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In vitro acaricidal activity of neem (*Azadirachta indica*) seed extracts with known azadirachtin concentrations against *Rhipicephalus microplus*

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ABSTRACT

The effect of four extracts from neem seeds (*Azadirachta indica*) containing 2000, 5000, 9000 and 10,000 ppm of azadirachtin A (AZA), quantified by high-performance liquid chromatography (HPLC) and diluted to 1.25%; 2.5%; 5.0%; 10.0% and 12.8% was verified by *in vitro* tests with engorged females and larvae of the cattle tick *Rhipicephalus microplus*. The results from the bioassays with the engorged females showed that the main toxic effect of the extracts was reduction of the reproductive parameters, with a sharp drop in the number of eggs laid and the hatching rate, mainly when the extracts were diluted to 10.0% and 12.8%. The product effectiveness (PE) calculations for all the solutions tested showed that the AZA solution at 10,000 ppm (N10) was the most effective. However, statistical analysis of the PE data obtained for the proportional AZA concentrations in the different diluted extracts showed significance ($P < 0.05$) of the effects included in the model (extract dilution, principle effect (classificatory) of the assay (extract) and the interaction between the two), indicating significant variations due to the dilution, the test and the interaction between the two factors in the tests with engorged females. For solutions N2, N5, and N9, it was not possible to estimate LC_{90} values in the dilution range tested. The lowest LC_{50} was observed for extract N5, and although extract N10 was the only extract for which the LC_{90} could be estimated within the range tested, the LC_{50} was higher than for N5 and N9. These results suggest that substances other than AZA present in the extracts influenced the efficacy, especially up to a certain LC range. In the tests with larvae, no mortality was observed, indicating zero effectiveness of all the extracts tested. The results of the tests with engorged females showed that the neem extracts had acaricide activity, inhibiting egg laying and the larval hatching rate. Complementary studies are necessary to develop new methods to isolate and/or identify other substances besides AZA contained in this plant, to enable using products made from it as acaricides.

1. Introduction

Rhipicephalus microplus is considered to be the main cattle parasite in Brazil, causing annual losses to dairy and beef herders of around US$ 2 billion (Grisi et al., 2002).

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These ticks cause damage directly due to herd irritability, blood spoliation, hide and udder injuries and inoculation of toxins, and indirectly by transmission of *Babesia* spp. and *Anaplasma marginale* to the cattle (FAO, 2004).

The indiscriminate use of insecticides has led to the rapid development of tick resistance, so control is currently hindered by the small number of effective molecules available in the market (Jonsson and Piper, 2007). The use of extracts from various plants is being studied because they are potentially less toxic to the animals and are safer for the environment (Santos et al., 2006; Albuquerque et al., 2007), although *in vitro* acute toxicities of neem-containing pesticides have been observed in experiments using hybridoma and oyster cells (Goktepe and Plhak, 2003).

Alternative insecticides can be developed from many compounds isolated from plants. These can act on parasites by reducing their development, survival and reproductive rate (Habluetzel et al., 2007). The neem tree (*Azadirachta indica*), of Asian origin, contains around 135 described compounds that can have action against arthropods, including the limonoids (tetranortriterpenoids), termed globally as azadirachtin. Among these, azadirachtin A (AZA), commonly referred to as azadirachtin (Liu et al., 2005), stands out (Mordue and Nisbet, 2000; Biswas et al., 2002; Neves, 2004).

The fruits of this plant contain the highest amounts of azadirachtin – its concentration can reach 40% in the extracted oil (Abdel-Shafy and Zayed, 2002). Azadirachtin has been shown to have inhibitory effect on vitellogenin during oogenesis of arthropods (Jonsson and Piper, 2007), acceleration of the hatching rate and mortality of *Hyalomma anatolicum excavatum* newly hatched larvae (Abdel-Shafy and Zayed, 2002) and decreased blood-feeding in *Dermacentor variabilis* (Landau et al., 2009).

Experiments conducted with neem have shown some acaricide properties of its extracts (Kalakumar et al., 2000; Benavides et al., 2001; Srivastava et al., 2008), but there is little information on the composition of the extracts used and the doses necessary for effective control of ticks, among other aspects (Jonsson and Piper, 2007).

Therefore, this study was developed to test neem extracts containing known concentrations of azadirachtin A (AZA), to facilitate standardization of the solutions used to control ticks and also to investigate the *in vitro* effects of these extracts against engorged females and larvae of *R. microplus*.

