiratory problems in *Tilapia zilli* (Omoregie and Okpanachi, 1997) and long exposure to sublethal concentrations of crude extract of this plant generally delays the growth of this cichlid fish (Omoregie and Okpanachi, 1992). There is no report of acute toxicities of neem on the mozambique tilapia. Therefore, the objective of the present study was to determine the acute toxicity of neem leaf extract to the different life stages of fresh water weed fish, *O. mossambicus* as they are commonly eradicated from the fish pond as unwanted ones prior to stocking during scientific aquaculture practice. Their behavioral responses were also observed in the present study during bioassay to assess the toxicity of neem leaf extract.

Materials and methods

Fresh leaves of neem plant (*Azadirachta indica*; Family: Meliaceae) were collected locally and cleansed with dechlorinated tap water to remove dust. Washed leaves were weighed by balance and then chopped and finely grounded in blender. Distilled water was added to the chopped leaves before grinding at the ratio of 2:1 by volume. To prepare an aqueous extract the residual leaves parts were separated by hand squeezing using fine cloths. The fresh extract was used immediately for the experiment at different concentrations. Different life stages of the fish species, *Oreochromis mossambicus* (Family: Cichlidae) were collected from the local ponds using hand net in the evening and brought to the laboratory with care avoiding minimum stress. Fry (mean length 1.09±0.166 cm, mean weight 0.427±0.045 g), juvenile (mean length 3.59±0.508 cm, mean weight 3.24±0.442 g) and adult (mean length 12.54±1.007 cm, mean weight 30.89±6.233 g) fish were separated. The fish used in the bioassay were treated with 0.1% $KMnO_4$ solution to avoid any pathogenic infection and acclimatized to the test condition for 72h before their use. The fishes were fed with fine mixture of rice bran and mustard oil cake (1:1) during the acclimatization period but not fed 24h before and during the bioassays.Static replacement bioassays were used for both acute toxicity tests for determination of 24, 48, 72 and 96h LC_{50} and recording behaviour changes. The underground water (temperature 27.3 ± 0.25 °C, pH 7.3 ± 0.14, free CO_2 8.6 ± 0.30 mg/l, DO 5.74 ± 0.22 mg/l, alkalinity 167 ± 8.30 mg/l as $CaCO_3$, hardness 115 ± 7.0 mg/l as $CaCO_3$) was used as a test medium. The test medium was replaced every 24h by freshly prepared test solution to avoid the interference of different abiotic factors with the animals' performance. Water chemical analysis and the bioassays were done following the methods outlined in American Public Health Association (APHA, 2005). Acute toxicity tests for fish irrespective of sex were conducted in 15L glass aquaria holding 10L of non chlorinated tap water in the laboratory. The selected test concentrations of neem leaf extract used for the determination of acute toxicity to *O. mossambicus* were 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 g/l for fry based on rough range finding tests. Similarly, selected test concentrations of the toxicant were 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 g/l for juvenile and 0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 g/l for adult fish. Each concentration was accompanied by three replicates. Ten organisms were used in each replicate. The number of dead fishes was counted every 24h and removed immediately from the test medium to avoid any organic decomposition and oxygen depletion.

Median lethal concentrations (LC_{50}) of neem

leaf extracts for different stages of *O. mossambicus* were calculated for different time of exposure (24, 48, 72 and 96h) using the data of mean mortality rate at different concentrations of the toxicant after Sprague (1975) and Dede and Kaglo (2001) following the formula:

$LC_{50} = LC_{100} – (\sum$ Mean death X Concentration difference of the toxicant / No. of test organisms per group).

On the basis of acute toxicity values toxicity factors at different exposure period were assessed following the formula after Ayoola et al., (2011):

Toxicity factor (TF) = LC_{50} at 24h / LC_{50} at any other exposure time.

The ethological changes in activities like cough (rapid and repeated opening and closing of mouth and opercular covering with partial extension of fins), yawn (wide opening of mouth and hyperextension of fins), fin flickering (repeated extension and contraction of dorsal fin), burst swimming (sudden and rapid forward movements), s-jerk (movement of body sequentially from head to tail) and partial jerk (movement of head or tail only) of the adult fish exposed to two different sub lethal concentrations of the neem leaf extracts (10 and 20% of 96h LC_{50}) were recorded at 24, 48, 72 and 96h intervals (Henery and Atchison, 1986). Each concentration was accompanied by three replicates containing 10 organisms in each. The observations were conducted for 30 minutes for each aquarium and the time of observation was rotated from the morning to evening to avoid the diurnal fluctuations in behaviour of the exposed fish.

Results

The median lethal concentration (LC_{50}) of neem leaf extracts at different time of exposure (24, 48, 72 and 96h) with 95% confidence limits and toxicity factors to different life stages of *Oreochromis mossambicus* are given in Table 1 and 2. No mortality was observed in the control group during the experiment.

