



Evaluation of seaweed extracts for the control of the Asian citrus psyllid *Diaphorina citri*

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Abstract

Insecticidal and repellent activities were evaluated on extracts from three macroalgae (*Caulerpa sertularioides*, *Laurencia johnstonii* and *Sargassum horridum*) against *Diaphorina citri* adults. The ethanolic extracts were obtained by maceration, filtration, and concentration under reduced pressure at 40 °C. Each extract was fractionated by solid-liquid methods, followed by column fractionation. The fractions obtained were analyzed by phytochemical tests to determinate their chemical composition. The three extracts showed alkaloids, terpenes, phenols, tannins, flavonoids, anthraquinones and saponins, which are associated with insecticidal and repellent activity. The repellency assay with *S. horridum* extract showed repellent activity over 24 h (Index of Behavioral Tendency, IBT = 0.376 ± 0.047), *L. johnstonii* extract showed repellency during 18 h (IBT = 0.240 ± 0.034). Although *C. sertularioides* extract at the beginning produced an attractant effect (during the first 4 h), it was followed by a repellent effect after 12 h (IBT = 0.297 ± 0.041). The repellent control used was Neemix 4.5 (azadirachtin) and it has a longer repellency of over the 24 h, however *S. horridum* had a higher repellent activity over the control during all the assay. In the insecticidal activity assay, lethal doses were calculated for each species: *L. johnstonii* (LD₅₀ = 284 µg mL⁻¹), *S. horridum* (LD₅₀ = 364 µg mL⁻¹), and *C. sertularioides* (LD₅₀ = 3703 µg mL⁻¹). Additionally, three terpenic compounds isolated from *L. johnstonii* were identified by GC/MS; debromolaurinterol, isolaurinterol, and laurinterol as potential insecticidal and repellent compounds. The results suggest that seaweed extracts represent an alternative in the development of agrochemicals for pest control.

Keywords Ethanolic extracts · Insecticide · Repellent · *Laurencia* · Laurinterol

Introduction

The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is the main insect vector of Huanglongbing (HLB) better known as citrus greening. It is a worldwide disease caused by a phloem-limited Gram-negative bacterium *Candidatus Liberibacter asiaticus* (Jagoueix et al. 1994; Bové 2006). The general symptoms include blotchy mottled leaves and premature fruit drop (Zhao et al. 2013), which leads to unmarketable fruit, yield reductions, tree dieback, and

eventual death of infected trees (Da Graca 1991). At present there is no control for HLB disease, except preventing trees from the infection (Bové 2006), therefore the search for effective alternatives to control psyllid population is essential.

Mexico is considered among the main citrus producers in the world (fifth place) with an annual production of 6.7 million tonnes. It is an activity of great economic and social impact and is carried out in 23 of 31 states of the country (Presidencia de la república 2018a), thus there is great effort by governmental institutions in order to achieve the reduction of the infestation levels of the psyllid, using chemical and biological control on regional control areas (Presidencia de la república 2018b). However, the excessive use of synthetic insecticides during the last decades has caused serious problems around the world, including widespread of environmental contamination, toxicity to non-target organisms (including human) (Isman 2008), and insect resistance (Hemingway et al. 2002). Hence, there is an increasing need for new sources of selective compounds with an insecticidal effect focused on the target pest. For this reason, some conventional pesticides were replaced by biorational pesticides, which act specifically on

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insect pests and are less harmful to humans and the environment (Ishaaya and Horowitz 2007).

Marine natural products have a large chemical diversity with great potential to provide leads for the development of insecticidal agents (El Sayed et al. 1997). Several studies have shown that seaweed extracts could be used to control several pests such as aphids (Argadoña et al. 2000), bugs (Sahayaraj and Kalidas 2011; Asha et al. 2012; Sahayaraj and Jeeva 2012), mosquitoes (Bianco et al. 2013; Yu et al. 2014, 2015; Salvador-Neto et al. 2016), moths (Argadoña et al. 2000; Abbasy et al. 2014), termites (Manilal et al. 2011; Ishii et al. 2017), and weevils (Manilal et al. 2011; Ishii et al. 2017). Also, the application of seaweed extracts on plant crops causes beneficial effects; resistance to pests and diseases (Stephenson 1966), growth stimulation of the plant (Khan et al. 2009), higher biomass yield, and improve seed germination and fruits quality (Gupta and Abu-Ghannam 2011; Hernández-Herrera et al. 2013). Therefore, over the last years, the use of biopesticides from seaweeds has gained attention due to eco-friendly approaches for the management of insect pests and plant pathogens (Sahayaraj et al. 2012). The objective of the study was to assess the insecticidal and repellent activity of ethanolic seaweeds extracts for the control of *D. citri* psyllids.