2. Materials and methods

2.1. Preparation of neem seed extracts

The extracts were prepared from neem seeds from trees planted in two Brazilian states: Paraná, in the south of the country, and Bahia in the northeast. The seeds were transported in isothermal boxes to the laboratory at the Department of Natural Products Chemistry of São Carlos Federal University (UFSCar) in São Paulo state, where they were kept in a freezer at −20 °C until processing. The seeds from Bahia were used to obtain neem oil, extracted by mechanical pressing. The oil obtained was filtered and the AZA levels were quantified by high-performance liquid chromatography (HPLC).

The seeds from Paraná were frozen in liquid nitrogen and ground to a powder. The 10 l of *n*-hexane was added to each kilogram and agitated for 8 cycles of 10 min each with 90 min rest intervals between cycles. The cake resulting from this process was mixed with ethanol in the same proportions described for the hexane extract and then agitated for 12 cycles of 10 min with rest periods of 12 h. This ethanol extract was concentrated in a rotoevaporator and the AZA concentration was quantified by HPLC. This extract contained high AZA concentrations and was used to enrich the neem oil. To enrich known volumes of neem oil, known masses of the freeze-dried ethanol extract with high AZA content were dissolved in ethanol and incorporated in the oil. The quantity of extract used was established by the azadirachtin level in the extract and the final desired azadirachtin concentration in the oil. The resulting enriched extracts were also analyzed by HPLC.

For chromatographic analysis we used the methodology described by Forim et al. (2010). The reagents used were HPLC grade (Mallinkrodt-St. Louis, MO, USA) and the water was purified using the Mili-Q purification system (Millipore, Bedford, MA, USA). The extracts were dissolved in acetonitrile and the AZA concentrations were determined in a Shimadzu Liquid Chromatograph System Model LC-10AD (Tokyo, Japan) with UV detector Model MSPD-10A, and interface with SCL-10AVP column Phenomenex Luna (II) C18 (150 × 4, 6 mm, 5 μm, Torrance, CA, USA). The samples were injected in a 20 μL volume at a flow rate of 0.8 mL/min. The development of the analytical method, the collection and data processing were optimized using the CLASS-VP software. The azadirachtin standard was technical grade (PS 2075, Supelco, Sigma–Aldrich, St. Louis, MO).

Four extracts were produced, containing 2000 (N2), 5000 (N5), 9000 (N9) and 10,000 (N10) ppm of AZA. All the extracts were kept frozen at −20 °C, in flasks covered with aluminum foil, until the bioassays.

2.2. Dilution of the extracts and preparation of the controls

Five dilutions were prepared for each extract (N2, N5, N9 and N10), using a solution of sterile distilled water, 0.66% Tween 80 as an emulsifier and 30% ethanol, to obtain the extract concentrations of 1.25%, 2.5%, 5.0%, 10.0% and 12.8%. Two controls were also prepared, one containing only water (C1) and the other containing water plus ethanol and Tween 80 (C2) at the above percentages. All the dilutions of these extracts and the controls were used in the tests both against the engorged females and larvae of *R. microplus*.

2.3. Bioassays

The engorged female ticks were collected from a naturally infested herd kept at the experimental farm of the Embrapa Southeast Research Station in the municipality of São Carlos, São Paulo state. These cattle had not been treated with acaricides for 50 days before collection of the ticks. Females with body lengths greater than 5 mm were

gathered and placed in polystyrene chests containing recyclable ice packs and taken quickly to the Animal Health Laboratory where they were washed, carefully dried on absorbent paper and weighed on an analytical scale. The bioassays were conducted on the same day the ticks were collected.

For the bioassays with engorged females, the adult immersion test (AIT) was used, as described in Drummond et al. (1973). The female ticks were separated into groups of 10, with homogeneous weights, using three repetitions for each extract concentration tested as well as for the controls. The ticks were immersed for 5 min in the solutions, placed in sterile Petri dishes and incubated in a BOD (biochemical oxygen demand) incubator at a controlled temperature of $27 \pm 1\,^{\circ}C$ and relative humidity above 90%. At the end of the laying period (15th day), the eggs were weighed and placed in transparent plastic syringes to verify the larval hatching rate.

The hatching rate was determined by counting the number of larvae and remaining unhatched eggs in a representative sample from the syringes, as described by Amaral (1993), with some modifications: the syringes containing the larvae and/or eggs were placed in a temperature controlled chamber at 40 °C for 24 h to kill the hatched larvae and thus facilitate the counting. The samples from each syringe were homogenized and randomly chosen portions were placed in Petri dishes to count the larvae under a magnifying glass. Three counts of 100 larvae or eggs were performed for calculation of the final average per treatment. The mean of egg weight for the groups C1 and C2 were 2.70 g and 2.69 g and the hatching rate was 100%, for both.

