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Biocide activity of *Annona coriacea* seeds extract on *Rhodnius neglectus* (Hemiptera: Reduviidae)

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Abstract: The use of synthetic insecticides for insect control may lead to different kind of problems, such as vector resistance to insecticides. To avoid these problems, a new research area to study botanical products as possible disease vectors controls, has become a feasible alternative. The purpose of this research was to evaluate the biocide activity of the ethanol extract of seeds of *Annona coriacea* on *Rhodnius neglectus* (Chagas disease vector) nymphs and adults. For this, different concentrations extracts were evaluated: 25, 50, 100 and 200mg/mL, and water in DMSO (20%) was used as control. The experimental design was completely randomized and we conducted the bioassay with nymphs and adults, with 10 nymphs and 10 adults (five males and five females) per treatment. Extract action was evaluated in both bioassays, in order to identify possible effects of mortality and life cycle interruption of nymphs and adults during a 28-day-period. The results obtained showed that the extract of *A. coriacea* was able to disrupt the development of nymphs and adults of *R. neglectus*, with a mortality rate of more than 90%, 36% and 100%, at the highest concentrations, respectively. There was also molting inhibition in nymphs, lower reproductive capacity in females, feeding deterrence and morphological changes in nymphs and adults. We concluded that the extract of *A. coriacea* has insecticide action on nymphs and adults of *R. neglectus*. Rev. Biol. Trop. 61 (1): 419-427. Epub 2013 March 01.

Key words: *Annona coriacea*, nymphicidal, adulticidal, seed extract, triatomines.

Chagas disease, originally identified as an enzootic disease, became a health problem due to the housing of the vector insects (Silveira 2000). Transmitting species of the protist *Trypanosoma cruzi* Chagas 1909, etiological agent of the disease, are hemathophagous insects with hemi metabolic development of the genus *Triatoma* Laporte 1832, *Rhodnius* Stal 1859 and *Panstrongylus* Berg 1879 (Hemiptera: Reduviidae), which have the highest epidemiological importance (Steindel *et al.* 2005).

Hemathophagy is essential for growing, molting and reproduction of triatomines (Rocha *et al.* 2004, Tartarotti *et al.* 2004). Blood meal followed by defecation is a relevant biological characteristic, when the vector may become infected or transmit *T. cruzi* (Galvão 2003, Rocha *et al.* 2004).

Currently, the elimination of the vector transmitting the Chagas disease is considered a strategy for control in Latin America countries by means of regular and systematic use of residual insecticide spraying in infested dwellings (Villela *et al.* 2005).

In Brazil, common insecticides being used for controlling triatomines are piretroids Deltamethrin, Cyfluthrin, Lambdacialotrina, Cypermethrin, and Alfa-cypermethrin (Oliveira Filho *et al*. 2000). Despite the efficiency of the chemical control, prolonged use of these

insecticides is questioned due to the possibility of resistance development and toxicity of these products (Dias 1997, Dias 2001, WHO 2002).

Due to the negative aspects of excessive use of synthetic insecticides, a new stimulus came up with the phytochemical study of plants, especially those with promising biocide activity and that can represent an alternative to vector control of Chagas disease (Costa *et al.* 2004).

Plant species that have been identified as promising in vector control have caused insect mortality, growth inhibition, molting process disruption, deformation of nymphs and adults, reduction in longevity, fertility and fecundity of adults (Schmeda-Hirschmann & Rojas de Arias 1992, Fournet *et al*. 1996, Valladares *et al*. 1999, Bobadilla *et al*. 2005, Coelho *et al*. 2006, Costa *et al*. 2006).

Species of the family Annonaceae have showed insecticidal action on triatomines, as related by Schmeda-Hirschmann & Rojas de Arias (1992) which tested the effect of *Annona reticulata* L. on nymphs of *Rhodnius neglectus* Lent 1954 and for Parra-Henao *et al*. (2007) that evaluated the effect of *Annona muricata* L. on fourth instar nymphs of *Rhodnius prolixus* Stal 1859 and *Rhodnius pallescens* Barber 1932.

