Insecticidal and antifungal effects of lemon, orange, and grapefruit peel essential oils from Argentina

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SUMMARY

The aim of the present work was to study the bioactivity of lemon, orange and grapefruit peel essential oils (EOs) obtained from Argentinian plantations on different agronomically important insect and fungal species. The chemical profile of EOs was determined by Gas Chromatography-Mass Spectrometry (GC/MS); the insecticidal activity was studied through contact and fumigant assays; the antifungal activity was evaluated with fumigant tests. Orange EO was the most effective against *Rhyzopertha dominica, Oryzaephilus* sp. and *Sitophilus granarius* in fumigation tests (LC_{50}= 89.39, 94.50, and 163.64 µL/L air, respectively); while the insecticidal effect of EOs was species-dependent in contact toxicity assays. Regarding antifungal activity, *Rhizoctonia solanii* was more susceptible than *Sclerotium rolfsii* to the tested EOs, with lemon from Industry 1 and orange being the most toxic EOs (MIC=10.77 µL/plate and 11.02 µL/plate, respectively). Conversely, *S. rolfsii* was strongly inhibited by lemon EO from Industry 2 (MIC= 52.40 µL/plate). Besides limonene, other compounds that could be responsible for these bioactivities were: linalool, carvone, α-pinene, β-pinene, β-myrcene, α-terpineol, terpinen-4-ol, limonene oxide, β-phellandrene, γ-terpinene, sabinene, neral, neryl acetate, β-caryophyllene and *p*-cymene. Citrus peel EOs could be used against different pests, contributing to the valorization of citrus residues.

Keywords: citrus peel waste, volatile organic compounds, insecticidal fumigant toxicity, insecticidal contact toxicity, antifungal effect.

Achimón, F., Leal, L. E., Pizzolitto, R. P., Brito, V. D., Alarcón, R., Omarini, A. B. y Zygadlo, J. A. Efecto insecticida y antifúngico de aceites esenciales obtenidos de la cáscara de limón, naranja y pomelo de Argentina. *Agriscientia 39*: 71-82

RESUMEN

El objetivo fue estudiar la bioactividad de los aceites esenciales (AE) extraídos de la cáscara de cítricos cosechados en plantaciones argentinas contra diferentes especies de insectos y hongos de interés agronómico. La

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composición química de los AE se determinó por cromatografía gaseosa y espectrometría de masas; la actividad insecticida se evaluó con ensayos de toxicidad fumigante y por contacto; la actividad antifúngica se determinó mediante ensayos fumigantes. El AE de naranja fue el más efectivo contra *Rhyzopertha dominica, Oryzaephilus* sp. y *Sitophilus granarius* por fumigación (LC_{50}= 89,39; 94,50 y 163,64 µL/L aire, respectivamente); mientras que la toxicidad por contacto de los AE varió según la especie de insecto. *Rhizoctonia solanii* fue más susceptible a los AE de limón Industria 1 y naranja (MIC=10,77 y 11,02 µL/placa, respectivamente) mientras que *S. rolfsii* fue más inhibido por el AE de limón Industria 2 (MIC= 52,40 µL/placa). Algunos compuestos presentes en los AE, que podrían ser responsables de estas bioactividades, fueron: limoneno, linalol, carvona, α-pineno, β-pineno, β-mirceno, α-terpineol, terpinen-4-ol, oxido de limoneno, β-felandreno, γ-terpineno, sabineno, neral, neril acetato, β-cariofileno y *p*-cimeno. Los AE obtenidos podrían usarse contra diferentes pestes, contribuyendo a la valorización de los residuos de la industria citrícola.

Palabras clave: cáscara de cítricos, compuestos orgánicos volátiles, toxicidad insecticida fumigante, toxicidad insecticida por contacto, efecto antifúngico.

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INTRODUCTION

The citrus industry plays an important role in the agro-industrial sector. Citrus are the most cultivated fruits worldwide (Chavan et al., 2018), with orange accounting for about 50-60% of the total citrus production (Singh et al., 2021); yet other fruits such as lemon, mandarin and grapefruit also have great industrial importance (Satari and Karimi, 2018). Argentina is among the top ten leading citrus fruit-producing countries of the world, with around 130,000 ha cultivated and more than 2.6 million tons produced per year (Salazar et al., 2018).

During processing, the citrus industry generates great amounts of solid/semisolid residues such as peel, pulp and seeds, which account for about 50% of the fruit weight (Singh et al., 2021). Traditional disposal strategies of waste include incineration, dumping on rivers and landfilling, which are associated with hazardous effects on the environment (Chavan et al., 2018). In this context, many studies have reported the contribution of different value-added products from citrus waste to promote a sustainable socio-economic development within the citrus industry. For example, citrus peel is a great source of several compounds

like carotenoids, flavonoids, dietary fibers, soluble sugars, polyphenols, amino acids and essential oils (EOs) (Chavan et al., 2018; Zema et al., 2018; Singh et al., 2021).

