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Toxic Effect of Citrus Peel Constituents on *Anastrepha fraterculus* Wiedemann and *Ceratitis capitata* Wiedemann Immature Stages

María J. Ruiz,*[,†,‡] María L. Juárez,[‡,§] Raúl A. Alzogaray,[‡,#,⊥] Federico Arrighi,[‡,∥] Lorena Arroyo,[∥] Gerardo Gastaminza,[†] Eduardo Willink,[†] Alicia del Valle Bardón,[‡,∥] and Teresa Vera[‡,§]

[†]Estación Experimental Agroindustrial Obispo Colombres, Av. William Cross 3150, Las Talitas (T4104AUD), Tucumán, Argentina

[‡]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, (C1033AAJ) C.A.B.A., Argentina

[§]Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, Florentino Ameghino s/n (4000), Tucumán, Argentina

[#]Centro de Investigaciones de Plagas e Insecticidas (CIPEIN-UNIDEF/CONICET), JB de La Salle 4397 (1603), Villa Martelli, Buenos Aires, Argentina

[⊥]Instituto de Investigación e Ingeniería Ambiental, UNSAM, 25 de Mayo y Francia, (1650) San Martín, Buenos Aires, Argentina

[∥]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Batalla de Ayacucho 471 (4000), Tucumán Argentina

ABSTRACT: The toxicity of essential oils from the citrus peel has been proposed as the major resistance mechanism offered by citrus to fruit fly infestation. We evaluated the insecticidal activity of the ether extracts from the lemon (*Citrus limon* [L.] Burm.) and grapefruit (*C. paradisi Macfadyen*) peel as well as from limonene and citral against *Anastrepha fraterculus* (Wiedemann) and *Ceratitis capitata* (Wiedemann) immature stages. We also evaluated the toxicity of the extracts at two ripening stages. Extracts proved toxic to *A. fraterculus* egg and larvae. The lemon and grapefruit extracts showed the same toxicity in both fruit fly species. For *A. fraterculus* eggs, citral was more toxic than limonene; for larvae, they showed equal toxicity. *Anastrepha fraterculus* eggs were more sensitive than *C. capitata* eggs. In conclusion, we provide evidence of chemical resistance mechanisms that could account for the nonhost condition of lemon for *A. fraterculus*.

KEYWORDS: *toxicity, citrus essential oils, plant resistance, fruit flies, immature stages, host status*

INTRODUCTION

Fruit flies (Diptera: Tephritidae) are important pests and represent a serious threat in fruit producing regions. Most species are highly polyphagous, and females lay their eggs in a wide variety of fruits, including many families of economic importance.[1,2] The impact of larval feeding and the occurrence of microorganisms causes the fruit falling from the tree and consequently impedes its commercialization. These economic losses are increased by the restrictions to access pest-free markets. Field sanitation, area-wide integrated pest management programs, quarantine treatments, and an accurate definition of the host condition allow overcoming this problem.[3−8] Under this scenario, a deep understanding of insect−plant interaction is mandatory.

In Argentina two species of fruit fly, the South American fruit flies, *Anastrepha fraterculus* (Wiedemann), and the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), generate losses of approximately 20% of the fruit production.[9] *Anastrepha fraterculus* is native to South America and restricted to tropical and subtropical areas.[10,11] The infestation of about 80 species of fruit from a wide range of families, such as Anacardiaceae, Annonaceae, Compretaceae, Euphorbiaceae, Fabaceae, Flacourtaceae, Juglandaceae, Moraceae, Myrtaceae, Oxalidaceae, Punicaceae, Rosaceae, Rubiaceae, Rutaceae, and Vitaceae, has been reported.[12,13] *Ceratitis capitata* is native to Africa and has a worldwide distribution[14] with high adaptability to different climates. It is a highly invasive species with a host range that exceeds 200 plant species.[15] In the areas in which the two species overlap, most of the fruit production is represented by citrus. In the northwest of the country, lemon and grapefruit are the main crops, while in the northeast, orange (*Citrus sinensis* [L.] Osbeck) and mandarins (*C. reticulata* Blanco) are the prevalent species.[16] Both *A. fraterculus* and *C. capitata* have been reported to naturally infest citrus species in Argentina, yet the infestation level varies according to the citrus species and the locality from where it was obtained.[17−19]

