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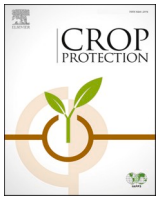

Microemulsion of essential oil of *Citrus aurantium* var. *dulcis* for control of *Aleurocanthus woglumi* and evaluation of selectivity against *Aschersonia aleyrodis* and *Ceraeochrysa cornuta*

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ABSTRACT

The aim of this study was to develop a microemulsion from the essential oil of *Citrus aurantium* var. *dulcis* (EOCA) as an alternative to synthetic pesticides, and to evaluate its efficacy against *Aleurocanthus woglumi* and selectivity towards the natural enemies *Aschersonia aleyrodis* and *Ceraeochrysa cornuta*. Chemical composition of the essential oil determined by gas chromatography coupled with mass spectrometry revealed limonene, myrcene, linalool and α-pinene as major components. The microemulsion was prepared using a ternary diagram illustrating the composition of water, essential oil, surfactant (procetyl AWS) and co-surfactant (ethanol). Selected microemulsions were characterized by polarized light microscopy, revealing to be isotropic with a Newtonian behaviour when measuring the shear stress against shear rate. The mean droplet size of microemulsions ranged from 53.69 nm to 2.22 μm. The insecticidal activity was evaluated by assessing three application routes, namely, contact, systemic and translaminar. Selectivity of microemulsions was determined by the percentage of growth inhibition (PGI) of fungi on *A. aleyrodis* and by contact on eggs and larvae of *C. cornuta*. The oil (LC_{50} = 36.07 mg/mL) in its pure form had a lower insecticidal effect on *A. woglumi* than the microemulsions (LC_{50} = 18.65 mg/mL). The microemulsions showed systemic insecticidal activity with approximately 90% mortality at 72 h (LC_{90} = 55.50 mg/mL). On *A. aleyrodis*, the microemulsion interfered with biomass production, which was concentration dependent. At a concentration of 15.68 mg/mL, a 91% inhibition of biomass production were observed. In the selectivity experiments against *C. cornuta*, the microemulsions were selective for eggs and larvae, and did not interfere with its predatory capacity.

1. Introduction

In recent years, there has been growing concern about the environmental and health impacts of synthetic pesticides used in agriculture. As a result, researchers and practitioners have been actively exploring more sustainable and eco-friendly alternatives to combat pests without compromising their efficacy. One such promising avenue is the use of botanical-based pesticides, which offer a greener and potentially safer approach to pest management (Ngegba et al., 2022). The production of biopesticides for agricultural pest control has had a growth rate of more

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than 70% yearly in Brazil (Global, 2023; Wang et al., 2019). Botanical pesticides, derived from plant extracts and essential oils, have emerged as a viable and attractive option due to their inherent biodegradability, low toxicity to non-target organisms, and reduced potential for developing resistance among target pests (Melanie et al., 2022). Among botanical products, essential oils from various plant sources have gained particular attention for their diverse chemical compositions and potent bioactive properties (Chenni et al., 2020; Wang et al., 2019). Citrus essential oils have gained considerable attention as promising biopesticides in the field of agriculture (Campolo et al., 2020). Extracted from various citrus fruits, such as oranges, lemons, grapefruits, and limes, these essential oils are rich in a diverse array of biologically active compounds (Saini et al., 2022).

Limonene, a monoterpene hydrocarbon, is one of the most abundant and well-studied components found in citrus essential oils, contributing significantly to their insecticidal properties (Assadpour et al., 2023; Sarma et al., 2019). It has been shown to be insecticidal against *Bemisia tabaci* Gennadius, 1889 (Hempitera: Aleyrodidae) (Zarrad et al., 2015). However, essential oils are volatile and unstable compounds, making them susceptible to oxidation and degradation when exposed to environmental factors (Hu et al., 2018). A widely used method to improve the physicochemical stability of these bioactive compounds and prevent degradation of the active ingredient is the emulsification process of essential oils (Cimino et al., 2021; Singh and Pulikkal, 2022; Souza et al., 2021).

Microemulsions are isotropic, transparent, thermodynamically stable liquid dispersions, usually formed by water, oil, surfactant and/or co-surfactant, with mean droplet size between 100 and 400 nm (Souto et al., 2022). Microemulsions have emerged as effective carriers for essential oils and other active ingredients (Cimino et al., 2021). Their unique properties, including increased stability, enhanced bioavailability of loaded bioactives, and improved penetration/absorption through biological barriers, make them an ideal candidate for the delivery of botanical-based pesticides (Jalali-Jivan et al., 2019).

Interest in the use of natural products for insect pest control, including to manage the citrus blackfly, *Aleurocanthus woglumi* Ashby, 1915 (Hemiptera: Aleyrodidae), is increasing (Carvalho et al., 2017). This pest is native to southwestern Asia and is widespread in much of the world (Africa, North, Central, and South Americas); it is of significant agricultural importance as it infests over 300 host plants, with citrus species being its primary host (Lopes et al., 2013). This insect poses a threat to Brazilian fruit farming due to the damage it causes, and the impacts that the adopted control measures can have on natural ecosystems, as its control has primarily been carried out using synthetic chemical insecticides (AGROFIT, 2023; Lemos et al., 2006).

In addition, when assessing the potential of bioactive substances, it is crucial to consider their selectivity towards non-target organisms, including natural enemies, for possible use in integrated pest management (Ngegba et al., 2022). The entomopathogenic fungus *Aschersonia aleyrodis* (Webber, 1897) and the predator *Ceraeochrysa cornuta* Navás, 1925 (Neuroptera: Chrysopidae) are examples of natural enemies. The former causes epizootics in citrus blackflies (Liu et al., 2012), whereas the second is commonly found in citrus orchards. *Ceraeochrysa cornuta* Navás is native to the Americas, with the majority of its species occurring in forests and agricultural areas of Neotropical regions (Tauber and Flint, 2010).