The data obtained from the assays with the engorged females at the various concentrations of each extract and controls were used to determine the oviposition reduction (% OR) and hatching reduction (% HR) using the equations described by Gonzales et al. (1993), and estimated reproduction (ER), for calculation of the product effectiveness (%PE), using the equations described by Drummond et al. (1973).

Oviposition reduction:

$$\%\ \mathrm{OR} = \frac{\mathrm{WEC} - \mathrm{WET}}{\mathrm{WEC}} \times 100$$

Hatching rate reduction:

$$\%\ \mathrm{HR} = \frac{\mathrm{HC} - \mathrm{HT}}{\mathrm{HC}} \times 100$$

Estimated reproduction:

$$\mathrm{ER} = \frac{\mathrm{WE} \times \%\mathrm{HT} \times 20,000}{\mathrm{WF}}$$

Product effectiveness:

$$\%\ \mathrm{PE} = \frac{\mathrm{ERC} - \mathrm{ERT}}{\mathrm{ERC}} \times 100$$

where %OR = percentage of oviposition reduction; WEC = mean weight of eggs in controls (g); WET = mean weight of eggs in treated group (g); %HR = percentage of hatching rate reduction; HC = hatching rate in controls; HT = hatching rate in treated group; ER = estimated reproduction; WE = weight of eggs (g); HT = hatching rate; WF = weight of females (g); 20,000 = is an estimate of the number of larvae in 1 g of eggs; PE = product effectiveness; ERC = estimated reproduction in controls; ERT = estimated reproduction in treated group.

For the assays with the larvae, the larval packet test (LPT) was used (Stone and Haydock, 1962). The larvae were obtained only from engorged female ticks that weighed between 160 and 300 mg, because this is considered the optimal weight range for obtaining viable eggs (Bennet, 1974). The female ticks were washed, placed in sterile Petri dishes and incubated in a BOD chamber at $27 \pm 1\,^{\circ}C$ and relative humidity above 90% to promote oviposition. After the laying period, the eggs were placed in sterile disposable syringes, identified, plugged with cotton balls and placed in the BOD chamber under the same temperature and humidity conditions to obtain the larvae. About 100 larvae were used in each test, with ages between 14 and 21 days. They were placed between two pieces of filter paper (2 cm × 2 cm) previously moistened with the solutions prepared, at the same concentrations described for the assays with the engorged females, and then closed to form packets, with three repetitions for each concentration. The packets were incubated in the BOD chamber under the same conditions described above. The readings were made with a magnifying glass and vacuum pump after incubation for 48 h, separating the live from the dead larvae. All larvae that showed no movement were considered dead.

2.4. Statistical analysis

For the statistical analysis of the product effectiveness (PE), the results from the assays of extracts N2, N5, N9 and N10 were considered together, with the test (extract) included in the model for analysis. After the preliminary analyses to verify the most adequate model, we decided to use a probit model, including as independent variables the extract concentrations and the main effect (classificatory) of the test (extract) as well as the interaction of the two. The extract concentration was transformed into base 10 logarithmic form for inclusion in the model:

$$f(Pij) = a + Fi + (b + b1i)Xj + eij,$$

where $f(Pij)$ = probit (Z score associated with the observed PE); a = intercept; Fi = effect of the extract i ($i = 1, \ldots, 4$); b = mean probit regression coefficient; $b1i$ = effect of interaction between the extract and the extract concentration in the solution (probit regression coefficient within the effect of the extract i); Xj = level of the extract on the solution; eij = residual.

The 50% and 90% lethal concentrations (LC_{50} e LC_{90}) of each of the extracts were estimated when the values remained in the range of the concentrations evaluated.

To present the results in graphical form, the proportional AZA concentrations in the solution (dilution of the

Table 1
Means and standard errors of the % oviposition reduction (OR) and % hatching reduction (HR) of adult females of *R. (B.) microplus* exposed to neem (*Azadirachta indica*) extracts quantified by HPLC and diluted to concentrations of 1.25%, 2.50%, 5.00%, 10.00% and 12.80%.

