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Effects of the hexanic extract of neem *Azadirachta indica* against adult whitefly *Bemisia tabaci*

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Abstract
The aim of this study was to evaluate the insecticidal and repellent efficiency of the hexanic extracts of seeds of neem *Azadirachta indica* on whitefly *Bemisia tabaci* adults. Bioassays were performed by testing seeds collected in 2007 (NEEM-1) and in 2008 (NEEM-2). Mortality was assessed with five levels (5-10%) and repellence at two levels (5 and 10%), for 8 hours. Water and Dichloromethane (0.1%) served as controls. The lethal median concentration of neem, analyzed by Probit, was 6.55%. The concentration of 10% (both, NEEM-1 and NEEM-2) produced 100% of mortality, NEEM-1 since the second hour of evaluation, whereas NEEM-2, since fifth hour of assessment. NEEM-1 and NEEM-2 (10%) produced 100% of repellent activity since seventh and sixth hour, respectively. Nevertheless, significant differences were not observed between the times of storage-collection for both extracts. It is recommended to achieve field studies that expand these results *in vitro*.

Keywords: Agricultural pest, Biological control, Mortality, Repellency.

Introduction
Habanero pepper (*Capsicum chinense* Jacq.) is widely demanded for domestic and international market, as fresh, dry or processed product [1]. Nevertheless, one of the principal pests that affect this species is the whitefly (*Bemisia tabaci* Genn.), that represents an important vector of several Begomovirus species [2, 3]. Moreover, there are records of economic loss generated by *B. tabaci* as result of diseases that insects cause in crops [4]. The main method to control whitefly has been the use of synthetic insecticides; nevertheless, this indiscriminate practice affects the biodiversity of the agro-ecosystems, generates injuries in the human health, affecting beneficial organisms (predators, parasitoids and pollinators) and pests generate genetic resistance [5, 6].

Thus, it is important to search the innovative methods for control of this pest, with lowest risks and compatibility with the environment [7]. Nowadays, one of the most effective strategies is the use of secondary metabolites of plants (alkaloids, terpenoids and phenols) with insecticidal effects against pest populations. This alternative has a low environmental impact and it is coincident with the methodological scheme of integrated pest management [8]. This tendency establishes the substitution of the synthetic pesticides in the agriculture for others, from natural origin, like neem (*Azadirachta indica* A. Juss) [9, 10].The variety of limonoid triterpenoids of this species presents a high effectiveness for control against several species of phytophagous insects including the behavior and the physiology of whitefly [11, 12, 13].

Though, in most of studies where extracts of neem were used for evaluating effects of bioactivity [13, 14, 15], the records were made in periods of 24 hrs, which lack the information about the results between these intervals.

In the present study, the records of bioactivity were verified every 60 min, from the beginning of bioassays until completing eight hours of evaluation, with a purpose, to assess the effects on adults of whitefly in shorter periods of time.

Similarly, few studies have evaluated the effects of seed storage times on bioactivity of plant extracts [16].

In consideration to the previous findings, the aims of this research were: 1) to evaluate the insecticidal and repellent efficiency of the hexanic extract of neem seeds on adult whitefly; and 2) to evaluate any change of the plant extract on the stored seeds.

Materials and Methods

Obtaining of *Bemisia tabaci*

Habanero pepper *Capsicum chinense,* Criolla Naranja variety, was cultivated in black bags of 5 kg of capacity, in the greenhouse of the Campus de Ciencias Biológicas y Agropecuarias (CCBA), of the Universidad Autónoma de Yucatán (UADY), at Merida, Mexico. Every bag contained substratum of soil mixed with lamb manure (3:1).

Two weeks after the transplant, and without chemical fertilizer added, the plants were infected by adults of whitefly *Bemisia tabaci* collected in crops of Habanero pepper, located in the town of X'matkuil, Yucatan. Forty-five days later, adults of whitefly were collected to perform the bioassays, which were initiated the same day of the collection.

Vegetable material

Neem fruits were collected manually from trees located in the plantation of the CCBA of the UADY. To evaluate the variability between the time of collection-storage of neem, the fruits collected in the period from July to August 2007, were called NEEM-1 and the collected ones in the period from July to August 2008, were called NEEM-2. The fruits were transported to the water-soil-plant laboratory of the CCBA, where the peel was eliminated the same day of the crop and then, rested for one day at room temperature (RT) to smooth the flesh.

