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Testing the Neem Biopesticide (*Azadirachta indica* A. Juss) for Acute Toxicity with *Danio rerio* and for Chronic Toxicity with *Daphnia magna*

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ABSTRACT.

Recently, some natural products have been used in the fields as alternative to synthetic compounds, to minimize the negative impacts to the environment. This study aimed to verify the effects of Neem-based bio-pesticide in causing acute toxicity for a fish and chronic toxicity for a microcrustacean. To this end, *Danio rerio* and *Daphnia magna* were exposed to various concentrations of a Neem-based oil formulation. In the first experiment, adults of *D. rerio* were exposed for 96 hours to different concentrations to determine the median lethal concentration ($LC_{50\text{-}96h}$). For *D. magna*, first an acute toxicity test was performed to determine the median effective concentration ($EC_{50\text{-}48h}$). Based on the EC_{50} established in the acute test, the concentrations for the 21-day chronic toxicity test were determined. Endpoints evaluated were reproduction (number of neonates produced) and size of *D. magna*. The median lethal concentration for the fish was 0.22 mL L^{-1}, and the median effective concentration ($EC_{50\ -\ 48h}$) for *D. magna* was 0.17 mL L^{-1}. In the chronic test, all concentrations affected reproduction and size of *D. magna*. The formulation tested may be hazardous to aquatic organisms.

Keywords: Aquatic organisms, Insecticide, Natural products, Toxicology.

INTRODUCTION

In order to achieve higher agricultural productivity, pesticides are being increasingly used worldwide. However, they have different effects on non-target organisms. In recent decades, contamination of aquatic environments has increased, and the growing use of synthetic pesticides ultimately causes environmental damage. Therefore, in an attempt to minimize these problems, natural products are used as an alternative.

The Neem tree (*Azadirachta indica*) of the family Meliaceae is native to India and was adapted to grow in Brazil a few years ago (Immich *et al.,* 2009). The plant contains an oil with insecticidal properties (Carneiro, 2003). Fruits are the most important source of oil, affecting insects in many ways, and leaves can also be used for pest control (Schmutterer, 1990). Plantations of these trees are growing rapidly in Brazil, to be used for timber production, for harvesting leaves and fruits as raw materials for extraction of insecticidal products, for medical and veterinary use, or for the cosmetics industry (Santos *et al.,* 2006). Neem contains many secondary plant metabolites, with the most biologically active being azadirachtin, a triterpenoid that is present in the oil from seeds, leaves, leaf extracts, Neem cake, and

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fruit (Koul *et al.,* 1990). Azadichtin has low toxicity against non-target organisms and low persistence in the environment (Schaaf *et al.,* 2000), both of which are desirable characteristics for a biocide.

The first commercial Neem product, i.e. Margosan-O® (W.R. Grace & Company, Columbia, MD, USA), was registered by the U.S. Environmental Protection Agency (U.S. EPA) for non-food crop insect pest control in 1985 (Stark and Walter, 1995). Several commercial and semi-commercial preparations are now available, including Azatin-EC™ (Agridyne Technology, Salt Lake, UT, USA), Bioneem™ (Ringer, Minneapolis, MN, USA), and Neemix™ (Thermo Trilogy, Columbia, MD, USA).

Studies on toxicity of Neem preparations and of pure azadirachtin have been conducted on laboratory animals and some non-target species (Gandhi *et al.,* 1988; Osuala and Okwuosa, 1993; Wan *et al.,* 1996; Mahboob *et al.,* 1998). In order to investigate Neem effects on non-target organisms, a study on the acute toxicity to the fish *Danio rerio* and on the chronic toxicity to the microcrustacean *Daphnia magna* were performed with a commercial formulation.

MATERIALS AND METHODS

Chemical

Bioneem oil (90% Neem oil and 10% emulsifiers and synergistic ingredients) was purchased from Universal Bioneem Company (Itinga District, Brazil).

Maintenance and Acute Toxicity Test with *D. magna*

Microcrustaceans (*D. magna*) were maintained in reconstituted water in laboratory according to Brazilian Technical Standard Association (ABNT, 2003) under the following conditions: 2000 mL container with 20 adults per liter with a photoperiod of 16:8 h light/dark cycle at 20±2°C. Reconstituted water was prepared using 18-MΩ deionized water and reagent grade-chemicals according to ABNT (2003). The culture medium was renewed twice a week. The animals were maintained to a maximum age of 21 days, ensuring conditions for production of healthy juveniles to be used in the tests. Food consisted of the green algae *Pseudokirchneriella subcapitata* given daily at a rate of 1.0×10^7 cells L^{-1}.

