

## GUAVA LEAF EXTRACTS AS REPELLENTS AGAINST *DIAPHORINA CITRI* KUWAYAMA

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(Received 12<sup>th</sup> Mar 2024; accepted 8<sup>th</sup> Jul 2024)

**Abstract.** The guava tree (*Psidium guajava* L.), a common plant in the Mexican citrus regions, has potential as a natural psyllid repellent. This study evaluated guava leaf extracts as repellents against *Diaphorina citri*, the Asian citrus psyllid. Guava leaves were collected, dried, and extracted with four solvents: hexane, dichloromethane, ethyl acetate, and methanol. The extracts were then tested for repellency and oviposition deterrence on adult psyllids. Hexane extracts at concentrations of 500 and 1000 ppm showed significant repellency and reduced psyllid egg-laying compared to controls. Conversely, dichloromethane extracts attracted psyllids and increased egg-laying. Gas chromatography-mass spectrometry analysis identified caryophyllene, caryophyllene oxide, and phenols as the most abundant compounds in the repellent hexane extract. These findings suggest that hexane-based guava leaf extracts have potential for managing *Diaphorina citri* populations in citrus orchards.

**Keywords:** Asian citrus psyllid, plant extracts, repellency, attraction of a pest, managing *Diaphorina*

### Introduction

Repellent compounds offer a promising approach to manage insect vectors like *Diaphorina citri*, the psyllid responsible for transmitting *Candidatus Liberibacter* bacteria, which causes Huanglongbing (HLB), a devastating citrus disease (Ling et al., 2022; Poerwanto and Solichah, 2020).

Guava, a readily available plant in Mexican citrus groves, has shown potential as a natural psyllid repellent (Onagbola et al., 2010; Poerwanto and Solichah, 2020 and 2022; Zaka et al., 2010). This study aimed to evaluate guava leaf extracts as repellents against *Diaphorina citri* and to identify their components using gas chromatography.

Among the plants that have repellent potential against *Diaphorina citri*, guava stands out (Onagbola et al., 2010; Poerwanto and Solichah, 2020, 2022; Zaka et al., 2010). This plant occurs naturally in Mexico in citrus-producing regions, making it easy to obtain for growers.

## Materials and methods

### *Guava leaf collection and extraction*

Mature guava leaves free from pests and diseases were collected from wild plants in Chicontepec, Veracruz (coordinates: 21° 03' 32.10" N, 98° 08' 53.99" W, elevation: 99 m). To facilitate future identification (optional), the trees were marked with paint.

### *Leaf processing and extraction*

Collected leaves were transported in coolers to the Postgraduate College's Phytochemistry Laboratory. Upon arrival, the leaves were shade-dried for three days on kraft paper. Afterward, they were ground using a hand mill, weighed, and stored in sealed jars.

### *Extract preparation*

For extraction, 800 grams of ground leaf material were divided into separate containers. To each container, 500 ml of a different solvent (hexane, dichloromethane, ethyl acetate, and methanol) was added sequentially, ensuring complete submersion of the leaves.

Following a 48-h soak, the solvent mixture was filtered. The solvent was then removed using a rotary evaporator with heat and vacuum. This extraction process was repeated four times for each solvent. Finally, the concentrated extracts from each solvent were collected in labeled vials, sealed, and stored under refrigeration (4-5°C) for subsequent bioassays. For extraction, 800 grams of ground leaf material were placed in separate containers and 500 ml of different solvents (hexane, dichloromethane, ethyl acetate, and methanol) were added sequentially to each container, ensuring the leaves were completely submerged.

After soaking for 48 h, the solvent mixture was filtered, and the solvent was removed using a rotary evaporator with heat and vacuum. This extraction process was repeated four times for each solvent. The final concentrated extracts from each solvent were collected in labeled vials, sealed, and stored refrigerated (4-5°C) for later bioassays.