Chemical and pharmacological investigations of species of the genus *Annona* have indicated important bioactive compounds, revealing the presence of alkaloids and acetogenin of Annonaceae, with evident insecticidal action on disease vectors (Alali *et al.* 1999, Boaventura 2003, Nascimento *et al.* 2006).

Research of He *et al.* (1997) with 44 species of Annonaceae showed pesticide properties of acetogenins on the mosquito that transmits yellow fever. The structure-activity relationships indicate that the compounds bearing adjacent bis-THF (tetrahydrofuran) rings with three hydroxyl groups were the most potent. This is the first report of pesticide structure-activity relationships for a series of annonaceous acetogenins which are known to act, at least in part, as potent inhibitors of mitochondrial NADH: ubiquinone oxidoreductase.

Considering the need of new strategies for triatomines control, the purpose of this research was to evaluate the nymphicidal and adulticidal potential of *Annona coriacea* Mart 1841 on *R. neglectus*, the vector.

MATERIAL AND METHODS

Extract preparation: We collected the fruits in the area of the Cerrado at "Fazenda Três Rios", located in Nova Marilândia/MT (14º23' S - 57º42' W) at an altitude of 467m. The specimens collected were identified and kept in the Herbarium TANG, Department of Biological Sciences-UNEMAT Tangará da Serra, MT, with registration number 269. The process of extract preparation was performed in the Laboratories of Entomology and General Chemistry, State of Mato Grosso University-UNEMAT Campus Tangará da Serra. Seeds were placed in an oven with air circulation at 40ºC for 72hr; afterwards, the seeds were ground until powder. The extract of the seeds of *A. coriacea* was obtained using 500g of seed powder with 1 500mL of ethanol solvent, and the mixture was kept in percolation during seven days. The suspension, that forms an only polar phase, was filtered on Büchner funnel, the solvent was removed throughout rotative evaporator and transferred to a drying greenhouse to complete solvent removal. The crude extract showed a yield of 30g. This crude extract was dissolved with a 20% Dimetil Sulfoxide (DMSO) solution.

Concentration determination: To determine the concentrations to be used in the experiments, some pre-tests for each instar were undertaken using the same methodology, thus determining the minimum concentration to be used. Pre-tests were also performed in order to select the appropriate concentration of DMSO to dissolve the extract and that would not affect insect development.

Rearing of *Rhodnius neglectus*: Insects rearing and experiments were conducted at the Insect/Plant Interaction Laboratory, the Center for Limnology, Biodiversity and Ethno Biology of the Pantanal (CELBE), State of Mato

Grosso University, UNEMAT, Cáceres. The insects obtained for the rearing were given by the Laboratory of Triatominae and Epidemiology of Chagas disease (LATEC), René Rachou Research Center (CPqRR)/Oswaldo Cruz Foundation (FIOCRUZ), Belo Horizonte-MG. The insects were placed in transparent polyethylene bottles with identification labels; having the bottom covered with sheets of filter paper. The bottle opening was sealed with thin cotton cloth and secured with adhesive tape and rubber bands. The bottles were kept in a B.O.D incubator at 28±1ºC, 60-70% relative humidity and with a photoperiod of 12hr as suggested by Schmeda-Hirschmann & Arias de Rojas (1992). As a food source for triatomine bugs, fowls of the *Gallus gallus* species were used being maintained immobilized with the aid of wooden plates during the blood meal of the insects (Schmeda-Hirschmann & Arias de Rojas 1992). We chose the species *R. neglectus* for the tests because of their short reproductive cycle in the laboratory, and because it is one of the most known species in the Mato Grosso region.

Nymph bioassays: Application of the ethanol extract was made on nymphs of fourth and fifth instars (15 to 20 days old) of *R. neglectus*, that were fed seven days before receiving the treatment; the nymphs that did not feed were discarded from the test. The fifth instar nymphs are recommended by the World Health Organization for laboratory tests, due to the fact that they are the more resistant developmental stage to chemicals (WHO 2002), and the fourth instars nymphs are the most commonly used by researchers in plant extract tests (Coelho *et al*. 2006). Bioassays were conducted in a randomized design and the concentrations tested were 25, 50, 100 and 200mg/mL and the control (DMSO in 20% - water) with three replicates, each stage consisted of ten insects. The amount of solution applied once on the abdominal tergites of each nymph was 1µL, according to methodology proposed by Schmeda-Hirschmann & Arias de Rojas (1992) and Coelho *et al*. (2006). The action of the extract was observed at different exposure times to assess the mortality rate, after the periods one, two, three, four, seven, 14, 21 and 28 days, as recommended by Coelho *et al*. (2006).