For the acute toxicity test, organisms aged between 2 to 26 hours were used. After a preliminary test, the definitive test was performed using the following concentrations of Bioneem oil: 0.0, 0.015, 0.031, 0.065, 0.125, 0.250, 0.50, 1.0, 2.0 mL L^{-1}. Four replicates (with five organisms) were used for each concentration. After 48 hours without feeding and illumination, the median effective concentration ($EC_{50\ -\ 48h}$) for immobility was determined.

Chronic Toxicity Test with *D. magna*

Concentrations for the chronic test were based on half of the EC_{50} determined in the acute test (OECD, 1998). The concentrations used were 0.0106, 0.0212, 0.0425, 0.0850, 0.17 mL L^{-1} and the control, with 10 replicates for each concentration. During the 21-day experiment, every two days, the test solution was renewed with only adults transferred to the new solution. During the renewal, organisms were fed with *P. subcapitata,* and the reproduction (number of produced neonates) was recorded. At the end of the test, the average number of neonates produced and size of adult organisms were compared between the control and Bioneem oil treatments.

Acute Toxicity Test with *D. rerio*

Adults of *D. rerio* were purchased from commercial suppliers (located in Piracicaba city, São Paulo state, Brazil) of good quality. Individuals were transported to the

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laboratory and placed under observation for acclimatization. Water with the following characteristics was supplied: dissolved oxygen concentration higher than or equal to 5 mg L^{-1}, pH between 7.4 and 7.8, and temperature of 25±1°C. The room temperature was 25ºC (OECD, 1992) with 16:8 h light/dark photoperiod. Fish food (Tretramin) was provided twice daily up until 24 hours before the start of the test.

Initially, a preliminary test was carried out followed by a definitive test at the following concentrations: 0.16, 0.2, 0.32, 0.4 and 0.8 mL L^{-1} and the control. Five organisms were placed in 2 L beakers with test solution and two replicates per concentration. The exposure system was static without feeding. The median lethal concentration (LC_{50}) was determined after 96 hours.

Statistical Analysis

The EC_{50} (48h) and LC_{50} (96h) to *D. magna* and *D. rerio,* respectively, were calculated using the Trimmed Spearman-Karber method (Hamilton *et al.,* 1977). For the chronic toxicity test, analysis of variance (ANOVA) was used. Data were transformed using Box-Cox transformation (Box and Cox, 1964), and the Hartley test was used to verify homogeneity (Hartley, 1950). A Tukey post-hoc test ($P< 0.05$) was used to compare the mean of reproduction and size between the groups treated with biopesticide and the control. A quadratic regression was carried out to verify the size behavior relative to the exposure concentrations and an exponential model for reproduction. For all analyses, the SAS program version 9.2 was used.

RESULTS AND DISCUSSION

In recent decades, many toxicity studies have been performed using extracts from different plant species instead of synthetic products in order to know their toxicological characteristics.

In the acute test with the microcrustacean *D. magna,* the EC_{50} (48 hours) value was 0.17 ml L^{-1}. In the chronic test, recurrent effects were detected on *D. magna*, such as reduced number of neonates and inhibition of size. It was observed that means of both parameters were significantly lower compared with the control, thus, demonstrating a toxic effect (Table 1). An exponential model for reproduction of *D. magna* is decribed in Figure 1.

Scott and Kaushik (1998) evaluated the toxicity of the commercial formulation Margosan-O and did not observe any effect on the size even at higher concentrations, which differs from the present study. Figure 2 shows a quadratic regression for size of *D. magna* and Figure 3 is an illustration of *D. magna* size of the control group and those treated with the product. It is clear that the size of the groups treated with bioneem is smaller than the control group as shown in Table 1. Also, in the chronic test, no mortality was observed in the control group, and any mortality in the bioneem treatment groups did not significantly differ from the

Table 1. Mean (±standard deviation) values of reproduction and size of *D. magna* exposed to different Bioneem concentrations during 21 days [a].

Concentration (mL L^{-1})	Reproduction	Size (mm)
Control	$154^{a} \pm 15$	$4.0^{a} \pm 0.2$
0.0106	$62^{b} \pm 15$	$3.0^{b} \pm 0.1$
0.0212	$37^{c} \pm 17$	$2.5^{bc} \pm 0.9$
0.0425	$28^{cd} \pm 9$	$2.4^{bc} \pm 0.2$
0.085	$18^{d} \pm 8$	$2.2^{cd} \pm 0.1$
0.17	$0.90^{e} \pm 1.19$	$1.6^{e} \pm 0.9$

[a] Means followed by same letters do not differ by the Tukey test ($P< 0.05$).