### *Repellency and oviposition bioassays*

To prepare the solutions for the repellency and oviposition bioassays, two concentrations (500 ppm and 1000 ppm) were made for each extract. The required amount of extract was weighed according to the target concentration and added to a test tube. A small amount of the corresponding solvent (hexane, dichloromethane, ethyl acetate, or methanol) was used to dissolve the extract. Then, 10 mL of a solution containing 3-6 drops of Tween 80, 1 mL of dimethyl sulfoxide (DMSO), and distilled water was added to the test tube. The mixture was thoroughly mixed using a water bath at 50-60°C and a manual stirrer. Control solutions were prepared identically, substituting the extract with distilled water.

At the National Reference Center for Biological Control (CNRCB), located in Tecmán, Colima (18° 55' 35" LN, 103° 53' 03" LO, 44 m above sea level), the bioassays were carried out with adult *Diaphorina citri*. *Murraya* plants (*Murraya paniculata*), 8 months old and 20 cm high, were used. Plants with 7 to 10 shoots 1 cm long were taken (Weathersbee and MacKenzie, 2005). Three plants were placed 70 cm × 70 cm × 70 cm cubes. The arrangement was in an equilateral triangle with 40 cm on

each side, one plant was a control, another was treated with 500 ppm and the last was treated with 1000 ppm.

The infestation was carried out with 20 adult psyllids per bucket, which were collected with manual vacuum cleaners in the breeding and maturing area and taken to the laboratory, where mature females were selected using the orange color of the abdomen as an indicator. They were placed in vials and taken to the experimental area.

The appropriate solution was applied to each plant with a manual sprinkler until it dripped, and the excess was allowed to dry for 1 h. The vial containing the adult *Diaphorina citri* was placed in the center of the triangle formed by the plants, and they were simultaneously uncovered. For six days, the number of insects perched on each plant was recorded at 9:00 am. On the seventh day, all the shoots were cut, and the number of eggs present in each was counted using a stereoscopic microscope.

The experimental design used was completely randomized, with 16 replicates for each extract and dose.

The extracts obtained with ethyl acetate, dichloromethane and hexane that showed the greatest response with *Diaphorina citri* adults were selected for analysis by gas chromatography, developed in the Chemical Ecology of Insects Laboratory of the Postgraduate College.

Two hundred milligrams of each extract was weighed and added to its corresponding solvent and, with the use of capillary tubes, distributed on glass plates (20 × 20 cm) covered with silica gel, and with an eluent solution, the extract was passed along the plate. A small fraction of the plaque was detected with the use of acid vanillin and a temperature of 110°C. The fractions detected with abundance of metabolites were marked, the silica gel was scraped from each fraction, placed in a flask, and covered with the hexane-ethyl acetate solution (4:1) and allowed to rest for 12 h. The silica gel was removed using Whatman 40 filter paper, and the solution was placed in vials that were labeled and sealed for reading on the chromatograph. Excess solvent was removed with liquid nitrogen prior to injection into the gas chromatograph.

The samples were analyzed using a gas chromatograph (GC) coupled to a mass-selective detector (CG/MSD 6890/5973; Agilent Technologies, Palo Alto, California, USA).

One microliter of sample was manually injected into the port, which was operated at 250°C. An HP-5 ms capillary column (Agilent, Santa Clara, California, USA) coated with a 0.250 µm phenylmethyl siloxane phase, 0.25 mm internal diameter, and 30 m long was used. Helium was used as the carrier gas at a column flow rate of 1 mL min<sup>-1</sup>. Each sample was injected into triplicate with the corresponding blank.

The relative abundance of compounds was calculated using the following formula:

$$\text{Relative abundance (\%)} = \frac{\text{peak area of compound } X}{\text{area of all peaks in the sample}} \times 100$$

### Data analysis

The frequency with which *Diaphorina citri* adults landed on the *Murraya* plants was obtained using the proc freq procedure of the SAS program. With the frequencies obtained, chi-squared tests were performed with  $\alpha=0.05$  to determine whether repellency to adults was present or not.

The number of eggs laid by psyllids on each plant was statistically analyzed using analysis of variance (ANOVA). This analysis was likely performed using the PROC

GLM procedure in SAS software (SAS, 2014). Following the ANOVA, mean comparisons between treatments were conducted using Duncan test with a significance level of alpha ( $\alpha$ ) set at 0.05.