The bioactivity of EOs extracted from citrus peel waste is a very popular field of research among many scientists. Citrus EOs have been classified as "generally recognized as safe" (GRAS), being used as flavoring agents in food, beverages and cleaning products, as well as in the pharmaceutical industry. In addition, citrus EOs have high concentrations of volatile organic compounds, such as terpenes and their oxygenated derivatives, which include esters, aldehydes, alcohols, epoxides and ketones (Singh et al., 2021). These compounds display many biological properties, conferring citrus EOs immense potential for the development of biopesticides. However, it should be considered that the chemical profile of EOs, and thus their bioactivity, is largely dependent on the cultivars origin and certain environmental factors, among others (Rahimmalek et al., 2017).

Currently, the control of pests depends on the application of synthetic pesticides. However, despite the efficacy of these chemical substances, a significant number of them can cause residual toxicity on food commodities, lead to the development of resistant populations and have adverse effects on the environment (Margni et al., 2002). Therefore, there is an increasing public demand for the development of new and safer pesticidal agents for the food industry. Even though the biopesticidal properties of citrus EOs have been widely studied against different species of insects and fungi (Jing et al., 2014; Oboh et al., 2017; Simas et al., 2017; Dosoky and Setzer, 2018), the use of EOs from citrus peel waste still remains as an emerging field. Additionally, to the best of our knowledge, the biopesticidal properties of citrus peel EOs from Argentinian plantations have not been previously reported.

The aim of the present work was to study the toxic effect of citrus peel waste EOs obtained from different plantations from Argentina on: i) the insects *Sitophilus granarius, Rhyzopertha dominica* and *Oryzaephilus* sp., which attack stored grains and ii) the filamentous fungi of agronomic interest *Rhizoctonia solani* and *Sclerotium rolfsii.*

MATERIALS AND METHODS

Extraction of the Essential Oils

Citrus peel for the extraction of EOs was obtained from two citrus processing industries from northwest Argentina: Industry 1 provided EOs from lemon, orange and grapefruit peel and Industry 2 provided lemon peel waste.

The EOs from Industry 1 were extracted through a Brown Oil Extractor (BOE: Brown International Corporation, Florida, U.S.A.), in which the removal of the EOs is achieved by gently puncturing the entire peel of the fruit. This stage takes place beneath the surface of a shallow pool of water to avoid the loss of EOs to the atmosphere. The oil sacks are ruptured, releasing the EO, which is subsequently captured in the water spray. After leaving the BOE, the EO/water mixture was centrifuged to separate the water and concentrate the EO (Zema et al., 2018). In the case of lemon EO from Industry 2, peel waste was dried at room temperature for three days and then subjected to hydrodistillation for 3 h in a Clevenger type apparatus.

Gas chromatography–mass spectrometry (GC-MS) analyses

Qualitative and quantitative analyses of the EOs were conducted with a Perkin Elmer Clarus 580 gas chromatograph coupled to a mass spectrometer (GC-MS). ADB5 column was used to separate the volatile compounds (30 m × 0.25 mm: film thickness 0.25 m; Elite 5 MS Perkin Elmer), and Helium was used as the carrier gas at a flow rate of 1 mL/s. The temperature of the injector was 200 °C. The oven temperature was set as follows: 60 °C for 5 min; ramped up to 170 °C at 4 °C/min; and then raised to 250 °C at 20 °C/min. The GC/MS interface temperature was 200 °C. Electron impact mode on mass spectrometer was set at 70 eV with a mass scan range of 40-300 atomic mass units (amu). Diluted samples (1/100 v/v in n-heptane) of 1 µL of each EO were manually injected using the splitless mode. Kovats retention indices (KI) were calculated after an analysis of C8-C21 alkane series (Sigma-Aldrich), under the same chromatographic conditions (Achimón et al., 2019). The identification of EO compounds was based on the comparison of their KI and mass spectra with those from the NIST-08 Mass Spectral Library (US National Institute of Standards and Technology; Stein et al., 2008) and literature data (Achimón, Krapacher et al., 2021). The amount of each compound was expressed as a relative percentage by peak area normalization (Achimón, Brito et al., 2021).

Insects

Insects were reared in sealed containers with wheat, under controlled conditions of temperature

(25 ± 1°C) and relative humidity (60 ± 5 %) and 12:12 h light:dark cycles. Unsexed adults of *S. granarius, R. dominica* and *Oryzaephilus* sp. were used for all the experiments.