The aim of this study was to formulate a microemulsion using the essential oil of sweet orange (*C. aurantium* var. *dulcis*) and to evaluate its potential to control the citrus blackfly (*A. woglumi*), while assessing its selectivity towards the natural enemies *A. aleyrodis* and *C. cornuta*. Overall, this study contributes with valuable insights in formulating an innovative microemulsion delivery system based on *Citrus aurantium* var. *dulcis* essential oil. Its potential as an effective and selective botanical-based pesticide against the citrus blackfly, while preserving populations of beneficial natural enemies, was assessed. These findings hold great promise for sustainable pest management strategies and contribute to reducing the need of synthetic pesticides in agriculture.

2. Materials and methods

2.1. Materials

Sweet orange (*Citrus aurantium* var. *dulcis*) essential oil (EOCA) was obtained through cold pressing of the peel of fruit purchased from Ferquima Indústria & Comércio LTDA (Vargem Grande Paulista, São Paulo, Brazil). The surfactants used in the study included Procetyl AWS (PPG-5-Ceteth-20) donated by Croda Internationals (Snaith, United Kingdom), Tween 80 (polysorbate 80) and pure ethyl alcohol commercially obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Gas chromatography (GC) coupled to mass spectrometry (MS)

The essential oil composition was analyzed by gas chromatography coupled to mass spectrometry (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan). The separations were carried out in a ZB-5 chromatographic capillary column (5% phenyl-95% dimethylpolysiloxane in dimensions of 30 m × 0.025 mm x 0.25 μm). The injection temperature was 200 °C (split mode). The oven temperature was programmed from 40 °C (isotherm for 1 min), increasing at 10 °C/min to 200 °C. For GC/MS, the molecules were ionized at a temperature of 250 °C and the same temperature was used at the interface in 17 min of run and injection of 1 μL. Data processing was performed with GCSolution software using the NIST library (NIST, 2023). The method used for the identification of the compounds was the comparison of the mass spectrum of the determined compound with the NIST chromatographic library, as well as the comparison of the retention indices of the compound in relation to the compounds listed in the literature and in the NIST virtual library.

2.3. Preparation of microemulsions

The ternary diagram shows the composition of water, EOCA, surfactant (procetyl AWS) and co-surfactant (ethanol) at each of the three vertices. The formulation concentrations ranged from 1% to 90% for water and essential oil, and from 9% to 85% for surfactant and co-surfactant (in a 2:1 ratio). The mixture of surfactant and co-surfactant was stirred for 24 h using a magnetic stirrer (Velp Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA). The essential oil was added to the surfactant mixture and stirred magnetically for 30 min, followed by the slow addition of the aqueous phase under stirring for 2 h. The mixtures were allowed to equilibrate for 48 h for subsequent visual analysis to assess the risk of phase transitions of the microemulsions (ME), i.e., from (i) transparent and fluid liquid; (ii) non-fluid liquid crystal; (iii) whitish emulsion to (v) phase separation. Fig. 1 shows the images of the microemulsions developed as depicted in Table 1, confirming that a transparent and fluid liquid was obtained for all four formulations. The observed results were plotted in SigmaPlot to visualize the possible system transitions (Souza et al., 2021).

The microemulsion formulations (Table 1) with the highest concentration of essential oil were diluted in water to observe their dispersion capacity at the time of application and their stability after dilution. Subsequently, samples containing a high amount of the citrus essential oil were subjected to characterization analyses.

2.4. Polarized light microscopy

The samples were characterized using Polarized Light Microscopy with an Olympus BX-51 microscope (Olympus, Tokyo, Japan), which was coupled to a digital camera (CL Color Evolution – model PL-A662), and image analysis software (model Pixer LINK) (Ferreira et al., 2015).

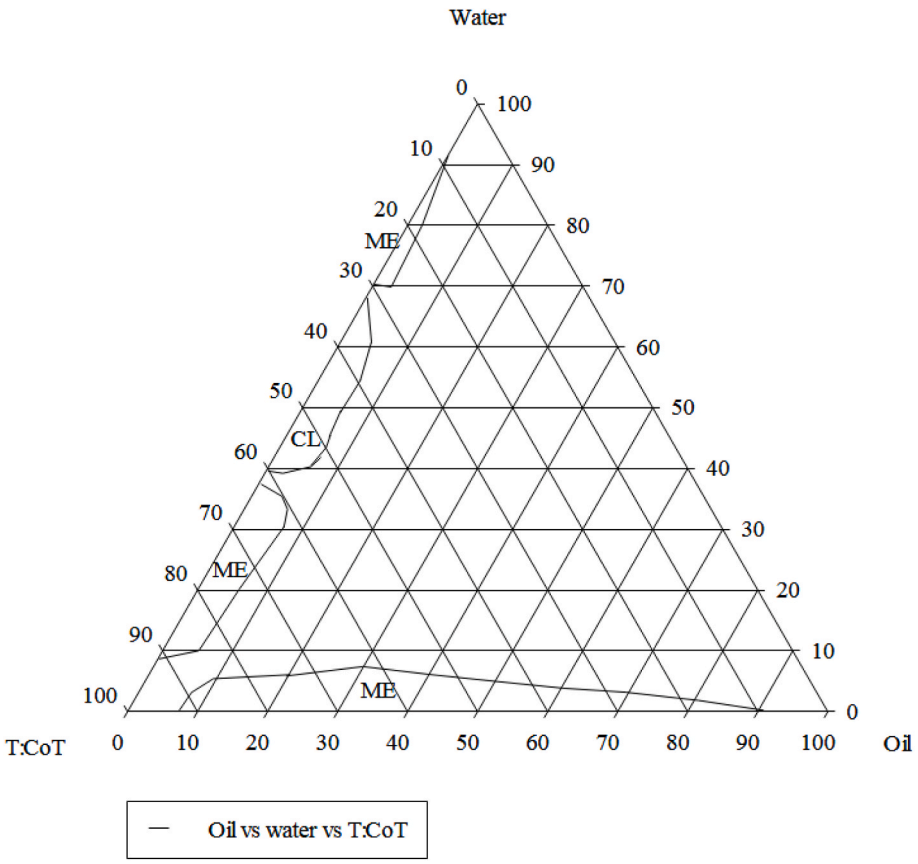

Fig. 1. Pseudo ternary diagram obtained from a combination of procetyl/ethanol 2:1, essential oil of *Citrus aurantium* var. *dulcis* and water (Captions: ME, microemulsion; LC, liquid crystal).