Materials and methods

Seaweed collection and preparation of extracts

Specimens of three species of seaweeds from Bahia de La Paz, Mexico, were collected by hand during low tide; *Laurencia johnstonii* were collected in April 2013 at Coyote beach (24° 21' 09.2" N–110° 16' 23.5" W), *Sargassum horridum* were collected in June 2013 at Tarabillas beach, San Juan de la Costa (24° 27' 37.4" N–110° 41' 07.8" W), *Caulerpa sertularioides* were collected in September 2013 at Calerita beach (24° 20' 53.9" N–110° 17' 59.9" W). All species were identified by Dr. Juan Manuel López Vivas at Marine Botany Laboratory, Universidad Autónoma de Baja California Sur. The fresh specimens were transported to the Seaweeds Chemistry Laboratory at CICIMAR-IPN and were washed with running tap water to remove sand, salt, and macroscopic epiphytes and then were air-dried.

Dried sample (2 kg) of each species were ground to 40 mesh size, sieved, and macerated with 2 L of distilled ethanol. The sample was extracted three times. The solvent was filtered and concentrated by evaporation in a rotary vacuum evaporator (Yamato R500) at 40 °C. The crude ethanolic extracts were stored at –20 °C until further use.

Insect collection

Adult psyllids were collected from the wild infected orange trees (*Citrus sinensis*) in La Paz, Baja

California Sur, México. The collected insects were maintained in screened cages under environmental conditions. For the assessment of the dose, ten replicate insects were used, the insects were anesthetized before the assay by being kept at –4 °C for 3 min.

Adult choice trial

The repellent activity was assessed in a choice trial with adult *D. citri* psyllids (1 h starved) in exposure chambers. The chambers contained freshly excised citrus leaves sprayed with either 2.5 mg mL⁻¹ seaweed extract or blank (distilled water). The leaves were air-dried for 1 h before the assay, then ten adult psyllids were release into the chambers. The number of insects that settled on the leaves with seaweed extract or blank were registered after 4, 8, 12, 18, and 24 h. The assay was replicated ten times with different insects.

The commercial repellent Neemix 4.5 (azadirachtin) was used as a positive control. For the assay, it was evaluated a standard concentration of 180 µg mL⁻¹ of azadirachtin. The repellent effect of each treatment was determined as an index of behavioral tendency (IBT) according to Ouyang et al. (2013):

$$IBT = \frac{\text{Diaphorina citri adults on the treatment}}{\text{Diaphorina citri adults on the treatment} + \text{Diaphorina citri adults on blank}}$$

Insecticidal activity

Seven stock concentrations (0.5, 2.5, 5.0, 7.5, 10, 12.5, and 15 mg mL⁻¹) of seaweed extract were assessed in an insecticidal assay. The extracts were applied under similar conditions as described before. For each treatment, ten adult psyllids were placed in a Petri dish containing the sprayed leaf. The assay was replicated ten times. Profidor 350 (imidacloprid) was evaluated as positive control with a standard concentration of 50 µg mL⁻¹ (LD₅₀ = 24.79; Boina et al. 2009) and distilled water as blank. Mortality was recorded at 24 h after exposure.

Phytochemical analysis

The major secondary metabolites obtained from the seaweeds were identified according to Harborne (1998). The qualitative screening was performed on silica gel TLC plates and developed in a dicloromethane:methanol (9:1) system.