Although fruit flies infest citrus, they have largely been recognized as poor hosts.[20,21] This unsuitability is mostly related to chemical resistance mechanisms present in the peel.[22,23] This involves gum secretions,[24] calluses in which eggs are drowned,[25,24] and toxic compounds present in the oils from the essential oil glands.[20,22,23,26] To a lesser extent, physical attributes such as peel elasticity and thickness also contribute to resistance.[21] In addition, it has been shown that citrus species from which flies emerge also affect female fecundity and adult longevity,[21] and essential oils at high doses are toxic to adults.[27,28]

Essential oils from citrus are composed primarily of monoterpenes and sesquiterpenes and some of their oxygenated derivates.[29] Monoterpene hydrocarbons are the most abundant group, and within it, limonene can be found in

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concentrations higher than 95% in some orange varieties. For other citrus species, such as grapefruit, the proportion can be lower (between 80−90%), and in lemon, it can be even lower (bellow 75%). In the case of lemon, the presence of other monoterpene hydrocarbons, such as β-pinene and γ-terpinene, allows attainment of similar amounts of monoterpene hydrocarbons, as in orange. Other compounds, such as oxygenated monoterpenes (alcohols and aldehydes), also show interspecific variation.

The biological activity of citrus essential oils has been widely recognized.[30] For fruit flies, larval toxicity of some of their compounds has been reported for *C. capitata*,[26,31] and in general monoterpene hydrocarbons show less toxicity than oxygenated monoterpenes.[31] In addition, the proportions of monoterpenes and other compounds of the essential oils vary according to senescence of the fruit. These changes have been associated both with host acceptance[32] and with a reduction in peel extracts toxicity.[26]

The interaction of all the resistance mechanisms offered by the plant results in a variation between species and cultivars in their suitability as hosts. For example, lemon has been reported as the poorest host citrus for larval survival of *C. capitata*[24,23] and the Caribbean fruit fly, *Anastrepha suspensa* (Loew).[22] For *A. fraterculus* populations from Argentina, the absence of larval development allows proposing that lemon is not a host,[33] yet the mechanisms involved have not been investigated. In addition, the inability of the Mexican morphotype of *A. fraterculus* to successfully develop in the Valencia orange and the Ruby Red grapefruit renders the status of Mexican *A. fraterculus* as a pest of citrus in Mexico unsubstantiated.[34] In contrast, grapefruit has been reported as a good host for *A. suspensa*[22] and the Mexican fruit fly, *Anastrepha ludens* (Loew.),[35] and it was found naturally infested by *A. fraterculus* and *C. capitata* in Argentina.[17,18] For the case of *A. ludens*, it has been argued that its long ovipositor allows the females to lay the eggs in the albedo area of the peel, thus preventing the contact of the newly emerged larvae with the toxic compounds present in the essential oil glands from the flavedo on their way to the pulp.[36] Moreover, the susceptibility of citrus to *A. suspensa* was found to vary according to the senescence of the fruit.[22,37]

Given the relevance of understanding the resistance mechanisms of the plants that can affect host use in fruit flies, we investigated the role of peel extracts of lemon and grapefruit on the survival of the immature stages of *A. fraterculus*. We used an integrative approach and evaluated the insecticidal activity of the ether extracts obtained from the peel of the two citrus species as well as the toxicity of the major constituent of the essential oil (limonene) and one oxygenated monoterpene (citral). In addition, we evaluated the impact of the ripening stage of the fruit on the toxicity. We also evaluated another fruit fly species, *C. capitata,* from which extensive research has been done for comparison. Finally, we characterized the chemical composition of the extracts to allow identification of putative responsible compounds in case we found a differential mortality.

