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Ecotoxicity and hematological effects of a natural insecticide based on tobacco (*Nicotiana tabacum*) extract on Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT. Natural insecticides from plant extracts represent an alternative to the highly toxic synthetic products in order to reduce environmental contamination; however some might also be toxic for non-target organisms. The present study determined the 50% lethal concentration (48h; LC50) for adults Nile tilapia (*Oreochromis niloticus*) exposed to the natural insecticide Fumydro[®], based on the tobacco plant (*Nicotiana tabacum*), and evaluated its effect on hematological variables. After preliminary tests, adult specimens of *O. niloticus* were exposed to four Fumydro[®] concentrations (200, 300, 400 and 500 $\mu\text{L L}^{-1}$). The 48h; LC50 of Fumydro[®] was determined at $370 \pm 50 \mu\text{L L}^{-1}$. The surviving fish after exposure to Fumydro[®] showed an increase in the number of red blood cells, hemoglobin concentration, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. The number of thrombocytes and leukocytes has not changed, unlike the differential leukocyte count that presented an increased percentage of neutrophils. The results indicated that the insecticide Fumydro[®] is highly toxic to Nile tilapia and changes in erythrocyte variables suggested the induction to hypoxemia with low effect on the immune system.

Keywords: toxicity, erythrocytes, leukocytes, teleost, thrombocytes.

Ecotoxicidade e efeitos hematológicos de um inseticida natural de extrato de tabaco (*Nicotiana tabacum*) em tilápia do Nilo (*Oreochromis niloticus*)

RESUMO. Inseticidas naturais produzidos a partir de extratos de plantas pode ser uma alternativa para diminuir a contaminação do ambiente por agrotóxicos sintéticos e altamente tóxicos, embora alguns deles possam também ser tóxicos para organismos não alvos. O presente estudo determinou a concentração letal 50% (CL50; 48h) para tilápia do Nilo (*Oreochromis niloticus*) expostas ao inseticida natural Fumydro[®], produzido a base de planta de tabaco (*Nicotiana tabacum*) e analisou o seu efeito nas variáveis hematológicas. Após testes preliminares, exemplares adultos de *O. niloticus* foram expostos a quatro concentrações de Fumydro[®] (200, 300, 400 e 500 $\mu\text{L L}^{-1}$). A CL50; 48h foi estimada em $370 \pm 50 \mu\text{L L}^{-1}$. Os peixes sobreviventes a exposição ao Fumydro[®] apresentaram aumento no número de eritrócitos, concentração de hemoglobina, hemoglobina corpuscular média e concentração de hemoglobina corpuscular média. O número de trombócitos e de leucócitos não foi alterado, mas ocorreu alteração na contagem diferencial de leucócitos ocorrendo um aumento na porcentagem de neutrófilo nos peixes expostos a 200, 300 $\mu\text{L L}^{-1}$ de Fumydro[®]. Estes resultados indicaram que o inseticida Fumydro[®] é extremamente tóxico para tilápia do Nilo e as alterações nas variáveis eritrocitárias sugerem que causa hipoxemia, mas tem pouca ação sobre o sistema imune.

Palavras-chave: toxicidade, eritrócitos, leucócitos, teleosteo, trombócitos.

Introduction

The environmental contamination by insecticide has recently increased due to the enlarged crop areas to produce food. Most of insecticides consist of broad-spectrum synthetic molecules which may result in numerous problems such as accumulation in the food product and intoxication in animal and human (ROEL et al., 2000). Most of these insecticides may directly reach aquatic systems by aerial spraying near the water and/or by runoff. Inputs from agricultural fields, and may thus affect

aquatic organisms, including fish (SANCHES et al., 2003).

Natural insecticides from plants are considered an alternative to synthetic ones due to easy degradation, no accumulation in the food, and absence of insect resistance (RODRÍGUEZ; VENDRAMIM, 1995). However, some might also be toxic for non-target organisms. The leaf extract of *Ricinus communis*, *Datura alba* and *Strychnos nux vomica* causes in fish erratic swimming, loss of reflexes, slow opercular movement and settling at the bottom

motionless (ASHRAF et al., 2010). The selectivity is an essential characteristic of insecticides, and it is necessary to evaluate if the natural insecticides are safe for non-target animals.

