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ORIGINAL ARTICLE

INSECTICIDAL ACTIVITY OF *Anacardium humile* (ANACARDIACEAE) NUT SHELL LIQUID AGAINST *Aedes aegypti* (DIPTERA: CULICIDAE)

Camila Aline Romano, Heloisa Helena Garcia da Silva, Marina Garcia and Ionizete Garcia da Silva

ABSTRACT

This work investigated the insecticidal activity of Cashew Nut Shell Liquid (CNSL) extracted from the species *Anacardium humile*, a native plant in the Cerrado biome, against *Aedes aegypti*. *A. humile* fruits were collected and kept in a forced ventilation kiln at 40ºC for seven days for CNSL extraction. Mortality tests were performed on third stage larvae, pupae and adults. In addition, female oviposition behavior, egg viability, and the residual effect of the solution on 3rd stage larvae were observed. The CNSL was diluted in decreasing gradient concentrations to obtain the Lethal Concentration (LC). In oviposition LC_{99} was used for larvae. Residual effect and oviposition tests were performed with LC_{99}, LC_{50} and LC_{90} were respectively obtained, 6.63 and 11.23 ppm for larvae. No mortality was observed in adults. The residual effect of the CNSL was five days in LC_{99}. A repellent effect of CNSL on the females was noted, with a significant reduction of egg numbers and a change in egg stratification patterns in the oviposition substrate. The larval hatching rate of the eggs exposed to the substrate moistened with CNSL was significantly lower when compared to the control. The results evidenced the insecticidal activity of *A. humile* CNSL, suggesting it as a promising product in the quest for new botanical insecticides.

KEY WORDS: Vector control; botanical insecticide; Cerrado.

INTRODUCTION

Insects are evolutionarily associated with plants which may produce active secondary metabolites against insects in general or specific to certain groups. These molecules act in the insects' organism by intoxication and repellency, in order to inhibit feeding (Mello & Silva Filho, 2002). Outstanding among the physiologically active molecules with toxicity properties are the

Laboratório de Biologia e Fisiologia de Insetos, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, 1ª. Avenida s/n, Setor Universitário, CEP 74605-050, Goiânia, GO, Brazil.

Corresponding author: Camila Aline Romano. E-mail: camilaalineromano@gmail.com

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alkaloids and cyanogenic glycosides. Other classes, such as tannins, have inhibitory action on the insect digestion of the plant (Mello & Silva Filho, 2002; Gullan & Canston, 2007; Ayoama & Labinas 2012). Metabolites can cause intoxication in mosquito larvae via inhibition of acetylcholinesterase and digestive enzymes as observed in *Moringa oleifera* (Garcez et al., 2013). Morphological modifications in the digestive tract can be observed per stratification of the intestinal epithelium and vesicle formation (Abed et al., 2007), and constrictions in the digestive tract as occurred in *Aedes aegypti* larvae submitted to the extract of *Magonia pubescens* (Arruda et al., 2003), *Sapindus saponaria* (Barreto et al., 2006) and *Copaifera reticulata* (Abed et al., 2007); as well as extrusion of the peritrophic matrix in assays with *M. pubescens* (Arruda et al., 2003; Valotto et al., 2010). In adults, the repellency of the extracts modified the reproductive behavior of the females (Aguiar, 2011).

Due to their metabolites, plants have been used as insecticides since ancient times but few originated commercially viable formulations. Research on insecticidal activity has been carried out with extracts and essential oils, and in recent decades compounds and active molecules against insects have been isolated (Carvalho et al., 2003; Mendonça et al., 2005; Santos et al., 2006; Geris et al., 2008; Prophiro, 2008; Govindarrajan et al., 2012; Valotto et al., 2014).