The ethanolic extract from *L. johnstonii* (115 g) was fractionated (Fig. 1) by solid-liquid extraction with silica gel (70–230 mesh) eluted with organic solvents on polarity gradient (*n*-hexane, dicloromethane, ethanol, and distilled water). The fractions were previously screened for biological activities and the most active fraction was applied in a silica gel (70–230 mesh) column eluted with *n*-hexane, dicloromethane, and

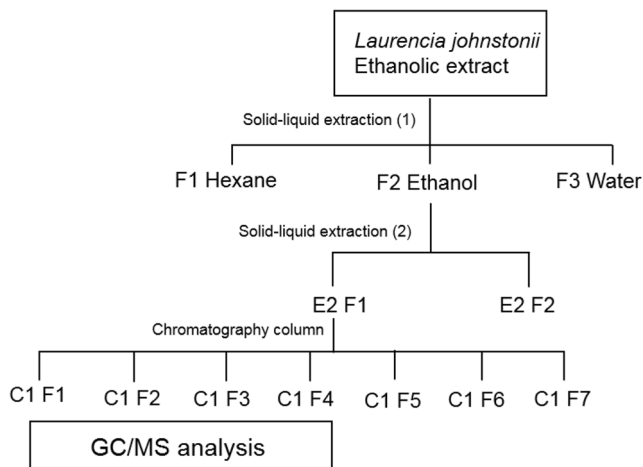


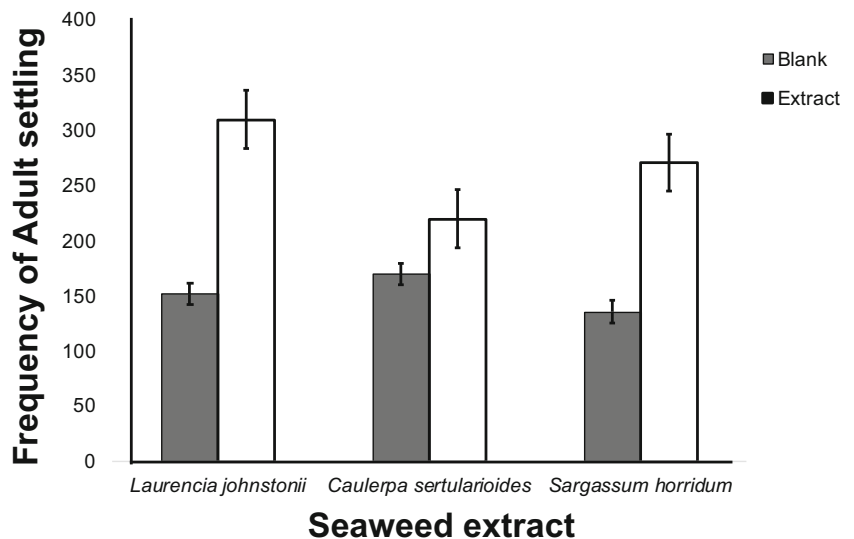
Fig. 1 Fractionation and chromatographic isolation of *Laurencia johnstonii* extract

ethanol (100% of each solvent) and yielded seven fractions. The fractions were analyzed by gas chromatography-mass spectrometry (GC/MS) and peak identification was carried out by comparison with published data on *Laurencia*.

Statistical analysis

Statistical analyses were carried out with Statistica 8.0 Statsoft for Windows. For the adult choice trial, the mean IBT index with standard error was calculated for each treatment. All data (including positive control) was subjected to two-way ANOVA with a confidence interval of 95% and Tukey post hoc test. For the insecticidal assay, the mortality was corrected using Abbott’s formula (Abbott 1925). The corrected mortality data were subjected to probit analysis (Finney 1971), the LC₅₀ was calculated with 95% confidence intervals on SPSS software.

Fig. 2 Effect of seaweeds extracts on the frequency of *Diaphorina citri* adults on a choice of treated or untreated citrus leaves. Blank control (distilled water). Counts were made after 24 h. Each treatment was replicated ten times



Results

Repellency activity

Seaweed extracts had a significant effect on the insect settling (Fig. 2) ($\chi^2 = 12.722$, $df = 2$, $p < 0.01$). It was recorded a higher frequency of organisms settled on the blank (distilled water), indicating there was repellency due to extracts during 24 h. This lack of acceptance or preference of insects to leaves with extracts may suggest that the presence of irritant or even toxic compounds for the tested organisms, so they choose the blank treatment (water) and avoid the contact with the seaweed extract.

Repellent activity was assessed with the index of behavioral tendency (IBT) (Table 1), where a low IBT indicates more repellency against the adults of *D. citri*. In the positive control, Neemix 4.5 showed a constant repellency during the 24 h assay, but the IBT values were higher than the seaweeds treatments, which indicates higher repellency with seaweed extracts.