Insecticides based on tobacco plants (*Nicotiana tabacum*) are the first natural insecticide used. They have high efficiency against several insects such as the diamondback moth, *Plutella xylostella* (DEQUECH et al., 2009), larvae and adults of yellow-margined leaf beetle, *Microtheca ochroloma* (DEQUECH et al., 2008) and larvae of mangrove buckeye, *Junonia evarete* (FERNANDES et al., 2006), and reduce the egg laying of the whitefly, *Bemisia tabaci* (QUINTELA; PINHEIRO, 2009). The leaf extract of *Nicotiana tabacum* has no influence on the behavior of many fish species (ASHRAF et al., 2010). However, there are only few studies on the toxicity of this insecticide to fish. Considering that the large scale application of natural insecticides may reach aquatic environments, it is important to determine the toxicity of tobacco-based insecticides to fish once they respond promptly to changes in the water and may be used as bioindicator of water quality (HEATH, 1995).

Bioassays to determine the 50% lethal concentration (LC50) provide important and rapid information about the acute toxicity of a given product and indicate possible sublethal concentrations (GOLDSTEIN et al., 1983) and blood variables provide information about the animal health (KAVAMOTO et al., 1983). Blood cells are the first to come into contact with xenobiotics and to present changes in response to biochemical or physiological disorders caused by the exposure to pesticides (CERQUEIRA; FERNANDES, 2002a and b; MAZON et al., 2002; RUAS et al., 2008; SILVEIRA-COFFIGNY et al., 2004). Blood changes depend on the action mechanism of the pesticide on biological systems, the contaminant concentration in the water, exposure time and species sensitivity (ALBERTO et al., 2005). Erythrocytes are essential for the transport of respiratory gases between the gills and the tissues; the leukocytes have high phagocytic activity and actively participate in the defense against foreign organisms being essential for the immunological response (SERPUNIN; LIKHATCHYOVA, 1998; TAVARES-DIAS; MORAES, 2004), and thrombocytes are involved in the blood clotting and also in the organism defense (PASSANTINO et al., 2005).

In this context, the main goal of this study was to (i) determine the 48h;LC50 of Fumydro®, a natural

insecticide based on the tobacco *N. tabacum*, for Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) and to (ii) analyze blood variables. This fish species is widespread in Brazilian aquatic ecosystems, being also intensively cultivated due to fast grow, hardness and high tolerance to environmental changes, such as temperature and dissolved oxygen (CHERVINSKI, 1982; FERNANDES; RANTIN, 1994; MONTEIRO et al., 2009).

Material and methods

Adults specimens of Nile tilapia, *O. niloticus* (Body weight = 79.0 ± 14.3 g; Total length = 16.3 ± 1.3 cm), were purchased from fish farming and kept in 500 L aquarium provided with continuous flow of dechlorinated and aerated water at $24^\circ\text{C} \pm 1$, pH 7.4 ± 0.2 and photoperiod 12D:12L, for 3 weeks. All fish were at early stage of gonadal maturation. No fish died during the acclimation period. Fish were fed with pellet feed with 36% protein (GuabiR, BR) every day to satiety, being the feeding suspended 24h before the start of toxicity tests. The control of fish sensitivity and sanity was conducted using an acute toxicity test with potassium chloride (KCl) (ABNT, 2006).

Acute toxicity tests (48h) were performed in duplicate ($n = 12$ in each concentration and control, not exceeding 1 g fish L^{-1}), to determine the 48h; LC50 of the natural insecticide Fumydro (HYFU 200907, Hydrofert LTDA) according to the recommendations of ABNT (2006). Preliminary acute toxicity tests were run to determine the concentration at which the insecticide causes 0 and 100% mortality. Afterwards, the experimental design consisted of four nominal insecticide concentrations (200, 300, 400 and $500 \mu\text{L L}^{-1}$) and a control, where fish were exposed to water. The tests were performed in glass aquaria using static system with continuous aeration at constant temperature ($24 \pm 1^\circ\text{C}$) and pH (7.4 ± 0.2). The fish were not fed during experiments. The number of dead fish was recorded every 12h and withdrawn. After exposure, the surviving fish were removed and anesthetized with 0.01% benzocaine. Blood samples were taken from the caudal vein using heparinized syringes in order to determine blood variables; white blood cells and thrombocytes were estimated using blood smears.

Hematological analyses

Hematocrit (Hct, %) was determined by centrifuging the blood sample contained in heparinized capillary tubes in a microhematocrit centrifuge (FANEN). Hemoglobin concentration ([Hb], g dL^{-1}) was determined by the

cyanmethemoglobin method (DRABKIN, 1948) in a spectrophotometer (SPECTRONIC GENESYS 5) at 540 nm, and the red blood cell counts (RBC, $n \mu\text{L}^{-1}$) was done with blood samples fixed in formol-citrate solution using an improved Neubauer chamber under a light microscope (400x magnification). Mean cell volume (MCV, fL), mean cell hemoglobin (MCH, pg) and mean cell hemoglobin concentration (MCHC, g dL^{-1}) were calculated using Hct, Hb and RBC measurements.