Botanical insecticide research emerges as an alternative for the control of insects, considering their biodegradability, selectiveness, low toxicity to animals and reduced environmental impact. *A. humile* is a native subshrub of the Cerrado biome, one of the 25 global diversity hotspots (Myers et al., 2000). The Cerrado region in Central Brazil is similar to Savanna regions with acid soils, small and scleromorphic trees and large shrubs (Haridasan, 2000). Popularly *A. humile* roots are used as a laxative and for diabetes and rheumatism treatments. The bark is used as an expectorant and anti-diarrheal medication. The pseudofruit is used in cookery and the nutshell oil extraction in the treatment of dermatological conditions and warts (Almeida et al., 1998; Lorenzi & Matos, 2002). Studies performed with the oil obtained from *A. humile* leaves showed larvicidal activity on *Ae. aegypti* (Porto et al., 2008), suggesting the need for further research both for the isolation of these molecules and for evaluation of other parts of the plant that may present biological activity. The present study examined the insecticidal potential of *Anacardium humile* (Anacardiaceae) nutshell liquid on *Ae. aegypti*, the main vector of dengue, Chikungunya, Zika and urban yellow fever viruses.

MATERIAL AND METHODS

Botanical material

A. humile fruits and pseudofruits were collected in September 2014 on the Santo Antônio farm, in Iporá/GO, Brazil (16º25'44.77"S and 51º19'49.82'O). According to *Köopen's* classification this is considered a tropical semi-humid region (Alves & Biudes, 2008).

An exsicata was authenticated by professor José Ângelo Rizzo from the Federal University of Goiás, Botanical Department, and further deposited at the Conservation Unit Herbarium.

Obtention of CNSL and test solutions

A. humile fruits (nut shell) were processed at the plant bioactivity laboratory in the Instituto de Patologia Tropical e Saúde Pública(IPTSP/UFG). Five hundred fifty kilograms of the nut shell were kept in a forced ventilation oven at 40°C for seven days to obtain the CNSL (Guissoni et al., 2013). A stock solution at a 1,000 ppm concentration was prepared by weighing 0.3 g of the CNSL that was later solubilized in 0.1% dimethyl sulfoxide (DMSO). The stock solution was diluted in decreasing series up to 3 ppm for the bioassays.

Bioassays

The mortality and residual effect on larvae, pupae and adults as well as adult repellency and egg viability bioassays were performed. Twenty L_3 stage larva were used in containers with 25 mL of the CNSL for each assay. Mortality was verified after 24h of exposure, confirmed by the absence of response to mechanical stimuli, stiffening and darkening of the body. A 0.1% water/DMSO solution was selected as negative control and 1 ppm temephos (Abate® Basf Chemical Goup) solution was used as the positive control. The same procedure was performed with the pupae. The adult mortality assay was performed following the protocol proposed by the World Health Organization (WHO, 2006) with adjustments.

The *A. humile* CNSL residual effect was evaluated with a LC_{99} solution found for the L_3 stage. The assays were performed by adding 20 L_3 into 50 mL of the CNSL solution in 200 mL polystyrene containers. Mortality was verified after 24h exposure. The larvae were removed from the solution for the mortality verification and subsequently new L_3 were placed in the same solution. Daily larval counting and replenishment continued until complete loss of the lethal effect. Water and DMSO 0.1% were the negative controls. All assays were performed in triplicate.

For the oviposition, fecundity and fertility assays 40 couples of newly emerged *Ae. aegypti* were kept in mating cages, according to the pre-established breeding methodology (Silva et al., 1998; Lima et al., 2009). They were offered a solution of water with 0.02% sucrose and blood meal in an artificial feeder containing 4 mL of defibrinated sheep blood. The oviposition behavior was analyzed by Simple Choice (SC), where the substrate with test solution and the control solution were placed in different cages; or by Multiple Choice (MC) where test and control solution were in the same cage (Aguiar, 2011). The oviposition substrate was composed of a 200 mL polystyrene dark color container simulating an ovitrap. In each container 60 mL of the test solutions were inserted. A filter paper marked with lines following the stratification model described by Silva et al. (2003) was used as substrate for oviposition. The solutions were changed daily, being replaced by ovitraps with fresh solution and substrate. The substrate removed from the cage remained in the biological chamber for 48h, and then the eggs were counted with the assistance of a stereomicroscope and a manual counter.

The eggs were individualized in polystyrene containers with 200 mL of water for incubation, and underwent daily observations, the life cycle was noted until the emergence of adults (Silva et al., 1998).

