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Article

Bio-Insecticidal Potential of Tangerine Peel Oil Against Greenhouse Whitefly: A Green Biopesticide Candidate

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Abstract: The excessive use of synthetic insecticides in modern agriculture has led to the contamination of the environment and the development of insect resistance. In this study, we evaluated the potential of tangerine (*Citrus reticulata* L.) peel essential oil (EO) as a natural insecticide against greenhouse whitefly (*Trialeurodes vaporariorum* W. (Homoptera: Aleyrodidae)), a common pest in greenhouse production. Petroleum ether (PET) and n-hexane (HEX) were used as solvents to extract essential oil (EO) from tangerine peels. The yield of EO was 1.59 % and 2.00 % (m/m) for PET and HEX, respectively. The insecticidal activity of EO was evaluated by measuring the mortality rate (MR) of greenhouse whiteflies at different time intervals. The results showed that PET and HEX extracts of tangerine EO effectively controlled the greenhouse whitefly. With both solvents, it was observed that with doses of 12.5% (v/v), similar results were achieved as with the positive control (corresponding to the commercial insecticide imidacloprid). Additionally, the FTIR analysis found that the EO contained d-limonene, which may be the source of its insecticidal properties. Therefore, tangerine peel essential oil is an excellent natural insecticide candidate for controlling greenhouse whiteflies effectively and sustainably.

Keywords: *Citrus reticulata* L. peels; essential oils; solvent extraction; petroleum ether; n-hexane; natural bio-pesticide; *Trialeurodes vaporariorum* W. (Homoptera: Aleyrodidae)

1. Introduction

Modern agriculture was sustained using chemicals for much of the 20th century [1,2]. Their uses increase yields and thus guarantee the growing demand for agricultural products that a constantly growing world population demands [3]. At a global level, this revolution led to the fact that, for the first time, food production worldwide could satisfy demand [4,5]. However, some researchers noted that the extensive and often abusive use of chemicals also caused some inconveniences for agroecosystems [2,6]. These problems are evident in the case of chemical insecticides, which, by controlling insect pests that affect crops, often have adverse effects on pollinating insects that guarantee the robustness and vitality of ecosystems [7–9]. Chemical biocides (like bactericides, fungicides, and nematicides) have also had similar effects, affecting the beneficial endophytic microflora for crops [10,11].

These long-term practices have resulted in the erosion of arable land [12,13] and the loss of the necessary balance between the crops' health and the soil's microflora[14]. They have also led to the appearance of resistance genes that allow different pests to survive and develop. Such is the case with chemical insecticides. There are reports of the emergence of resistance genes among numerous insect species that have acquired various

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resistance genes to multiple types of chemical insecticides, forcing farmers to use higher doses of these chemicals and worsening the problem [15,16].

Strategies have been devised to mitigate the impact of the extensive use of chemical fertilisers and pesticides, such as integrated pest management, where chemicals are used, and other control strategies, such as biologicals and other natural products [17,18]. Among the latter are the so-called "natural" pesticides, products commonly obtained from plants [19–21]. Additionally, if the sources of these botanical pesticides are low-value agricultural or agro-industrial residues, this would aid in reducing the issues associated with excessive chemical use and these wastes and residues. Additionally, the latter would lessen the expense of treating these residues and positively impact environmental contamination [22].

Numerous studies have demonstrated the bactericidal, fungicidal, antioxidant, and insecticidal activity of essential oil extracts from citrus peel [23–27]. These qualities make it suitable for aromatherapy and homoeopathic treatments for various pathologies [28–33]. Additionally, in agriculture, when growing flowers, fruits, and vegetables, as well as when transporting and storing agricultural products, pest damage is prevented.

On the other hand, some natural substances present in fruits and plant tissues have evolved to become repellents for insects and other pests [19,20]. Such is the case with some terpenes, such as d-limonene [34–38]. This monoterpene has demonstrated its biocidal and insecticidal effects against various pests [35,36]. D-limonene is found in the peels of many citrus fruits [39]. The beneficial effects of d-limonene on human and animal health have also been reported [40,41].