Table 1
Composition of the developed microemulsions based on orange essential oil selected for further characterization (Captions: EOCA, essential oil of *Citrus aurantium* var. *dulcis*).

Formulation code	EOCA (%)	Aqueous phase (%)	Surfactant/co-surfactant 2:1 (%)
F4	40	6	54
F5	50	5	45
F6	60	4	36
F7	70	3	27

2.5. Rheology

Rheological parameters were determined using a Compact Modular Rheometer (MCR-302, Anton Paar, Ostfildern-Scharnhausen, Germany) equipped with a conical geometry (1° angle, 49.97 mm diameter and 96 μm plate spacing). Shear stress was evaluated by applying a shear rate ranging from 0.1 to 200 s^{-1}. All analyses were performed at room temperature and in triplicate.

2.6. Mean particle size and polydispersity index

Average particle size (z-Ave) and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer series (Malvern Instruments, Malvern, UK). Samples were diluted 100-fold with Milli-Q water adjusted to a conductivity of 50 μS/cm and analyzed in triplicate (n = 3) (10 runs per measurement, 30 in total). Data were expressed as arithmetic mean ± standard deviation (SD).

2.7. Biological assays

2.7.1. Estimation of lethal concentration (LC) on citrus blackfly

The bioassays were conducted at 25 ± 2 °C and relative humidity of 70 ± 10%. To estimate the LC of essential oil and microemulsion on citrus blackfly, infested leaves were collected from a sweet orange orchard located in the municipality of Estância (Sergipe, Brazil). Preliminary experiments were conducted to determine the concentration range of samples that achieved a mortality range between 0% and 100% of citrus blackfly nymphs. A minimum of five concentrations is needed to estimate the LC (Ejeta et al., 2021; Silva et al., 2018). For the essential oil, concentrations of 12.65, 16.86, 25.29, 33.72, 46.37 and 50.59 mg/mL were firstly prepared, which were then diluted in an aqueous solution of two surfactants (polysorbate and PPG-5-Ceteth-20) at a concentration of 0.4% in water. For the determination of LC of essential oil, the control group consisted of the aqueous solution of surfactants in distilled water. For the estimation of the LC of microemulsion, concentrations of 4.48, 8.96, 17.92, 26.88 and 44.8 mg/mL were prepared in distilled water. For this sample, the control group consisted of leaves without any treatment. Each treatment/concentration used 10 leaves each infested with a minimum of 20 2nd instar citrus blackfly nymphs, collected from a sweet orange orchard. To ensure that only 2nd nymphs were present, the leaves infested with the insects went through a sorting and cleaning process with a stylus and soft bristle brush to remove impurities and other stages of insect development. Each leaf served as a single replicate. Prior to the experiments, the infested leaves were cleaned under a stereoscope to remove dead insects and field debris. The leaves were then immersed in 15 mL of the respective treatment solution, removed, allowed to dry naturally and placed in pots with the petiole in contact with distilled water to prevent dehydration. Mortality was assessed 24 h after application and confirmed by piercing the

nymphs with an entomological needle.

2.7.2. Translaminar and systemic effect

The LC_{90} of citrus oil-loaded microemulsion was applied to the adaxial side of citrus leaves infested on the abaxial side with 2nd instar citrus blackfly nymphs. Application was made by spraying with an electric "Paasche Airbrush" micro-atomizer (ASW-18 model, Sagyma pro, Manaus, Brazil) at 5 pounds of pressure on infested leaves with an average of 30 insects/leaf. In the control group, 10 leaves were sprayed individually with distilled water and, in the treatment group, 10 leaves were sprayed with the microemulsion LC_{90} (55.50 mg/mL), each leaf being considered a replicate. After spraying, the petiole was placed in plastic microtubes (2 mL) in contact with distilled water.

The systemic effect of the microemulsion was also evaluated. For this purpose, 20 leaves infested with 2nd instar citrus blackfly nymphs were used. In the treatment, the petiole of 10 leaves remained in contact with the microemulsion (concentration of 55.50 mg/mL) in plastic microtubes (2 mL) until evaluation, while in the control the petiole of 10 leaves remained in contact with distilled water. Nymph mortality was assessed after 24, 48 and 72 h of exposure, with nymphs with dehydrated tegument considered dead.

2.7.3. Selectivity of the microemulsion to the entomopathogenic fungus Aschersonia aleyrodis

The entomopathogenic fungus *Aschersonia aleyrodis* was obtained from Emdagro's Bank of Entomopathogenic Fungal Isolates, located at the Laboratory of Biotechnological Pest Control (LCBiotec). To evaluate the selectivity, five concentrations of citrus oil-loaded microemulsion (1.12, 6.72 and 11.2, 13.44 and 15.68 mg/mL), previously set by preliminary experiments (Ejeta et al., 2021; Silva et al., 2018), were used, in addition to a control receiving no application. Each concentration of the microemulsion was added to 4 mL of commercial liquid medium (potato dextrose broth) with antibiotics (0.25 g/L chloramphenicol and 0.20 g/L tetracycline) and transferred to flat-bottomed glass tubes (25 mL). The culture medium containing the microemulsion was inoculated with 1 mL of the entomopathogenic fungus suspension containing 0.02 g biomass per mL. The tubes were kept on a shaker under stirring (150 rpm and 25 $\pm$ 1 °C) for 7 days. For each tested concentration of the microemulsion, this procedure was done five times (with each tube considered a replicate), whereas the control consisted of the culture medium without the addition of the microemulsion. After 7 days, the fresh biomass of the fungus was quantified. To quantify the fresh biomass of the fungus each treatment was centrifuged in a Falcon tube (15 mL) and weighed on a precision balance (Cubis®II, Sartorius, Göttingen, Germany), eliminating the weight of the empty tube. From the biomass results, the percentage of growth inhibition (PGI) of fungi was determined using Equation (1).

$$PGI = \left(\frac{GC\text{-}GT}{GC}\right) \times 100 \qquad \text{(Equation 1)}$$

where GC (growth control) stands for the mean diameter of fungi grown in broth, and GT (growth treatment) represents the mean diameter of fungi exposed to microemulsions.