Fumigant toxicity against insects

Fumigant toxicity of EOs was performed as described by Brito et al. (2021). Ten adults of each insect species were placed in 30 mL-glass vials sealed with plastic caps. A 2 cm-diameter filter paper disk with different amounts of the EOs was placed on the underside of each cap covered with nylon gauze to avoid direct contact between the insects and the tested EOs. The concentrations tested ranged between 66.66 and 500.00 µL/L air (7 concentrations), and filter paper discs without EO were used as controls. The glass vials were placed in a rearing chamber under controlled conditions of temperature (25 ± 1 °C) and relative humidity (60 ± 5 %). Insects were considered dead when no movements were observed, and the mortality of the insects was determined after 24 h. Five replicates were performed for each treatment, and the experiment was repeated twice.

Contact toxicity test against insects

Contact toxicity of Citrus EOs was evaluated following the methodology proposed by Arena et al. (2020): dilutions of the EOs were prepared in acetone, and aliquots of 200 µL of each dilution were applied to 5.5 cm-diameter filter paper discs placed on the bottom of Petri plates of the same diameter. The concentrations tested ranged from 100 and 800 µg/cm^2 (7 concentrations). After 2 min of solvent evaporation, ten adult insects were placed in each Petri plate. The Petri dishes were placed in a rearing chamber under controlled conditions as described above. The insects of the control treatment were kept under the same conditions but only with acetone. Insects were considered dead when no movements were observed, and mortality of insects was determined after 24 h. Five replicates were performed for each treatment, and the experiment was repeated twice.

Fumigant antifungal activity

The strains of *R. solani* and *S. rolfsii* used in the experiments were field isolates provided by the Laboratory of Plant Pathology, Faculty of Natural Sciences (National University of Salta). The fungal species were cultivated in Petri dishes with Potato Dextrose Agar (PDA; Britania) for 7 days at 22 °C in the dark.

The antifungal activity of the EOs was studied as described by Vilela et al. (2009) with some modifications. Filter paper disks were separately moistened with each EO and placed on the upper lid of Petri plates (9 cm) containing 20 mL of PDA. The tested EOs doses were 0 (control), 2, 4, 6, 10 and 20 µL/plate for *R. solani* and 0, 5, 10, 15, 20, 35 and 50 µL/plate for *S. rolfsii.* A 5 mm-diameter mycelial plug of the fungal species was placed in the center of each Petri plate, the plates were sealed with Parafilm and incubated in the dark at 22 °C. The antifungal activity was calculated as the percentage of inhibition of fungal growth according to the following formula: *% Inhibition = [(CT) / C] x 100*, where *C* is the average diameter (mm) of the control colonies and *T* is the average diameter of the treatment colonies. The colony diameters of the treatment plates were measured using a caliper after a 7-day incubation period, when the fungal colonies from the control treatment completely covered the plate surface. Five replicates were conducted for each treatment, and the experiment was repeated twice (Alarcón et al., 2012).

Data Analyses

Lethal concentrations causing 50 % mortality (LC_{50}) and Chi-square statistic values (X^2) of the adequacy of fit ($p < 0.05$) were calculated according to Finney (1971) and subjected to probit regression analysis using the POLO-PLUS Software at 95 % confidence interval. The LC_{50} values were considered to be significantly different if the 95 % confidence limits did not overlap. Regarding antifungal activity, differences between treatments were tested by a Kruskal-Wallis test using the InfoStat Software (Di Rienzo et al., 2017). In addition, the minimum inhibitory concentration (MIC), namely the lowest concentration at which no fungal growth was observed, was calculated for each EO. The inhibition percentage was plotted against the concentration of each EO and a linear regression was conducted ($y = a + bx$) to obtain the slope of the line (b) and the intercept (a); then, the following formula was applied $MIC = (100 - a)/b$ (Brito et al., 2019).

RESULTS AND DISCUSSION

Essential Oil Composition

Citrus peel is the main residue of the citrus processing industries and is characterized