The tangerine (*Citrus reticulata* L.) is a citrus fruit highly appreciated by consumers for its pleasant flavour and as a source of vitamins and minerals [42,43]. In Ecuador, in the Pimampiro canton, Imbabura province, there are relatively large mandarin plantations, highly appreciated for their quality. According to recent estimates, several hundred hectares are planted in the canton to provide enough of this popular fruit. It is even estimated that nearly 20-30% of them are wasted at the peak of the mandarin harvest, as it occurs elsewhere [44]. The overproduction of some foods, such as tangerines, brings unpleasant effects on health and the environment, especially in the areas surrounding municipal landfills. Therefore, finding some alternatives to recover these crop wastes is necessary [45–47]. In the case of mandarin, it might be convenient to find economically feasible ways to use the tangerine peel to produce essential oils with high contents of bioactive substances such as d-limonene [48].

Finally, the northern Andean zone of Ecuador (formed by the provinces of Pichincha, Imbabura, and Carchi), whose economies depend on agriculture, has recently extended the crops protected in greenhouses in recent years [49,50]. As a result, meaningful and very profitable productions, such as the cultivation of flowers, have spread throughout this region [49]. Ecuadorian Andean flowers from this region enjoy a well-earned prestige for their beauty and quality, as evidenced by demanding customers from North America, Europe, and Asia [49,51,52]. One pest affecting these crops is the greenhouse whitefly (*Trialeurodes vaporariorum* W. (Homoptera: Aleyrodidae)), which must be adequately controlled [53,54].

Chemical insecticides like neonicotinoids and organophosphorus are frequently used to control this pest. For the control of greenhouse whiteflies, imidacloprid is among the most widely used and efficient neonicotinoid insecticides.

However, organophosphorus and neonicotinoid insecticides in closed environments, such as greenhouses, have been associated with occupational diseases in greenhouse workers exposed to them [55,56] and damage from pollinating insects such as bees [57].

Studies among the children of the regular employees of the flower greenhouses in the highlands of Ecuador's Andean region found that their anxiety and school tardiness were related to their mothers' exposure to low doses of organophosphate insecticides over an extended period when the pesticides were used in greenhouses [58–63].

This work aims to test the effect of the essential oil from tangerine peels, used at various doses, on the greenhouse whitefly on a laboratory scale and thus prove the potential of using tangerine peel EO as a suitable candidate to be a natural and ecological bioinsecticide.

2. Materials and Methods

2.1. Location and origin of the tangerine fruits used.

The tangerine fruits (*Citrus reticulata* L. var. clementine) used in this work come from the Pimampiro Canton, located in the extreme east of the Imbabura province in northern Ecuador (Figure 1a, 1b, and 1c). The harvested fruits were in their optimal state of maturity when the fruit peel was easily separated from the rest of the fruit (Figures 1d, 1e, 1f, and 1g).

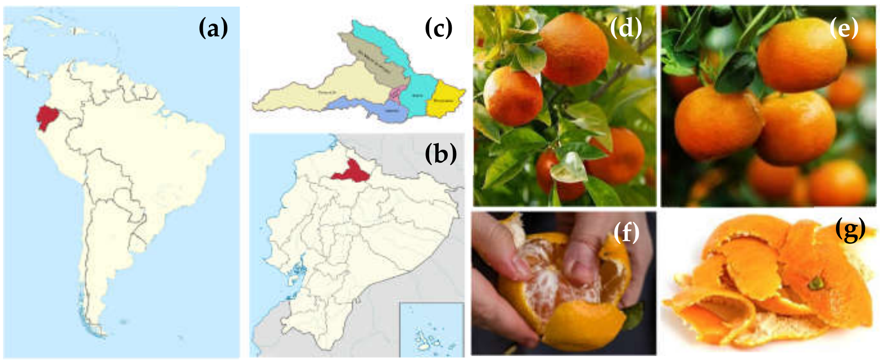

Figure 1. (a) Location of the Republic of Ecuador in South America. (b) Location of the province of Imbabura, in the northern part of Ecuador. (c) Canton Pimampiro is located in the extreme east of the Imbabura province. (d) and (e) Tangerine fruits (*Citrus reticulata* L.) in their optimal state of harvest. (f) Easy manual removal of the tangerine peel. (g) Tangerine peels.