MATERIALS AND METHODS

Insects. Eggs and first instar larvae of *A. fraterculus* and *C. capitata* were obtained from colonies established at the Agriculture Zoology laboratories of Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina. *Anastrepha fraterculus* colony was initiated in 1997 with pupae obtained from infested guavas, collected in the vicinity of Tafí Viejo, Tucumán province (northwest Argentina).[38] Wild individuals were introduced into the laboratory and were maintained following standard procedures.[38,39] *Ceratitis capitata* colony was initiated with wild individuals obtained from infested oranges collected at different localities in northwest Argentina (Salta and Jujuy provinces) and held under artificial rearing conditions using standard procedures. For the bioassays, eggs were collected from the rearing cages for 4 h. To evaluate the egg stage, eggs were used right after collection. For the larval stage, eggs were incubated for 48 to 72 h in a chamber at 25 ± 2 °C and 60 ± 20% relative humidity to obtain the larvae. All the experiments were conducted in the laboratory.

Plant Material. The citrus varieties used were lemon, *C. limon* var. Eureka, and grapefruit, *C. paradisi* var. Foster Seedless. Fruit were collected in May 2011 and in August 2012 from the experimental orchard at EEAOC (26°47′15,45″/65°11′23,72″) in Las Talitas, Tucumán, Argentina. These periods corresponded to two different ripening stages of the fruit; the fruit from May was at the stage in which it is harvested for its commercialization (the fruit is already ripening and is turning from green to yellow) while the fruit from August corresponded to the overripe stage. Fruits were randomly selected from different plants; however, special precaution was taken to avoid damaged fruit or with symptoms of illness or pests.

Extraction of Ether Extracts. One day after harvest, the fruits were washed with tap water and dried at room temperature. The flavedo was removed from the peel with a metal grater and placed in a glass Erlenmeyer. Peel compounds were extracted with ethyl ether by immersion. The flask was covered with a cotton plug and was placed on a shaker for 40 min. Ether extracts were filtered, and the solvent was evaporated using a rotary evaporator at room temperature.

Chemical Compounds. Two compounds were also evaluated. The monoterpene hydrocarbon limonene was selected, since it is the predominant compound of essential oil citrus extracts; the oxygenated monoterpene citral was selected because it is a component that exhibited high toxicity against *C. capitata* larvae.[26,31] (*R*)-Limonene (98%) and citral (mixture of neral and geranial) (95%) were purchased from Sigma-Aldrich, Buenos Aires, Argentina.

Chemical Characterization of the Ether Extracts from the Peel. The chemical characterization of the extracts was performed at the Laboratory for Research and Analytical Services (LISA) from Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (FBQF-UNT) (Tucumán, Argentina). The ether extracts were analyzed by gas chromatography (GC) using an Ultra Trace gas chromatograph with DB-1 column-MS 25 mm × 0.25 mm ID, temperature ramp of 60 to 300 °C (3 °C/min), and an injection temperature of 270 °C. The mass spectrometer used was a Polaris Q, EI (+) 70 eV with an ion trap analyzer as detector. Individual peaks were identified by the retention time and retention rates. At least two independent analyses were performed for each extract. The results were processed to obtain the percentage of the area occupied by each compound, and this value was averaged in each extract. The components were identified by the comparison of their retention index (RI) with reference to a homologous series of *n*-alkanes (C9−C25), by comparing their mass spectra with those reported in the literature, and by computer matching with the Adams 31[40] library.