Blood smears were air-dried and stained using polychromatic differential staining (Fast Panotic LB, Laborclin). Leukocyte types and thrombocytes were identified according to Tavares-Dias and Moraes (2004). The numbers of erythroblast, leukocyte and thrombocyte were counted and indirectly calculated based on McKnight (1966). Briefly, the number of erythroblast, leukocytes and thrombocytes found in 5000 erythrocytes was recorded in adjacent fields in the monolayer portion of the blood smear. The total number of leukocytes per μL^{-1} blood was calculated as follows:

$$\text{Leukocytes } \mu\text{L}^{-1} = \frac{\text{number of leukocytes} \times \text{erythrocytes } \mu\text{L}^{-1}}{5000}$$

the total number of erythroblasts and thrombocytes were calculated using the same formula.

The differential counts of leukocytes were made with the same blood smear. All leukocyte types were counted until the sum of lymphocytes, monocytes and various granulocytes totaled 200, regardless of the number of thrombocytes. Each leukocyte type was divided by the total number of leukocytes counted (200) and multiplied by 100 to provide the percentage of each type of leukocyte.

Statistical Analyses

The 48-h LC50 were calculated using the Trimmed Spearman-Kärber method and the LC50 Program JSpear test (HAMILTON et al., 1977) with 95% confidence limits. The hematological data were presented as mean \pm standard deviation (SD). Data normality was tested and a one-way ANOVA was applied to identify differences between control and Fumydrö[®] exposed-fish. Differences between groups were detected applying the Dunnett post hoc test with 95% confidence limits, using the GraphPad InStat software.

Results and discussion

Acute toxicity

No fish died in the control group; but mortality occurred in fish exposed to Fumydrö[®] and increased

with increasing concentrations of the insecticide in the water, indicating a dose-dependent response. All fish died when exposed to 500 $\mu\text{L L}^{-1}$. After 24h exposure, fish has begun to die when exposed to 200 and 300 $\mu\text{L L}^{-1}$ Fumydrö[®]. However, at higher concentrations, fish mortality occurred during the first 24h exposure reaching up to 33% when exposed to 500 $\mu\text{L L}^{-1}$ Fumydrö[®]. The Figure 1A shows the total percentage of fish mortality for each concentration of Fumydrö[®] and the cumulative mortality for every 12h exposure. The 48h; LC50 to *O. niloticus* after exposure to Fumydrö[®] was calculated as $370 \pm 50 \mu\text{L L}^{-1}$ (Figure 1B). Fish exposed to Fumydrö[®] presented lethargy and aquatic surface respiration (ASR); before dying fish remained motionless on the bottom of the aquarium.

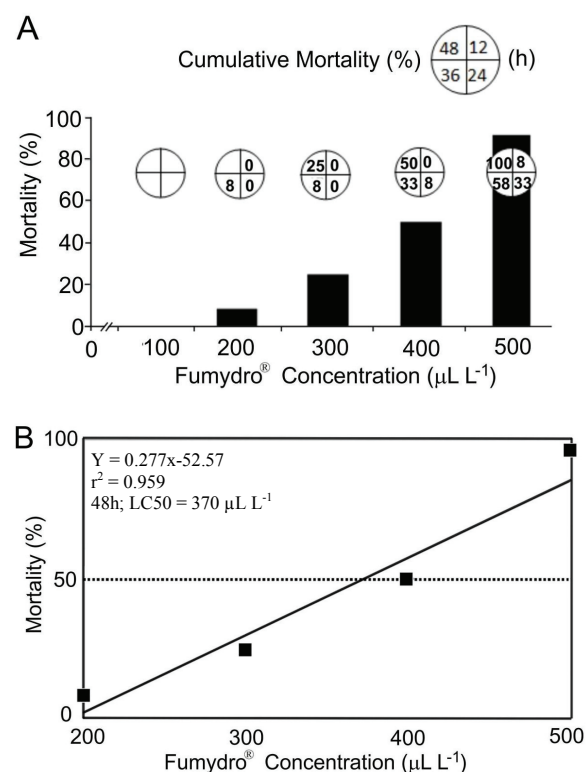


Figure 1. A. Percentage of mortality for *O. niloticus* ($n = 12$ in each concentration) exposed to Fumydrö[®]. The pie charts indicate, clockwise direction, the cumulative percentage of mortality during the exposure to the insecticide every 12h; B. Relationship between Fumydrö[®] concentration and mortality to determine the 48h; LC50 of the insecticide to *O. niloticus*.