In the present study exposed fish showed alteration in behaviour in addition to discolouration of the body. During acute toxicity test the fish under exposure showed erratic swimming, loss of equilibrium and hyperactivity with the advancement of time of exposure and concentration.

Table 1. Mean lethal concentrations (LC_{50}) of neem leaf extracts to different life stages of *Oreochromis mossambicus.*

Life stages of the fish	LC_{50} (g/l)			
	24h	48h	72h	96h
Fry	3.28	2.62	2.19	1.67
Juvenile	4.96	3.56	2.74	2.27
Adult	7.58	7.00	6.28	5.83

Table 2. Toxicity factor of neem leaf extracts to *Oreochromis mossambicus* under different exposure period.

Exposure time (h)	Toxicity factor value		
	Fry	Juvenile	Adult
24	1.00	1.00	1.00
48	1.25	1.39	1.08
72	1.50	1.81	1.21
96	1.97	2.19	1.30

Initially rapid fin movement and swimming rate of the treated juvenile and adult fishes were gradually decreased at 96h of exposure at higher concentrations of neem leaf extracts. Treated fish also exhibited various distressed signs like reduced movement, lost of balance and bottom settling in a motionless condition at 96hr of exposure at higher concentration of neem extracts.

Table 3. Changes in frequencies of ethological parameters of adult *Oreochromis mossambicus* exposed to two sub-lethal concentrations (10 and 20% of 96h LC_{50}) of neem leaf extracts under different exposure period (24, 48, 72 and 96h). Data are the mean frequencies (± SD) of the observation period of 30 minutes at every 24h interval. Here C, T_1 and T_2 stand for control (0.00 g/l), 10 and 20% of 96h LC_{50} values of the toxicant respectively.

Ethological parameters	72h			96h		
	C	T_1	T_2	C	T_1	T_2
Cough	9.33±0.58	12.66±1.53	11.33±1.53	9.66±0.58	11.33±1.15	13.66±0.58
Yawn	4.66±0.58	11.66±0.58	15±1	5.33±1.53	13.33±0.58	14.33±0.58
Fin flickering	61.66±2.081	94±3	126±4	54.66±3.06	88±2	147.33±2.52
Burst swimming	2±1	5.33±1.15	6.66±1.53	1.33±0.58	3.33±1.154	2.66±0.58
S-jerk	2.33±1.53	8.66±1.53	9.33±0.58	2.66±1.15	7.66±1.53	8.66±1.53
Partial jerk	4.33±1.53	5.33±2.52	7±1	3.33±1.53	7±1	7.66±1.15

Ethological parameters	24h			48h		
	C	T_1	T_2	C	T_1	T_2
Cough	9.66±0.58	10.33±1.15	10.66±0.58	11.33±1.15	12.33±0.58	11.66±0.58
Yawn	2.66±1.53	14±1	18±1	3.66±0.58	16.66±1.15	17.66±1.53
Fin flickering	57.66±.08	108.33±3.51	140±5	65.33±2.52	99.33±4.16	130.66±5.03
Burst swimming	1.66±0.58	2.66±1.53	4.66±2.31	1.33±0.58	5±2	5.33±2.52
S-jerk	1.66±1.15	4±1.73	6±1	2±1	4±1	8.33±1.15
Partial jerk	4.33±1.53	5.33±2.52	7±1	3.33±1.53	7±1	7.66±1.15

Fish fry showed swirling movements at higher concentrations of the toxicant. However, the ethological responses of the adult fish under exposure of two sub-lethal doses of neem leaf extracts were recorded in Table 3. Initially rapid fin movement and swimming rate of the treated juvenile and adult fishes were gradually decreased at 96h of exposure at higher concentrations of neem leaf extracts. Treated fish also exhibited various distressed signs like reduced movement, lost of balance and bottom settling in a motionless condition at 96h of exposure at higher concentration of neem extracts. Fish fry showed swirling movements at higher concentrations of the toxicant. However, the ethological responses of the adult fish under exposure of two sub-lethal doses of neem leaf extracts were recorded in Table 3. Throughout the exposure period cough and yawn of the exposed fish did not affect severely than control fish but the frequencies of other ethological responses like fin flickering, burst swimming, partial and S-jerk altered remarkably as compared with the control (Table 3). The dose dependent changes in response were more pronounced in the exposed fish initially showing their hyperexcitability but gradually decreased with the time of exposure.

