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Effect of *Eugenia winzerlingii* Extracts on *Bemisia tabaci* and Evaluation of its Nursery Propagation

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Abstract: The development of plant-derived products to control *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae) is an urgent need for production of horticultural crops. Plant extracts and essential oils of several species of the genus *Eugenia* (Myrtaceae) have shown insecticidal activity. In southern Mexico, leaf extracts from *Eugenia winzerlingii* showed nematicidal effect but its insecticidal properties have not been explored. Therefore, the objective of this study was to evaluate the insecticidal effect of aqueous and organic extracts from *E. winzerlingii* leaves on *B. tabaci* egg, nymph and adult stages, and else to explore its nursery propagation. Then, extracts of this species were obtained by maceration with different polarity solvents. Bioassays were carried out on *Capsicum chinense* leaves. Mortality assays showed that aqueous and total crude ethanol (TCE) extracts necrosed the eggs (LC_{50} = 0.21% w/v and 4.68 mg/mL, respectively), whereas hexane, ethyl acetate (ETA), residual ethanol and TCE extracts affected the nymphs (LC_{50} = 0.25 - 4.85 mg/mL). In adults, oviposition inhibition by free choice assay indicated that TCE and ETA extracts had major activity (EC_{50} = 14.62 and 27.86 µg/cm^2, respectively). On other hand, the sexual and vegetative propagation of *E. winzerlingii* showed that this species can be easily cultivated by seeds. In conclusion, extracts of *E. winzerlingii* leaves are highly effective in controlling *B. tabaci*. TCE extract, in particular, was toxic to three stages of *B. tabaci*. This plant could be a potential alternative to develop a novel botanical insecticide to manage this destructive pest.

Keywords: *Bemisia tabaci*; bioassays; *eugenia winzerlingii*; oviposition inhibition; plant propagation

1 Introduction

The sweet potato whitefly (*Bemisia tabaci* Gennadius, Hemiptera: Aleyrodidae) is a highly destructive pest attacking a wide range of crops, including horticultural and ornamental species grown in greenhouse and field worldwide. Whitefly has particularly caused high economic losses in solanaceous crops like tomato, pepper, potato and sweet potato. In general, these losses are associated not only with the direct effects of this insect through feeding, but also with its ability to transmit a wide variety of Geminivirus species [1,2]. Currently, synthetic insecticides are used to control the whitefly. However, the repeated use of chemical insecticides has caused the emergence of insecticide resistant population of whitefly, as well as, intoxication to non-target organisms, such as pollinators and other beneficial insects [3]. Therefore, a need for the development of alternatives to chemical pesticides for the management of this plant pest. These alternatives include the application of insect growth regulators, entomopathogenic fungi, and the exploration of plant extracts, which are well known strategies to manage pest in various

crops and considered environmentally friendly methods [1,4,5]. Aqueous and organic extracts of plant species from different families have demonstrated insecticidal effects when applied directly to *B. tabaci* or deterrent effects when applied to the leaf surface [6-10]. Plant extracts and essential oils (EOs) of several species of the genus *Eugenia* (Myrtaceae) have shown activity on various insect species. For example, organic extracts from *Eugenia candollena*, *E. jambolana* and *E. uniflora* were highly toxic to *Aedes aegypti* larvae [11-13]; EOs from *E. sulcata* are lethal to *Dysdercus peruvianus* and *Oncopeltus fasciatus* [14]; extracts and EOs from *E. caryophyllata* have insecticidal effects by contact against *Attagenus unicolor japonicus* larvae and *B. tabaci* adults [9,15]. In a previous work, we observed that ethanol extract of *Eugenia winzerlingii* Standl. leaves, a shrub endemic to Southern Mexico and Central America, has a significant toxic effect on the plant parasitic nematode *Meloidogyne incognita* [16]. However, little is known of the insecticidal activity and reproduction of this plant species. Therefore, the objectives of the present study were to evaluate, under laboratory conditions, the insecticidal effects of aqueous and organic extracts of *E. winzerlingii* leaves against eggs and nymphs of *B. tabaci*, as well as the oviposition inhibition effect on adults. In addition, vegetative and seed propagation was also evaluated with the long-term goal of promoting the preservation and sustainable use of this endemic plant.