2.7.4. Selectivity of the microemulsion to the predator Ceraeochrysa cornuta

The selectivity of the sweet orange oil-loaded microemulsion was assessed on eggs, larvae and on the predatory capacity of the predator *C. cornuta*, originating from a colony at the Laboratory of Biotechnological Pest Control (LCBiotec/EMDAGRO), using the LC_{90} (55.50 mg/mL) of the microemulsion as treatment. To test whether the microemulsion affected egg viability, lacewing eggs were collected on three consecutive days and used in the bioassays. On each day of collection, 96 eggs were individualized in an Elisa-type plate covered with perforated polyvinyl chloride (PVC) film with an entomological pin for ventilation, thus forming the control group. Meanwhile, another 96 eggs were treated with the LC_{90} microemulsion by spraying with an electric "Paasche Airbrush" micro-atomizer (ASW-18 model, Sagyma Pro, Manaus, Brazil) at 5 pounds of pressure. After natural drying, these eggs were also placed in an Elisa-type plate as in the control group. In the evaluation, eggs were considered viable when larvae were observed to emerge, with each line of the plate (12 eggs) considered as a replicate, giving a total of 8 replicates per day in each group.

In the larval selectivity test, 84 first instar larvae, between 12 and 24 h after hatching, were separated and sprayed with a micro-atomizer at 5 pounds of pressure. In the control group, 42 larvae were sprayed with distilled water only, while in the treatment group 42 larvae were sprayed with the microemulsion. All larvae were individualized in Petri dishes (Ø 6.5 × 1.5 cm), fed with eggs of *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: *Pyralidae*) and evaluated after 24 and 48 h to check for the presence of dead larvae. To test the predatory capacity of lacewing larvae after treatment with the microemulsion, 26 first instar lacewing larvae were individually sprayed between 12 and 24 h after hatching in petri dishes (Ø 6.5 × 1.5 cm) with a micro-atomizer at 5 pounds pressure. In the control group, 13 larvae were sprayed with distilled water and in the treatment group, 13 larvae were sprayed with the microemulsion. After spraying, the larvae were individually placed in Gerbox-type boxes (11 x 11 × 3.5 cm) to which the prey, 2nd instar citrus blackfly nymphs were offered. In this bioassay, each lacewing larva represents a replicate and in each group the prey was offered at two densities, 80 and 130 nymphs. Thus, for the density of 80 prey, 9 lacewing larvae/replicate were used per group and, for the density of 130 prey, 4 replicates were used. The assessment was made after 12 and 24 h by counting the number of preys consumed.

2.8. Statistical analysis

To estimate the lethal concentration (LC) of citrus oil and microemulsion on citrus blackfly, mortality data obtained from bioassays were submitted to probit analysis using PoloPlus software (PROBIT; PoloPlus version 1.0). Insect mortality over time (24, 48 and 72 h) were compared using an ANOVA with translaminar and systemic treatments as fixed effects, followed by. mean comparisons using Tukey's test at 5% significance using GraphPad Prism 8.0.1 statistical software. Selectivity data for the entomopathogenic fungus *A. aleyrodis* were subjected to regression analysis using GraphPad Prism 8.0.1. For this analysis, the biomass content of the fungus was set as the dependent variable and the concentration of the microemulsion was set as the independent variable. Selectivity data for the predator *C. cornuta* were compared using t-tests [SAS PROC TTEST procedure: pooled or Satterthwaite, considering equality of variances]. For this analysis, egg viability, larvae mortality, and predatory capacity were set as the dependent variables.

3. Results

3.1. Physicochemical characterization

3.1.1. Gas chromatography (GC) coupled to mass spectrometry (MS)

The chromatographic analysis of orange essential oil showed eight components identified by the retention time and percentage of relative area (Table 2). The concentration of the identified components in orange essential oil ranged from 0.14% to 95.14%, corresponding to 100% of the oil composition. The main constituents present in orange oil were limonene (95.14%) as the major one, followed by myrcene (2.44%), linalool (0.66%) and α-pinene (0.54%), which correspond to 98.78% (Table 2).

Other studies reported the variation in the concentrations of the main components of orange oil, but have also observed limonene as the main compound, confirming the result obtained in our present study (de Araújo et al., 2020; Giunti et al., 2019; Zarrad et al., 2015). This

Table 2
Compounds identified in orange essential oil by gas chromatography (GC) coupled to mass spectrometry.

Peak	Name	Retention Time	Relative Area%
1	α -Pinene	8.459	0.54
2	Sabinene	9.639	0.46
3	Myrcene	10.143	2.44
4	n-Octanal	10.500	0.28
5	α-Terpinene	10.767	0.14
6	Limonene	11.458	95.14
7	Linalool	13.509	0.66
8	n-Decanal	16.697	0.34

variation may be related to several factors, such as plant genetics, collection technique, processing, cultivation techniques and geographical area of planting (Ferronatto and Rossi, 2018).

3.1.2. Preparation of microemulsion

The pseudo ternary diagram built to obtain the microemulsion is displayed in Fig. 1, in which only two regions - microemulsion region and liquid crystal region – are observed.

3.1.3. Polarized light microscopy

To identify the isotropy or anisotropy of the system, samples (F4 – F7) were analyzed by polarized light microscopy (Fig. S1, Supplementary Material). The formulations showed isotropy (dark field), which means that the samples under the plane of polarized light do not deviate the light, which is a typical behavior of microemulsion-based systems.