Oreochromis niloticus presented a high sensitivity to Fumydrö[®] whose component nicotine is highly toxic to numerous insects (FOSTER et al., 2003). This natural insecticide Fumydrö[®] affected the Nile tilapia health resulting in fish mortality at high concentration in the water, impacting thus the

aquatic environment. According to the classification of Helfrich et al. (2009), this insecticide may be considered highly toxic for Nile tilapia as the 48h; CL50 is between 0.1 and 1 mL L⁻¹. However, this toxicity may be related not only to the insecticide potential of the *N. tabacum* extract, but also to the associated substances included in the commercial formulation.

Studies using only the leaf powder of the same plant showed a lower toxicity than Fumydró®. Agbon et al. (2002) observed that the 48h; LC50 to *O. niloticus* was 109.6 mg L⁻¹ of tobacco leaf powder, and Omoniyi et al. (2002) reported values as high as 626.0 mg L⁻¹ for 48h; LC50 to the African catfish *Clarias gariepinus*. For 96h; LC50 the toxicity of tobacco leaf powder was 751.2 mg L⁻¹ to *Heteroclinas*, an hybrid between *Heterobranchius bidorsalis* and *Clarias gariepinus* (ADAMU, 2009); and no toxicity was verified by Ashraf et al. (2010) to *Tilapia* sp, *Colisa lalia*, *Channa Punctatus*, *Ambassis ranga*, *Catla catla*, *Ctenopharyngodon idella* in toxicity tests up to 100 mg L⁻¹.

Comparing the toxicity of Fumydró® with other pesticides to *O. niloticus*, Fumydró® is less toxic than the organochlorines, triodan (LC50 = 3.8 µg L⁻¹) and lindane (LC50 = 173 µg L⁻¹) (GURURE, 1987) and copper (LC50 = 7.98 mg L⁻¹) (SEDDEK, 1990).

Hematological variables

The mean values of Hct, Hb, RBC and the hematimetric indices (MVC, MHC, MCHC) of *O. niloticus* exposed to Fumydró® are illustrated in Figure 2. The Hct and MCV of exposed fish were not significantly different from the control. RBC increased only in fish exposed to 200 µL L⁻¹ and the MCH increased in those exposed to 400 µL L⁻¹. The [Hb] and MCHC increased with increasing concentrations of Fumydró® in the water. The blood variables of *O. niloticus* from the control group was similar to those found for this species in fish culture ponds, except for the RBC that was greater than by Azevedo et al. (2006).

The ASR behavior and the increased hematological variables in fish exposed to Fumydró® suggested a response to an increased O₂ uptake by the gills, and O₂ carrying capacity by the blood to prevent tissue hypoxia. According to Cerqueira and Fernandes (2002a), pesticides may induce morphological changes in the gills, such as cell proliferation, edema and mucus secretion to prevent xenobiotics reach the blood stream. These changes may cause respiratory dysfunction due to the increase in the water-blood diffusion distance which reduces the O₂ uptake by the gills (FERNANDES et al., 2007). Conversely, Omoniyi et al. (2002) reported that the exposure to the tobacco leaf

powder led to a decrease in the Hct, RBC, [Hb], VCM and HCM in *C. gariepinus*, suggesting a possible anemic effect.

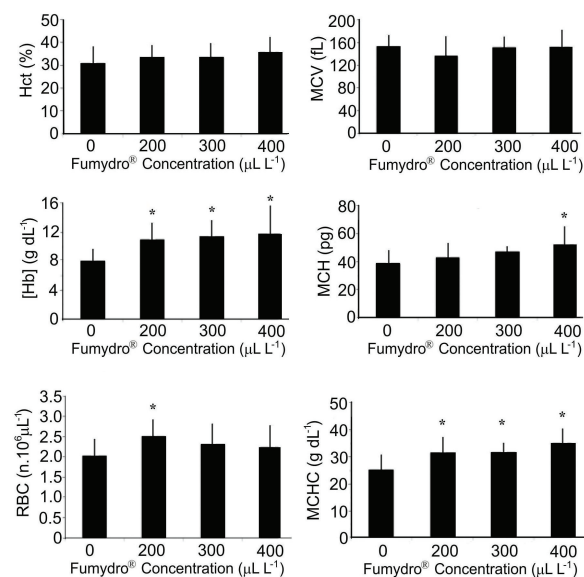


Figure 2. Hematological variables: hematocrit (Hct); red blood cells (RBC); hemoglobin concentration ([Hb]) and hematological indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of *O. niloticus* (n = 8) exposed to the natural insecticide Fumydró® for 48h. * indicates significant difference from the control (p < 0.05).