2 Material and Methods

2.1 Plant Material

Fresh leaves of *E. winzerlingii* were collected around Xpuhil, Campeche, Mexico (coordinates 12° 36’ 15” SE 49° 16’ 0.7” W). A voucher sample (PS3009) was deposited in the herbarium of the Natural Resources Unit at the Centro de Investigación Científica de Yucatán (www.cicy.mx). Leaves were dried (50-60°C) under artificial light for three days and were triturated by hand.

2.2 Extracts Preparation

2.2.1 Aqueous Extract

For the aqueous extract (AE), boiling distilled water (100 mL) was added to a sample (3 g) of triturated leaves of *E. winzerlingii*. After 20 min, the suspension was filtered through paper (Whatman No. 1) [17]. Finally, the suspension a room temperature was sterilized by a 0.22 µm Millipore membrane (Merck Millipore, MA, USA). This AE (3%) was stored (-20°C) until required for bioassays.

2.2.2 Organic Extract

The total crude ethanol extract (TCE) was obtained as described by Cristobal-Alejo [16]. Briefly, dried triturated *E. winzerlingii* leaves (10 g) were added to ethanol (20 mL) for 24 h. The suspension was filtered and the solid residues were macerated two more times. The solvent was separated by filtration and eliminated under low-pressure at 40°C to obtain a dry extract. The yield was 8% of the respective dry mass used. For hexane (HEX), ethyl acetate (ETA) and residual ethanol (RETH) extracts, a second sample of triturated leaves (10 g) was sequentially macerated with each solvent (20 mL, three times each) as previously described for TCE extract. Solvents were eliminated in the same way as in the TCE extraction protocol. The yield of each extract relative to the initial dry mass used was 0.6%, 1.6% and 12.5% for HEX, ETA and RETH extracts, respectively. All organic extracts were stored at 4°C until required.

2.3 Bioassays

2.3.1 Insects

The colony of *B. tabaci* was reared on habanero pepper plants (*Capsicum chinense* Jacq.) in a greenhouse at 25-35ºC and 55-85% relative humidity at the Instituto Tecnológico de Conkal, in Yucatan, Mexico.

2.3.2 Toxicity on Eggs and Nymphs of Bemisia tabaci

Bioassays were carried out on eggs and third instar nymphs as described by Cruz-Estrada [18]. Groups of 16 *B. tabaci* adults were confined for 48 h in clip cages (2 cm diameter) set on habanero pepper leaves. After removing the adults from the clip cages, plants were incubated for three or 15 days for bioassays with eggs or nymphs, respectively. Then, circular sections of leaves (2 cm diameter approx.) with at least 30 eggs or nymphs, were cut and transferred to Petri dishes (6 cm diameter) with a 5-mm-deep 2 % agar bed in the base. All organic extracts were dissolved (10 mg/mL) in ethanol:dimethyl sulfoxide (DMSO)-0.6% of tween 20 (99.5:0.5, v/v) and afterwards suspended with water (1:1, v/v). An aliquot (10 µL) of each extract suspension was deposited on the surface of the leaf sections fully covering the eggs or nymphs. Imidacloprid® (0.7 µg mL^{-1}) was used as positive control; distilled water and the solvent mixture used to dissolve organic extract, were used as negative control. Petri dishes containing the leaf sections were incubated at 24 ± 3ºC, 75 ± 8% relative humidity on a 12:12 h light:dark cycle. Mortality (percentage of necrotic eggs or nymphs) was recorded 72 h after extract application. Each treatment was set with five replicates. The most active extracts were assessed to determine their Lethal Concentrations (LC_{50} and LC_{90}) using several dilutions for organic extracts (10, 5, 2 and 1 mg/mL) and AE (3, 1.5, 0.75 and 0.35 % w/v).