by a high concentration of EOs. The chemical composition of the EOs extracted from citrus peel waste is provided in Table 1 according to their elution order in a DB-5 capillary column. A total of 40 volatile organic compounds were detected in the chromatographic analyses, with monoterpenes being the most predominant class. As expected, limonene was the prevalent component in the four EOs evaluated, particularly in orange and grapefruit EOs (89.49 % and 84.32 %, respectively). Orange EO was the one with the highest amount of limonene, followed by β-myrcene (3.87 %), α-pinene (1.66 %), decanal (1.22 %) and linalool (1.03 %), along with other minor constituents (Table 1). In grapefruit EO, β-myrcene (6.46 %) and α-pinene (2.76 %) were the next most abundant compounds after limonene, followed by the sesquiterpenes β-caryophyllene and germacrene D (1.64 %), and decanal (1.54 %). Regarding lemon EOs, similar and lower amounts of limonene were detected (44.88 % and 44.99 % for lemon EOs from Industry 1 and Industry 2, respectively). In addition, lemon EOs showed high amounts of β-pinene (17.77 % and 13.24 % for Industry 1 and Industry 2, respectively). Lower quantities of β-myrcene were detected in lemon EOs (1.93 % and 1.61 % for Industry 1 and Industry 2, respectively) compared to grapefruit and orange EOs. It is interesting to note that, although lemon EOs were extracted from the same plant species, there were significant differences in their chemical composition. For example, the aromatic monoterpene *p*-cymene was more abundant in lemon EO from Industry 2 (16.50 %) compared to lemon EO from Industry 1 (1.42 %), and γ-terpinene was more abundant in lemon EO from Industry 1 (16.07 %), with lower values for that from Industry 2 (1.37 %). Furthermore, the presence of the minor constituents was rather different between lemon EOs (Table 1). These variations in the chemical composition between these EOs could be due to differences in their extraction processes. The drying stage to which lemon peels were subjected before EO extraction would lead to the changes in the composition of lemon EO from Industry 2. Indeed, the oxidation of lemon EO increases the formation of *p*-cymene, the loss of γ-terpinene and the chemical transformation of limonene into limonene oxide (Nguyen et al., 2009).

Fumigant toxicity test against insects

Orange was the most effective EO against the three insect pests evaluated in fumigation tests, with LC_{50} values of 89.39 µL/L air (79.08-99.33), 94.50 µL/L air (53.77-119.56) and 163.64 µL/L air (144.10-182.26) for *R. dominica*, *Oryzaephilus* sp. and *S. granarius*, respectively (Table 2). Orange EO had limonene as the prevalent compound (89.49 %) (Figure 1). The fumigant toxicity of limonene was previously reported in several stored product insects, and it would exert its toxic effects by inhibiting the acetylcholinesterase (AChE) activity (Abdelgaleil et al., 2009), a target enzyme of neurotoxic insecticides. Moreover, this monocyclic monoterpene hydrocarbon (Figure 1) would act by dissolving the lipidic cuticle of the insect exoskeleton, thus allowing the penetration of other toxic volatile organic compounds (Bravim dos Santos et al., 2021). As shown in Table 1, grapefruit EO also presented high amounts of limonene (84.32 %). However, even though their 95 % confidence intervals overlapped, grapefruit EO showed higher LC_{50} values compared to orange EO for the three species of insects evaluated (Table 2). The bioactivity of an EO is usually attributable to its major components; however, the presence of minor constituents can lead to synergistic, antagonistic or additive effects. In general, oxygenated monoterpenes are more toxic compared to monoterpene hydrocarbons (Kordali et al., 2017). Other volatile compounds present in orange EO (but not in grapefruit EO) were the oxygenated monoterpenes linalool and carvone (Table 1). Hence, the insecticidal activity of limonene could have been enhanced by its demonstrated synergetic effects when combined with other volatile compounds such as linalool (Pavela, 2014). Linalool is an aliphatic monoterpene alcohol with two double bonds in its structure (Figure 1). This compound exhibited potent insecticidal activity against *R. dominica*, *Tribolium castaneum*, *Sitophilus oryzae* and *S. granarius* in fumigation toxicity assays (Rozman et al., 2007; Abdelgaleil et al., 2009; Kordali et al., 2017).

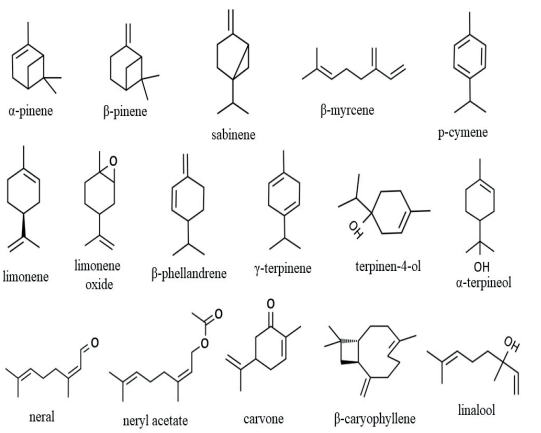

Figure 1. Chemical structures of the main compounds of lemon, orange and grapefruit peel EOs from Argentina

