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Coleoptericidal activity of an enzymatic extract of Neem (*Azadirachta indica*) on *Aethina tumida*

Actividad coleoptericida de un extracto enzimático de Neem (*Azadirachta indica*) sobre *Aethina tumida*

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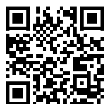

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ABSTRACT

This work aimed to obtain enzymatic extracts from green, dried, and frozen neem seeds and to evaluate the coleoptericidal effect on *Aethina tumida* in the larval stage. In the first phase, the effect of freezing (–20 °C) and dehydration (50 °C) of the green seed on the release of azadirachtin A was evaluated. Subsequently, the enzymatic extraction was carried out using the Crystalzyme® PLMX preparation, followed by an 80 % alcohol extraction (v v-1). The alcoholic extract was roto evaporated and its coleoptericidal activity on *Aethina tumida* larvae and adults was evaluated using the Burgerjon Tower with concentrations of 0, 0.05, 0.5, 50, and 500 ppm of azadirachtin A. The results indicated that there is no statistically significant difference in the concentration of azadirachtin A when dehydrating or freezing the neem seeds, likewise, the extracts with 500 ppm of azadirachtin A promoted 100 % mortality of larvae at 48 h, the LC50 and LC90 calculated were 0.58 and 77.67 ppm, respectively. According to these results, it is concluded that the enzymatic extracts of neem seeds are a viable alternative for the biological control of *Aethina tumida* in the larval stage.

KEY WORDS: Azadirachtin A, Bioinsecticide, Median lethal concentration, Larval stage, Terpenoids.

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RESUMEN

El objetivo de este trabajo fue obtener extractos enzimáticos de semillas de neem verdes, secas y congeladas y evaluar el efecto coleoptericida sobre *Aethina tumida* en etapa larvaria. En la primera fase, se evaluó el efecto de la congelación (–20 °C) y deshidratación (50 °C) de la semilla verde sobre la liberación de azadiractina A. Posteriormente, se realizó la extracción enzimática empleando el preparado Crystalzyme® PLMX, seguida de una extracción alcohólica al 80 % (v v-1). El extracto alcohólico se rotoevaporó y se evaluó su actividad coleoptericida sobre larvas y adultos de *Aethina tumida* utilizando la Torre de Burgerjon con concentraciones de 0, 0.05, 0.5, 50 y 500 ppm de azadiractina A. Los resultados indicaron que no existe diferencia estadística significativa en la concentración de azadiractina A al deshidratar o congelar las semillas de neem, asimismo, los extractos con 500 pmm de azadiractina A promovieron 100 % de mortalidad de larvas a las 48 h, las LC50 y LC90 calculadas fueron de 0.58 y 77.67 ppm respectivamente. De acuerdo con estos resultados, se concluye que los extractos enzimáticos de semillas de neem es una alternativa viable para el control biológico de *Aethina tumida* en etapa larvaria.

PALABRAS CLAVE: Azadiractina A, Bioinsecticida, Concentración letal media, Etapa larvaria, Terpenoides

Introduction

Bees are susceptible to various diseases, parasites, and pests that affect the development and productivity of the colony. Currently, one of the most critical pests in Mexico is *Aethina tumida,* known as the "small hive beetle", a parasite native to tropical and subtropical regions south of sub-Saharan Africa that can cause colony collapse disorder (hive abandonment), honey fermentation, and the formation of foam with the characteristic odor of oranges when it rots (Ellis *et al.,* 2003). The most commonly used control method is the application of synthetic pesticides. Still, it has been shown that these products induce genotypic and phenotypic changes in pests, promote their adaptability, require higher doses to control them, and generate resistance (Borges *et al.,* 2005), so it is necessary to explore the use of biopesticides that are effective and do not contaminate the environment. Neem is one of the most studied biopesticides due to its toxic effects on pests and represents a potential alternative as a substitute for synthetic pesticides due to its components with insecticidal and antifeedant activity (Denardi *et al.,* 2011; Giglioti *et al.,* 2011), among which azadirachtin A (AzaA) stands out (Soni *et al.,* 2012). However, a common problem in obtaining these metabolites is the variability in their extraction depending on the method used and their stability under storage conditions (Aguilar-Acosta *et al.,* 2020). One of the alternative methods used in the industry to improve the extraction of metabolites of interest from plant sources is cellulolytic

