THE USE OF SOME GENETIC AND BLOOD PARAMETERS TO ASSESS THE NEEM SEED OIL TOXICICTY IN TILAPIA (*OREOCHROMIS NILOTICUS*) FISH

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ABSTRACT

The current study was conducted to investigate the effect of two sublethal doses of neem seed oil (NO) on somegenetic and blood variables in tilapia fish species *Oreochromis niloticus*. The 96 h LC_{50} value was 1230 mg/L. For chronic exposure, $^1/_{10}$ and$^1/_{20}$ of 96 h LC_{50}value wereused in experimental treatments. At the end of chronic exposure (three weeks), some blood parameters including RBCs, Hb, Hct, and MCHC were significantly decreased. However, a significant increase in WBCs,MCV and MCH values were observed in exposed fish. The changes occurring in DNA characteristics of fish following chronic toxicity at intervals of 7,14, and 21days included variations in band intensity, loss of normal bands and appearance of new bands.The changes were dose-dependent. Also, the results indicated that genomic template stability (a qualitative measure reflecting changes in DNA profiles) was significantly affected at the high dose. Conclusively, the DNA polymorphisms detected by PCR analysis associated with some deviated blood parameters could be used as a useful biomarker assay for the detection of natural pesticide pollution in fish.

Keywords: Neem seed oil, Tilapia *(O. niloticus)*, Blood , genotoxicity.

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INTRODUCTION

Fish and other aquatic organisms are directly influenced by pesticides through different pollution of aquatic surface [1] in many fish organs and vital systems [2].Pesticides of plant origin are less toxic than synthetic to fish.The synthetic pesticides are applying as pesticide for cleansing the culture fishpond [3][4].Pesticides application mostly associated with different types of harmfulness in fish as hematological changes [5]and alteration in genetic system [6][7]. Massive use of plant pesticides that could reach water streams may also have an adverse effect on non-target organisms in an appropriate time [8][9].

Recently, the use of eco-friendly botanical pesticides is considered as pests control [10]. Neem, *Azadirachtaindica* A. Juss, provides many useful compounds that could be used as pesticide in the form of oil extract[11], one of them is azadirachtin which is the principal active ingredient of neem seed oil [12]. Also, neem has been used successfully in aquaculture systems to control fish marauders [13].

Although neem extract is considered target specific and of low toxicity towards non-target aquatic life [14], water extracts of the bark of the neem plant caused respiratory problems in redbelly tilapia, *Tilapia zilli* (Gervais) [15], while long exposure to low concentrations of the crude extract of *A. indica* delayed the growth of this cichlid fish [16].

Previous study reported that neem extracts have toxic effects at higher dose. An extensive acute toxicity study in rats and rabbits using neem seed oil showed that the neem leaves extract at 200 mg/kg concentration was foundto be non-toxic to rabbits [17]. Neem extracts are also harmful to the aquatic species on a certain level and their concentration 0.005% aqueous emulsion the neem extract was non-toxic to insectivore's fish, Gambusia species, 100% of them were killed in 24hr by a concentration of 0.04%. This concentration killed 80% of tadpoles in 24hr and 100% in 2 days. However, this extracts arenontoxic to tadpoles at 0.01% concentration [18].

DNA changes have become attractive and useful for monitoring environmental quality, water pollution and the health condition of aquatic organisms [19]. The entry of toxicants into aquatic media may affect the water quality parameter which in turn leads to certain hematological and DNA changes [20]. So, the aim of this study was to use some blood and genetic parameters for monitoring chronic sublethal toxicity induced by neem seed oil in Tilapia fish *(O. niloticus).*

MATERIALS AND METHODS
Fish
A total of 290 fish sample of *O. niloticus* with an average weight of (35.2±4.8 g) werecollected from the local nursery pools of South Fish Farm, Makkah, Kingdom of Saudi Arabia.Fish were transferred and placed in glass aquaria. They were adapted and fed on 25% protein pelleted diet two times daily for one week.Physicochemical parameters of studied water are measured according to [21] and presented in table (1). The physicochemical parameters of tested water were supported throughout the study period. Ventilation was provided throughout the study.