Table 1. Chemical composition of lemon, orange and grapefruit peel EOs

KI	Compound	Grapefruit	Lemon (Industry 1)	Orange	Lemon (Industry 2)
928	α-thujene	-	0.61	-	0.38
934	α-pinene	2.76	3.03	1.66	2.05
951	camphene	-	0.12	-	0.10
974	sabinene	-	3.37	-	-
983	β-pinene	-	17.77	0.45	13.24
992	β-myrcene	6.46	1.93	3.87	1.61
1005	octanal	-	0.16	0.37	0.20
1011	δ-3-carene	-	0.18	-	-
1018	α-terpinene	-	0.21	-	0.12
1026	*p*-cymene	-	1.42	-	16.50
1031	limonene	84.32	44.88	89.49	44.99
1035	β-phellandrene	-	-	-	2.08
1049	(E)-β-ocimene	-	0.18	0.37	-
1060	γ-terpinene	-	16.07	-	1.37
1091	terpinolene	-	0.70	-	0.18
1101	linalool	-	-	1.03	0.52
1105	nonanal	-	0.24	-	0.32
1131	pinocarveol	-	-	-	0.24
1135	limonene oxide	-	-	-	1.35
1151	verbenol	-	-	-	0.48
1153	citronellal	-	0.14	-	0.20
1164	terpinen-4-ol	-	-	-	0.78
1165	pinocarvone	-	-	-	0.23
1189	α-terpineol	0.76	0.50	0.33	1.84
1212	decanal	1.54	-	1.22	-
1221	trans-carveol	-	-	0.55	0.66
1234	cis-carveol	-	-	-	0.40
1241	neral	-	1.46	-	1.27
1245	carvone	-	-	0.46	1.20
1353	neryl acetate	-	1.25	-	0.50
1382	geranyl acetate	-	0.47	-	0.50
1418	α-cis-bergamotene	-	0.08	-	-
1425	β-caryophyllene	1.64		0.51	0.19
1435	α-trans-bergamotene	-	0.96	-	1.33
1445	aromadendrene	-	-	0.20	0.54
1454	α-humulene	0.02	-	-	-
1482	germacrene D	1.64	-	-	-
1492	valencene	-	-	-	0.54
1513	β-bisabolene	-	1.45	-	1.90
1577	spathulenol	0.86	-	-	-

KI: Kovats retention indices. The volatile content of each EO is expressed as relative percentage (%) by peak area normalization.

Indeed, it was reported that the toxicity of linalool could be related to its AChE inhibitory activity (Praveena and Sanjayan, 2011). On the other hand, the α,β-unsaturated monoterpene ketone carvone (Figure 1) also showed good insecticidal activity towards the stored products beetles *S. oryzae*, *T. castaneum*, *R. dominica* (Tripathi et al., 2003; Abdelgaleil et al., 2009) and *S. zeamais* (Herrera et al., 2015) in fumigation toxicity assays. The presence of an extra double bond between the alpha and beta carbon (α,β-unsaturation) increases the polarizability of the molecule, which is associated with stronger intermolecular attractive forces (Figure 1). Consequently, α,β-unsaturated ketones can bind with nucleic acids and amino acids, targeting several metabolic pathways of the insect (Herrera et al., 2015).

Table 2. Fumigant toxicity of lemon, orange and grapefruit peel EOs against *S. granarius*, *Oryzaephilus* sp. and *R. dominica* adults at 24 h after exposure

EO	Insect	LC_{50} (µL/L air)	95 % Confidence interval	Slope ± S.E.	X^2
Lemon (Industry 1)	*S. granarius*	240.35	216.67- 260.53	7.87 ± 1.21	2.63
	Oryzaephilus sp.	165.82	146.65 - 179.26	9.74 ± 2.01	2.93
	R. dominica	100.92	101.09 - 124.38	5.61 ± 0.60	1.89
Lemon (Industry 2)	*S. granarius*	237.34	217.40 - 255.83	7.99 ± 1.23	0.05
	Oryzaephilus sp.	201.03	111.29 - 244.53	5.46 ± 0.97	4.38
	R. dominica	139.34	102.56 - 170.97	3.95 ± 0.48	3.49
Orange	*S. granarius*	163.64	144.10 - 182.26	4.79 ± 0.58	0.48
	Oryzaephilus sp.	94.50	53.77 - 119.56	5.60 ± 0.76	7.21
	R. dominica	89.39	79.08 - 99.33	6.37 ± 0.81	0.35
Grapefruit	*S. granarius*	175.05	131.79 - 219.63	3.66 ± 0.38	8.81
	Oryzaephilus sp.	171.67	141.72 - 212.09	5.87 ± 0.55	11.71
	R. dominica	105.30	86.57 - 122.04	3.36 ± 0.43	2.42

LC_{50}: concentration that caused 50 % of the mortality; X^2: Chi-square value, significant at $p < 0.05$ level.