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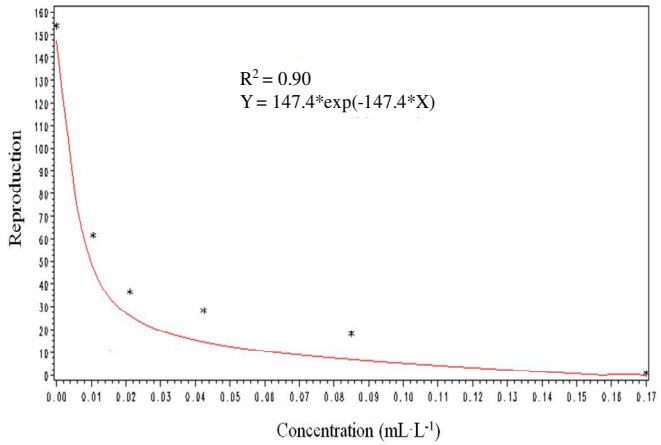

Figure 1. Exponential model for reproduction of *D. magna* exposed to Bioneem concentrations (mL L^{-1}) for 21 days.

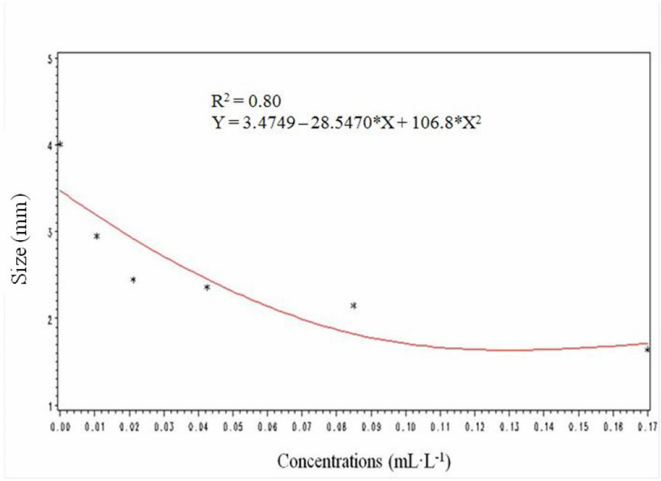

Figure 2. Quadratic regression for size of *D. magna* exposed to Bioneem concentrations (mL L^{-1}) for 21 days.

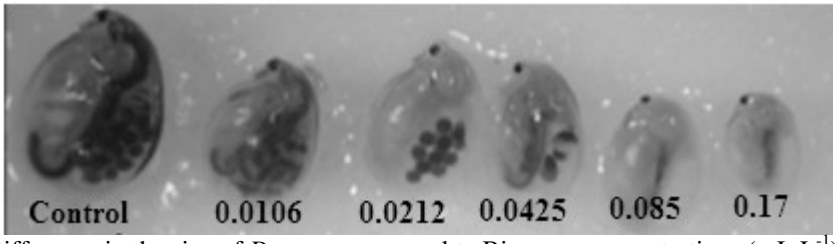

Figure 3. Difference in the size of *D. magna* exposed to Bioneem concentrations (mL L^{-1}) for 21 days.

controls. Table 2 shows the estimated parameters of the quadratic regression model for the size and an exponential model for reproduction of *D. magna*.

In the acute test for fish, the LC_{50} (96h) was 0.22 mL L^{-1}. Ahmad and Ansari (2011) tested a neem-based insecticide (Azacel) on embryos of *D. rerio* and the value of LC_{50} (72 hours) was 0.06 µg L^{-1} (0.0004 mL L^{-1}), showing that embryos are more sensitive than adults. In the same study, the LC_{50} to fingerlings for 96 hours was 0.05 µg L^{-1} (0.00034 mL L^{-1}). The authors concluded that the pesticide addressed may affect embryos and fingerlings in defined concentrations. Comparing the results of the

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Table 2. Estimated parameters of the quadratic regression model for size and exponential model for reproduction of *D. magna*.

Parameter	DF	Estimates (Reproduction)	Estimates (Size)	Confidence Limit (95%) (Reproduction)	Confidence Limit (95%) (Size)	*P* value
β_0	2	147.40	3.47	[131.10; 163.50]	[3.42; 3.73]	< 0.0001
β_1	2	-	-28.55	-	[-37.37; -25.98]	< 0.0001
β_2	2	-	106.80	-	[103.60; 168.60]	< 0.0001

present work with those of Ahmad and Ansari (2011) shows that the Azacel formulation was more toxic than Bioneem to *D. rerio*.