Figure 3 shows the morphological characteristics of erythrocytes, erythroblasts, lymphocytes, neutrophils, monocytes, eosinophils, special granulocytic cells (SGC) or PAS-positive granular leukocytes (PAS-GL) and thrombocytes in the blood of *O. niloticus*.

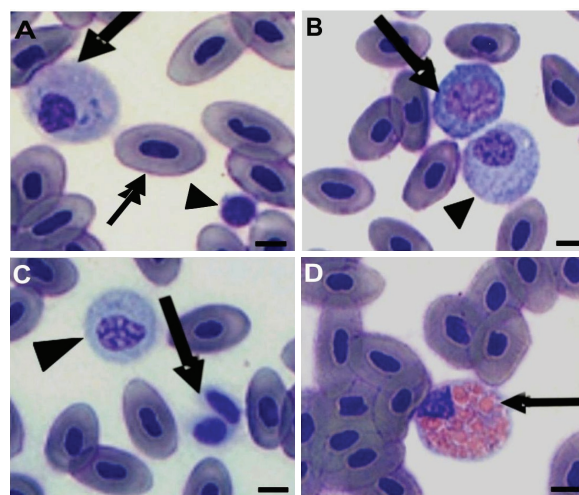


Figure 3. Blood cells of *Oreochromis niloticus*. A- Erythrocyte (double arrow), lymphocyte (arrowhead) and special granulocytic cells or PAS-positive granular leukocyte (arrow). B- Monocyte (arrow) and special granulocytic cells (arrowhead). C- Neutrophil (arrowhead), thrombocytes (arrow); D- Eosinophil (arrow). Scale bar = 10 µm.

The number of erythroblasts exhibited a large variation between the groups and was not significantly different from the control, as well as leucocytes and thrombocytes (Table 1).

The percentage of neutrophils in fish exposed to 200 and 300 $\mu\text{L L}^{-1}$ Fumydro[®] has increased, on the other hand the percentage of lymphocytes, monocytes and SGC did not present any change (Table 2).

Table 1. Mean values of erythroblasts, leukocytes and thrombocytes (number. $10^3 \pm \text{SD}$) of Nile tilapia *Oreochromis niloticus* (n = 8) exposed to different concentrations of the natural insecticide Fumydro[®] for 48h.

Blood Cells	Control	Fumydro [®] Exposure ($\mu\text{L L}^{-1}$)		
		200	300	400
Erythroblasts	79 \pm 26	65 \pm 32	60 \pm 72	71 \pm 50
Thrombocytes	34 \pm 27	34 \pm 13	26 \pm 12	28 \pm 12
Leukocytes	25 \pm 6	31 \pm 9	27 \pm 24	33 \pm 18

Table 2. Differential leukocyte percentage ($\pm \text{SD}$) in the blood of Nile tilapia, *Oreochromis niloticus* (n = 8) exposed to the natural insecticide Fumydro[®] for 48h.

Leukocytes (%)	Control	Fumydro [®] Exposure ($\mu\text{L L}^{-1}$)			
		200	300	400	
Lymphocytes	82.18 \pm 15.08	57.23 \pm 17.70	60.93 \pm 22.58	78.87 \pm 6.69	
Eosinophils	-	0.10 \pm 0.22	-	-	
Neutrophils	10.85 \pm 8.57	34.30 \pm 13.92*	31.73 \pm 15.29*	19.44 \pm 5.71	
Monocytes	5.17 \pm 6.22	6.39 \pm 7.02	6.73 \pm 7.39	1.49 \pm 0.60	
Basophils	-	-	-	-	
SGC	1.79 \pm 2.66	1.99 \pm 2.76	0.59 \pm 0.64	0.20 \pm 0.44	

*significantly different from the control group (p < 0.05).

The lack of significant changes in lymphocytes and other white blood cells, except the increased percentage of neutrophils in fish exposed to 200 and 300 $\mu\text{L L}^{-1}$, revealed that this insecticide had little influence on the immune system of Nile tilapia and probably did not stimulate the cortisol stress response. In general, the stress response to xenobiotics through increased cortisol levels in the blood reduces the lymphocyte percentage (IWAMA; NAKANISHI, 1996).

Conclusion

The natural insecticide Fumydro[®] based on the tobacco plant (*Nicotiana tabacum*) extract is highly toxic to Nile tilapia. The increased erythrocyte variables suggested a possible tissue hypoxia, which may affect the detoxification and/or tissue repair processes and may lead fish to die during the exposure to high insecticide levels.

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