3.1.4. Rheology

The results of the rheological characterization of the microemulsions are shown in Fig. 2 and Table 3. The formulations followed a Newtonian behavior highlighted by the linearity in response to shear stress (Pa) against shear rate (s^{-1}), as well as against the flow behavior of the microemulsions, which was equal to or very close to 1. These formulations also showed low viscosity indicated by the low consistency index (K), with formulation 4 (F4) being more viscous. It was observed that the viscosity decreased as the oil concentration increased and the T/CoT mixture decreased. In F4, the value of n was slightly above 1 (n = 1.0016), indicating a very slight shear thickening behavior. This means that as the shear rate increases, the orange essential oil formulation may exhibit a slight increase in viscosity. The K was 0.0240, which suggests

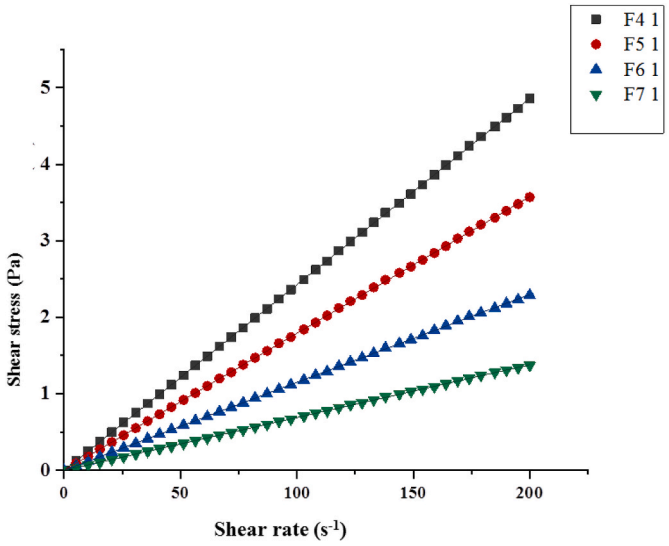

Fig. 2. Rheological behaviour of orange essential oil formulations (F4–F7), recording the shear stress (Pa) in response to the shear rate ranging from 0.1 to 200 s^{-1}.

Table 3
Flow behaviour (n) and consistency index (K) of orange essential oil-loaded microemulsions.

Formulation code	n	K
F4	1.0016	0.0240
F5	0.9976	0.0181
F6	0.9988	0.0115
F7	1.0018	0.0068

Each value represents the average of the triplicates.

that the formulation has moderate resistance to flow. A higher K value indicates higher viscosity, so this formulation may have a moderately thicker consistency compared to other samples.

In F5, the value of n was very close to 1 (n = 0.9976), indicating almost a Newtonian flow behavior. This means that the orange essential oil formulation has a constant viscosity regardless the applied shear rate. The K was 0.0181, suggesting that this formulation has lower resistance to flow compared to F4. It may have a slightly thinner consistency due to its lower K value.

F6 was similar to F5, i.e., the value of n was also close to 1 (n = 0.9988), indicating nearly Newtonian flow behavior for this formulation as well. The K was 0.0115, which is lower than the values of F4 and F5. This suggests that F6 has the lowest resistance to flow among the presented samples and is likely to have the thinnest consistency.

In F7, the value of n was slightly above 1 (n = 1.0018), indicating a slight shear thickening behavior for this formulation, similar to F4. The K was 0.0068, which is the lowest among all samples. This indicates that F7 has the least resistance to flow and is likely to have the thinnest consistency among all the formulations.

3.1.5. Mean particle size and polydispersity index

The mean droplet size (z-Ave) of the formulations ranged from 53.69 nm to 2.22 μm. While all formulations were monomodal with respect to size distribution, formulation F6 had the smallest average droplet size 53.69 nm with a PDI of 0.491 (Table 4).

Formulation F4 had a z-Ave of 2.22 μm, with a PDI of 0.552, which translates a moderate droplet size distribution, in comparison to F6 and F7, with F7 having the lowest PDI (0.312 ± 0.118). F6 was found to be highly polydispersed, since PDI was close to 1. F4 was then selected for further studies. To assess the droplet size of F4 by the time of application, the microemulsion was firstly diluted to the LC_{90} concentration for citrus blackfly, and then DLS analysis was performed. The average droplet size was reduced to 352.6 ± 6.108 nm and the PDI was reduced to 0.398 ± 0.019. The water content influenced the droplet size, and its distribution showed a significant decrease in comparison to the non-diluted sample (2222.0 ± 160.2 nm for z-Ave, and 0.552 ± 0.232 for PDI).

3.2. Biological assays

The LC_{50} and LC_{90} of the population of 2nd instar nymphs of citrus blackfly, estimated for the sweet orange essential oil and for the microemulsion based on this oil, ranged between 18.65 and 66.98 mg/mL (Table 5). When comparing the two treatments to achieve the same level of nymph mortality after 24 h of exposure, the microemulsion generally required lower concentrations (LC_{50} = 18.65 mg/mL, LC_{90} =

Table 4
Mean droplet size (z-Ave ± standard deviation (SD) and polydispersity index (PDI) of orange essential oil-based microemulsions.

Formulation code	z-Ave ± SD (nm)	PDI ± SD
F4	2222.0 ± 160.2	0.552 ± 0.232
F5	365.5 ± 105.7	0.941 ± 0.058
F6	53.69 ± 16.74	0.491 ± 0.057
F7	73.79 ± 37.22	0.312 ± 0.118

Table 5
Estimated lethal concentration (LC), in mg/mL, of essential oil of *C. aurantium* var. *dulcis* and microemulsion on 2nd instar nymphs of *A. woglumi* after 24 h.

Treatments	N[(1)]	DF [(2)]	LC_{50} (CI 95%) [(3)]	LC_{90} (CI 95%)[(3)]	Slope (β ± SE)[(4)]	χ^{2}[(5)]	H[(6)]
Essential oil	3588	4	36.07 (34.07–37.81)	66.98 (62.80–72.82)	4.77 ± 0.37	2.95	0.73
Microemulsion	2032	3	18.65 (16.85–20.41)	55.50 (48.24–66.58)	2.70 ± 0.21	2.25	0.75

Captions.
(1) Total number of nymphs.
(2) Degree of Freedom for chi-square (χ^2) test.
(3) Lethal Concentration with Confidence Interval at 95% probability.
(4) Angular coefficient of the line ± standard error (SE).
(5) Chi-square.
(6) H - Heterogeneity.

55.50 mg/mL) than the pure essential oil (LC_{50} = 36.07 mg/mL, LC_{90} = 66.98 mg/mL). From the recorded LC_{50}, the microemulsion was found to be more toxic than the pure essential oil; yet less conclusive results were obtained with LC_{90} since confidence intervals overlap.

For the translaminar route of action, no significant difference was observed between the microemulsion and the control in relation to insect mortality during the three evaluations [F(2,45) = 5.046; p = 0.9307]. The mortality of citrus blackfly nymphs by systemic action was 46.92, 76.38 and 87.32% after 24, 48 and 72 h of exposure of the leaf petiole to microemulsion, respectively (Fig. 3).