Recently, many studies have been conducted to assess the toxicity of extracts from different plant species. Goktepe and Plhak (2002) tested two neem-based commercial formulations, namely, Nimix and Bioneem, on *Daphnia pulex* and found an EC_{50} (48 hours) of 0.028 µL mL^{-1} and 0.033 µL mL^{-1}, respectively, showing that *D. pulex* is more sensitive to Bioneem than *D. magna*, since this value was lower than that found in the present study.

Also, Dunkel and Richards (1998) studied the toxicity of the commercial formulation of Azadirachtin on six species of aquatic macroinvertebrates and observed toxicity on all of them. Botelho *et al*. (2010) reported on the toxicity of the same formulation used in the present study for *Ceriodaphnia dubia* and found an EC_{50} (48 hours) of 0.032 mL L^{-1}, demonstrating that *C. dubia* was more sensitive to Bioneem than *D. magna*. Saucke and Schmutterer (1992) found EC_{50} (48 hours) of 0.19 µL L^{-1} for *D. magna* using the commercial formulation Margosan-O. In the same study, other commercial formulations were tested and EC_{50} values ranged from 0.04 to 3.38 µL L^{-1}. They also observed that toxicity of other formulated products was 10 times more than the aqueous extract of neem seeds, thus, concluding that the ingredients in the formulation were probably the main causes of toxicity. In a study on fish *Lepidocephalichthys guntea*, Mondal *et al*. (2007) reported the toxicity of two neem-based commercial formulations (Nimbicidini and Nim Gold).

The use of plant-based substance for agricultural purposes may be useful to replace those already considered toxic to non-target organisms. However, it is important to know all the characteristics of the molecule, including its physical and chemical properties and toxicity to aquatic and soil organisms.

CONCLUSIONS

The toxicity tests showed that *D. magna* had high sensitivity to the bio-insecticide evaluated in this study, even the lowest concentrations showed toxicity on reproduction and size of organisms. Based on the LC_{50} (96 hours) to *D. rerio,* the compound was also toxic to fish, suggesting that even at low concentrations this product may cause adverse effects to aquatic organisms.

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آزمون شیرابه میوه چریش (*Azadirachta indica* Adr. Juss) به عنوان آفت کش زیستی ازنظر ایجاد مسمومیت حاد در ماهی *Danio rerio* و مسمومیت مزمن در *Daphnia magna*

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چکیده

در سال های اخیر، به منظور کمینه کردن اثرات منفی روی محیط زیست، کار برد برخی مواد طبیعی در مزارع جایگزین مواد ساخت بشر شده است. هدف پژوهش حاضر بررسی اثر فرمولاسیون روغن میوه درخت جریش در ایجاد مسمومیت حاد برای یک ماهی و مسمومیت مزمن برای یک ریزجاندار سخت پوست بود.به این منظور، ماهی *Danio rerio* و *Daphnia magna* در معرض *غلظت های مختلف یک فرمولاسیون روغن میوه* درخت چریش قرار داده شدند.در آزمون نخست،برای تعیین غلظت کشنده میانی ($LC_{50\text{-}96h}$) ، ماهی های بالغ *D.rerio* به مدت ۹۶ ساعت در معرض غلظت های مختلف قرار داده شدند. در مورد *D. magna*، نخست یک آزمون مسمومیت حاد انجام شد تا غلظت موثر میانی ($EC_{50\text{-}48h}$) مشخص شود. بر مبنای EC_{50} به دست آمده در آزمون مسمومیت حاد، غلظت های آزمون مسمومیت مزمن ۲۱روزه تعیین شدند. ضوابطی که مورد ارزیابی قرار گرفتند شامل تولید مثل (تعداد نوزادان تولید شده) و اندازه *D.magna* بود. غلظت کشنده میانی برای ماهی ۰/۲۲ میلی لیتر در لیتر و غلظت موثر میانی ($EC_{50\ -\ 48h}$) برای *D. magna* *برابر* ۰/۱۷ میلی لیتر در لیتر بود.در آزمون مسمومیت مزمن همه غلظت ها بر تولید مثل و اندازه *D.magna* اثر داشتند. نتیجه اینکه فرمولاسیون آزمون شده ممکن است برای جانداران آبی خطرناک باشد.

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