Contact toxicity test against insects

The insecticidal effect of EOs was species-dependent in contact toxicity assays (Table 3). Lemon EO from Industry 2 was more effective against *S. granarius* with a LC_{50} value of 367.40 µg/cm^2 (307.56 - 409.52); orange EO and lemon EO from Industry 1 were more toxic to *R. dominica* with LC_{50} values of 200.33 µg/cm^2 (119.25-230.72) and 227.79 µg/cm^2 (178.26-261.41), respectively; and orange EO and grapefruit EO were more effective against *Oryzaephilus* sp., showing similar LC_{50} values of 221.85 µg/cm^2 (184.25-286.81) and 226.42 µg/cm^2 (204.39-247.56), respectively. These findings indicate that the insecticidal activity of monoterpenes, as well as other toxic compounds, depends on several factors including not only the applied doses and insect species involved, but also on the methods of application and routes of penetration of the compounds. In the present study, the EOs were allowed to enter the insect body through inhalation (fumigant toxicity assays) or by direct contact (contact toxicity assays). In this regard, it was reported that certain EO pure components act more efficiently when applied in fumigation tests compared to contact assays, and vice versa (Abdelgaleil et al., 2009; Velázquez-Nuñez et al., 2013; Sun et al., 2020).

In addition to limonene, grapefruit and orange EOs presented higher amounts of α-pinene, β-myrcene and β-caryophyllene compared to the remaining EOs, which could be responsible for their toxic activity against *Oryzaephilus* sp. (Table 1; Figure 1). In fact, the contact toxicity of β-myrcene and β-caryophyllene was previously reported against several insect pests (Sun et al., 2020). As it was mentioned before, different interactions can occur among the compounds of an EO, enhancing its bioactivity. For example, a significant synergistic effect was reported when different species of insects were treated with binary mixtures of limonene/β-myrcene and limonene/α-pinene (Pavela, 2014).

Lemon EOs from Industry 1 and Industry 2 presented similar amounts of certain components, such as limonene, β-pinene, α-pinene, β-myrcene, neral, α-bergamotene and β-bisabolene (Table 1; Figure 1). However, there were important differences in the quantities of other volatile compounds, such as *p*-cymene and α-terpineol, which were from 4 to 12 times higher in lemon EO from Industry 2, while other components, such as carvone, β-phellandrene, limonene oxide and terpinen-4-ol were only present in lemon EO from Industry 2. These compounds, alone or combined, could be responsible for the higher toxic activity observed against *S. granarius*. This is in agreement with previous studies that reported 100 % mortality of *S. granarius* adults treated with the monoterpene alcohols α-terpineol and terpinen-4-ol after 12 h of exposure (Kordali et al., 2006). Furthermore, lemon EO from Industry 2 was the only one that presented the epoxide monoterpene limonene oxide (Figure 1). Epoxides are also part of a group of compounds recognized as active principles with insecticidal activities (Justino et al., 2005). These molecules are susceptible to reactions with electrophiles and nucleophiles, being able to react with cellular macromolecules. Even though this compound was present in small amounts (1.35 %), its contact toxicity against other species of *Sitophilus* has already been established by Yildirim et al. (2013). In addition, lemon EO from Industry 2 also

Table 3. Contact toxicity of lemon, orange, and grapefruit peel EOs against *S. granarius*, *Oryzaephilus* sp. and *R. dominica* adults at 24 h after exposure

EO	Insect	LC_{50} (μg/cm²)	95% Confidence interval	Slope ± S.E.	X^2
Lemon (Industry 1)	*S. granarius*	480.26	465.20 - 564.69	7.52± 1.02	7.17
	Oryzaephilus sp.	277.79	254.77 - 296.18	9.74± 1.33	2.10
	R. dominica	227.79	178.26 - 261.41	9.15± 1.43	3.91
Lemon (Industry 2)	*S. granarius*	367.40	307.56 - 409.52	8.78± 1.13	3.07
	Oryzaephilus sp.	230.05	197.35 - 284.87	3.57 ± 0.75	0.33
	R. dominica	282.02	259.35 - 302.19	8.51± 1.10	1.63
Orange	*S. granarius*	501.66	465.45 - 536.49	6.55± 0.90	1.76
	Oryzaephilus sp.	221.85	184.25 - 286.81	2.77 ± 0.61	1.34
	R. dominica	200.33	119.25 - 230.72	4.07± 0.52	5.45
Grapefruit	*S. granarius*	500.41	469.38 - 529.66	7.88± 1.05	2.26
	Oryzaephilus sp.	226.42	204.39 - 247.56	7.72 ± 1.87	1.73
	R. dominica	258.09	239.39 - 275.36	9.64 ± 1.24	0.97