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enzymes such as Crystalzyme® PLMX, which was used by Pardío *et al*. (2018) to increase the extraction of vanillin in green vanilla bean extracts and by Aguilar-Acosta *et al*. (2020) to obtain neem extracts with a higher concentration of AzaA. These enzymatic complexes hydrolyze the cellulolytic structures of the plants and allow the extraction of the metabolites of interest in higher proportions due to the combination of enzymatic activities they present (cellulases, pectinases, hemicellulases, and arabinases). This work aimed to obtain enzymatic extracts from green, dry, and frozen neem seeds, quantify the concentration of extracted AzaA, and evaluate the coleoptericidal effect on *Aethina tumida* at the larval stage.

Material and Methods

Seed collection and treatment

Seeds of neem (Azadiracta indica) were collected from fruits 110 days after flowering, in trees at least 15 years old, established in the ranch El Naranjal, municipality of Jamapa, Veracruz, located at the coordinates 18º 59' 40.2" N; 96º 15' 11.5" W, altitude of 57 masl. The seeds were collected according to their yellow-green color (characteristic of mature fruits), mixed, and randomly divided into three groups, each of which was divided into 4 subsamples. One group of seeds was frozen at -20°C, another group was subjected to slow dehydration in a forced-air oven at 50°C until constant weight, and the third group was directly subjected to enzymatic extraction.

Azadirachtin A extraction

The enzyme preparation Crystalzyme® PLMX (CPL-MX) (Valley Research Inc. Fresno, California USA) [pectinases (EC 3.2.1.15), cellulases (EC 3.2.1.4), hemicellulase (not reported), arabinase (EC 3.2.1.99)] with an enzyme activity of 5.94 FPU/mL determined by the filter paper unit technique proposed by Eveleigh *et al*. (2009). 0.59, 1.78, 2.97, and 5.94 FPU of enzyme g^{-1} of seed (dry basis) were evaluated to determine the optimal enzyme concentration as a function of hydrolysis of cellulosic structures of neem seeds Aguilar-Acosta *et al*. (2020). For AzaA extraction, 35 g of seeds from each subsample were homogenized with phosphate buffer (pH 5.0) and subjected to hydrolysis with CPL-MX, ratio 1:10 (dry base seed: phosphate buffer) for 18 h at 40 °C. At the end of the enzymatic hydrolysis, alcoholic maceration was performed for 48 h (80% v v^{-1}). The alcoholic extract was rotary evaporated and stored for AzaA quantification. This procedure was carried out in triplicate.

Quantification of AzaA

AzaA concentration was performed with HPLC (Waters 525 Milford, Massachusetts, USA) following the technique designated by Kaushik (2002), using a photodiode array detector (Waters 2996), 4 mm NovaPak C18 column (3.9 x 150 mm) and AzaA standard (Sigma Aldrich®, Darmstadt, Germany). The extracted samples were centrifuged and diluted 1:1 in HPLC grade acetonitrile and filtered through a 0.22 µm Acrodisc (Millipore®), 20 mL were injected with a flow rate of 1 mL min-1,

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using acetonitrile: water (40:60) as mobile phase. The retention time of AzaA was 3.1 min and the concentration was read at 217 nm.

Coleoptericidal Activity of Neem Enzyme Extract

Obtaining larvae

Aethina tumida beetles were collected from beehives in the southern zone of the Veracruz state, placed in plastic containers with airtight lids to which a mesh-covered vent was adapted, and transferred to the biochemical laboratory of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Veracruzana. A portion of the hive with honey and pollen was introduced into the containers as food to avoid death by starvation and they were kept in coolers with cold gel to maintain the temperature at 25 °C. In the laboratory, the beetles were kept at 25 °C until they oviposited. Hatched larvae were fed with a pollen and honey mixture for 1 week. Five larvae were placed in 14 mm diameter Petri dishes (10 dishes per treatment) with a 2 to 3 cm hole in the top lid (sealed with mesh cloth) to allow aeration. A mixture of honey and pollen was added as food and a cotton swab moistened with distilled water to maintain internal humidity and was stored at 28-30 °C for 24 h. Once the time had elapsed, the percentage mortality of larvae and beetles was identified to determine the effect of storage.