Laurencia johnstonii showed an IBT of 0.361 after 4 h, but it reached the highest repellent effect after 18 h (IBT = 0.240) and eventually decreased (IBT = 0.889) after 24 h. It suggests that protection time of the volatile compounds is active during the first hours and then it was dissipated. The treatment with *C. sertularioides* extract showed no repellent effect during the first 4 h (IBT = 0.6098); on the contrary, it was observed that more insects settled on the leaves with the seaweed treatment. This preference indicates that the extract could contain some attractant compounds, which quickly volatilizes and therefore the beneficial effect decreases noticeably. After 8 h, a repellent effect was identified, and it was constant until 12 h (IBT = 0.297), however, the protection time did not exceed 18 h, and at the end of the assay, the insects did not make a preference in particular. *Sargassum horridum* showed a repellent activity

Table 1 Index of behavioral tendency (IBT) of *Diaphorina citri* adult psyllids on the choice trial with seaweeds extracts [2.5 mg mL⁻¹] and positive control Neemix 4.5

Exposure time	IBT ± standard error (n = 10)			
	<i>L. johnstonii</i>	<i>C. sertularioides</i>	<i>S. horridum</i>	Neemix 4.5
4 h	0.361 ± 0.036 abc	0.589 ± 0.091 c	0.310 ± 0.066 ab	0.471 ± 0.062 abc
8 h	0.357 ± 0.058 abc	0.397 ± 0.053 abc	0.314 ± 0.043 ab	0.515 ± 0.090 abc
12 h	0.299 ± 0.042 ab	0.297 ± 0.041 ab	0.348 ± 0.049 abc	0.581 ± 0.072 bc
18 h	0.240 ± 0.034 a	0.403 ± 0.065 abc	0.364 ± 0.036 abc	0.538 ± 0.076 abc
24 h	0.889 ± 0.068 abc	0.700 ± 0.057 abc	0.376 ± 0.047 abc	0.661 ± 0.091 c

IBT values were analyzed by two-way ANOVA; means followed by the same letter are not significantly different (P < 0.05, Tukey's multiple comparison test)

from the first hours (IBT = 0.310), it was observed a strong preference for the leaves sprayed with water, this behavior remained constant over 24 h (IBT = 0.376). The protection time was higher compared with other extracts evaluated, including positive control Neemix 4.5.

The two-way ANOVA analysis showed statistical differences between seaweeds extracts and exposure time ($p < 0.05$, $F_{3,155} = 11.219$), where *S. horridum* and *L. johnstonii* showed significant higher protection, compared to *C. sertularioides* and positive control.

Insecticidal activity

After 24 h of exposure, 100% mortality was observed with the three extracts at the highest concentration used. All concentration-mortality data were used to determine lethal doses for each seaweed (Table 2). *Laurencia johnstonii* extract caused mortality of the insects during the first 12 h of exposition; because of this higher activity, it was necessary to reduce the concentrations (μg) of the extract to calculate the lethal dose ($\text{LD}_{50} = 284 \mu\text{g mL}^{-1}$). These positive results suggest the presence of insecticidal compounds, therefore chromatographic methods were used to isolate and identify the possible active compounds. Insect mortality was followed by *S. horridum* extract with a $\text{LD}_{50} = 364 \mu\text{g mL}^{-1}$ possibly because the active compounds are at lower concentration on the ethanolic extract. The lowest mortality was obtained with *C. sertularioides* extract ($\text{LD}_{50} = 3703 \mu\text{g mL}^{-1}$).

Identification of active compounds

Phytochemical screening revealed the presence of alkaloids, triterpenes, phenols, tannins, flavonoids, and saponins on the

seaweed extracts. Since *L. johnstonii* showed higher insecticidal and repellent activities, their chemical composition was assessed by gas chromatography-mass spectrometry (GC/MS) analysis. The GC/MS profile of *L. johnstonii* C1F3 fraction revealed three peaks at 17.53, 18.07, and 20.56 min (Fig. 3). Fraction C1F2 revealed five peaks at 16.33, 18.06, 19.74, 20.57, and 34.02 min (Fig. 4). Additionally, some similarities were found on fractions, C1F2, C1F3, and C1F4, where peaks at 18.06 and 20.56 were present in all fractions, according to comparison with molecular masses and literature data (Table 3), these compounds were identified for isolaurinterol and laurinterol, respectively. The peak at 17.53 present in C1F3, C1F4, and C1F6 was identified as debromolaurinterol (Fig. 5).