Table 1. Physicochemical characteristics of water used in the experiment.

Parameters	Parameters
pH	Bicarbonate (HCO_3^-)
Temperature	Carbonate (CO_3^-)
Dissolved Oxygen	Sulphate (SO_4^-)
Alkalinity ($CaCO_3$)	Chloride(Cl^{-1})
Total Hardness ($CaCO_3$)	Calcium (Ca^{++})
Ammonia (NH_3)	Magnesium (Mg^{++})
Ammonium (NH_4^+)	Potassium (K^+)
Nitrite (NO_2^-)	Sodium (Na^+)
Nitrate (NO_3^-)	Electric conductivity
Total soluble salts	

Neem seed oil (NO)

Neem seed oil (Trade name: Trilogy oil) was purchased from Trifolio-m GmbH Company, Germany, in the form of Oil containing 0.3% active ingredient of azadirachtin.Its chemical structure is presented in figure (1)
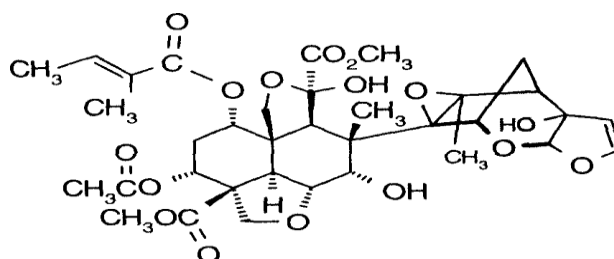

Figure 1. Structure formulation of azadirachtin

Chemicals:

All chemicals used in the present study were obtained from Sigma-Aldrich company. DNA Tag polymerase (Promega, USA), dNTPs (Boehringer Mannheim), Nucleic acid (DNA) purification and extraction reagents, and Agarose gel (QIAGEN), Oligonucleotides as random primers (Operon Tech. Inc., USA), DNA Maker for agarose gel electrophoresis (Gibco BRL), loading dye solution (Fermentas, Lithvuania), ethidium bromide staining solution(Bio-Rad) were also purchased.

Determination of 96 hours (LC50) for neem seed oil

The lethal concentration (LC_{50}) of NO was determined according to the method of **[22]**and Environmental Protection Agency [23].110 healthy fish were equally distributed in eleven aquaria with 100-liter capacity (Ten fish for each aquarium). The aquaria were supplied with dechlorinated tap water, kept at stable ventilation, temperature and pH, and feeding. Ten doses of NO (700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, and 1600 mg/L).were distributed in the aquaria, respectively, the aquarium 11 was kept as control. Fish were observed for 96 hours to record the number of dead and active fish in each aquarium.

The **LC50** was calculated according to the following formula [24].
LC50 = highest lethal dose - ∑axb/n as mg/L, where: (a) is a value of thedifferencesbetween two consecutive doses, (b) is the mean of the mortality caused by two consecutive doses and (n): is the number of fish in each group (10 fish). The dead fish were removed immediately. Behavioral changes, signs of vital were closely followed up and recorded daily for treated fishes.

Experimental design and treatment

The experimental fish (180) were divided into three groups, the first was the control group (60 fish/three replicates aquaria/group); the second group consisted of 60 fish/three replicates aquaria/group and subjected to$^{1}/_{10}$ of LC_{50}(NO1) andthe third group consisted of 60 fish/three

replicates aquaria/group and subjected to $^1/_{20}$of LC$_{50}$ (NO2) for three weeks. Fish were kept under observation along the period of experiment.Samples were taken at the end of the experimental time.