Statistical analysis

Data obtained from mortality versus concentration (ppm) were analyzed by Statistica 12.0 software (Statsoft, 2013) using the Probit graph for the determination of lethal concentrations (LC_{50} and LC_{90}). Repellency was evaluated using the Active Oviposition Index (AOI) determined by the Kramer & Mulla (1979) formula (Aguiar, 2011): AOI = [NOt – NOc]/[NOt + NOc] where NOt refers to the total eggs in the substrate with test solution and NOc to the number of eggs in the control substrate. Data from the oviposition assay were also analyzed by the *Friedman* (Fr) ($\alpha = 0.05$) test (Aguiar, 2011; Tampe et al., 2016). This analysis was performed via BioEstat 5.0 software (Ayres et al., 2007).

RESULTS AND DISCUSSION

Assays with immature forms

The extraction process resulted in 130 g of CNSL yield with a typically dark coloration. In the third instar larvae assays 100% mortality was observed in serial dilutions of 100 to 13 ppm. The first larvae mortality records occurred after 10 minutes of exposure to the 100 ppm solution. For the 20 ppm solution dead larvae appeared after a two-hour exposure. The LC_{50}, LC_{90}, LC_{99} for larvae were respectively 6.63, 11.23, and 12.00 ppm (Figure 1).

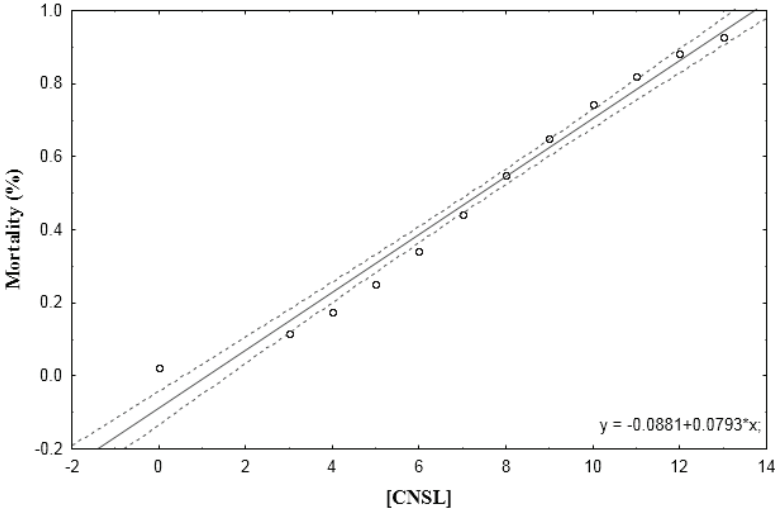

Figure 1. Mortality curve estimated by PROBIT for CNSL bioactivity against *Aedes aegypti* larvae.

The lethal concentrations obtained in the larvicidal assay were similar to those found by Guissoni et al. (2013) in the evaluation of the mortality of larvae exposed to *A. occidentale* CNSL. The LC_{50} and LC_{90} were respectively 6.65 and 10.98 ppm. The results obtained in this study are higher those found by Porto et al. (2008) with essential oil extracted from *A. humile* leaves (LC_{50} and LC_{90} of 20.9 and 39.8 ppm respectively) because the efficiency of CNSL is noticeable due to the lower concentration for the same mortality. This difference may be related to the presence and/or concentration of certain classes of secondary metabolites in plants, in addition to edaphic, climatic conditions, herbivore attacks and genetic factors (Schulz et al., 2002; Gobbo-Neto & Lopes, 2007). These variations maintain the lethality and concentration modifications required for the existence of this activity when oils or extracts from different parts of the same plant are used.

In residual effect sampling with CNSL using LC_{99}, total larval mortality was obtained only in the first 24h; after that the lethality of the solution gradually decreased to non-existence on the fifth day of experimentation. The residual effect (Figure 2) shows the values obtained in the assays as well as the data from the control sample where the absence of larval death can be observed.