## Results and discussion

### *Repellency of plant extracts against Diaphorina citri in Murraya plants*

The presence of *Diaphorina citri* psyllids was consistently lower in *Muraya* plants treated with guava extracts prepared using ethyl acetate, compared to untreated control plants. At a concentration of 500 ppm, the guava extracts significantly reduced psyllid landings by 57% compared to the control. Interestingly, increasing the concentration to 1000 ppm only provided a 26% reduction, suggesting that repellency did not increase proportionally with dose. Repellent activity persisted throughout the 6-day experiment ( $X_{2c} = 68.63 > X_{2t} = 5.99$ ), although it gradually diminished over time.

In contrast to guava extracts, dichloromethane extracts exhibited an attractive effect on *Diaphorina citri*. Control plants had significantly fewer psyllids compared to plants treated with 500 ppm of dichloromethane extract. The psyllid presence was 2.7 times higher with 500 ppm and a staggering 3.7 times higher with 1000 ppm compared to the control ( $X_{2c} = 32.56 > X_{2t} = 5.99$ ).

Hexane extracts, on the other hand, demonstrated repellent activity against *Diaphorina citri* ( $X_{2c} = 20.15 > X_{2t} = 5.99$ ). Compared to the control, psyllid landings were reduced by 32% and 36% on plants treated with 500 ppm and 1000 ppm of hexane extract, respectively. However, this repellency was not consistent throughout the six days of the experiment.

Finally, methanol extracts did not show any repellent effect against *Diaphorina citri* at either 500 ppm or 1000 ppm concentration according to the Chi-square test ( $X_{2c} = 4.46 < X_{2t} = 5.99$ ).

When there was a repellent effect of *Diaphorina citri* on *Muraya* plants with guava leaf extracts, it was attributed to the presence of volatile compounds, both in the present study and in those of Ling et al. (2022); Poerwanto and Solichah (2020, 2022) and Onagbola et al. (2011). For their part, Bhadra and Singh (2023) pointed out that guava is also rich in flavonoids, which are also associated with plant protection; for example, De Sousa et al. (2022) quantified pentahydroxflavone in guava leaves.

In this regard, Savoldi et al. (2020), in *Psidium cattleianum* extracts, determined that the most important compounds were sesquiterpenes, particularly caryophyllene, with 14.8%, and verified that they had antimicrobial activity against eight fungi and six bacteria. This compound was also identified in the present study, although as caryophyllene oxide and in concentrations of 17.78%.

It is important to mention that different effects of guava extracts have been found. According to Binyamenn et al. (2021), guava fruit volatiles attracted *Bactrocera* spp. For their part, Díaz et al. (2023); found that guava leaf extracts acted as repellents against *Anastrepha fraterculus* and *Bactrocera* sp.) It is important to mention that there are several studies that evaluate the action of plants against different insects, such as those of Binyameen et al. (2021); Delgado-Ortiz et al. (2023); Gómez et al. (2020) and Magallán-Hernández et al. (2023).

One of the extracts with repellent effect was the one obtained with hexane, which is a nonpolar solvent and mainly extracts lipids, carotenes and terpenoids, and the extracts obtained with different plants with this solvent have shown repellency against different

insects; Pantoja-Pulido et al. (2020) obtained cholinesterase activity against *Atta* spp. with dichloromethane extracts from *Tithonia diversifolia*; for their part, Teke and Mutlu (2021), using essential oil from some plants, obtained good control against *Sitophilus granarius* and *Tribolium castaneum* (Herbst). Khoshrafter et al. (2020) and Ramos et al. (2022) found repellency of *Melia* spp. against *Spodoptera* spp.

### ***Effect of extracts on oviposition of Diaphorina citri***

The repellent effect of guava leaf extracts was confirmed by counting the number of eggs laid by *Diaphorina citri* on *Murraya* plants after seven days of evaluation. Duncan's test was used ( $\alpha=0.05$ ) (Table 1).