Application of the Extract

From the extracts evaluated with different enzyme activity units, the one with the highest AzaA concentration was selected and from this extract, dilutions were made with distilled water until concentrations of 0.05, 0.5, 5.0, 50, and 500 ppm of AzaA were obtained. For the control treatment (0 ppm), distilled water was used (González-Gómez *et al*., 2006). The extracts were applied using Burgerjon's Spray Tower, which simulates field-applied pesticides. The amount of product used for spraying per unit area was 1-2 mg/cm2, applying 15 mL of the solutions at a pressure of 0.703 kg/cm2, leaving it for 1 minute to promote sedimentation of the droplets on the larvae (Rodríguez *et al*., 2005).

Determination of Coleoptericidal Activity

After the application of the extracts, the larvae were transferred to Petri dishes (8 cm Ø) with adapted lids with a hole in the center (4.5 cm Ø) covered with a fine mesh and were placed on maintenance substrate (pollen and honey) and incubated at 32 ºC and 70 % relative humidity. Mortality was evaluated at 24 and 48 h (Rodríguez *et al*., 2005, González-Gómez *et al*., 2006), and the lethal concentrations 50 (LC50) and 90 (LC90) were determined with the data obtained.

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Experimental Design and Statistical Analysis

Data were analyzed using a completely randomized design. For enzymatic extraction, 3 treatments (fresh, dry, and frozen seeds), 6 replicates (subsamples), and 3 replicates per replicate (n = 54) were used. For the coleoptericidal activity test, 5 concentrations (0, 0.05, 0.5, 0.5, 50, and 500 ppm AzaA) were used with 10 replicates (Petri dish) per treatment (n = 50). An arcsine transformation of the percentage of dead larvae mortality was applied. Mortality was compared between groups by ANDEVA and a comparison of means by Tukey's test ($p < 0.05$) using SAS statistical software (2022). The PC probit statistical method was used to determine LC50 and LC90 (González-Gómez *et al*., 2006).

Results and Discussion

Firstly, different units of CPL-MX activity were evaluated and it was shown that using 1 mL (5.94 FPU/mL) per gram of neem seed (dry basis), promoted the highest concentration and rate of release of reducing sugars derived from the hydrolysis of the cellulosic structures of the neem seed (Table 1). From the next phase, this enzyme concentration was used to hydrolyze the neem seed before alcoholic maceration.

Table 1. Evaluation of the enzyme concentration on neem seed hydrolysis.

Enzyme	Volume (mL)	Activity (FPU mL)$^{-1}$	*k* (h)$^{-1}$	RS (mg mL)$^{-1}$
	0.1	0.59	0.47 ± 0.03^{a}	4.15 ± 0.20^{a}
CPL-MX	0.3	1.78	0.64 ± 0.05^{b}	4.85 ± 0.11^{b}
	0.5	2.97	1.15 ± 0.07^{c}	4.47 ± 0.09^{c}
	1.0	5.94	1.43 ± 0.68^{d}	4.49 ± 0.32^{abc}

FPU: Filter Paper Units; k: speed constant of reduced sugars liberation; RS: reduced sugars in equilibrium. Values with different letters between rows are significantly different ($p > 0.005$).

The concentrations of AzaA obtained after the alcoholic maceration are shown in Figure 1. AzaA concentration was statistically higher ($p < 0.05$) in the extracts obtained from the dried seeds treated with the enzyme preparation CPL-MX. This extract was selected to evaluate the coleoptericidal activity on *Aethina tumida* larvae.