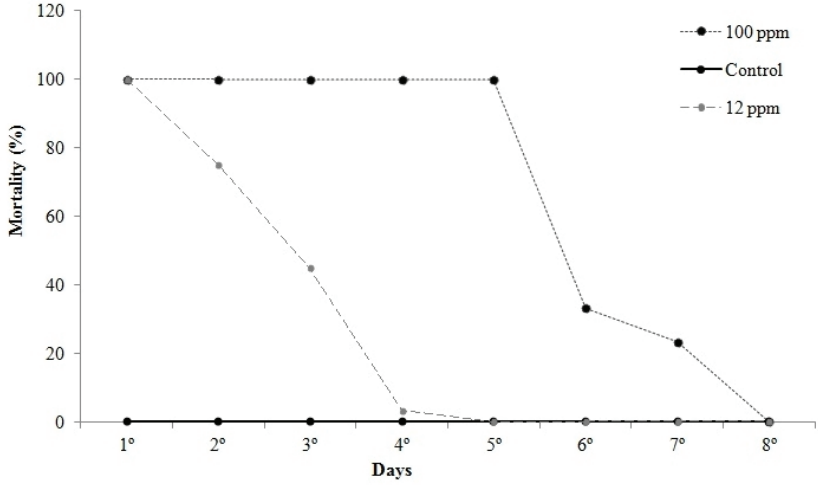

Figure 2. Residual effect of CSNL solution at 12 ppm on 3rd stage larvae of *Aedes aegypti.*

Regarding the duration of CNSL activity, Champakaew et al. (2007) observed the total mortality of the larvae up to the 9th day for a solution of *Curcuma zedoaria* (curcumin fake). Likewise Dill et al. (2012) obtained the same mortality during eight days of exposure to a 50 ppm ethanolic extract of *Annona coriacea* (araticum-do-campo) seeds. Guissoni (2011) obtained total mortality of larvae until the 6th day of observation exposed to a solution of 200 ppm *A. occidentale* CNSL. Guarido (2009) verified that the dichloromethane extract of *Annona foetida* (graviola-do-mato) was able to induce 100% mortality of larvae up to the 12th day and the action decreased gradually until extinction on the 25th day. Concerning the duration of the lethal effect with moderate mortality, Prophiro (2008) reported mortality up to the 32nd day of exposure. Dill et al. (2012) obtained results up to the 45th day and Guissoni (2011) observed mortality up to the 14th day. In this sense it is shown that the activity of *A. humile* CNSL presents reduced residual effect when compared to other vegetable oils. However, the efficiency period of approximately one week presented in the pilot test can be considered favorable since this period is relatively similar to the development period of the larvae until adult phase.

In this study, the pupae assay showed a LC_{50} of 276 ppm and a LC_{90} of 728.62 ppm. Mortality of approximately 48% of the pupae exposed to the solution at 100 ppm was observed with darkening of the cephalic portion. The winged specimens presented deformities, visibly reduced size, mal formation of the wings and darkened abdomen. In contrast all pupae exposed to the control with water and DMSO solution were able to complete the metamorphosis. Only one winged pupa died in the control. As the concentration decreased, an increase in the number of adult animals and decrease in pupal mortality were observed. At the 25 ppm concentration the first viable adults were observed, and in the CNSL solution at 5 ppm, 99% of the adults were viable, characterizing loss of activity. No mortality of pupae was observed in the positive control.

The pupicidal activity observed in the CNSL tests required high concentrations. Carvalho et al. (2011) reported 100% mortality of pupae at 158.70 ppm when exposed to the crude ethanolic extract of *Persea americana* (avocado), a concentration lower than the LC_{50} obtained in the CNSL trials. However, reasonably low concentrations (100 ppm) were efficient in stopping the cycle, as the surviving pupae did not develop viable adults. As for morphology, the dead pupae exposed to the treatments showed similarity to the studies of Saranya et al. (2013) in assays with aqueous extract of *Spathodea campanulata*. Prophiro (2008) also obtained similar results in assays performed with sub-lethal doses of *Copaifera* sp. oil in which the pupae surviving the doses preceded the incomplete emergence of the adult.

Assays with adults

Female behavior evaluation revealed repellent effect of the CNSL of *A. humile* both in the test solution as in LC_{99} (Table). This behavior is illustrated in the table, where females opted for oviposition at the extremities of the substrate, differing from the pattern observed in both the control ovitraps and the available literature (Figure 1). Previous studies on the oviposition of *Ae. aegypti* showed female preference for oviposition approximately 1.5 cm above water (Silva et al., 2003). In addition, there was a significant difference in oviposition averages in all tests when compared to the control. Aguiar (2011) observed repellence in the behavior of females during oviposition in oils from *Foeniculum vulgare* (fennel) and *Cymbopogon witerianus* (citronella) observed by the significantly lower number of eggs than in the water control. According to the author, females tend to ovulate more in places where conditions are favorable to the development of spawn since repellent substances present in essential oils may indicate a disadvantage for the survival of the immature.