Dilution	Extract							
	N2		N5		N9		N10	
	% OR	% HR	% OR	% HR	% OR	% HR	% OR	% HR
1.25%	2.67 ± 2.67	0.00 ± 0.00	14.00 ± 9.07	3.33 ± 3.33	3.33 ± 2.40	0.00 ± 0.00	6.67 ± 2.33	1.00 ± 0.58
2.50%	14.33 ± 7.42	6.67 ± 6.67	35.33 ± 19.7	20.00 ± 15.28	47.67 ± 1.20	26.67 ± 3.33	9.33 ± 3.18	13.00 ± 6.81
5.00%	37.00 ± 6.81	23.33 ± 18.56	51.67 ± 6.06	20.00 ± 10.00	61.33 ± 10.14	16.67 ± 3.33	16.33 ± 6.96	18.67 ± 7.88
10.00%	53.67 ± 10.93	20.00 ± 15.28	70.00 ± 12.50	38.33 ± 28.92	61.33 ± 13.17	33.33 ± 8.82	52.67 ± 7.69	88.00 ± 2.31
12.80%	59.00 ± 8.19	26.67 ± 12.02	79.67 ± 7.06	58.33 ± 25.22	82.67 ± 9.82	43.33 ± 29.63	71.33 ± 9.87	78.00 ± 4.73

AZA concentrations: N2 = 2000 ppm; N5 = 5000 ppm; N9 = 9000 ppm; N10 = 10,000 ppm.

Table 2
Mean product effectiveness (PE) and standard errors obtained in tests with adult females of *R. (B.) microplus* exposed to neem (*Azadirachta indica*) extracts quantified by HPLC and diluted to 1.25%, 2.50%, 5.00%, 10.00% and 12.80%.

Dilution	Extracts			
	N2	N5	N9	N10
1.25%	2.67 ± 2.67	17.33 ± 8.76	3.33 ± 2.40	7.67 ± 2.19
2.50%	21.00 ± 6.11	51.00 ± 17.69	61.67 ± 2.40	20.33 ± 9.17
5.00%	54.00 ± 8.89	62.67 ± 2.33	67.33 ± 9.91	31.00 ± 12.10
10.00%	60.00 ± 14.93	79.00 ± 12.50	72.67 ± 12.45	94.67 ± 0.88
12.80%	67.67 ± 9.67	88.33 ± 8.41	86.00 ± 7.21	93.33 ± 2.91

AZA concentrations: N2 = 2000 ppm; N5 = 5000 ppm; N9 = 9000 ppm; N10 = 10,000 ppm.

extract multiplied by the AZA concentration in the extract) were also calculated. All the statistical analysis were performed using the SAS statistical package (SAS, 2002/2003).

3. Results

The experiments performed in the laboratories of the Natural Chemical Products Department of UFSCar showed it is possible to obtain extracts with high AZA concentration from neem seeds and that these extracts do not deteriorate when kept frozen at −20 °C and protected from light.

The tests to assess the effects of extracts N2, N5, N9 and N10 on the engorged females showed that the percentage of oviposition reduction (OR) and hatching reduction (HR) varied among the different extracts tested, with the greatest reductions being found at the highest extract concentrations (Table 1). The greatest OR was observed for extract N9 (82.67%) and the greatest HR for extract N10 (88.00%).

The average effectiveness percentages (PE) obtained according to Drummond et al. (1973) from the observed values are shown in Table 2. Extract N10, diluted to 10% (94.67%) and 12.80% (93.33%), was most effective, followed by extracts N9 (86.00%) and N5 (88.33%), both diluted to 12.80%.

It can be seen from Fig. 1, which plots the predicted PE values as a function of the extract concentration for each of the extracts according to the probit model utilized, that at the same concentrations extract N5 was most effective at lower dilutions, while N10 was the only extract to attain estimated PE values above 90%. Fig. 2 shows the same pre-

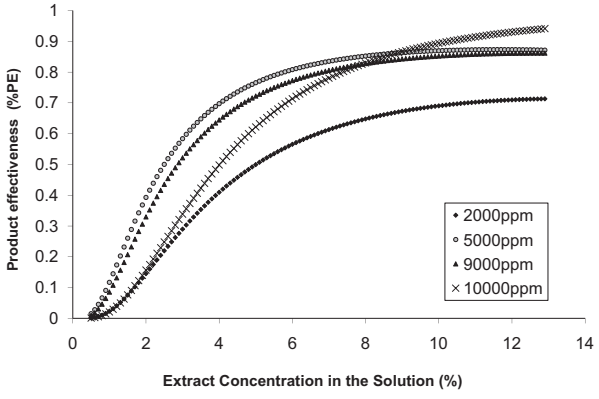

Fig. 1. Product effectiveness (%PE) obtained in tests with adult females of *R. (B.) microplus* exposed to neem (*Azadirachta indica*) extracts quantified by HPLC: N2 = 2000 ppm of AZA; N5 = 5000 ppm of AZA; N9 = 9000 ppm of AZA; N10 = 10,000 ppm of AZA.