Blood examination

Whole blood samples were withdrawn from the caudal vein using a syringe containing sodium citrate as an anticoagulant. Total erythrocytic count (RBCs) and total leukocytic count (WBCs) were estimated using improved Neubauer-haemocytometer according to [25]. Hemoglobin (Hb) content was estimated using cyanomethemoglobin method described by [26]. Haematocrit (Hct) was carried out using micro-hematocrit method according to [25]. Blood indiciesnamely Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC) were calculated according to [27].

DNA extraction

Ten caudal fin tissue sample (1cm^2) was collected from each Treatment and stored in 95% ethanol for preservation until DNA analysis. DNA extraction and purification were carried out according to [28], with slight adjustments. Purity of DNA were estimated through fluorometric analysis, usingBio-photometer (Germany) and through detecting EB-stained bands with DNA ladder of 3 kb.

Polymerase Chain Reaction (PCR)

a thermocycler (Perkin-Elmer model 480) with heated lid was used for DNA amplification according to [29] with some minor amendments. PCRs were performed in a total volume of 20 μl of the PCR ingredients which consisted of 2 μl of template DNA (10 ng), 0.2 μl forward primer 0.2 μl reverse primer (Table 2) originally developed for tilapia by [30], 0.5 μl dNTP (0.2 mM; nucleotides), 4 μl (SBD) 5× reaction buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl$_2$, 500 mM KCl, 0.1 mM EDTA, 5 mM DTT, 50% glycerol, 0.1% Triton X 100), 13.6 μl dd-H2O, and 2.2 U of Taq DNA polymerase. Five primers were used of 20-21 bp in length (Table 1). The bandsproduction protocol consisted of an initial denaturing step of 2 min at 96 °C, followed by 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 60 s (annealing) and 72 °C for 60 s (extension), with an additional extension period of 10 min at 72 °C. A negative control, without genomic DNA, was run with every set of samples to confirm that no contaminating DNA was present in the reactions.

Amplification mixtures were stored at 4 °C before use. The reproducibility of the PCR–technique in detecting NO-induced DNA changes was also determined using three replicates for each samples. The samples were stored at -20°C until separation on agarose gels (1%agarose gel, at 80 V for 2 h in a 1 × TBE buffer). The gel was stained with ethidium bromide and visualized in a UV transilluminator.

Genomic template stability (%) was calculated as $100 - (100\ a/n)$, where (*a*) was PCR-polymorphic profiles detected in each treated samples and (*n*) was the number of total bands in the control. Polymorphism observed in PCR- profiles included intensity of bands, disappearance of a normal band and appearance of a new band in comparison to control PCR- profiles [31].

Table 2. Sequences of 5 primers used in the experiment

Primers	Sequences of primers (5′ → 3′)
GM211	GCAAGTTGAGAGGCTACTGT
GM538	CAGCATGTTGTCTGGATCTTG
UNH104	GCAGTTATTTGTGGTCACTA
UNH123	CATCATCACAGACAGATTAG
UNH146	CCACTCTGCCTGCCCTCTAT

Statistical analysis:

Duncan's multiple range tests was used to determine differencesamong means byusing software program of Statistical Analysis [32]. One-way ANOVA test was used for comparing the treated and untreated control groups.

RESULTS

Water quality characteristics

The mean values of experimental water are presented in table (3). The examined parameters were within the permissible levels of growing fishes [33].

Table 3. Records of physicochemical characteristics of water used during experimental period.

Parameters	Mean	Parameters	Mean
pH	6.7	Bicarbonate (HCO_3^-)	118.7 mg L^{-1}
Temperature	27 ^{o}C	Carbonate (CO_3^-)	0.02 mg L^{-1}
Dissolved Oxygen	5.6 mg L^{-1}	Sulphate (SO_4^-)	86.3 mg L^{-1}
Alkalinity ($CaCO_3$)	145.8 mg L^{-1}	Chloride(Cl^{-1})	14.9 mg L^{-1}
Total Hardness ($CaCO_3$)	128.7 mg L^{-1}	Calcium (Ca^{++})	29.6 mg L^{-1}
Ammonia (NH_3)	0.3 mg L^{-1}	Magnesium (Mg^{++})	6.4 mg L^{-1}
Ammonium (NH_4^+)	0.6 mg L^{-1}	Potassium (K^+)	4.9 mg L^{-1}
Nitrite (NO_2^-)	0.0 mg L^{-1}	Sodium (Na^+)	35. 3 mg L^{-1}
Nitrate (NO_3^-)	0.8 mg L^{-1}	Electric conductivity	0.2 Mmohs cm^{-1}
Total soluble salts	264 mg L^{-1}		