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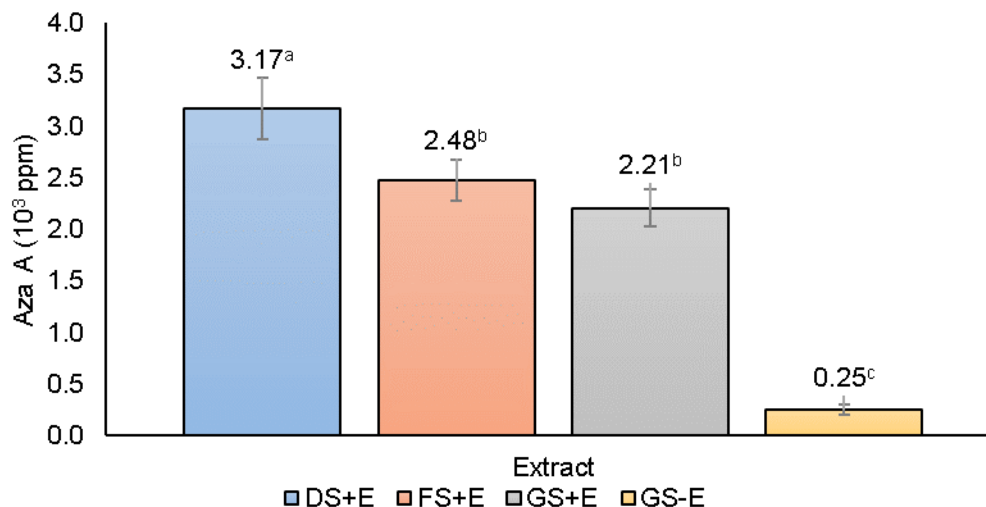

Figure 1.- Azadirachtin A concentration in Neem seed extracts.

DS+E: Dry seed with enzyme preparation; FS+E: Frozen seed with enzyme preparation; GS+E: Green seed with enzyme preparation; GS-E: Green seed without enzyme preparation. [a b]Different literals in the column indicate significant statistical differences ($p<0.05$).

At 48 hours after the application of 0.5 ppm of Aza A, extracted from dry seed with CPL-MX, 50 % mortality of larvae was observed, and the estimated LC were LC_{50}: 0.58 ppm and LC_{90}: 77.67 (Table 2).

Table 2. Estimated lethal concentration of AzaA for *Aethina tumida* larvae.

AzaA (ppm)	Log ppm	Dead larvae proportion	Proportion corrected	Probit
0		0.2	0.0	
0.05	-1.30103	0.4	0.25	4.326
0.5	-0.30103	0.6	0.50	5.00
50	1.69897	0.9	0.88	6.150
500	2.69897	1.0	1.0	
Slope	Intercept	Test value	LC_{50}	LC_{90}
0.6035	5.1391	5	0.5881	
0.6035	5.1391	6.28		77.67

The lethal concentrations found in this assay are within the ranges reported with insecticidal activity, Izadi *et al*. (2012) reported doses of 0.22 ppm for the LC50 in the control of Agonoscena pistaciae, Ghazawi *et al*. (2007) found that the LD50 in Hetaracris littoralis was reached with the application of 101.2 ppm, both cases in the larval stage. The coleptoricidal activity of AzaA on larvae could be due to several effects, it is an antagonist of 20-hydroxyecdysone hormone and juvenile hormone (JH), modifies or suppresses ecdysteroid hemolymph and JH titers; inhibits the secretion of morphogenetic peptide hormone and allatotropins of the corpus cardiacum complex causing reduced pupation, malformation or failure of adult emergence (Mordue and Blackwell, 1993; Bezzar-Bendjazia *et al*., 2017). In its structure it contains an acetate, a tiglate ester, two methyl esters, secondary and tertiary alcohol, an epoxy, and a vinyl ether, which is part of an acetal and a hemiacetal (Ley *et al*., 1993), this complexity of functional groups act on insect growth regulation and cell biosynthetic mechanisms, altering various metabolic pathways necessary for growth and development, in adult insects affecting cuticle development and ecdysis (Mordue *et al*., 2010). Another advantage of using AzaA extracts obtained from Neem is that insects hardly generate resistance (Mordue *et al*., 2010), which makes it an effective biological control method. Feng and Isman (1995) report that in the peach potato aphid (Myzus persicae) it was until generation 40 after initiating the application of purified AzaA that they found resistance, however, when it was applied in the form of extract obtained from neem seed, resistance did not occur.

Conclusions

The use of AzaA obtained by enzymatic extraction with Crystalzyme® PLMX from dehydrated neem seeds is a viable and efficient alternative for the biological control of *Aethina tumida* in the larval stage.

Authors contribution

Conceptualization AFP; methodology development, SRDR, RDR, ELG, KRE; experimental validation, SRRD, RDR, ELG, KRE; analysis of results, AFP, SLA; data management, AFP, SLA; manuscript writing and preparation, AFP, SLA; drafting, revising and editing, SRDR;

“All authors of this manuscript have read and accepted the published version of this manuscript.”

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Conflict of interest

"The authors declare that they have no conflict of interest."

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