Discussion

Piscicidal plants contain different active compound which are responsible to kill fish. The lethal toxicity values of neem leaf extracts to *Oreochromis mossambicus* in the present study indicates its potent piscicidal activity. Probably azadirachtin-A, a most potent tetranorterpenoid found in neem with pesticidal properties is the active ingredient which is responsible to kill fish (Winkaler et al., 2007). The acute toxicity of neem leaf extracts to fish is very much pervasive (Saravanan et al., 2011). The piscicidal effects of neem leaf extracts to fish *O. mossambicus* expressed as 24h LC_{50} values found in the present study (3.28, 4.96 and 7.58 g/l for fry,

juvenile and adult respectively) is manifold higher than the findings of Saravanan et al., (2011) in *Cirrhinus mrigala* (1.035 g/l). Similarly, 96h LC_{50} value (5.83 g/l) for the adult fish in the present observation was higher than the value for *Clarias gariepinus* (4 g/l) as reported by Mousa et al., (2008). The median lethal value for fry mozambique tilapia (1.67 g/l) found in the present experiment may be compared with the value for *O. niloticus* (1.80 g/l) as recorded earlier. Cruz et al., (2004) observed 96h LC_{50} value of 4.80 g/l for *Prochilodus lineatus*. This finding was almost similar with 24h LC_{50} value (4.96 g/l) for juvenile but little lower than the 96h LC_{50} value (5.83 g/l) for adult fish in the present study. Such variation in the lethal toxicity probably due to variation in fish species used, their age, sex and size, test methods and water quality (Don-Pedro, 1996). Differences in the sensitivity of fish species to neem may also be attributed to the variation in the amount of active compound present in neem depending on its plant parts, its origin or even the individual tree (Isman et al., 1990; Lue et al., 1999). The median lethal concentrations of neem leaf extracts to fish showed increasing values in relation to age of fish suggesting that resistance of the fishes increased with age (Sharma and Sharma, 1995; Ganesh et al., 2000). Such increase in resistance to neem toxicity with age may be attributed as a size effect or some other factor closely related to size or age (Chapman, 1978). Development of gill mechanism in fish with age may also play an important role to overcome toxic effect in connection with oxygen uptake (Sharma and Sharma, 1995). With the progress of time of exposure toxicity factor for neem leaf extracts as a toxicant increases to *O. mossambicus* in the present investigation. This finding may be used as tool to establish toxicity scale for neem leaf extracts as well as to establish its environmental safety limit in the fish farm for controlled management practice (Mason, 1992). The study revealed that the neem leaf extracts can cause marked ethological changes in fish and can be identified as a sensitive indicator of physiological stress in fish (Asraf et al., 2010). It is an indicative of internal disturbances of the body functions due to toxicant induced cumulative deleterious effects at various metabolic sites of the fish body or due to disruption of nervous system function. Ethological responses such as body imbalance, restlessness, loss of equilibrium were observed by Saravanan et al., (2011) in *Cirrhinus mrigala* under toxic stress of neem leaf extract. Time and dose dependent behavioural changes as recorded in the present study were in conformity with the observation of Mousa et al. (2008) in Nile tilapia and African cat fish exposed to neem leaf extract. Ashraf et al., (2010) recorded discolouration, erratic swimming, loss of reflex and settling at bottom in a motionless condition in different fishes under the exposure of various plant extrascts of different piscicidal plants. Some of these behaviours were consistent with those found in the present study in *O. mossambicus*. The fish under exposure did not show significant changes in the frequencies of cough and yawn in the present study but their hyperexcitation, jerking movement may be in order to get relieved from the stressful environment. Initial hyperactivity in the exposed fish was probably an early indication of their avoidance reaction from the toxicant which may be related to narcotic effects or to change in sensitivity of chemo receptors (Suterlin, 1974). It is quite obvious that the elevated muscular activity in connection with changes in fin flickering and jerking

movement of the neem extract treated fish requires considerable amount of energy but cessation of movements indicates its less supply with the progress of time. With the progress of time the fish exhibited sluggish movement and cessation of swimming indicating the effects of neem on the central nervous system or it may be to compensate the demand of energy (Al-Kahem, 1995). The active ingredient present in the neem probably interfere with the membrane transport of Na^+, K^+, Ca^{2+}, or Cl^- ions, inhibit selective enzyme activities, and contribute to the release and/ or the persistence of neurotransmitters at synaptic junctions which leads to hyperactivity, swimming in imbalanced manner and lethargy (Ecobichon, 1991). The present findings highlight the toxicity of neem leaf extracts to *Oreochromis mossambicus* indicating its potentiality as phytopiscicide. Fish at their different life stages exposed to different concentrations of fresh neem leaf extracts exhibited toxic responses which eventually lead to death. The lethal toxicity values of the extracts to *O. mossambicus* thus serve as baseline information on its toxicity which may be helpful in formulating the dose of neem extracts as organic piscicide in aquaculture management. The ethological responses in exposed fish are the most sensitive parameters to measure the neurotoxicity (Doving, 1992). Thus ethological changes in fish treated with neem leaf extracts focus on the nature of toxicity as well as physiological state of the fish under exposure. However, the importance of this study is to determine the safer level of the aqueous extracts of *Azadirachta indica* leaves in respect of different life stages of fish in the fish farm.

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