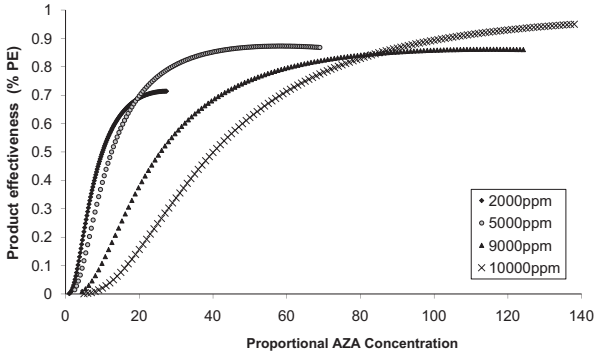

Fig. 2. Product effectiveness (%PE) in relation to the effects of the concentration of AZA in the solution on engorged females of *R. (B.) microplus*. N2 = 2000 ppm of AZA; N5 = 5000 ppm of AZA; N9 = 9000 ppm of AZA; N10 = 10,000 ppm of AZA. Proportional AZA concentration = dilution of the extract multiplied by the AZA concentration in the extract.

Table 3
Lethal concentration (LC) for 50 and 90% mortality of adult females of *R. microplus* exposed to neem extracts. N2 = 2000 ppm of AZA; N5 = 5000 ppm of AZA; N9 = 9000 ppm of AZA; N10 = 10,000 ppm of AZA.

LC	Extracts			
	N2	N5	N9	N10
50	5.0	2.5	2.9	4.0
90[a]	–	–	–	10.3

[a] Points refer to extracts that did not attain LC_{90}.

dicted PE values, now as a function of the proportional AZA concentrations (PAC) in the solution (concentration of the extract multiplied by the AZA concentration in the extract) for each extract. It can seen that for each extract, although the increase of the PAC had a direct relation with the product effectiveness, the effect of the PAC was lower in the extracts with higher AZA concentrations, except N10 at the highest concentrations.

The LC_{50} and LC_{90} values for all the tested extracts are shown in Table 3. The lowest LC_{50} value for the engorged females was observed for extract N5 (2.5%) and the lowest LC_{90} was obtained for N10 (10.3%).

In the tests on the larvae, none of the extracts tested caused mortality.

4. Discussion

The tests of the different neem seed extracts prepared in this experiment show this part of the plant concentrates the highest levels of the active ingredients that can be used as bioinsecticides.

The results obtained from the tests with engorged females showed that the main toxic effect produced by the extracts was to inhibit this parasite's reproduction. The highest OR and HR rates were observed when testing extracts containing AZA concentrations greater than 2000 ppm and in solutions with higher concentrations of the extracts. Similar effects were also observed for the tick *H. anatolicum excavatum* by Abdel-Shafy and Zayed (2002), who tested commercial neem seed oil (Neem Azal F). These authors observed reductions in the hatching rate of 35%, 34%, 52% and 60% on the fifth day after treatment with the oil diluted at 1.6, 3.2; 6.4 and 12.8%, respectively. They further observed that on the first day after treatment of the eggs, there was an increase in the hatching rate and suggested that AZA was responsible for this effect, by stimulating hatching before the larvae were fully viable and thus preventing continuation of the parasite's life cycle. In the present study we did not observe any early hatching of the larvae.

The results obtained for the product effectiveness (PE) show that in general the higher the quantity of the extract in the diluted solution, the greater the PE. The results were more variable with respect to the AZA concentration in the extract, but there was a general tendency for increased effectiveness in function of rising AZA concentration of each extract. Similar observations were made by Ndumu et al. (1999) studying the tick *Amblyomma variegatum*. They observed that two main factors influenced the efficacy of the neem extracts studied by them: time of exposure to the oil and quantity of oil in the extract. According to them, the greater the exposure time to the oil and the higher the quantity of oil in the extract, the more effective the products were. In the present study the time of exposing the females to the different extracts was equal, so we could only observe the effects of different quantities of AZA in the extracts and of extracts in the dilutions prepared. High efficacy levels were also reported by Abdel-Shafy and Zayed (2002), using the commercial neem seed extract (Neem Azal F) on engorged females of the tick *Hyalomma anatolicun excravatum*. They worked with various dilutions of a single extract and observed that mortality increased with an increase in the relative concentration of the product.