Adult bioassays: Applications of the ethanol extract were made on adults of *R. neglectus* following the same protocol of the previous bioassay (WHO 2002). Each experimental group consisted of five males and five females of *R. neglectus*. The bioassay was conducted in a randomized design with three replicates. The amount of solution applied on the abdomen of each insect was 5µL as indicated in the WHO protocol (2002). The concentration and length of action of the extract were evaluated as described for the nymphs.

Criteria for repast, morphological changes and mortality assessment (passing over): The food source (blood) was offered on the eighth day of the experiment, and was provided once a week until the end of the test, to avoid the possibility of dying due to lack of food. To determine whether the insects fed, we observed them during a 30min period, to confirm if they introduced the proboscis in the food source, and if there was any increase of their abdominal size, after blood intake.

In order to determine mortality of insects affected by the extracts evaluated, we used the method following WHO (2002). An insect was considered dead, when it did not show any locomotive activity when placed on a filter paper. In order to evaluate the locomotive activity, we observed the insect movements during a daily 30min period, always comparing with the control.

We also recorded the morphological changes, antifeedant activity, ecdysis inhibition, female fecundity and fertility of eggs, for the different concentrations during the 28-day-trial.

Data were submitted to ANOVA with Poisson distribution with correction for excessive dispersion because samples were dealing with

count data. The average concentration of the extracts were compared by Tukey test ($p<0.05$), using the program R version 2.9.0.

RESULTS

Effect of *A. coriacea* different concentration extracts on fourth instar nymphs of *R. neglectus*: When analyzing the effect of different concentrations on the mortality of the fourth instar nymphs we observed that the effect of the extract of *A. coriacea* was statistically significant for the different concentrations ($p=0.006$, GL=4.119).

The concentrations of 100 and 200mg/mL showed the highest numbers of dead nymphs, with no differences between them, with mortality rates of 80 and 93.3%, respectively (Table 1). The concentrations 25 and 50mg/mL showed lower mortality values, differing from those of 100 and 200mg/mL. All concentrations, except 25mg/mL, differed from the control treatment (Table 1).

At concentrations of 25 and 50mg/mL, some morphological changes such as irregular abdomen and proboscides, deformed legs and antennae occurred were observed, with a total percentage of 36.6 and 20% of insects affected, respectively; for other concentrations, only 3.3% of insects had changes in their morphology (Table 2). Much of the changes occurred even on the instars of testing, in the fourth instar nymphs, and those that had achieved the molting of fifth instars also showed morphological abnormalities, mainly

TABLE 1
Average mortality of 4th and 5th instar nymphs of *Rhodnius neglectus* at different extract concentrations of *Annona coriacea*

Concentration (mg/ml)	Dead individuals				
	4th instar nymphs (n)	4th instar nymphs (%)	5th instar nymphs (n)	5th instar nymphs (%)	Total (n)
Control	0 a	0	0 a	0	30
25	5 ab	16.6	1 a	3.3	30
50	10 b	33.3	1 a	3.3	30
100	24 c	80.0	8 ab	26.6	30
200	28 c	93.3	11 b	36.6	30

Note: Values followed by the same letter in column did not differ from each other at 5% probability.

TABLE 2
Percentage of 4th and 5th instar nymphs of *Rhodnius neglectus* that fed, molted and underwent morphological changes after the application of extracts of *Annona coriacea*

Concentration (mg/ml)		Nymphs that feed	Nymphs that molted	Nymphs with morphological changes
4th instars	Control	100	100	0
	25	3.3	26.6	36.6
	50	3.3	3.3	20
	100	3.3	0	3.3
	200	0	3.3	3.3
5th instars	Control	100	100	0
	25	0	16.6	20
	50	0	0	30
	100	0	0	40
	200	0	0	33.3

in proboscides. Dead insects presented very evident signs of dehydration.