2.3.3 Oviposition Inhibition on Bemisia tabaci Adults

The oviposition inhibition (OI) of whitefly adult was tested in free choice experiments, as described by Baldin [19], with some modifications. Two separated leaf sections (ca. 1 cm^2) were placed on the agar and treated as described by the assay for eggs or nymphs. One leaf section contained the extract (100 µg/cm^2), while the other contained the solvent (negative control). After solvent evaporation, 16 *B. tabaci* adults were placed in each Petri dish and incubated at 26 ± 2ºC, 75 ± 8% relative humidity under natural light. The number of eggs laid on each leaf section was recorded 24 h after adult transfer. The percentage of OI was calculated using the equation: $OI = [1 - (T/C)] \times 100]$, where T and C are the number of eggs on the treated and control leaf sections, respectively. Each treatment was set with five replicates. The Petri dishes were set in an upside-down position and incubated at 26 ± 2ºC, 75 ± 8% relative humidity and natural light. The numbers of eggs on each leaf section was recorded after 24 h of extract application. The extracts with the highest OI were tested to determine their Effective Concentration (EC_{50} and EC_{90}) using serial dilutions (100, 50, 25 and 12.5 µg/cm^2).

2.4 Nursery Propagation of Eugenia Winzerlingii

2.4.1 Sexual Propagation

Fruits of *E. winzerlingii* were collected in Calakmul, Campeche, Mexico. These were separated into three batches by their ripening stage: unripe, semi-ripe and ripe. Seeds were sown within 20-23 days of fruit collection. Following mechanical removal of fruit's pericarp, seeds were sown in plastic trays (400 cm^3) filled with commercial substrate (Cosmopeat®). Trays were kept in the nursery under natural conditions (35.1 ± 2.3°C, 64.2 + 7.8% relative humidity). Throughout the experimental period (three month), the substrate was irrigated at field capacity.

Seeds were treated with gibberellic acid (Biogib 10 ps, 250 µg/ml of AI) for 48 or 72 h. Control seeds were immersed in water only. Each treatment had five replicates of 10 seeds each. After exposing to gibberellic acid, seeds were washed with distilled water. Seeds were uniformly sown to an approximate depth of 1 cm. Germination percentage (GP) was recorded 40 days after sowing. GP was calculated by the following equation: *GP = (number of germinated seeds/total number of seeds tested) × 100*.

2.4.2 Vegetative Propagation

Hardwood and semi-hardwood cuttings were taken from healthy and uniform sized trees of *E. winzerlingii* from Calakmul, Campeche, Mexico. Based on the assumption that the auxins promote rooting, indole-3-butyric acid (IBA) at 0.06% (Raizone®, Faxsa México) was used [20]. For IBA

treatment, cuttings were dipped directly three seconds in the powder of the commercial product Raizone® (0.06% of IBA). Control cuttings were not treated with IBA. Four replicates of 10 cuttings were set per treatment. Cuttings were planted to an approximate depth of 2 cm into plastic trays containing substrate compounded of soil:commercial substrate (Cosmopeat®):sand (3:2:1 v/v). Temperature within the nursery was 35.1 + 2.3°C and humidity was 64.2 + 7.8%. Cuttings were watered every other day. Percentage of rooted cuttings (PCR) was recorded 60 days after sowing by the following equation: *PCR = (Number of rooted cuttings/total number of rooted cuttings) × 100*.

2.5 Data Analysis

The experiments were set in a completely randomized design. All data on percentages was transformed to square root functions to homogenize variance. Lethal and effective concentration (50 and 90) were calculated by Probit analysis. All analysis of variance and Tukey mean comparison ($P < 0.05$) were performed using Statistical Analysis System (SAS) software, version 8.1 for Windows.

3 Results and Discussion

Results showed that the aqueous (3 % w/v) and organic extracts (10 mg/mL) of *E. winzerlingii* leaves had differential effects on *B. tabaci*. All extracts were active at least against one of the *B. tabaci* stages. The aqueous and TCE extracts resulted in the highest mortality of eggs (> 89%), indicating they have strong ovicidal activity, where "strong" is defined as OI > 70% [21]. The other organic extracts (HEX, ETA and RETH) and negative control had negligible effects on egg mortality (< 30%). It is worth noting that the mortality caused by AE was not significantly different to imidacloprid®, a widely used chemical insecticide (Tab. 1).