After 24 hrs, the seeds were extracted, washed with drinking water and then dried one day at RT. The dry seeds were stored in paper bags for conservation at RT before were labelled with the information of sampling (date and site of collection).

Obtaining of the extract of neem

To obtain the extracts of NEEM-1 and NEEM-2, the method described by Ramos *et al*. (2004) [17] was followed. Briefly, every sample of 5 g of dry seeds softened in a porcelain mortar; later 1 g of the grinding was degreased in 20 mL of ether of oil during 20 hrs, with constant agitation. Immediately the residue was placed in a separation funnel of 125 mL and was separated to the rest to isolate the aqueous phase of the ethereal phase.

The aqueous residue was kept in agitation for 20 hrs in 20 mL of methanol RG for the extraction of azadirachtin. The liquid fraction was recovered by means of decantation, and the solvent was eliminated with a rotary evaporator Büchi model R-114 at 45 °C. The extract was re-suspended in 15 mL of methanol-water (2:1), later the methanolic-aqueous extract was separated with separation funnel by 20 mL of Dichloromethane, three times. The aqueous fraction was eliminated and hence the organic phase was recovered by evaporation at 40 °C in the rotar vapor.

For identification and quantification, the residue obtained of the organic phase was re-suspended in acetonitrile (3 mL), and then filtered in acro-discs with membrane of polytetrafluoroethylene (PTFE) with pores of diameter 0.2 µm.

Quantification of azadirachtin for HPLC

The quantification of azadiracthinin neem seeds was done by High-Performance Liquid Chromatography (HPLC) with an equipment Perkin-Elmer (series 200, USA). Briefly, 20 µL of samples of NEEM-1 and NEEM-2 were injected, by duplicate, with a column C18 of 25 cm by 46 mm. The eluent consisted of acetonitrile-water (70:30), with a flow of 0.5 mL/min, with readings at 210 nm of wave length at RT.

Chromatograms of samples were compared with a curve of calibration elaborated with a standard solution of azadirachtin (SIGMA; purity 95%). Standard concentrations were 10, 20, 30, 40, 60 and 100 ppm, obtained by dilution of 0.5 mg of the standard in 1 mL of acetonitrile (concentration of 500 ppm) [17].

Obtaining of neem extract

To obtain the hexanic extract of NEEM-1 and NEEM-2, methodology established by Romero y Vargas (2005) [18], with some modifications, such as samples quantity and reflux times, was completed. The extraction was performed by the method Sohxlet, briefly, 10 g of every sample of seeds were weighed by duplicate. Before, outer coat was grinded in a porcelain mortar, trying that the size of the particles was homogeneous.

Later, every sample was transferred to a cassette of cellulose extraction and was placed in Sohxlet. Extractions were realized by reflux by 4 hrs using n-hexane as solvent. Later, the solvent was evaporated and the obtained extract was stored in amber bottle and kept in refrigeration (4 °C) until use in bioassays.

Experimental design

For bioassays of mortality and repellency, a complete randomized experimental design with four repetitions for every treatment was undertaken. Controls were distilled water (C) and dichloromethane (DCLO, 0.1%). The time of evaluation was 8 hrs, for both bioassays, making readings every hour until end of trial.

Bioassays of mortality and repellency

The concentrations of the hexanic extraction of NEEM-1 and NEEM-2 were prepared according to its relation weight /volume (w/v); for example, for the concentration of 10%, 2 g of the extract were placed in a volumetric flask of 20 mL, with Dichloromethane as solvent. For the bioassay of mortality, five concentrations of the total extract (5%, 6%, 7%, 8%, and 10% w/v) were evaluated. In contrast, for the bioassay of repellency, two concentrations (5% and 10% w/v) were tested.

For both bioassays, groups of 10 adults of whitefly were conformed. Insects were transferred to a plastic flask of 150 mL, at a temperature of 25±2 °C. Bottle caps were perforated, and a piece of fabric Tricot was placed as base of the flask. Above this, a filter paper Whatman No. 1 was placed and on the filter 0.3 mL of each concentration evaluated of neem were applied. The paper filter was left 30 min at RT before transferring it to the flasks. A leaf of Habanero pepper was added to each flask, as food for the insects. Finally, 10 adult insects of whitefly were introduced into the flasks.