Determination of 96 hours (LC50) for neem seed oil

The results given in table (4) show that the death of all fish (100% mortality) was recorded at concentration of 1600 mg/L. The dose of 700 and 800 mg/L were negatively affected doses. whereas The effective dose of (NO) that cause 50% fish death (LC50) was 1230mg/L, comparing with the control.

Table 4. The LC50 of *Oreochromis niloticus* after treated with neem seed oil for 96 h.

Doses (mg/L)	No. of exposed fish	No of dead fish				Overall deaths at 96 h	A	B	AB
		Day1	Day2	Day3	Day4				
0	10	0	0	0	0	0	0	0	0
700	10	0	0	0	0	0	700	0	0
800	10	0	0	0	0	0	100	0	0
900	10	0	1	2	3	3	100	1.5	150
1000	10	2	3	4	4	4	100	3.5	350
1100	10	1	2	3	3	3	100	3.5	350
1200	10	2	4	5	5	5	100	4	400
1300	10	2	3	4	4	4	100	4.5	450
1400	10	5	6	6	6	6	100	5	500
1500	10	6	6	7	7	7	100	6.5	650
1600	10	8	9	9	10	10	400	8.5	850
								$\sum$AB =3700	

Where A = differences between the two consecutive doses and B = arithmetic mean of the mortality caused by two consecutive doses.

Blood Examination

Table (5) shows that a significant decrease in RBCs, Hb, HCT and MCHC values in fish treated with (1/10LC50) and (1/20LC50) was recorded. In contrast, there was a significant increase in WBCs, MCV and MCH values as compared with their control groups (*P*<0.05).

Table 5. Effects of NO (1/10 LC50 and 1/20 LC50) on some blood parameters of *Oreochromis niloticus*

Parameters	1/10 LD$_{50}$		1/20 LD$_{50}$	
	Control	Treated	Control	Treated
RBC$_S$ (X 10^6 mm^{-3})	1.38±0.09 [a]	1.18±0.10 [b]	1.38±0.09 [a]	1.45±0.07 [b]
WBC$_S$ (X 10^3 mm^{-3})	26.01±1.15 [a]	35.06±1.79 [b]	26.01±1.15 [a]	32.26±1.99 [b]
Hb (g/dl)	7.25±0.25 [a]	6.12±0.14 [b]	7.25±0.25 [a]	7.06±0.38 [b]
HCT (%)	20.99±1.14 [a]	15.48±0.90 [b]	20.99±1.14 [a]	17.95±1.00 [b]
MCV (fl)	11.33±1.33 [a]	13.89±1.11 [b]	11.33±1.33 [a]	14.17±1.09 [b]
MCH (pg)	38.32±2.63 [a]	41.82±2.33 [b]	38.32±2.63 [a]	44.27±3.08 [b]
MCHC (g/dl)	29.95±2.22 [a]	27.70±1.85 [b]	29.95±2.22 [a]	25.34±2.11 [b]

- Values are mean ± SE, a: significant at P > 0.05, b: significant at P > 0.01

Polymerase Chain Reaction (PCR)

Table (6) and Figure (2) illustrate the results of PCR. Results showed asignificantdifference between unexposed and exposed fish, with obvious changes in both the number and the intensity of produced DNA-bands. The reduction in the intensity of the bands was observed after treatment with both doses with all primers except primer 2and 3 (Table 6),While an increase in band intensity was occurred at all primers. As well, the number of disappearing DNA-bands was increased by both doses for all primers except primer 2and 3 for (1/10 LC50) and primer 3 for