Discussion

The marine environment is a rich source of diverse chemically and biologically active natural products (Choudhary et al. 2017) synthesized by seaweeds, invertebrates, and bacteria. Several studies demonstrate the large potential of the seaweeds extracts to replace synthetic pesticides and agrochemicals (El Sayed et al. 1997; Yu et al. 2014), Seaweeds have compounds that cause mortality, interrupt growth (Cetin et al. 2010), development, and reproduction of some insects (Asha et al. 2012; Sahayaraj and Jeeva 2012; Asharaja and Sahayaraj 2013). Additionally, these compounds could have specificity, less toxicity to non-target organisms, and rapid biodegradation (Isman 2008), which provides an ecological advantage.

The present research revealed that crude seaweeds extracts have insecticide and repellent activity against *D. citri* insect,

Table 2 Insecticidal activity of seaweeds extracts against *Diaphorina citri* adults

Extract	LD50 ($\mu\text{g mL}^{-1}$)	Slope ($\pm\text{SE}$)	95% confidence interval ($\mu\text{g mL}^{-1}$)	χ^2
<i>Laurencia johnstonii</i>	284	1.50 (0.12)	249–322	90.06
<i>Sargassum horridum</i>	364	0.36 (0.06)	179–587	93.34
<i>Caulerpa sertularioides</i>	3703	0.59 (0.08)	3007–4502	64.66

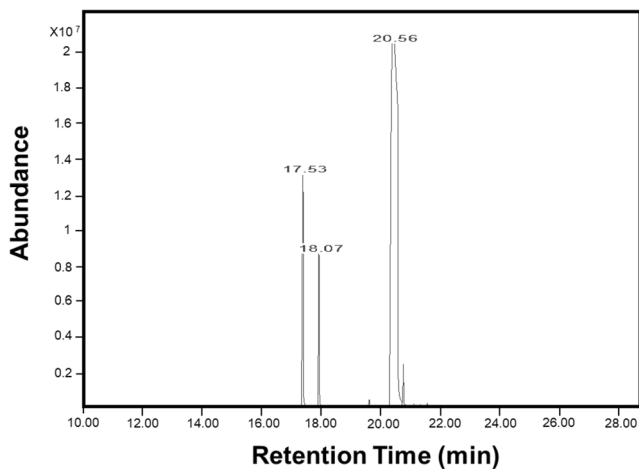


Fig. 3 GC/MS chromatogram of *Laurencia johnstonii* C1F3 fraction

and these positive results are related to the chemical composition of each macroalgae. In the phytochemical screening, we detected the presence of several groups of compounds associated with insecticidal activities on several studies that used terrestrial plant extracts, containing alkaloids, phenols, terpenes (Rattan 2010), and saponins (De Geyter et al. 2007). A unique feature of active compounds from marine sources is the presence of halogenated compounds, mainly found in red and brown algae (Smit 2004; Sharma 2011), especially in *Laurencia*. From that genus brominated sesquiterpenes with larvicidal activity against mosquitoes have been isolated, such as laurepinnacin, isolaurepinnacin (Fukuzawa and Masamune 1981), deoxyrepacifenol, *Z*-laureatin, *Z*-isolaureatin (Watanabe et al. 1989), elatol (Bianco et al. 2013), and obtusol (Salvador-Neto et al. 2016).

Repellent and insecticidal effects shown from *L. johnstonii* extract led us to the isolation of three active sesquiterpenes.