For bioassays of repellency, two flasks of 150 mL were joined by means of a tube of transparent plastic of 4 cm of length and 6 mm of diameter. In one flask the insects were placed and a leaf with the concentration of the extract of neem to evaluate was added; in another flask a leaf without extract was added, this procedure was done with the aim to observe the movement of the insects to place whereas in one or another flask.

The index of repellency was determined according with the following formula [7]:

$$Index\ of\ repellency = \frac{2G}{G+P}$$

Where:

G = percentage of insects in each treatment

P = percentage of insects in the control

The index of repellency, classifies to the substance evaluated like neutral if the result is equal to 1, attractively if it is bigger than 1 and repellent if it is minor to 1.

Statistical analysis

Results of bioassays were analyzed by Kruskal Wallis test because data did not observe the hypotheses of normality and homogeneity of variances. Probit analysis was performed to determine the CL50 in *Bemisia tabaci* due to the extracts of *Azadirachta indica*. Analyses were performed using Statgraphics 5.0. Differences among means were evaluated by Tukey analysis (P≤0.05).

Results and Discussion

There were not significant differences in the concentrations of NEEM-1 versus NEEM-2 (P≤0.05). By this, it was decided to combine the results of both extracts to obtain the lethal median concentration (LC_{50}) by Probit analysis which was 6.55% (I.C. 95% = 2.59-9.50) (P=0.01, $x^2 = 35.39$).

The values of the total extract of both, NEEM-1 and NEEM-2, in grams, are shown in Table 1, with a performance of extraction of 29.1% and 24.7%, respectively. These values are similar to those obtained by Esparza-Díaz *et al*. (2010) [19], who found performances between 22 and 26% for the hexanic extraction of seeds of *A. indica.*

The hexanic extraction, as indicated by Esparza-Díaz *et al*. (2010) [19], offers an optimal percentage weight/weight (% w/w), but that is not correlated with a proportionally high percentage of azadirachtin. Those authors obtained similar proportions of this metabolite in hexanic, methanol and aqueous extractions.

In the present investigation, the analysis for HPLC revealed for NEEM-1 and NEEM-2, total concentrations of azadirachtin ranged from 0.35 to 0.40 mg g^{-1} (Table 1), similar values obtained by other authors [17, 19, 20]. It is necessary to emphasize that Ramos *et al*. (2004) [17], found that the concentration of azadirachtin in the fruits of neem modifies with the age, since for his study increased from 0.4 mg g^{-1} at 31 days to 2.8 mg g^{-1}at 127 days after the flowering (physiological maturity), then it decreased to the half, 20 days later (147 days after the flowering).

Other studies have demonstrated that does not exist relation between the quantity of the extract and the time of storage, for instance, Abou-Fakhr Hammad *et al*. (2001) [21] found that the extracts of leaves of *M. azaderach* collected and stored throughout one year are similar, and there was not affecting in the concentration of the secondary metabolites in this time.

For the present study, it was not consider associating the contents of azadirachtin with the age of maturity of the fruits, for this it is not possible to affirm if the difference in the contents of azadirachtin between the investigations of Ermel *et al*. (1987) [20], Ramos *et al*. (2004) [17] and our study could be influenced by this relation. It will be important to consider this association in further studies, to do more conclusive comparisons.

Table 1: Extract obtained and concentration of azadirachtin in seeds of neem

Sample	Extract obtained (Mean ± S. D.)	Azadirachtin concentration
		(mg/g)
NEEM-1	2.91 g	0.35
NEEM-2	2.47 g	0.40

Bioassays of mortality

Results in Figure 1 show mortality of *B. tabaci* with the different concentrations of azadirachtin for NEEM-1 (1a) and NEEM-2 (1b). For both cases, the observed trend was a higher mortality corresponding to a higher concentration. Also was observed that the mortality increased with the course of the time of evaluation in every bioassay. For NEEM-1, the highest concentration (10%) caused the mortality of 90% of the insects from the first hour of the bioassay. For the same concentration, in the second hour, the mortality was increased until 100%.

The mortality induced by the extract of NEEM-2 was less vigorous. Thus, for concentration of 10%, the mortality of insects at first hour was 30%. The 100% of mortality was reached until the fifth hour of evaluation.