Molting observations showed that only a small percentage could perform the ecdysis: 26.6% at concentration of 25mg/mL and 3.3% at concentrations of 50mg/mL and 200mg/mL. We observed that only 3.3% (equivalent to an individual) had achieved the blood meal at the concentrations above mentioned, demonstrating the deterrent effect in fourth instar nymphs (Table 2).

Effect of concentrations of *A. coriacea* on fifth instar nymphs of *R. neglectus*: On the basis of exploratory variations, we found that the number of dead individual specimens showed significant differences in function of the concentrations tested (p=0.028, GL=1.119), therefore, the number of dead individual specimens was higher with the increase of extract concentration (Table 1).

At concentrations of 100 and 200mg/mL there was a significant mortality rate, showing the effectiveness of plant extracts as well as its toxicity in higher concentrations (Table 1). The comparisons of the average (significant) number of dead individual specimens, between the treatments of 100 and 200mg/mL did not differ among themselves, as well as the concentrations of 25 and 50mg/mL that did not differ from control (Table 1).

At all concentrations, *A. coriacea* extracts resulted in morphological changes in mouthparts, legs, antennae and abdomen in 20-40% of the insects tested. These deformations were: raised or bent proboscides, legs bent in an arc shape, brittle antennae and irregularly shaped abdomens showing recesses in the abdominal tergites. Most insects were unable to perform the ecdysis, and all nymphs presented antifeedant activity at all extract concentrations (Table 2).

When analyzing the results of mortality rates in the tests with fourth and fifth instars nymphs, we observed that even at higher concentrations, 100 and 200mg/mL the death percentage of fifth instars insects resulted lower (26.6 and 36.6%), than the results obtained for fourth instar nymphs (80 and 93.3%), respectively (Table 1).

Effect of concentrations of *A. coriacea* on adults of *R. neglectus*: The effect of different concentrations on the mortality rate of adult insects was observed, showing that the extract of *A. coriacea* was statistically significant for the different concentrations (p=0.01, GL=1.159).

The analysis of the average number of deaths observed at different extract concentrations revealed that the concentrations of 25, 50, 100 and 200mg/mL did not differ among them, except when compared to the control (Table 3). Thus, we can say that the lowest concentration of 25mg/mL caused a mortality equivalent to the higher concentration evaluated, killing 90% of adults, while 100 and 200mg/mL killed

TABLE 3
Average mortality, fecundity and fertility of adults of *Rhodnius neglectus* after the application of extracts of *Annona coriacea*

Conc. (mg/ml)	Dead adults		Fecundity (n)	Fertility (hatched eggs)		Blood meal (%)	Total (n)
	(n)	(%)		(n)	(%)		
Control	1a	2.5	530a	517a	97.5	100	40
25	36b	90	332ab	288b	86.4	0	40
50	37b	92.5	124c	90c	72.5	0	40
100	40b	100	67d	11d	16.4	0	40
200	40b	100	45d	6d	13.3	0	40

Note: Values followed by the same letter in column did not differ from each other at 5% probability.

100% of the insects, explaining the interaction between the concentrations (Table 3).

Fecundity evaluation during the 28 days period, showed that all concentrations did differ in relation to the control, except for the concentration of 25mg/mL, noting that the greater concentrations, 100 and 200mg/mL, did not present significant difference among themselves (Table 3). As to the fertility of the eggs we observed that with the growing increase of the concentrations, occurred a significant reduction of this variable where at the concentration of 25mg/mL differed statistically from the averages of 50, 100 and 200mg/mL, while 50mg/mL differed from all concentrations.

The concentrations of 100 and 200mg/mL were similar. Just as in the case of fecundity, fertility also decreased with increasing concentrations (Table 3). We can observe that fertility and fecundity showed different results at different concentrations, and the lower ovipositions (egg positioning), 67 and 45 eggs, were obtained at the higher concentrations, 100 and 200mg/mL. At these concentrations hatching was observed only in 16.4 and 13.3% of eggs, respectively (Table 3). We can note that from the lowest concentration, 25mg/mL to the highest, 200mg/mL the extract of *A. coriacea* was able to inhibit feeding in 100% of adult insects, and they failed to detect the source of blood at the time of blood meal (Table 3). The insects of the control group fed normally.