LC_{50}: concentration that caused 50% of the mortality; X^2: Chi-square value, significant at $p < 0.05$ level.

contained β-phellandrene which demonstrated a strong contact toxicity against *S. granarius* adults, accompanied by low respiration rates, which is an indicator of physiological stress (Plata-Rueda et al., 2018). On the other hand, lemon EO from Industry 1 showed higher insecticidal effect than lemon from Industry 2 against *R. dominica.* The monoterpene γ-terpinene was 12-fold higher and sabinene was only present in lemon EO from Industry 1 compared to that obtained from Industry 2. These results are in agreement with previous studies that reported the insecticidal effects of γ-terpinene against *R. dominica* and other stored product insects (López et al., 2010). According to the authors, γ-terpinene would probably affect the activity of detoxifying enzymes of the microsomal monooxygenase system. The insecticidal activity of sabinene against different stored products insects was previously reported in contact toxicity assays (Wang et al., 2011; Liu et al., 2020). Moreover, sabinene showed synergistic effects with limonene on the inhibition of AChE activity (Liu et al., 2020).

Antifungal activity

The fumigant toxicity of EOs was evaluated against *R. solanii* y *S. rolfsii*. Even though the mechanisms of antifungal activity of monoterpenes are not fully understood, it has been established that they exert their effect at the level of the membrane and membrane embedded enzymes due to their lipophilicity (Marei et al., 2012). Indeed, it has been reported that these compounds change the fatty acid composition of cell membrane, affecting its permeability and inhibiting respiration (Marei et al., 2012). For this reason, the antimicrobial activity of EOs is usually higher in fumigant assays than in direct contact assays because the presence of monoterpenes in vapor phase facilitates their solubilization in fungal cell membranes (Velázquez-Nuñez et al., 2013). The EOs evaluated showed antifungal activity by reducing or totally inhibiting fungal growth in a dose-dependent manner (Table 4).

Rhizoctonia solanii proved to be more susceptible than *S. rolfsii* to the tested EOs since higher inhibition values were observed with lower concentrations of the EOs (Table 4). For example, 100% growth inhibition was observed in *R. solanii* treated with 10 μL/plate of lemon EO from Industry 1 while the same EO and concentration produced only 5.92% inhibition in *S. rolfsii*. In fact, none of the EOs tested in this study inhibited the growth of *S. rolfsii* by 100%. In the case of *R. solanii*, the four EOs evaluated significantly inhibited fungal growth at 6 and 10 μL/plate. Lemon EO from Industry 1 and orange EO showed the highest antifungal activities, with 100% of mycelial growth inhibition at 10 μL/plate and MIC values of 10.77 and 11.02 μL/plate for lemon and orange EO, respectively. As it was stated above, there were important differences in the chemical profile between these EOs, with limonene being the prevalent component. The antifungal effect of limonene against *R. solani* has been widely reported (Marei et al., 2012; Feng et al., 2020). Regarding modes of action, Marei et al. (2012) found that limonene is a potent inhibitor of pectin methyl esterase (PME), an enzyme involved in the methylesterification of pectins, the main components of fungal cell walls. Such changes in pectin structure are associated with changes in cellular adhesion, plasticity, pH and ionic contents of the cell wall, which influence membrane integrity,

Table 4. Fumigant antifungal activity of lemon, orange and grapefruit peel EOs against *R. solani* and *S. rolfsii*