Before separating the peels, the tangerine fruits were washed with tap water to remove dust that might have adhered during transportation. After removing the peels (Figure 1g), they were cut into small 5-10 mm pieces to facilitate the essential oil extraction.

2.2. Solvent extraction at laboratory scale

The extracts were obtained in a 250 ml Soxhlet apparatus, where 50 g of tangerine peel was added, and petroleum ether or n-hexane was used as the solvent, similar to that reported elsewhere [64]. Approximately 4 hours after the start of the extraction, it was considered finished. Next, the extract obtained was removed from the solvent in a vacuum rotary evaporator until it was observed that no traces of solvent condensed. This last process did not exceed, in any case, an hour and a half (Figure 2).

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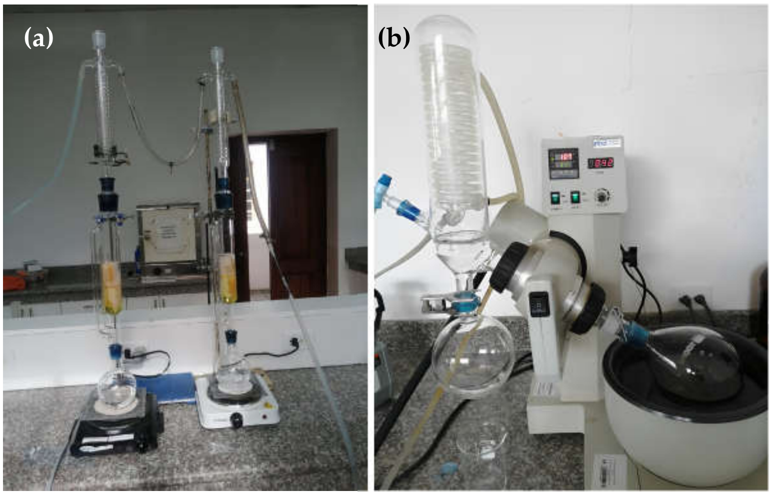

Figure 2. Experimental set for the extraction with solvents of the essential oil of the tangerine peel (*Citrus reticulata* L.). (a) 250 mL Soxhlet apparatus. (b) vacuum rotary evaporator for the removal of the solvents used.

2.3. Determination of the rate of mortality of the greenhouse whitefly

To determine the rate of mortality that the different treatments could exert on the greenhouse whitefly, an entomological box (30 x 30 x 48 cm) was built with one side covered by a white mosquito net cloth, where the experiments were carried out on the different treatments and their controls (Figure 3a).

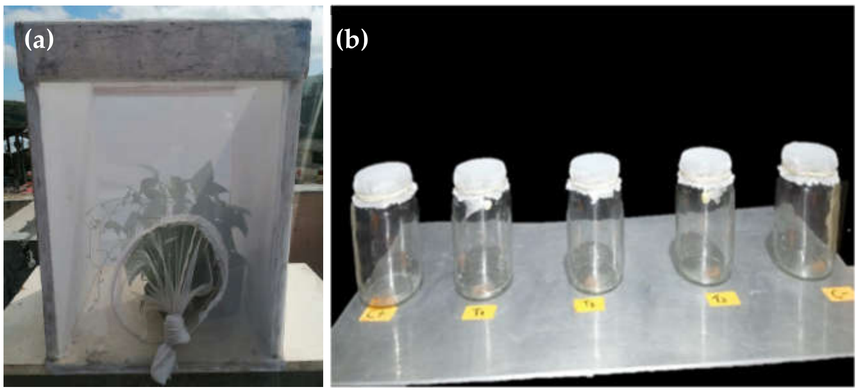

Figure 3. Devices for culturing larvae and carrying out experiments to determine the dose-effect relationship of the different treatments. (a) Entomological box to cultivate greenhouse whitefly larvae; (b) The creation of an experimental block with the greenhouse whitefly larvae, made up of the controls (negative and positive) and the three treatments with EOEs (T1 - 12.5 %, T2 - 25.0 %, and T3 - 33.3 % (v/v)).