(1/20 LC50), whereas, the molecular sizes of disappearing bands wereshown from 157 to 895 bpapproximately (Figure 2). Finally, extra bands appeared with primer 1, 4 and 5 (two to three new bands) for (1/10LC50), and primer 1, 2,4 and 5 (one to two new bands) for (1/20 LC50). Molecular sizes of extra bands were indicated from 265 to 1580 bp approximately. Results indicated that tow new bands in figure (2) were highly intensity, stable and specific for primer 1 and 4. Alternatively, intensity of new PCR bands changed with the increase of (NO) concentration.
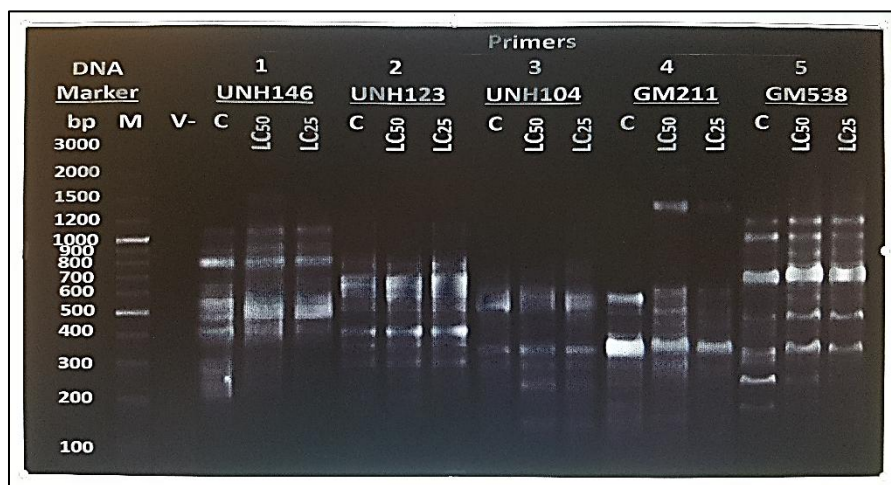

Figure 2. PCR characters of genomic DNA from *O. niloticus* exposed to1/10 LC50 and 1/20 LC50, in the top of the picture show that, M: DNA marker, V⁻˙Gel control, C: Control of DNA-bands.

Table 6. Changes of total bands in control, and of polymorphic bands and varied bands in *O. niloticus* after treatment with neem seed oil (1/10 LC50 and 1/20 LC50).

| primers No. (Name) | Control | (NO) concentration | | | | | | | |
| | | 1/10 LC50 | | | | 1/20 LC50 | | | |
		a	b	c	d	a	b	c	d
Primer 1 (UNH146)	11	2	0	2	2	2	2	1	1
Primer 2 (UNH123)	10	0	0	0	1	1	0	0	2
Primer 3 (UNH104)	7	0	0	0	3	0	0	0	3
Primer 4 (GM211)	7	2	2	1	2	1	1	1	1
Primer 5 (GM538)	9	3	1	2	2	2	0	2	3
Total bands	44	7	3	5	10	6	3	4	10
a + b		10				9			
a + b + c + d		25				23			

a: indicates appearance of new bands, b: disappearance of normal bands, c: decrease in band intensities, and d: increase in band intensities. a + b denotes polymorphic bands, and
a + b + c + d, varied band.

Meanwhile, five primers weregiving the forty-four bands, Molecular weight ranging from146 bp in primer (5) to 2174 bp in primer (1) comparing with the control as illustrated in (Figure2). The polymorphic bands were distinguished at each concentration of (NO) for different primers. Values of genomic stability of *O. niloticus*after treated with (NO) were*P* (%) = 43.2% and47.7%

for (1/10 LC50 and 1/20 LC50), respectively. At all treatments that polymorphisms were resulted as the present and/or absent of PCR- bands production against the control. Repeated tests have confirmed that the difference in the intensity of the band (results not shown) was devoted and there has been no significant change in the nuclear DNA template concentration within a certain scale and /or a change in the concentration of PCR reagents.