In contrast, *B. tabaci* nymphs displayed that all organic extracts caused high mortality (> 90%), whereas the AE and negative control had no effect. Interestingly, application of imidacloprid® had little impact on nymph mortality (Tab. 1).

Table 1: Mortality of *Bemisia tabaci* caused by organic (10 mg/mL) and aqueous extracts (3 % w/v) of *Eugenia winzerlingii* leaves after 72 h exposure

Extract	Mortality (%)	
	Eggs	Nymphs
Aqueous (AE)	99.20 ± 1.79 a	4.51 ± 8.43 d
Total crude ethanol (TCE)	89.46 ± 4.91 b	100.00 ± 0.00 a
Hexane (HEX)	29.34 ± 7.67 c	91.99 ± 2.93 b
Ethyl acetate (ETA)	9.23 ± 4.78 d	97.53 ± 2.84 ab
Residual ethanol (RETH)	3.34 ± 2.84 de	100.00 ± 0.00 a
Imidacloprid®	99.25 ± 1.68 a	26.45 ± 8.97 c
Negative control	1.0 ± 2.24 e	10.77 ± 6.46 d

Negative control: ethanol: DMSO-0.6% tween 20 (99.5:0.5, v/v) and water (1:1, v/v)

For eggs: df = 5, F = 105.1, $p < 0.0001$; for nymphs: df = 5, F = 66, $p < 0.0001$.

Mean mortalities (± Standard error) followed by the same letter within the same column are not significantly different (Tukey, $p > 0.05$).

For eggs, estimates of LC_{50} and LC_{90} for AE were 0.21 and 1.29 % w/v, respectively; whereas for TCE extract the values were higher (Tab. 2). Furthermore, this AE was more toxic than other plant species reported, such as AE from leaves of *Acalypha gaumeri, Annona squamosa* and *Petiveria alliaceae* (LC_{50}

= 0.36 - 0.42 % w/v); or the concentrations (10 % w/v) of the extracts used were higher than that of the present work (3 % w/v) [18,22].

Table 2: Toxicity (LC_{50} and LC_{90}) of extracts from *Eugenia winzerlingii* leaves on *Bemisia tabaci* eggs after 72 h exposure

Extract	n	Slope ± SE	LC_{50} (CL)	LC_{90} (CL)	P
Aqueous	5	1.63 ± 0.15	0.21 (0.14 - 0.28) *	1.29 (1.17 - 1.41) *	< 0.0001
Total crude ethanol	5	3.63 ± 0.17	4.68 (4.46 - 4.90) **	10.54 (9.75 - 11.54) **	< 0.0001

n = number of groups tested containing 30 individuals each

CL: Confidence limits * LC_{50} = %w/v ** LC_{50} = mg/mL

The most active extracts from *E. winzerlingii* against nymphs of *B. tabaci,* were the ETA and TCE (LC_{50} = 0.25 - 0.78 and LC_{90} = 3.10 - 3.90 mg/mL). These were significantly lower than those calculated for HEX and RETH extracts (Tab. 3). When compared, the nymphicidal effect was greater than other organic extracts reported from or *P. alliaceae* and *Trichillia arborea* (LC_{50} = 1.27 - 1.61 mg/mL), and also, with [18].

Table 3: Toxicity (LC_{50} and CL_{90}) of organic extracts from *Eugenia winzerlingii* leaves on *Bemisia tabaci* nymphs after 72 h exposure

Extract	N	Slope ± SE	mg/mL		P
			LC_{50} (CL)	LC_{90} (CL)	
Total crude ethanol	5	1.83 ± 0.16	0.78 (0.66 - 0.93) b	3.90 (3.43 - 4.61) a	< 0.0001
Hexane	5	1.82 ± 0.12	3.19 (2.89 - 3.49) c	16.02 (13.45 - 20.04) c	< 0.0001
Ethyl acetate	5	1.17 ± 0.18	0.25 (0.08 - 0.47) a	3.10 (2.50 - 3.74) a	< 0.0001
Residual ethanol	5	5.82 ± 0.29	4.85 (4.67 - 5.04) d	8.06 (7.65 - 8.56) b	< 0.0001

n = number of groups tested containing 30 individuals each CL: Confidence limits

LC_{50} and LC_{90} values followed by the same letter within the same column are not significantly different.