*Murraya* plants treated with guava extracts prepared using 500 ppm ethyl acetate, 1000 ppm methanol, 1000 ppm hexane, and 500 ppm hexane had significantly fewer *Diaphorina citri* eggs compared to control plants. Application of 500 ppm ethyl acetate resulted in 64% fewer psyllids landing compared to controls, while there was no significant difference in repellency observed at 1000 ppm (Table 1).

**Table 1.** Total number of *Diaphorina citri* eggs per *Murraya* (*Murraya paniculata*) plant, with and without application of guava extracts

Treatment	Eggs per <i>Murraya</i> plant
Dichloromethane 500 ppm	126.44 a
Control	77.63 b
Methanol 500 ppm	65.56 bc
Dichloromethane 1000 ppm	56.06 bc
Ethyl acetate 1000 ppm	51.31 bc
Ethyl acetate 500 ppm	50.13 c
Methanol 1000 ppm	47.56 c
Hexane 1000 ppm	19.38 d
Hexane 500 ppm	8.69 d

Mean values with different letter indicate statistical difference ( $P < 0.05$ ) according to Duncan's multiple range test. SD = 49.3283

*Murraya* plants treated with 500 ppm of dichloromethane extract, showed the highest number of *Diaphorina citri* eggs, followed by the control plants (Table 1). This suggests that dichloromethane may not only fail to repel psyllids but might even attract them.

Plants treated with 500 ppm hexane guava extract exhibited strong repellency against *Diaphorina citri*, resulting in a significantly lower number of eggs compared to the control and dichloromethane treatments. Dichloromethane, on the other hand, had the highest number of recorded eggs (Table 1).

Hexane extracts showed inconsistent repellency against adult psyllids. This suggests that hexane extracts may not prevent adult psyllids from landing on the plants but might inhibit their egg-laying behavior. Similar observations were made with 1000 ppm methanol extract, which displayed some repellency and was also associated with a lower number of eggs.

In *Diaphorina citri*, the inhibition of oviposition was also observed with different doses of *Artemisia*, although the highest dose was not the one that obtained the greatest reduction in oviposition (Rizvi et al., 2023), as in the present study. with the extracts with ethyl acetate and hexane.

### Identification of compounds in the extracts studied

The identification of the secondary metabolites found in the selected extracts is presented in Table 2. As expected, due to the polarity of the solvents, there are differences in what was extracted with each of them.

**Table 2.** Compounds found in extracts evaluated against adult *Diaphorina citri*

Compound	Relative abundance (%)		
	Ac. ethyl	Dichloromethane	Hexane
<b>Heneicosane (C<sub>21</sub>)</b>	4.25	3.36	-
<b>Octadecane (C<sub>18</sub>)</b>	10.79	4.90	4.12
<b>Styrene</b>	10.95	8.29	-
<b>Docosane (C<sub>22</sub>)</b>	6.72	9.64	16.82
<b>1-Heptadecene (C<sub>17</sub>)</b>	2.86	-	-
<b>Hexacosane (C<sub>26</sub>)</b>	-	3.02	4.21
<b>Caryophyllene</b>	-	3.66	2.75
<b>3-methyl-heptadecane (C<sub>17</sub>)</b>	-	2.95	-
<b>1-Hexadecene (C<sub>16</sub>)</b>	-	-	11.24
<b>E-15-Heptadecenal</b>			5.34
<b>Cariophyllene oxide</b>	-	-	17.79
<b>(S)-1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-ciclohexene</b>	-	-	4.51

The extract with hexane and to which the observed response was attributed were caryophyllene, caryophyllene oxide and phenols that have been reported in other extracts that had a repellent effect on insects (Muro et al., 2004; Neves et al., 2021; Villamarín et al., 2022); the presence of a sulfur compound was also detected, which has been reported to be responsible for repellency, especially in guava leaves (Rouseff et al., 2008; Ling et al., 2022; Poerwanto and Solichah, 2020, 2022).

Other compounds of interest present in the hexane extract were the undecyl ester of pentafluoropropionic acid, which is a fatty acid that can inhibit the development of fungi and bacteria; dichloromethane extracts of *Tithonia diversifolia* had cholinesterase activity against *Atta* spp. (Pantoja-Pulido et al., 2020).