These results present percentages of response similar to those obtained for Dimetry *et al*. (1996) [22], who observed levels of mortality between 83 and 95% in adults of whitefly exposed to three commercial formulations of neem (concentrations of 0.125%, 0.25%, 0.5% and 1%). It is necessary to emphasize that in contrast to the present study; those authors observed the maximum values of mortality in the first hour and then, lethality was decreasing every 24 hrs up to completing 96 hrs.

In other studies [14, 15], effects of 100% in the mortality, using commercial formulations of neem have been registered. These values, obtained to higher times of reading (1, 2, 3 or 4 days). These times could be cause a masking of the information, not observable due to the long period of evaluation. For this reason, in the present experiment the percentages of mortality were recorded every hour during a total of eight hour period, to visualize the short-term effect of the extract. The relevancy of this decision was demonstrated because relevant trends in this short term of time were observed.

In agreement to these results, the utilization of the hexanic extracts of neem with a concentration of 10% in times from two to 5 hours is convenient for the control of whitefly, in plantations of Habanero pepper, according to the criteria of Lagunes (1994) [23].

However, it is recommendable to make more studies that validate these effects. For instance, it would be to project them to field level and with different forms of application (aspersion, in soil, etc.). At the same time, potentially collateral effects on the plants must be evaluated.

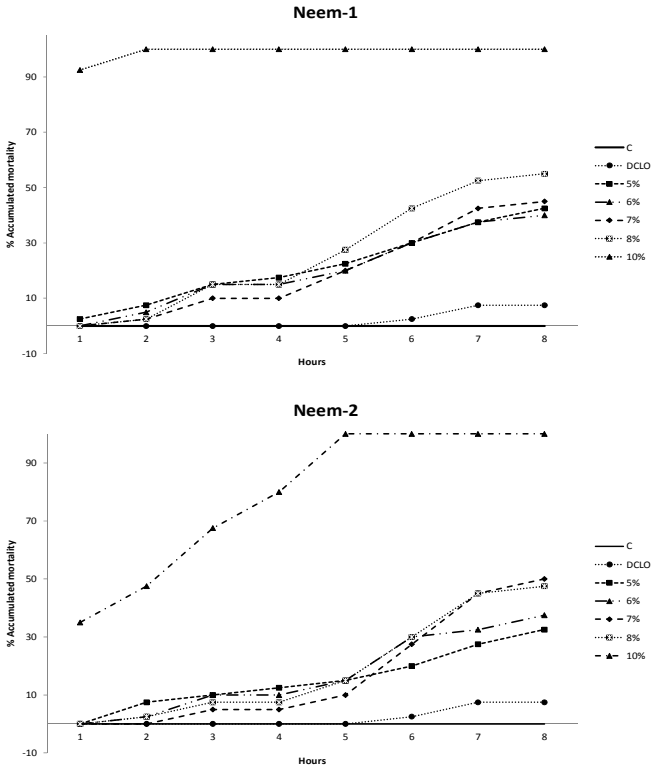

Fig 1: Mortality of whitefly with different concentrations of NEEM-1 (a), and NEEM-2 (b), in an interval of 8 hrs.

Bioassays of repellence

In bioassays of repellence, the concentration of the extracts (NEEM-1 and NEEM-2) showed significant differences with respect to the controls (P≤0.01). In contrast, there were not observed significant differences between concentrations of NEEM-1 and NEEM-2 themselves (P≤0.01).

Thus, it was evident that at the highest concentration of the extract of neem (10%), corresponded a maximum number of repelled organisms, as well as highest percentages and indexes of repellency. It was detectable that for NEEM-1 and NEEM-2, in the lowest concentration evaluated (5%) there was observed a repellent effect in 75% of the individuals. Furthermore, with the concentration of 10% of the extracts (NEEM-1 and NEEM-2) a level of repellence of ~ 100% there was registered (Table 2).

Table 2: Index of repellence in whitefly exposed to the hexanic extract of neem.