In the selectivity of the microemulsion on the entomopathogenic fungus *A. aleyrodis*, the biomass production of the fungus was found to be dependent on the concentration of the microemulsion (Fig. 4), ranging from 0.032 g/mL to 0.341 g/mL ($P < 0.0038$). The percentage of biomass inhibition of the fungus at concentrations of 1.12, 6.72, 11.2 and 13.44 mg/mL was 4.5% (0.341 g/mL), 59.25% (0.145 g/mL), 72.46% (0.098 g/mL) and 90.62% (0.033 g/mL), respectively, reaching 91% (0.032 g/mL) at the concentration of 15.68 mg/mL, while the control produced 0.357 g/mL of biomass ($P \leq 0.0038$) (Fig. 4).

The treatment with the microemulsion did not affect the viability of *C. cornuta* eggs, with a total of 80.79 ± 2.26 and 78.12 ± 2.87 % (t1.46 = −0.73, P > 0.4697) hatching of the lacewing larvae from the eggs of the control and treatment groups, respectively. Similarly, no significant difference was observed in the proportion of hatched larvae between the

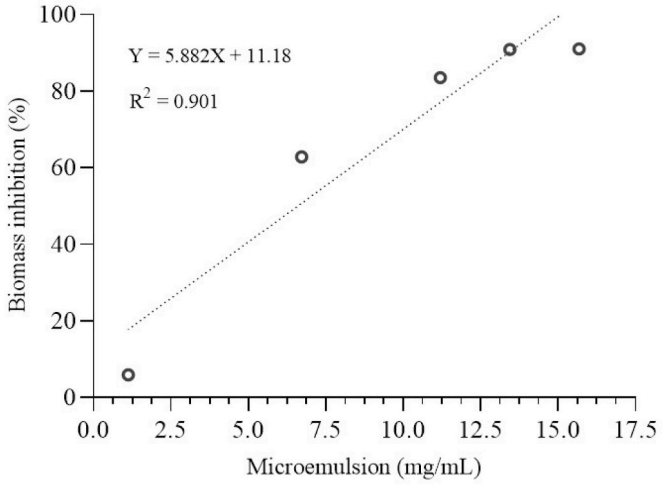

Fig. 4. Percentage of inhibition of the mycelial growth of *Aschersonia aleyrodis* treated with the microemulsion based on the essential oil of *Citrus aurantium* var. *dulcis*.

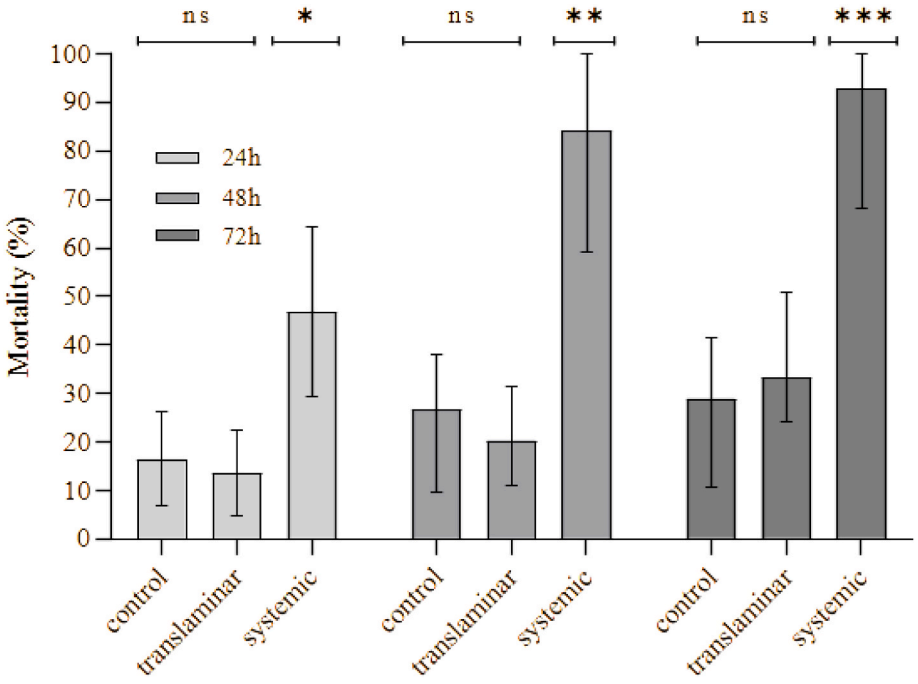

Fig. 3. Effect of LC_{90} of citrus essential oil microemulsion via systemic action and translaminar action on 2nd instar nymphs of citrus blackfly with exposure at 24, 48 and 72 h.

control and treatment group on any of the three days of egg collection and evaluation.

In the selectivity test on 1st instar larvae of *C. cornuta*, the microemulsion based on essential oil of *C. aurantium* var. *dulcis* was highly selective, with 100% survival of the predator larvae even after treatment with the microemulsion.

Regarding the predatory capacity test of 1st instar larvae of *C. cornuta* treated with the microemulsion on the citrus blackfly, *A. woglumi*, the microemulsion did not interfere in the predation capacity regardless of the density of prey offered and the time of exposure of the prey to the predator (Fig. 5). Only an increasing consumption of blackfly nymphs by the predator was observed with increasing supply/exposure time in the control and treatment groups, regardless of the density of prey offered. Thus, at the end of 24 h of exposure to the predator, at the density of 80 prey, 44.55 ± 4.59% of the prey had been predated by the larvae of the lacewing treated with the microemulsion, while in the control group a total of 38.66 ± 3.72% was observed, which were statistically identical ($t1.16 = 1.00$, $P > 0.3340$). For the density of 130, the 1st instar larvae of *C. cornuta* treated with the microemulsion predated a total of 42.8 ± 8.23% of prey and in the control group they predated 44.5 ± 10.43% of prey after 24 h of exposure, which were statistically identical ($t1.7 = -0.13$, $P > 0.9003$).

