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 (LC₅₀-96h). For D. magna, first an acute toxicity test was performed to determine the median effective concentration (EC₅₀-48h). Based on the EC₅₀ 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 (EC₅₀-48h) 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). Azadirachtin 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-ECTM (Agidyne Technology, Salt Lake, UT, USA), BioneemTM (Ringer, Minneapolis, MN, USA), and NeemixTM (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 1.0×10⁷ 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 (EC₅₀ - 48h) for immobility was determined.

**Chronic Toxicity Test with D. magna**

Concentrations for the chronic test were based on half of the EC₅₀ 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
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 25°C (OECD, 1992) with 16:8 h light/dark photoperiod. Fish food (Tetramin) 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 described 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>
<thead>
<tr>
<th>Concentration (mL L(^{-1}))</th>
<th>Reproduction</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>154(a) ± 15</td>
<td>4.0(a) ± 0.2</td>
</tr>
<tr>
<td>0.0106</td>
<td>62(b) ± 15</td>
<td>3.0(b) ± 0.1</td>
</tr>
<tr>
<td>0.0212</td>
<td>37(c) ± 17</td>
<td>2.5(c) ± 0.9</td>
</tr>
<tr>
<td>0.0425</td>
<td>28(d) ± 9</td>
<td>2.4(d) ± 0.2</td>
</tr>
<tr>
<td>0.085</td>
<td>18(d) ± 8</td>
<td>2.2(d) ± 0.1</td>
</tr>
<tr>
<td>0.17</td>
<td>0.90(e) ± 1.19</td>
<td>1.6(e) ± 0.9</td>
</tr>
</tbody>
</table>

\(a\) Means followed by same letters do not differ by the Tukey test (P< 0.05).
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
Table 2. Estimated parameters of the quadratic regression model for size and exponential model for reproduction of D. magna.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DF (Reproduction)</th>
<th>Estimates (Reproduction)</th>
<th>Estimates (Size)</th>
<th>Confidence Limit (95%) (Reproduction)</th>
<th>Confidence Limit (95%) (Size)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_0$</td>
<td>2</td>
<td>147.40</td>
<td>3.47</td>
<td>[131.10; 163.50]</td>
<td>[3.42; 3.73]</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>2</td>
<td>-</td>
<td>-28.55</td>
<td>-</td>
<td>[-37.37; -25.98]</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>2</td>
<td>-</td>
<td>106.80</td>
<td>-</td>
<td>[103.60; 168.60]</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

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.

ACKNOWLEDGMENTS

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REFERENCES

آزمون شیاره میوه گریش (Azadirachta indica Adr. Juss) به عنوان آفت کشی زیستی از ایجاد سمومیت حاد در ماهی Danio rerio و سمومیت مزمن در Daphnia magna

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چکیده
در سال های اخیر، به منظور کمیته کردن اثرات منفی روی محیط زیست، کار برخی مواد طبیعی در مزرعه گیاهگزاری مواد ساخت بشر شده است. هدف از پژوهش حاضر بررسی اثر فرمولاسیون رونگ میوه درخت گریش در ایجاد سمومیت حاد برای یک ماهی و سمومیت مزمن برای یک رژیم شکن پوست بود. این منظور، ماها و ماهی Daphnia magna و Danio rerio در معرض غلتک های مختلف یک فرمولاسیون رونگ میوه درخت گریش قرار داده شدند. در آزمون نخست، برای تعیین غلتک کشنده به مدت ۹۶ ساعت در معرض غلتک های مختلف قرار داده شدند. در مورد میانه (EC50-96h) مایلی D. rerio از ماهی بالغ D. magna به مدت ۹۶ ساعت در معرض غلتک های مختلف قرار داده شدند. در مورد میانه EC50 (EC50-48h) مشخص شد. بر مبنای ۱/۵ EC50، غلتک های آزمون سمومیت حاد غلطت های در آزمون سمومیت حاد غلطت های بیشتر میانه EC50 (EC50-48h) غلطت های در آزمون سمومیت حاد غلطت های بیشتر میانه EC50 (EC50-48h) غلتک های آزمون سمومیت حاد غلطت های غلطت کشنده میانه برای ماهی ۱۲۷/۴ میلی لتر در دمای D. magna غلطت موثر میانه (EC50) برای D. magna از ماهی بالغ D. magna مسومیت همه غلتک ها بر تولید مثل و اندام‌های دیگر. نتایج اینکه فرمولاسیون D. magna مسومیت همه غلتک ها بر تولید مثل و اندام‌های دیگر را اثر داشته. نتیجه اینکه فرمولاسیون D. magna مسومیت همه غلتک ها بر تولید مثل و اندام‌های دیگر را اثر داشته. نتیجه اینکه فرمولاسیون D. magna مسومیت همه غلتک ها بر تولید مثل و اندام‌های دیگر را اثر داشته.