Fig. 4 Gas chromatogram of *Laurencia johnstonii* C1F2 fraction

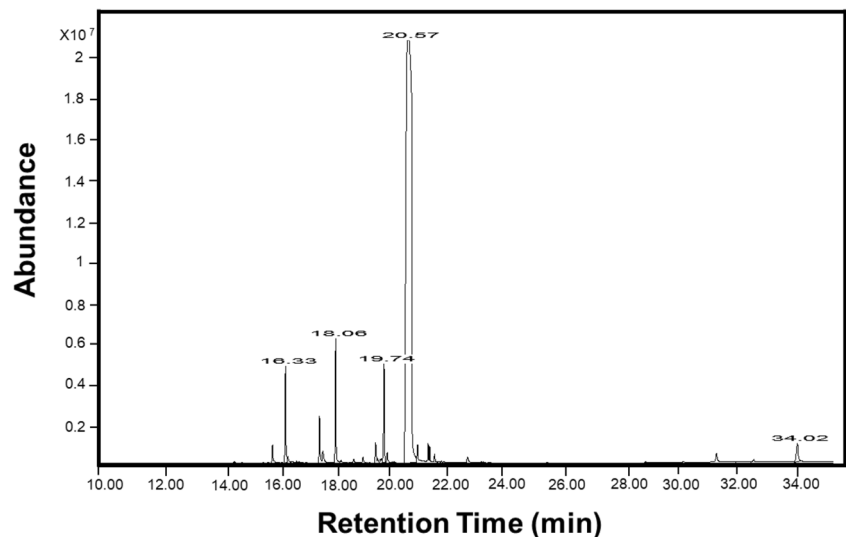


Table 3 GC/MS analysis of main peaks of *Laurencia johnstonii* fractions and comparisons with literature data

Peak RT (min)	Found on fraction	Molecular mass	Possible compound
15.76	C1F6	216	Debromoaplysin
16.32	C1F2	216	Debromoisolaurinterol
17.04	C1F6	232	Debromoaplysinol
17.53	C1F3, C1F4	216	Debromolaurinterol
17.81	C1F6	232	Laur-11-en-1,10-diol
18.06	C1F2, C1F3, C1F4	294	Isolaurinterol
19.73	C1F2, C1F6	310	Aplysinol
20.56	C1F2, C1F3, C1F4, C1F6	294	Laurinterol

Laurinterol and isolaurinterol were recently isolated and evaluated on *Acanthamoeba castellanii* parasite (García-Davis et al. 2018). However, there is only one report of repellent and insecticide activity with laurinterol. This major secondary metabolite was isolated from *Laurencia nidifica*, and it showed repellency against corn weevil *Sitophilus zeamais* and insecticide activity against termites *Reticulitermes speratus* with a $LD_{50} = 2.2 \mu\text{g}$ per insect topic application (Ishii et al. 2017). Although the mechanism of action is still unknown, on the repellent assay, it was identified a lower number of psyllids settled on the leaves with water. This may suggest the presence of toxic compounds, feeding inhibitors type, so the psyllids died of starvation at 24 h. *Sargassum horridum* and *C. sertularioides* extracts showed higher lethal dose values ($LD_{50} = 364 \mu\text{g mL}^{-1}$ and $LD_{50} = 3703 \mu\text{g mL}^{-1}$, respectively), however, the isolation of the active compounds as next step of this research is necessary.

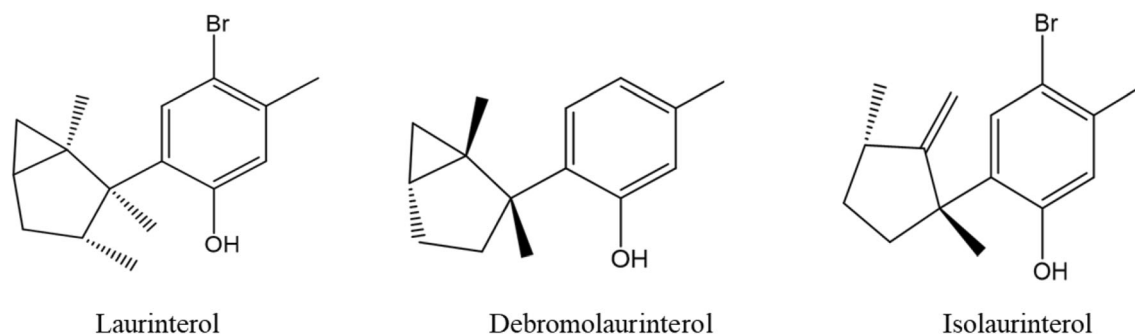


Fig. 5 Sesquiterpenes isolated from *Laurencia johnstonii* C1F3 fraction

According to our results, the use of macroalgae as insecticides and repellents represents a viable alternative in the future. Seaweeds products provide additional protection to the plant and stimulated growth. It makes them attractive candidates for agriculture. Also, these products could have better ecological acceptance compared to chemical compounds. Chemical diversity in the marine environment remains a resource unexplored for the development of new agrochemicals (Peng et al. 2003).

Conclusion

Laurencia johnstonii extract showed the highest repellent and insecticidal effect against HLB vector *Diaphorina citri*. The main active metabolite isolated was laurinterol, although the mechanism of action is still unknown.

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