DISCUSSION

In the present study, the 96 h LC_{50} value of neem seed oilfor the freshwater fish *Oreochromis niloticus*was found to be 1230 mg/L which indicates that neem seed oil is toxic to tilapia fishat higher doses. These data are consistent with the conclusion reached by Winkaler *et al.,* [8] that the lethal conservation (LC_{50}) of neem leaves extract on *Prochilodus lineatus* was 4.8 g/L. This result agrees well with that of Mamdouh *et al*., [34] who found that the 96-h LD_{50} values of neem leaf extract for Nile tilapia and cat fish were 1.8 and 4 g/l, respectively.

The differences in the LC_{50} value of various parts of plant species to fish could be attributed to the differences in chemicals present in the plants, fish size, age, species and also the sensitivity of the fish used for the experiment [20].

Blood indices have been proven to be a valuable tool for fisheries biologists for monitoring changes in fish health, behavior and visible lesions [35][36]. In the current study the decreased levels of RBCs count, Hb content and Hct value in fish treated with neem seed oil might have resulted from hemolysis caused by this treatment. Similarly, (Saravanan *et al.,* [37] found that the RBC, Hct, Hb, MCHC, MCH and MCV levels were significantly decreased in fish (*Cirrhinus mrigala*) after treatment with neem leaves extract, whereas WBC count was significantly increased. Moreover, Omoniyi *et al.*, [38] and Adeyemo [39] have recorded similar observation in *Clarias gariepinus* exposed to leaf extracts of tobacco, *Nicotiana tobaccum* and cassava effluents. In contrast, an increase in RBCs count, Hb and Hct content in *O. niloticus* exposed to aqueous extracts of *Moringa oleifera* seeds [40].

Atamanalp and Yanik, [41] attributed the decrease in Hb content during stress condition that mostly associated with pollutants to the decrease in the rate of Hb synthesis which leads to impaired oxygen supply to various tissues resulting decrease in the number of RBC through hemolysis. Moreover, the lysing of erythrocytes leads to a reduction in hematocrit value [42].

The obtained results demonstrate a significant increase inWBC in fish exposed to both sublethal doses of NO. This might be due to stimulated lymphopoiesis and/or enhanced release of lymphocytes from lymphomyeloid tissue as a defense mechanismof fish to tolerate the neem seed oil toxicity. Ates *et al.*, [36] mentioned that the increase in leucocyte count indicates the stimulatory effect of the toxicant on immune system that depends on the toxicant stress.

Previous studies have shown that changes in DNA fingerprint (i.e. band patterns) observed reflect DNA alterations in genome from single base changes (point mutations) to complex chromosomal rearrangements [43]. Savva [44] reported that DNA fingerprinting offers a useful biomarker assay in ecotoxicology. Similarly, in this study, DNA damage induced by NO

treatment in *O. niloticus* was reflected by changes in DNA profiles; variation in band intensity, disappearance of bands, and appearance of new bands (Figure2 and Table 6).

The obtained results indicated that genomic template stability in *O. niloticus* was significantly affected by NO- intoxication stress. Modifications of band intensity and change in appearance/ disappearance bands are likely to be due to one or a combination of the following events: (1) changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations and DNA damage in the primer binding sites (because the binding site is only 10 base long whereas genomic rearrangements occur in much longer fragments), and (2) affective of DNA-polymerase in *O. niloticus* with damaged DNA. These actions could work to stop or decrease production of DNA in the reaction of PCR [45][46].

CONCLUSION

It could be concluded that application of neem seed oil in aquatic activities need more investigations before its widespread uses. Also, the use of DNA measurements of polymorphism in association with deviated blood parameters can be used as an effective practical tool to detect environmental toxicants. Finally, using battery of tests for screening toxicity in biological systems can be useful as early warning system.

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