Thus, the microemulsion of the essential oil of *C. aurantium* var. *dulcis* was found to be selective to the eggs and larvae of the lacewing *C. cornuta* and did not affect the predatory capacity of this predator.

4. Discussion

The mean droplet size (z-Ave) and polydispersity index (PDI) provide insights into the size distribution of droplets of the orange essential oil-loaded microemulsions, as well as their long-term stability. F4 had the largest mean droplet size whereas F7 had the lowest PDI, indicating a less polydispersed size distribution with a narrower spread of droplet sizes. These results are important for the understanding of the stability and efficacy of the formulations, as droplet size and distribution can affect factors such as absorption, surface area, and dispersibility. This finding aligns with previous studies highlighting the ability of microemulsion formulations to achieve small droplet sizes and improve the efficacy of pesticide delivery (Shao et al., 2018; Souza et al., 2021). Additionally, the stability analysis demonstrated that formulation F4 maintained the characteristics of a microemulsion after dilution, indicating its suitability for further bioassays.

Citrus essential oil and the microemulsion obtained from citrus oil were toxic to citrus blackfly. These results are in agreement with previous studies, which showed that the insecticidal action of orange essential oil is related to its major constituent, limonene, which can interact with the neurotransmitter system (Giunti et al., 2019; Leite-Andrade et al., 2022; Liu et al., 2019), resulting in increased toxicity. The dominating compound in most citrus species is limonene, with other common compounds including linalool, pinene, β-caryophyllene, β-myrcene, terpinene and citral (Bulbuli et al., 2021). The insecticidal action of citrus oil is often related to the activity of major constituent of citrus oil present in the crude oil. For instance, limonene can inhibit the enzyme acetylcholinesterase (AChE), which causes constant stimuli from the neurotransmitter acetylcholine ACh, leading to hyperexcitation, paralysis and death (Santos et al., 2021; Zarrad et al., 2015).

According to the LC_{50}, the microemulsion exhibited higher toxicity, in comparison to the pure essential oil. This increased toxicity can likely be attributed to the improved solubility and bioavailability of the active compounds in the microemulsion formulation. The greater efficacy of microemulsified oil compared to pure oil was also shown for a microemulsion based on *Citrus sinensis* essential oil, which caused 90% mortality to *Galleria mellonella* Linnaeus, 1758 (Lepidoptera: Pyralidae) larvae *in vivo*, while pure oil killed only 45% (Souza et al., 2021). The greater toxicity of the microemulsion may be related to the increased contact surface due to the decrease of the size of its droplets, thus improving permeability and action on the target organism (Tiburtino et al., 2015). For the translaminar route of action, there was no significant difference in insect mortality between the microemulsion and the control group, indicating that the microemulsion did not exhibit enhanced translaminar activity. However, the microemulsion showed systemic insecticidal activity, with increasing mortality rates of citrus blackfly nymphs over 24, 48, and 72 h of exposure to the treated leaf petioles. This systemic activity suggests that the active compounds in the microemulsion can be absorbed and translocated within the plant, providing prolonged insecticidal effects. In the translaminar effect, the physicochemical properties of the active ingredient are responsible for determining the degree of its redistribution in the leaf mesophyll (Klittich et al., 2020). Systemic pesticides, on the other hand, are translocated by the plant through the sap conducting vessels, in an upward direction (Elbert et al., 2008). Sap-sucking pest insects, such as the citrus blackfly, when feeding on the plant, can ingest the active ingredients of the product, thereby reducing pest populations (Branco et al., 2023).

Our study successfully formulated a microemulsion using the essential oil, and the results showed isotropic behavior under polarized light microscopy, indicating the formation of a stable microemulsion system. This aligns with previous research that demonstrated the successful formulation of essential oil-based microemulsions with enhanced stability and efficacy (Shao et al., 2018; Souza et al., 2021). Microformulations are able to induce systemic activity due to the smaller droplet size, allowing the microemulsion to be translocated by the plant through the xylem or phloem. Systemic activity in products with insecticidal activity is desirable, since only phytophagous insects will directly consume the active ingredients, reducing their population without directly affecting beneficial insects (Saito, 1969; Sánchez-Bayo, 2021). In addition, it is possible to decrease spray coverage, enabling the

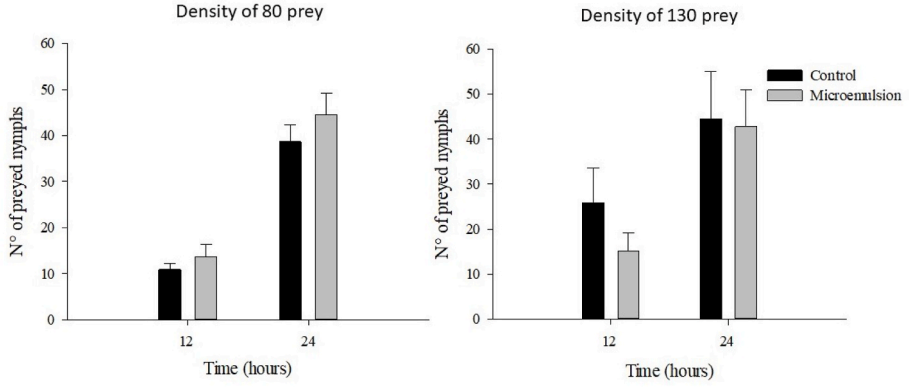

Fig. 5. Predatory capacity of *Ceraeochrysa cornuta* larvae treated and untreated with the microemulsion of sweet orange essential oil *C. aurantium* var. *dulcis*, on 2nd instar nymphs of *A. woglumi* at densities of 80 and 130 prey, after 12 and 24 h of exposure.

use of lighter application equipment, as well as decrease the cost of application (Saito, 1969). The rheological characterization of the microemulsions revealed a Newtonian behavior, characterized by a linear response to shear stress and a consistency index close to 1. The flow behavior index (n) and consistency index (K) are rheological parameters that describe the viscosity and flow characteristics of the orange essential oil formulations. Out study showed that F4, F5, and F7 had n values slightly above 1, indicating a slight shear thickening behavior. On the other hand, F6 had an n value close to 1, suggesting almost a Newtonian flow behavior. Additionally, F4 had the highest K value, indicating the highest resistance to flow and likely a moderately thicker consistency compared to the other formulations. F7, with the lowest K value, had the least resistance to flow and is expected to have the thinnest consistency. These rheological properties are essential for understanding how the formulations will behave during processing, storage, and application.