Treatment	Repelled individuals	Percentage of Repellency	Index of repellency*
C	2±0.4[a]	5[a]	1[a]
DCLO	3±0.3[a]	7.5[a]	0.99[a]
5% (NEEM-1)	30±1.2[b]	75[b]	0.42[b]
10% (NEEM-1)	39±1.6[c]	97.5[c]	0.05[c]
5% (NEEM-2)	30±1.1[b]	75[b]	0.42[b]
10% (NEEM-2)	40±1.7[c]	100[c]	0[c]

*Repellency according to criteria of Salvadores *et al.*, 2007. C= Water Control; DCLO= Solvent control, Dichloromethane. Numbers with distinct index are significate different (P≤0.01).

The information obtained in this bioassay, is comparable with the obtained for Gajmer *et al*. (2002) [24] who observed that the methanolic extracts of neem with concentration of 10% can serve like repellent, since they avoid the oviposition of *Earias vittella* (Fabricius), natural pest of the cotton crops.

On the other hand, in the evaluation by times of the repellency, percentages of efficiency were observed from the first hour for both concentrations of *A. indica*. Nevertheless, the concentration of 10% had a faster effect than that of 5% (Figure 2). This way, for the concentration of 5%, was observed that during the first hour the repellent effect ranged from 5% to 20% (NEEM-1 and NEEM-2, respectively). Instead, for the concentration of 10%, also in the first hour, the percentage of repellency reached levels of 35-40% (Figure 2).

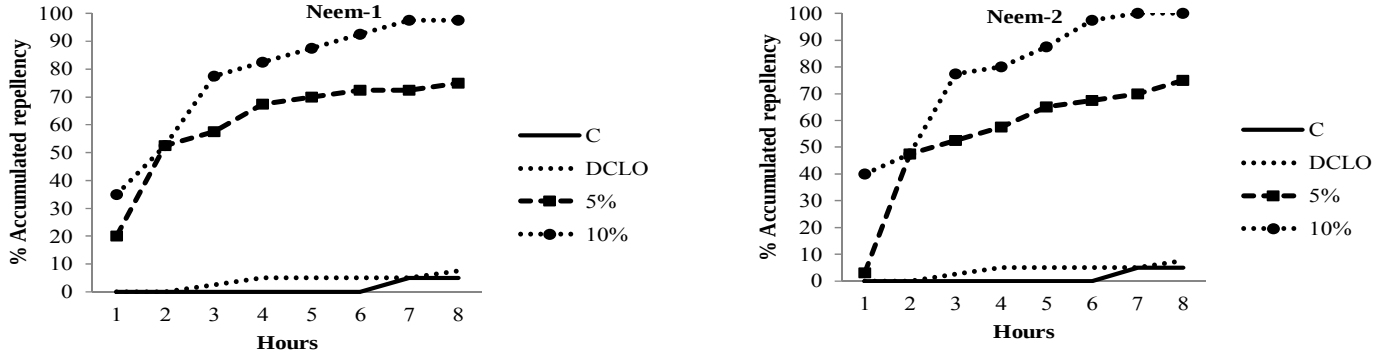

Fig 2: Repellency of whitefly in time intervals of 8 hrs.

The increasing trend of accumulated repellency against *B. tabaci* was similar between the extracts of NEEM-1 and NEEM-2 (without significant differences, P≤0.01). The better percentages of repellency were reached in the concentration of 10%, in the seventh hour of evaluation (97.5 and 100%, for NEEM-1 and NEEM-2, respectively) (Figure 2).

These results are different from the obtained for González-Gómez *et al*. (2006) [16], who evaluated extracts of *A. indica* against *V. destroyer*, finding optimum indexes of repellency, until 72 hours of study. It is possible to suggest that besides the intrinsic susceptibility of the involved species, the higher values of concentration and method of administration of the extracts of neem used, have originated the indexes of repellency more efficient in our study.

In later studies, it would be necessary to evaluate the residually of the extract, because it is probable that the degradation of active ingredients in the time and, in consequence, the effect is higher at first hours of exposition [16]. To complement, it is necessary to base from the results obtained in this study and to evaluate the extract in field conditions to determine their impact on the crops in situations most near to the needs of the producers.

Conclusions

The results of this study can offer a great value for the formulation of the Integrated whitefly Managing, because the extracts of neem evaluated had optimal effects, both insecticide and repellent, in concentrations of 10% and times from 2 to 5 and 7 hours, respectively, on *B. tabaci* adults. There was not observed differences, with respect to the times of storage between the extracts of NEEM-1 and NEEM-2, since both presented similar effects of mortality and repellence.

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