The evaluation of the OI effects of the organic extracts from *E. winzerlingii* exhibited that these had differential effects. Only the ETA and TCE extracts showed strong effects on this variable, where the OI values recorded were higher than 90% (Fig. 1). The lowest estimates of EC_{50} of OI was displayed by TCE extract with LC_{50} values of 14.62 and LC_{90} of 51.45 µg/cm^2 (Tab. 4). Based on the LC_{50} of both extracts, our results were lower than those using other plants. For example, the aqueous, ethanolic and acetone extracts from *Pluchea sericea* leaves, demonstrated a moderate repellent index (values range from 0.52 to 0.7) on *B. tabaci* adults [6]; and ethanol extracts from *Capsicum chinense* fruits caused repellency and mortality, with a LC_{50} values of 29.4 % (w/v) [23]. Furthermore, EOs and extracts from *E. caryophyllata* have insecticidal effects by contact against *B. tabaci* adults with LC_{50} value of 0.22 ml/cm^{-3} [9].

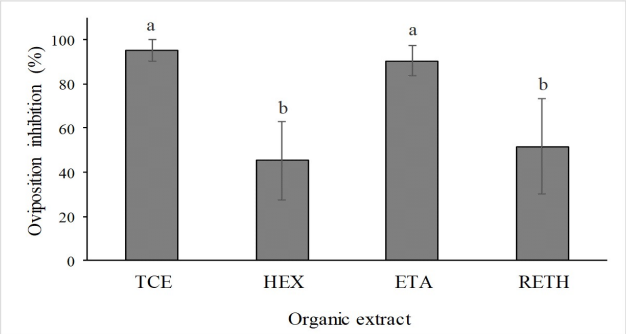

Figure 1: Oviposition inhibition on *Bemisia tabaci* adults caused by the application of organic extract (100 µg/cm^2) from *Eugenia winzerlingii* leaves after 72 h of adult release. Values are means ± standard error (df = 3, F = 3.15, $P < 0.05$)

Table 4: Effective concentration (EC_{50} and EC_{90}) of the oviposition inhibition on *Bemisia tabaci* exposed to organic extracts from *Eugenia winzerlingii* for 24 hours

Extract	N	Slope ± SE	µg/cm^2		P
			EC_{50} (CL)	EC_{90} (CL)	
Total crude ethanol	5	2.35 ± 0.16	14.62 (12.52 - 16.56) a	51.45 (46.80 - 57.50) a	< 0.0001
Ethyl acetate	5	1.17 ± 0.18	27.86 (26.01 - 29.67) b	74.63 (68.80 - 81.92) b	< 0.0001

n = number of groups tested containing 10 individuals each

EC_{50} and EC_{90} values followed by the same letter within the same column are not significantly different

CL: Confidence limits

In general, our results showed that extracts from *E. winzerlingii* leaves had high activity on *B. tabaci* eggs, nymphs and adults. We observed that the eggs were the most resistant stage and were necrosed only by more polar extracts (AE and TCE). These results suggest that the ovicidal activity of extracts is attributed mainly to polar metabolites. On other hand, medium polarity compounds in ETA and TCE extracts produced toxic effects on nymphs and oviposition deterrence in adults. The only extract toxic to all three stages of *B. tabaci* was TCE, with good effects. This indicates that biological activity could be attributed to several metabolites biosynthesized by *E. winzerlingii* leaves which could jointly or independently contribute to produce biotoxic and repellent activity on *B. tabaci*. This complex active mixture makes the plant more effectivity on pests [24].