Fungi	Concentration (µL/plate)	Mean values of mycelial growth (cm) and inhibition percentage (%)			
		Lemon (Industry 1)	**Lemon (Industry 2)**	**Orange**	**Grapefruit**
R. solanii	0	9.50 ± 0 (0)	9.5± 0 (0)	9.5±0 (0)	9.5±0 (0)
	2	8.82±0.16 (7.16)	6.3 ± 0.14 (33.68)	9.17±0.24 (3.47)	9.38±0.09 (1.26)
	4	8.41±0.49 (11.47)	5.99 ± 0.81 (44.84)	8.34± 0.65 (12.21)*	9.33±0.18 (1.79)
	6	6.98± 0.13 (49.37)*	4.13 ± 0.73 (56.53)*	5.57±2.03 (41.37)*	8.21±0.95 (13.58)*
	10	0± 0 (100)*	3.05 ± 0.53 (67.89)*	0±0 (100)*	3.21±0.22 (62.21)*
	MIC	10.77	13.85	11.02	18.01
S. rolfsii	0	9.5± 0 (0)	9.5± 0 (0)	9.5±0 (0)	9.5±0 (0)
	5	9.5± 0 (0)	9.38±0.14 (1.32)	9.38±0.14 (1.32)	9.44±0.13 (0.66)
	10	8.94±0.24 (5.92)	9.03±0.10 (5)	9.08±0.30 (4.47)	9.05±0.17 (4.74)
	15	8.20± 0.27 (13.68)	7.94±0.40 (16.45)	8.46±0.35 (10.92)	8.69±0.06 (8.55)
	20	7.24± 0.60 (23.82)*	6.95 ± 0.49 (26.84)*	7.86± 0.26 (17.24)*	8.36± 0.25 (11.97)*
	35	4.94 ± 0.07 (48.03)*	2.74± 0.14 (71.18)*	5.83± 0.36 (38.68)*	6.13± 0.24 (35.53)*
	50	2.37± 2.04 (78.55)*	0.5 ± 1 (94.74)*	3.64± 0.46 (61.71)*	3.66± 0.15 (61.45)*
	MIC	65.46	52.40	84.56	82.07

*Significant differences with respect to the control ($p \leq 0.05$). The numbers in brackets indicate the percentage of fungal growth inhibition.

affecting fungal development. The bioactivity of lemon EO from Industry 1 and orange EO against *R. solani* cannot be fully attributed to limonene because grapefruit EO and lemon EO from Industry 2 also had limonene as their major component and reported weaker antifungal effects. Some other compounds that are only present or present in higher amounts in the most fungicidal EOs and that might be also exerting an effect against *R. solani* were: sabinene, γ-terpinene, linalool, neral and neryl acetate (Figure 1). For example, linalool and neryl acetate reported a strong toxicity to *R. solani* growth (Kordali et al., 2007); sabinene and γ-terpinene were also toxic to different species of filamentous fungi either acting alone or in binary mixtures (Espinosa-García and Langenheim, 1991). In addition, several studies reported the fumigant toxicity of neral, an α,β-unsaturated aldehyde, against several species of phytopathogenic fungi (Wuryatmo et al., 2003) (Figure 1).

Sclerotium rolfsii was more inhibited by lemon EO from Industry 2 followed by lemon EO from Industry 1, with MIC values of 52.40 and 65.46 µL/plate, respectively (Table 4). On the other hand, grapefruit and orange EOs presented higher MIC values, both EOs causing 61 % inhibition of mycelial growth at 50 µL/plate. In addition to limonene, other volatile compounds present in high amounts in lemon EOs that might be responsible for their bioactivity to *S. rolfsii* were α-pinene and β-pinene. Both structural isomers of pinene showed antifungal activity against different species of fungi (da Silva et al., 2012; Ložienė et al., 2018) probably through the interference with cell wall fungal enzymes (de Macêdo Andrade et al., 2018). Furthermore, the aromatic hydrocarbon *p*-cymene is well represented in lemon EO from Industry 2 and proved to be toxic against different molds species. A molecular docking study revealed that its antifungal effects might be related to its interaction with the enzymatic domain of glucosamine-6-phosphate synthase, a target enzyme for antifungal agents (Dutta et al., 2020). Moreover, the monoterpene alcohol α-terpineol, which was present in higher amounts in lemon EO from Industry 2, produced distorted and collapsed hyphae and irreversible damages to cell membrane and organelles in another species of filamentous fungus (Park et al., 2009).

CONCLUSION

To conclude, the insecticidal activity of EOs was highly dependent on the species of insects involved and the application method used. We showed that lemon EO from Industry 2, grapefruit EO, and lemon EO from Industry 1 led to higher contact toxicity against *S. granarius*, *Oryzaephilus* sp. and *R. dominica,* respectively. In contrast, in fumigant toxicity assays, orange was the most effective EO against the three insect pests evaluated, probably because orange EO bioactive components may be more toxic when penetrating the insect body via the respiratory system. These results are encouraging since the application of EOs through fumigation has two advantages: first, fumigation allows the homogeneous distribution of the EOs, reaching a large number of insects; and second, EOs applied in vapor phase are able to penetrate

inside the grains, affecting larvae and adults in the interior. Regarding antifungal activity, different EOs proved to be more effective against *R. solani* and *S. rolfsii*. Still, we were able to identify certain pure compounds that could be responsible for the toxic effects of EOs, such as limonene, sabinene, γ-terpinene, linalool, neral, neryl acetate, α-pinene, β-pinene and α-terpineol. Future studies evaluating the antifungal property of these pure compounds either alone or combined are necessary in order to achieve a bioactive antifungal formulation of natural origin.

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