After 5-10 days, the population of greenhouse whitefly larvae was sufficient to carry out each of the blocks of experiments, which were carried out in 250 mL glass jars, whose metal lid was replaced by a piece of mosquito netting tightly fastened to prevent the exit or entry of insects. In each jar, ten flies were placed, which, having been collected from the entomological box, had similar physiological ages, as shown elsewhere [65].

Before, the moistened sterile cotton was placed with a total volume of 177.8 µL, according to the treatments under study and their controls (Figure 3b).

Each experimental treatment, as well as the positive or negative controls, was carried out in triplicate.

The essential oil extracts (EOEs) obtained using petroleum ether (PET) and n-hexane (HEX), from which all the solvent was removed, were used in three treatments. Additionally, two controls were used, one negative, where the volumes used of essential oil were replaced by deionised water, and another positive, where the commercial insecticide (Cigaral 35 SC®, a chemical insecticide based on a concentrated suspension of imidacloprid ($C_9H_{10}ClN_5O_2$, CAS Number: 138261-41-3) at 350 $g·L^{-1}$) was used at a dilution of 1/1000. The procedure was as follows ~5-7 g of commercial sterile sanitary cotton was taken, and the total volume was poured onto the cotton, ensuring it was uniformly dispersed over the surface (Table 1).

Table 1. Different treatments were used in each experimental block.

Treatment	The volume of AC (μL) [1]	Characteristic of AC
Positive control (C^+)	0.178	$C_9H_{10}ClN_5O_2$
T1 - 12.50 % (v/v)	22.2	EOEs [2] w/PET or HEX
T2 - 25.00 % (v/v)	44.4	EOEs w/PET or HEX
T3 - 33.33 % (v/v)	59.3	EOEs w/PET or HEX
Negative control (C^-)	none	Water

[1] AC: active component; [2] EOEs: essential oil extracts.

Once the experiment started, the dying flies were counted every three hours. The mortality rate was calculated as follows:

$$MR = \frac{number\ of\ dead\ larvae}{number\ of\ total\ larvae} \quad (1)$$

2.4. Statistical analysis and comparison between treatments

The statistical comparison of the mortality rate, for each of the treatments and its controls, both between treatments and for each time that elapsed, was carried out using the open-access statistical R-package, version 4.0.5 (2021-03-31).

2.5. FTIR Analysis of EOEs from tangerine peels

EOEs from tangerine peels obtained using solvent extraction were analysed by IR spectrometry using an Agilent Cary 630 FTIR (Agilent Technologies Inc., Santa Clara, CA, USA) in a wavenumber range between 400 and 4000 cm^{-1} over 32 scans with a resolution of 4 cm^{-1}. Moreover, a single rebound diamond crystal was sampled using an ATR sampling technique.

3. Results

3.1. Mortality rate experiments

The results of the experiments on the mortality rate of greenhouse whiteflies using tangerine oil extracts and petroleum ether (PET) and n-hexane (HEX) solvents were very similar (Figure 4).

However, the mortality rate values (n = 180) did not follow a normal distribution, according to the Shapiro-Wills test performed.