Here we studied four different extracts and observed that as the quantity of extract in the solution increased, so did the OR, HR and PE indices. The statistical analyses of the PE showed that the effects included in the model – extract concentration in the solution, the extract (test) and the interaction between the two – significantly influenced ($P < 0.05$) this variable. There was substantial variation in the results regarding the AZA concentration in the extracts, with N5 being the most efficient in the solutions with lower extract quantity and N10 being the most efficient at the highest concentrations. We must stress, however, that the effect of the bioassay was partly combined with the effect of the AZA concentration, since each test was performed with a different extract, diluted in the same form. Since the effects of the extract and the interaction were significant, the effectiveness of each extract analyzed was different. From Fig. 2 it can be inferred that the higher the proportional AZA concentration of the extract, the lower its relative efficacy was at the lowest extract concentrations in the solution.

The statistical analyses showed that the higher the quantity of the neem extract in the solution, the more effective it became, irrespective of the AZA concentration of the extract. This finding indicates that other limonoids present in the seeds might have influenced the results. A plausible hypothesis is that since the extracts were enriched only considering the AZA concentrations, there might have been a gradual reduction in the quantity of other limonoids present in the basic extract produced. Generally the precision of the effects of various types of neem extracts to control pests is hard to specify, since the complexity of the compounds and their various modes of action make it hard to discern the mechanisms involved (Mossini and Kemmelmeier, 2005).

Other limonoids present in neem extracts have proven to be toxic to parasites, as found by Cohen et al. (1996). They noted two other limonoids in the extracts studied by them, called nimbolide and epoxiazadiradion, both of which appeared to have cytotoxic potential.

The use of hexane as the solvent to produce the extracts permitted production of compounds with high AZA levels. Because this is a solvent with low polarity (Forim et al., 2010), limonoids with medium to high polarity remained in the neem cake. The solutions varied in extract quantity, depending on the quantity of the extract with high AZA content required to enrich the oil produced.

However, since N10 was more effective when the extract's concentration in the solution was higher, there is room to speculate about the influence of AZA on the PE.

We should point out, however, that the difference between the effects of N9 and N10 were considerable despite the small AZA concentration difference. If N9 is ignored, it can be supposed that other limonoids present in the extracts (in decreasing quantities from N2 to N10) were responsible for the increased PE until a determined maximum value (maximum PE), from where the increasing extract concentration in the solution did not produce further results. On the other hand, the AZA started to have more effects at higher concentrations, as in the case of N10. Consideration must go to the method used to produce the extracts, whereby the higher the AZA concentration was, the greater the quantity was of the purified extract added and the smaller the quantity of the basic extract, which contained most of the limonoids with low, medium and high polarity. In general, the extract that contained the lowest AZA concentration had higher quantities of other limonoids, which might have favored the acaricide effect. In contrast, as the AZA concentration increased in the extracts, the quantity of the other limonoids with low polarity decreased, causing a relatively smaller effect than expected.

According to the statistical analyses, extract N5 was the most effective until a concentration of approximately 8.0% (Fig. 1). This extract had moderate quantities of the basic oil and AZA when compared with extracts N9 and N10, unlike extract N2, which should presented a higher quantity of other low-polarity limonoids and a higher concentration of AZA and other limonoids of medium and high polarity. All these factors might have influenced the acaricidal effect of the extracts tested.

We observed that extract N5 had the lowest LC_{50} (Table 3). Few results on the lethal concentration of neem solutions have been reported in the literature. Benavides et al. (2001) found that the LD_{99} for inhibition of oviposition in *R. microplus* females after immersing them in neem seed extracts was 28.1%.

None of the extracts tested caused mortality among the larvae. These findings can be related to the type of test employed (using impregnated sheets of tissue paper). Passos (1994) observed that the cellulose in paper diminishes the toxicity of some insecticides because it retains the active ingredient. Besides this, tests with impregnated paper may not allow full contact of the larvae with the chemical compounds tested (Chagas et al., 2003).

In closing, we can conclude that extracts prepared from neem seeds can contain high concentrations of AZA A and other limonoids with deleterious action on the reproduction of *R. microplus* females. Complementary studies are needed to establish the nature of the other limonoids contained in neem seeds that have acaricidal effect.

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