Regarding the selectivity of the microemulsion towards *Aschersonia aleyrodis*, the biomass production of the fungus was significantly inhibited at various concentrations of the microemulsion. The inhibition increased with higher microemulsion concentrations, reaching 91% inhibition at 15.68 mg/mL. These findings indicate that the microemulsion may have a negative impact on the entomopathogenic fungus, which could potentially affect the natural control of the citrus blackfly population. However, the microemulsion did not interfere with the viability and hatching of *Ceraeochrysa cornuta* eggs. The survival rate of the predator larvae after treatment with the microemulsion was 100%, indicating the selective nature of the microemulsion towards the lacewing eggs and larvae. Furthermore, the microemulsion did not affect the predatory capacity of *C. cornuta* larvae towards the citrus blackfly nymphs, indicating its compatibility with the predator's biological control activity. Interestingly, the microemulsion exhibited higher efficacy against *Aleurocanthus woglumi* compared to the pure oil. This enhancement in insecticidal effect could be attributed to the improved solubility and bioavailability of the active compounds in the microemulsion formulation. The citrus essential oil-based microemulsion was not selective to the entomopathogenic fungus *A. aleyrodis*. Microorganisms when subjected to high concentrations of active ingredients, may have an inhibition of mycelial growth, which is not necessarily an indicator of reduction in sporulation or conidial viability and vice versa (Coster and Zimmermann, 1975). *In vitro* studies have the advantage of exposing the microorganism to higher concentrations of the tested product, i.e., it can be possible to examine potential acute effects, which is more rare under field conditions. Therefore, the high toxicity of a product *in vitro* does not always indicate its high toxicity in the field. Field trials are necessary to further examine the toxicity of the microemulsion on *A. aleyrodis*.

The selectivity of the microemulsion of the essential oil of *C. aurantium* var. *dulcis*, to eggs and larvae of the predator indicates that the product is also safe for this predator, a desirable characteristic in a bioinsecticide. Farias et al. (2020), when evaluating the selectivity of the essential oil of Tahiti acid lime (Persian lime 58) at concentrations of 5.8 and 12.5 mg/mL on 1st instar larvae of *C. cornuta*, still found mortality of 10 and 20.4% respectively. From these finding, we may anticipate the potential use of microemulsions based on essential oils to minimise toxic effects on non-target organisms (Lucia and Guzmán, 2021).

The safety of the *C. aurantium* var. *dulcis* essential oil microemulsion towards the generalist predator *C. cornuta* becomes more evident when comparing our results with selectivity tests using insecticides. For example, the viability of eggs of *Ceraeochrysa cubana* Hagen, 1861 (Neuroptera: *Chrysopidae*) subjected to 11 synthetic insecticides, among them products registered for the control of the citrus blackfly (e.g., triazophos, flufenoxuron), was reduced by an average of 70% (Carvalho et al., 2011). High mortality rates and decreased predatory capacity of second-stage larvae of *Chrysoperla sinica* Tjeder, 1936 (Neuroptera: *Chrysopidae*) were also recorded when treated with synthetic insecticides (Shan et al., 2020).

5. Conclusions

In this study, we confirmed that the major compound of *Citrus aurantium* var. *dulcis* essential oil was limonene. Citrus essential oil and its microemulsion formulation were effective in laboratory bioassays in the control of citrus blackfly, with the microemulsion having a systemic effect. The results of this study highlight the potential of microemulsions formulated with *Citrus aurantium* var. *dulcis* essential oil as effective botanical-based pesticides against citrus blackfly nymphs. The microemulsion exhibited enhanced insecticidal activity compared to the pure oil, attributed to improved solubility and bioavailability of active compounds. The microemulsion demonstrated systemic insecticidal activity, making it a potential valuable tool for prolonged pest control. However, caution is needed regarding its impact on the entomopathogenic fungus *A. aleyrodis*, as the microemulsion showed inhibitory effects on biomass production. These findings emphasize the importance of assessing the potential effects on beneficial organisms to ensure the preservation of natural control agents. On a positive note, the microemulsion displayed excellent selectivity towards *C. cornuta* eggs and larvae, while maintaining the predator's predatory capacity. This favorable characteristic supports the product's safety and compatibility with this predator.

Overall, this study provides valuable insights into the formulation and efficacy of essential oil-based microemulsions as environmentally friendly alternatives to synthetic pesticides. Further research is needed to optimize the microemulsion formulation, evaluate its efficacy in the field, and address any potential concerns related to impacts on non-target organisms. The development of alternatives to the use of synthetic chemical formulations for pest and disease control in crops is essential. The results obtained in our study are a preliminary step toward obtaining a commercial product based on byproducts of high economic value. Further research is nevertheless needed to explore the economic costs of this type of formulation and to analyze crop yields through pest management with this sustainable approach of using formulations derived from plant oils and extracts. With continued efforts and advancements in botanical-based pesticide development, we are moving closer to achieving sustainable and eco-friendly pest management practices in agriculture.

Authors contributions

Joseane de Jesus Oliveira: Conceptualization, Investigation, Draft and final versions of the manuscript. **Eliana M. dos Passos:** Investigation and Data curation. **Suely M. Alves:** Investigation and Formal analysis. **Victor H. V. Sarmento:** Investigation and Methodology. **Thiago R. Bjerk:** Investigation and Methodology. **Juliana C. Cardoso:** Investigation and Resources. **Cristina Blanco-Llamero:** Validation and Draft version of the manuscript. **Eliana B. Souto:** Validation, Research, Supervision, Project administration, Funding acquisition, Draft and final versions of the manuscript. **Patrícia Severino:** Validation, Research, Supervision, Project administration, Funding acquisition, Draft and final versions of the manuscript. **Marcelo da Costa Mendonça:** Validation, Research, Supervision, Project administration, Funding acquisition, Draft and final versions of the manuscript. All authors made a substantial contribution to this work and approved the final version of the manuscript.

Ethics issues

This work does not raise ethics issues.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2024.106586.

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