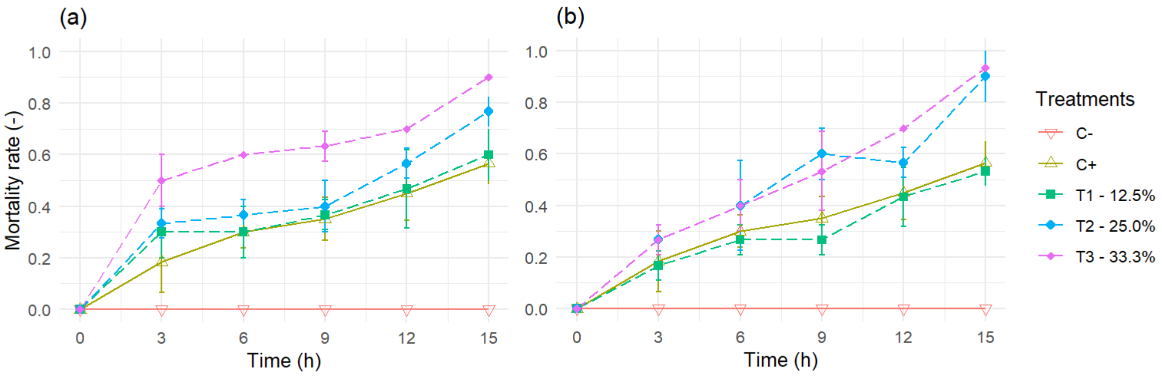

Figure 4. Mortality rate (MR, Eq. 1) of greenhouse whitefly larvae placed in contact with the controls (positive and negative) and the three treatments used (T1 - 12.5%, T2 - 25.0%, and T3 - 33.3% (v/v)) of extracts of essential oils (EOEs), obtained from tangerine peels, using (a) petroleum ether (PET); (b) n-hexane (HEX).

Additionally, the presence of both the essential oil components of the tangerine peel and imidacloprid, the active ingredient in the commercial insecticide, must have had a lethal effect on the greenhouse whitefly during the experiment's duration, as evidenced by the fact that all treatments plus the positive control (C+) produced significantly different and higher values than the negative control.

On the other hand, when the treatments were compared among themselves and with C+ for each of the extracts obtained with PET and HEX, employing a Wilcoxon non-parametric paired rank sum test, it was found that there were no significant differences ($p < 0.05$) between treatments T1 to T3 and between these and C+, for EOE obtained with HEX. In contrast, for the EOE obtained from PET, differences were observed between T3 and T1, T2, and C+, but not between T1, T2, and C+ among themselves ($p < 0.05$).

3.2. FTIR-characterisation of EOEs from tangerine peels

The IR spectroscopy analyses of the final samples of the EOEs of tangerine peels obtained by extraction with PET and HEX show remarkable similarities (Figure 5).

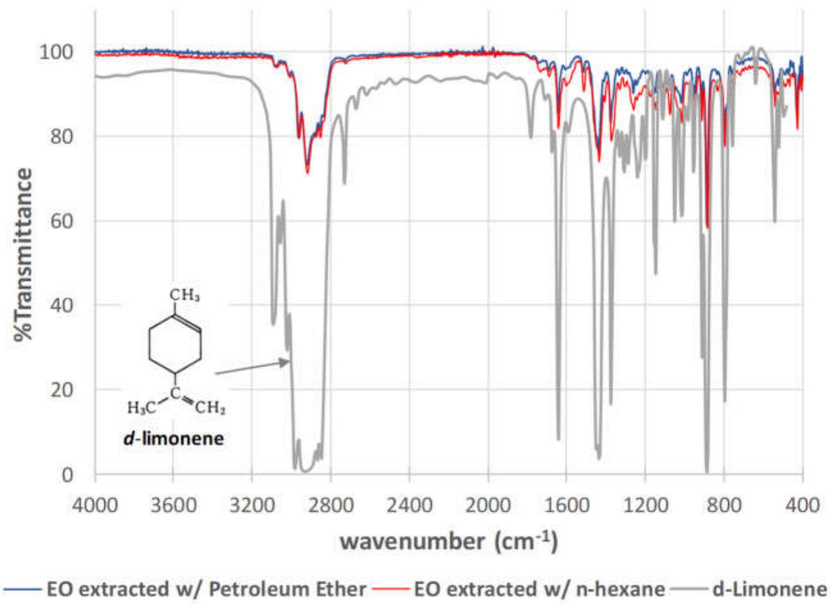

Figure 5. FTIR spectra of the essential oil extracts of tangerine peels extracted with petroleum ether (**blue** line) and n-hexane (**red** line) and their contrast with the FTIR spectrum of d-limonene.

The peaks shown in the FTIR spectra represent the main interactions between the atoms present mainly in d-limonene. Thus, for example, the broad peak between 3100 and 2800 cm^{-1} represents the asymmetric stretching of the C-H bond in the methyl ($-CH_3$) and methylene ($-CH_2-$) groups. The broad peak between 1600 and 1620 cm^{-1} defines the stretching of the C=C bond in the cyclohexene ring. Peaks at approximately 1450 cm^{-1} describe the doublet stretching of the C-H bond in the methylene groups. Whilst peaks at around 1160 cm^{-1} and 990 cm^{-1} represent the C-C bond in the methylene and methyl groups and the cyclohexene ring present in the molecule, respectively.