DISCUSSION

In this research, the results obtained with the topical application of the extract of *A. coriacea* showed that a promising tool for *R. neglectus* control was found, with positive effects for all concentrations. Costa *et al*. (2004) have already mentioned that the effects of plant extracts are able to cause mortality, as well as to disrupt the cycle of insect development. Besides the significant mortality rate effects such as antifeedant activity, disruption of the molting process, morphological changes, effects on fertility and fecundity were observed in tests with fourth and fifth instar nymphs and adults of *R. neglectus*.

The extract of *A. coriacea* caused a mortality rate of 33.3% of fourth instar nymphs of *R. neglectus* at the concentration of 50mg/mL. Similar results were obtained by Schmeda-Hirschman & Rojas de Arias (1992) with *A. reticulata*, as they reported that caused a mortality rate of 35% in the nymphs of this species. Alves (2007) evaluated the insecticidal activity of some species of the Cerrado region and recorded a variation in the mortality rate from 12.5 to 42.5% for *Rhodnius milesi* Carcavallo, Rocha, Galvão & Jurberg. Additionally, Fournet *et al*. (1996), in research with *Minsthostachys andina* Brett reported that the mortality rate of *Triatoma infestans* Klug 1834 and *R. neglectus* ranged from 30 to 50%.

Vilaseca *et al*. (2004) analyzed the insecticidal activity of *Hedeoma mandoniana* Wedd for controlling *T. infestans* and *R. neglectus* recording a mortality rate of 33.3%. Parra-Henao *et al*. (2007) also evaluated the toxic effect of plant species, and related that *Annona muricata* L. was the species that caused the highest mortality rate (56%) at a concentration of 70mg/mL on fourth instar nymphs of *R. prolixus* and *R. pallescens*. There are no publications available that explain the toxicity of *A. coriacea* on bugs, but the insecticidal activity is explained due to the fact that genus *Annona* have active molecules such as the acetogenins, that have various biological activities, including the insecticide (Boaventura 2003, Nascimento *et al*. 2003).

A survey carried out with fractions of *Annona squamosa* showed adulticide and larvicide activity, as well as oviposition inhibition in the different development instars of *Aedes albopictus.* The component responsible for the insecticidal activity has been reported as the composite oleate isooctyl phthalate (Kempraj & Bhat 2011).

The extract of *A. coriacea* was able to inhibit the molting process in *R. neglectus*, unlike the results obtained with *A. reticulata* by Schmeda-Hirschman & Rojas de Arias (1992) that did not observe changes in the molting

process, on the fourth instar nymphs of *R. neglectus*. However, these authors assessed the species *Mangifera indica* L., *Spilanthes* sp., *Tagetes erecta* L., *Mintostachys* sp. *Salvia cardiophylla* Benth, *Cassia* sp. Azederach, *Senna occidentalis* L. and *Melia* finding that they inhibited ecdysis with rates ranging between 22.2% and 33.3%.

Despite all the research presented that indicated the effects of plant extracts on the fourth instar nymphs, we assessed also in this experiment tests with fifth instar nymphs, because they are the most recommended by WHO (2002) due to higher resistance of such instars in development.

The results obtained with adult mortality of *R. neglectus* were similar to those obtained by Lima *et al*. (1992), with aquatic suspension of hexachlorocyclohexane (HCH), registering 100% of mortality for the adult insects and fifth instar nymphs. We observed that fifth instar nymphs were more resistant to the insecticide and died only after exposure for several days.

Changes or morphological abnormalities observed in both fourth and fifth instar nymphs of *R. neglectus*, are probably related to antifeedant activity caused by the extract, leading to nutritional deficiency in the nymphs, as reported by Costa *et al*. (2004). Although suffering higher mortality rate, fourth instar nymphs showed fewer morphological changes than fifth instar nymphs. It is concluded, therefore, that fifth instar nymphs were more susceptible to the bioactive effect that caused morphological changes, even after showing a higher resistance to the toxic effect of the extract; further studies on the active principles of *A. coriacea* are required.