This study is the first contribution on insecticidal properties of *E. winzerlingii*. The genus *Eugenia* shows wide range of biological activity including antifebrile, antidiabetic, antirheumatic, anti-inflamatory and antimicrobial effects. However, insecticidal activity of extracts or metabolites of *Eugenia* species have not been studied in detail. Phytochemical works on this regard have mainly identified metabolites like flavonoids, triterpenes, chalcones, tannins and EOs [25-26]. Overall, EOs of various botanic families have high activity on *B. tabaci* [27]. The most abundant components (≥ 10%) of EOs of *E. caryophyllata* includes acetyleugenol, eugenol, α-cubenene, β-caryophyllene and methyl eugenol. Eugenol, methyl eugenol, and minor recurrent components acetyl eugenol, isoeugenol and methyl isoeugenol have showed insecticidal effects on pests [28-29]. Whereas, EOs of *E. uniflora* contain mainly curzerene and γ-elemene [30], and EOs of *E. candollena* contain the sesquiterpene β-elemene (35.87%) as the most abundant component [12]. Therefore, active principles responsible for insecticidal effects from *E. winzerlingii* might be related to these types of compounds, but studies should be carried out to find the most abundant components of the evaluated extracts.

Extraction of metabolites from plants should be accompanied by the massive production of the species. Of particular interest are plant species that are endemic and that naturally occur in conserved areas. In an attempt to explore the feasibility for nursery propagation of *E. winzerlingii*, sexual and vegetative reproduction of this species was evaluated. The percentage of seed germination of *E. winzerlingii* at 40 days after sowing was higher than 80 % in all treatments. No significant differences were observed on seed germination between those treated with gibberellic acid (Biogil 10 ps, 250 μg ml^{-1}) and those of the control (Tab. 5). There was also no effect ($P < 0.05$) of fruit ripening stage on percentage of seed germination.

Table 5: Effect of gibberellic acid treatment on the germination percentage of *Eugenia winzerlingii* seeds obtained from fruits at different ripening stages

Fruit ripening stage	Exposure time (h)	Germination rate (% ± SD)
Unripe green	0	96.0 ± 5.48 a
	48	90.0 ±10.0 a
	72	90.0 ± 4.47 a
Semi ripe	0	90.0 ± 4.47 a
	48	96.0 ± 5.48 a
	72	98.0 ± 4.47 a
Fully ripe	0	92.0 ± 4.47 a
	48	88.0 ± 10.95 a
	72	84.0 ± 5.48 a

% germination rate (± standard deviation) followed by the same letter within the same column are not significantly different (Tukey, $P > 0.05$)

These data showed than *E. winzerlingii* can be easily propagated by seeds, even though we observed no effect of auxin to enhance germination rate. Studies on propagation of other *Eugenia* species have also reported the feasibility of propagation by seeds. For example, in *Eugenia dysenterica*, *E. uniflora* and *E. stipitata* found high germination rate (> 80%) [31-33]. They also observed that the degree of fruit ripeness affects this process, as seeds from fully ripe fruits showed higher dormancy than those from partially ripe fruits. Our results, however, exhibited that in *E. winzerlingii* the germination of seeds did not depend either on the fruit ripeness stage or on the seed treatment with auxin. On the other hand, vegetative propagation of *E. winzerlingii* was not successful, as adventitious roots failed to develop in cuttings. This is important to mention that shoots appeared two weeks after cuttings were planted, but dried off gradually, likely as consequence of no root formation. Various works on vegetative propagation of Myrtaceae species showed similar outcomes to our work. For example, Franzon [34] reported failure on the vegetative propagation with hardwood and semi-hardwood cuttings of *Eugenia involucrata*, *E. uniflora* and *E. brasiliensis*. Our results then argue in favor of seed propagation of *E. winzerlingii* when massive reproduction is required. The contribution in the knowledge of potential uses of *Eugenia* genus leads us to strengthen the conservation and sustainable preservation of this plant [35].

All results obtained with *E. winzerlingii* leaves, indicating that it is important to continue the studies with this species. The propagated plants need to be evaluated from different perspectives, the presence of the active ingredients in their extracts, the insecticidal effect at greenhouse field, as well as studying the agronomic aspects.

In conclusion, extracts from *E. winzerlingii* leaves have a direct effect when applied to immature *B. tabaci* or indirectly on oviposition when leaves are treated. The AE was the most effective ovicidal agent, ETA extract was the most toxic for nymphs, and TCE extract caused high OI in adults. ETC extract is the most recommendable to control the three *B. tabaci* stages. Propagation of *E. winzerlingii* can be carried

out by seeds. The present work shows that extracts from *E. winzerlingii* leaves, a renewable source, are good candidates to develop a botanical insecticide to control *B. tabaci*.

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