4. Discussion

The challenges of the near future are enormous; on the one hand, to continue satisfying the growing demand for food of the world population, and on the other hand, to maintain sufficient balance to stop the environmental deterioration that chemical products can exert on ecosystems.

Some studies report the larvicidal and insecticidal activity of some citrus essential oil extracts. Thus, for example, its larvicidal activity has been registered against *Bactrocera tryoni* [66], *Aedes aegypti* [67], and *Culex pipiens* [68].

However, there are few reports on using EOE from *Citrus reticulata* L. peels to control greenhouse whiteflies. Only the use of lemon (*Citrus aurantifolia* H.) peels EOE is reported, with observations of insecticidal activity at all stages of the life cycle of the species of *Trialeurodes vaporariorum* W. (egg, nymph, and adult stages) [69].

On the other hand, other aromatic plant extracts with monoterpenes, such as d-limonene, have been reported for their insecticidal activity. In this way, for example, the repellent and anti-oviposition activities of five aqueous plant extracts (*Foeniculum vulgare* (seed), *Achillea millefolium* L. (leaves), *Cuminum cyminum* L. (seed), *Thymus vulgaris* L. (leaves and flowers), and *Citrus sinensis* L. (peel)) on adult greenhouse whiteflies are suggested. In all cases, the presence of monoterpenes in the EOEs is indicated. In this study, however, the lowest repellent and oviposition effects were observed in *C. sinensis* peels, which could suggest that water is not a suitable extraction solvent to extract the active principles [70].

Although the exact mechanism by which d-limonene kills greenhouse whitefly larvae is not entirely known [71], it involves several different modes of action, which could favour its long-term use in the pest control of insects in greenhouses without promoting the appearance of resistance in them.

The extraction of essential oils and d-limonene from citrus peels has been done with different yields using different methods and techniques. Hydro-distillation by stripping with steam [72], the use of the Soxhlet apparatus and organic solvents such as petroleum ether, n-hexane, and ethyl alcohol [64,73], the use of supercritical fluid of CO_2 [74], as well as extractions assisted by microwaves [75] and ultrasound [76].

The yields obtained in this work (1.6 and 2.0 % (m/m) for PET and HEX, respectively) are close to those reported by other authors, who write that d-limonene yields of 3 and 5 % (m/m) were obtained using PET and HEX as solvents [64].

Interestingly, some authors report better extraction yields for d-limonene, using ethyl alcohol as an extraction solvent, whose polarity is greater than that of n-hexane and petroleum ether [64,73]. For example, in one of the studies, a d-limonene yield of 78% was achieved using ethyl alcohol as a solvent, a significantly higher yield than that obtained with the previous non-polar solvents [64].

The difference that has been observed with ethanolic extraction is that the colour of the ethanolic extract is dark brown instead of the typical yellow hue of the n-hexane extract, which could indicate that together with terpenes, such as d-limonene, other components are being extracted with the ethanol, such as pectin, which is also abundant in the tangerine peel (results not shown).

Finally, the FTIR spectra of the essential oil extracts of tangerine peel obtained here suggest the presence of d-limonene in the majority. This result agrees with other similar studies, where for the EOE from *C. reticulata* peels, it was obtained that there was 88.9% [77], whilst another study points to d-limonene, α-farnesene, and β-elemene as the fundamental components of EOE [78].

5. Conclusions

The tangerine peel, a by-product of the tangerine agro-industry, is an excellent example of the valorisation of agricultural waste [45,79,80]. Obtaining an essential oil from the peel through extraction with organic solvents and its subsequent removal by distillation provides a product of natural origin that has very low phytotoxicity in potatoes (*Solanum tuberosum*) (results not shown), which allows one to obtain from it a candidate for a botanical, natural, and environmentally friendly insecticide [35,81].

Despite its relatively lower availability than traditional chemical insecticides [82], a natural bio-insecticide based on extracting essential oil from the tangerine peel and used in closed environments such as greenhouse crops could provide more benefits for controlling some insect pests in long-term agricultural practices. Additionally, it could improve the health and working conditions of hundreds of Ecuadorian women workers currently employed there.

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