The deterrent action of the extract of *A. coriacea* at all concentrations prevented the blood meal of fourth and fifth instar nymphs and adults. The effective feeding after defecation is considered very important to complete the cycle of disease development, and if there is no blood meal there is no infection, nor the transmission of the etiological agent of Chagas disease (Garcia & Azambuja 2004, Tartarotti *et al*. 2004). The deterrent effect or anti-feeding can also be caused by the active compounds of *A. coriacea* that makes more difficult the normal development of the insect causing deformities and preventing the full metamorphosis. Thus, as defined by Cabral (1999) and observed in both experiments with *A. coriacea* in fourth and fifth instar nymphs, a negligible amount of insects were able to feed themselves.

The results obtained at the different observation times showed a gradual increase in mortality of fourth instar nymphs, which is considered a relevant result. In the tests with fifth instar nymphs, mortality also showed a gradual increase until the end of the experiment, and the concentrations that showed significant mortality were 100 and 200mg/mL. Despite the fact that we did not observe mortality at lower concentrations, the effects caused by the plant extracts, such as antifeedant activity, morphological changes and inhibition of ecdysis were sufficient to prevent the development of fifth instar nymphs at all tested concentrations.

In adult insects, the gradual increase of the mortality rate occurred at the concentration of 25mg/mL, where the percentage of dead individuals was equivalent to the one observed in fourth instar nymphs in the highest concentration, indicating that the adult insects are more susceptible to toxicity than the fourth and fifth instar nymphs. Just as observed by Costa & Perondini (1973) the resistance increased up to the fifth instars decreasing in the adult instars. According to WHO (2002) fifth instar nymphs have a higher resistance to chemicals, while adult insects are less resistant.

The reduction in fecundity and fertility rates of *R. neglectus* recorded during the 28 days of observation, probably occurred due to antifeedant activity caused by food deprivation, as also observed by Braga & Lima (2001) that evaluated the effect of food deprivation in females of *Panstrongylus megistus* Burmeister 1835, and concluded that fertility in females is related to the amount of blood intake and that food deprivation is responsible for the reduction of the fertility.

We concluded therefore that the extract of *A. coriacea* showed nymphicidal effect,

altering the development of fourth and fifth nymphs of *R. neglectus*, showing a significant mortality rate in fourth instar nymphs, inhibiting the molting process, causing morphological changes and antifeedant activity in both tests, thus disrupting the development of the nymphs of *R. neglectus*. In adulthood, the extract proved to be an effective biocide even at the lowest concentration.

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RESUMEN

La enfermedad de Chagas se convirtió en un problema de salud debido a su importancia epidemiológica, es producida por el protista Trypanosoma cruzi, cuyos insectos vectores son del género Triatoma y Panstrongylus. El objetivo de este estudio fue evaluar la actividad biocida del extracto de *Annona coriacea* en las ninfas de *Rhodnius neglectus* y en sus adultos. Se evaluaron 14 concentraciones de 25, 50, 100 y 200mg/ml del extracto etanólico, así como el control, en este caso agua de DMSO (20%). Se utilizo un diseño completamente aleatorizado con tres repeticiones para el bioensayo con 10 ninfas y 10 adultos (cinco machos y cinco hembras) para cada tratamiento. La acción del extracto se observo durante 28 días en ambos bioensayos. Los resultados obtenidos mostraron que el extracto de *A. coriacea* fue capaz de interrumpir el desarrollo de las ninfas y adultos de *R. neglectus*, con una mortalidad de más del 90%, 36% y 100%, correspondiendo a las concentraciones más altas. También hubo inhibición de la muda de las ninfas, una menor capacidad reproductiva de las hembras, disuasión alimentaria y cambios morfológicos en las ninfas y adultos. Se concluye que el extracto de *A. coriacea* presentó acción insecticida en ninfas y adultos de *R. neglectus.*

Palabras clave: *Annona coriacea*, ninficida, adulticida, extracto semilla, triatominos.

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