The ethyl acetate extract was also repellent to adult *Diaphorina citri*. The results obtained with the extracts obtained with this solvent also vary according to the plant and the type of insect, as pointed out by Pantoja-Pulido et al. (2020), when evaluating dichloromethane extracts from *Tithonia diversifolia*, they found that they had cholinesterase activity against *Atta* spp. The compounds reported to have repellent activity and extracted from guava leaves with methanol in the present study were: eicosane and 2, 6, 10, 14-tetramethylhexadecane (Villamarín et al., 2022). Also noteworthy in Table 2 is the presence of alkanes with a high content of carbon atoms, such as mineral oils, used to control *Diaphorina citri*. In fact, it has been observed that there is a correlation between the presence of alkanes C22, C23, C24 and their combinations and their repellent and anti-oviposition potential for the psyllid (Poerwanto and Solichah (2022) (Table 2).

Methanol is a polar solvent and metabolites such as sugars, peptides, saponins, and glycosylated and sulfated flavonoids are extracted. The lack of response with methanol

extracts may be related to the type and amount of substance used, as other studies have observed that extracts with alcohols have a strong repellent effect on pests (Barre and Jenber, 2022; Jiménez-Galindo et al., 2023 and Sserunjogi et al., 2021). But where there has been no response, it has been suggested to explore other parts of the plant to obtain compounds that exert repellent activity.

This property of repellent and anti-ovipositor effect shown by the extracts obtained with hexane and ethyl acetate is what is sought for the management of *Diaphorina citri*, since it reduces the spread of bacteria and the reproduction of the psyllid (Grafton-Cardwell et al., 2013). However, work must continue in this direction because, although there are positive results in the field, the problem is the short duration of the effect, which requires the development of devices or mixtures that prolong the release of the extracts to make their use in orchards viable.

Secondary metabolites were extracted with dichloromethane, which attracts the insect, since it is common for pests to use the volatile compounds released by plants to find food or places to reproduce.

In guava it has been reported that there are flavonoids (Vargas-Álvarez et al., 2006), but also sesquiterpenes, such as  $\alpha$ -copaene, which act as general attractants (Liu, 2021), but also, Savoldi et al. (2020) determined in *Psidium cattleianum* extracts that the most important compounds were sesquiterpenes, particularly caryophyllene, at 14.8%. They found that they had antimicrobial activity against eight fungi and six bacteria. In this document, both caryophyllene and caryophyllene oxide were found in concentrations of 2.75% and 17.78%, respectively, when extraction was done with hexane.

In the present study (Table 2), the compound that stands out and that could be exerting the attracted activity detected is acetic acid, which has been evaluated and tested as an attractant for different pests, with promising results.

In evaluations with acetic acid, either alone or in combination with other attractants, several species of *Lepidoptera* have been captured (Frewin et al., 2022; Landolt and Alfaro, 2001; Knight et al., 2019; Whitfield et al., 2019).

Although acetic acid and 1-Heptadecene were also detected in the extracts with ethyl acetate, in that case there was repellency, which could be attributed to the combination of compounds and the concentration that existed in each of them, since the repellency or attraction It is not given by a single compound or its concentration (Neves et al., 2021; Villamarín et al., 2022).

Ethyl acetate and dichloromethane, which in general extracted similar compounds, the response is totally opposite, since while the first is repellent, the second attracts, which is associated with the abundance of the metabolites they extract and the mixture of these in the extract.

It will be important to evaluate the behavior of *Diaphorina citri* against compounds such as acetic acid, either with the preparation of guava extracts or with the use of synthetic compounds that have been used with other insects and that could provide data that allows its application with traps. yellow that attracts the insect or its application in trap crops.

## Conclusion

The ethyl acetate and hexane extracts showed repellent and anti-ovipositor effects on *Diaphorina citri*; while dichloromethane extracts were attractive and favored oviposition.

**Conflict of interests.** The authors declare that they have no conflict of interests.

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