Table. Oviposition of *Aedes aegypti* on substrate moistened with CNSL at 12 and 100 ppm in the Single Choice (SC) and Multiple Choice (MC).

		Total eggs per stratum						p			General	
		1.5 cm	3.0 cm	4.5 cm	Total	AOI	Fr	1.5 cm	3.0 cm	4.5 cm	Fr*	p
SC	CNSL12 ppm	1370	425	188	870	-0.280	-	-	-	-	1.6	0.205
	NC	3393	129	53	3574	-	-	-	-	-	-	-
MC	CNSL 12 ppm	486	102	282	2010	-0.173	40.468	ns	<0.05	<0.05	4.455	<0.05
	NC	2534	1242	215	2851		-	-	-	-	-	

Legend: *Values obtained by comparison with results obtained in the respective negative controls. AOI – Active Oviposition Index; Fr – Friedman test; p – p value; SC – Single choice; MC – Multiple choice; NC – Negative control; ppm - parts per million.

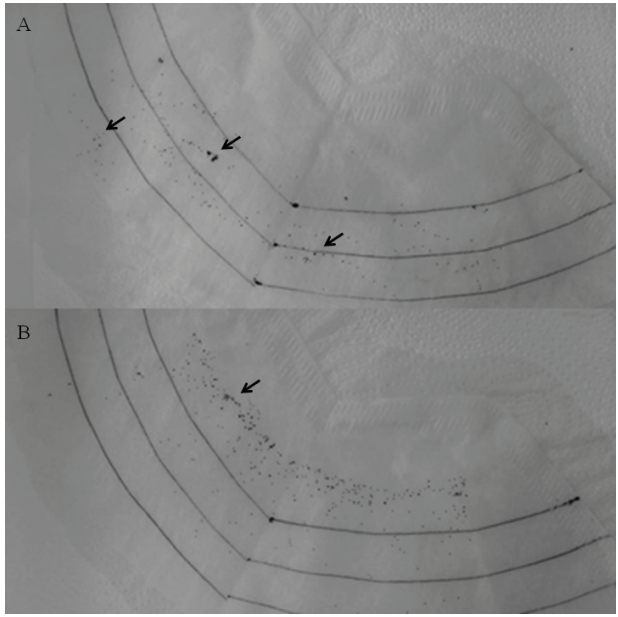

Figure 3. Oviposition stratification of *Aedes aegypti*. Image A) dampened substrate with CNSL; Image B) control substrate.

Secondary metabolites in sub-lethal doses promote changes in the biological cycle of insects sometimes slowing the development of immatures, sometimes causing incomplete ecdysis in adulthood. In addition, some studies also show changes in food and reproductive competence without a predictable pattern (Shaalan et al., 2005). Karmegam et al. (1997) propose that the morphological changes and variation in the ecdysis process may originate from hormonal changes triggered by the extracts. Some chemical larvicides such as pyriproxifen, for example, act on the chitin synthesis of larvae acting similarly on the regulating hormones of insect development (Valle et al., 2015).

Regarding the viability of the eggs, 2,739 larvae were quantified from eggs from the oviposition test. A significantly higher hatching rate was obtained in the eggs of the control substrate ($p = 0.01$). The average hatching was 79.4 % in the control samples and 25.0 % in the CNSL samples at 12 ppm. Thus, although oviposition was not significantly influenced by the presence of CNSL, the egg viability exposed to this solution was reduced to much lower rates.

In the assays on adult mortality the activity of the CNSL on insects was not recorded. A pilot study providing CNSL as food was also carried out simultaneously with this research (unpublished data); however, no lethal effect or changes in the reproductive behavior of females were noted.

Results indicate that the CNSL of the species *A. humile* presents potential for the development of products to be used in the control of *Ae. aegypti* since the LC_{50} values were lower than 49 ppm, as defined by the literature (Silva et al., 2004). Thus, further studies should be carried out to investigate the mechanisms that generate mortality in immatures as well as chemical analysis for determination, isolation and identification of the molecules involved in this process. Assays to verify the toxicity of CNSL to other groups of organisms are also recommended.

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