Bioassays. *Fumigant Toxicity Assessment. Eggs.* Fumigant toxicity was determined by exposing the eggs to the volatiles of the ether extracts or to the pure compounds. For each experimental unit, 20 eggs were deposited with the aid of a fine brush on a piece of black filter paper (2 cm × 2 cm) which was in turn placed over a dampened cloth (5 cm × 5 cm) inside a glass Petri dish of 10 cm diameter (90 cm^3 volume). The cloth was used to avoid the dehydration of the eggs. On the other side of the Petri dish, and without being in contact with the damp cloth, a square of filter paper (3 cm × 3 cm), previously impregnated with the extract or the corresponding compound, was placed. The Petri dish was sealed with parafilm and incubated at 25 °C for 24 h. Then the damp cloth and the filter paper with the eggs were placed in an untreated plastic Petri dish of 10 cm diameter and left for incubation. After 5 days, the number of chorions (corresponding to those individuals that survived the embryonic developmental stage)

Table 1. Chemical Compositions of the Ether Extracts from Citrus Peel of Ripe and Overripe Fruit of Lemon and Grapefruit, and Their Relative Proportions (% Area)

		ripe		overripe	
compound	RI[a]	*C. limon*	*C. paradisi*	*C. limon*	*C. paradisi*
α-thujene	935	0.25 ± 0.02[b]			
α-pinene	942	1.20 ± 0.17	0.22	0.69 ± 0.17	
camphene	957	1.27 ± 0.17	0.33 ± 0.06		
sabinene	983			1.13 ± 0.23	0.28 ± 0.06
β-pinene	988	6.08 ± 0.73	0.21 ± 0.05	5.94 ± 0.59	0.23 ± 0.31
myrcene	1000	1.54 ± 0.17	1.36 ± 0.12	1.11 ± 0.21	1.30 ± 0.26
octanal	1010	0.02 ± 0.01	0.58 ± 0.3		
pseudolimonene	1018	tr[c]			
o-cimene	1032	0.18 ± 0.25		0.66 ± 0.13	0.07
d-limonene	1042	71.50 ± 1.37	82.70 ± 0.98	64.3 ± 4.58	88.5 ± 0.88
(ε)-β-ocimene	1055	0.11	0.23 ± 0.05		
γ-terpinene	1068	9.38 ± 0.43	0.10 ± 0.01	11 ± 0.6	0.34 ± 0.49
cis-sabinene hydrate	1075	0.08 ± 0.02	0.33 ± 0.44		
terpinolene	1097	0.32 ± 0.01	0.07 ± 0.05		
linalool	1107	0.27 ± 0.05	0.43 ± 0.28		
nonanal	1111	0.09 ± 0.01	0.12 ± 0.08		
camphor	1158	0.04 ± 0.01	0.12 ± 0.06		
(ε)-isocitral	1190	0.38 ± 0.05	0.43 ± 0.31	0.56 ± 0.12	0.12 ± 0.02
decanal	1209	0.02 ± 0.01	0.71 ± 0.3		
nerol	1229	0.32 ± 0.03	0.14 ± 0.09	0.86 ± 0.2	0.02
neral	1240	1.25 ± 0.11	0.17 ± 0.11	1.13 ± 0.19	0.05 ± 0
geraniol	1252	0.23 ± 0.02	0.10 ± 0.08	0.9 ± 0.2	0.04 ± 0.04
geranial	1267	1.82 ± 0.16	0.36 ± 0.22	1.7 ± 0.29	0.08 ± 0.03
undecanal	1299	0.03 ± 0.01	0.07 ± 0.04		
neryl acetate	1357	0.44 ± 0.02	0.02	1.53 ± 0.25	0.03 ± 0.01
α-copaene	1368		0.27 ± 0.03		
geranyl acetate	1375	0.25 ± 0.01	0.16 ± 0.07		
β-elemenene	1381	0.01	0.23 ± 0.03		
dodecanal	1398		0.06 ± 0.03		
β-caryophillene	1409	0.33 ± 0.01	0.65 ± 0.01	0.39 ± 0.08	1.05 ± 0.22
trans-α-bergamotene	1425			1.17 ± 0.16	0.02
α-humulene	1442	0.03 ± 0.01	0.11 ± 0.02		
bicyclo germacrene	1482	0.05 ± 0.02	0.07 ± 0.03		
β-bisabolene	1494	0.72 ± 0.03		1.88 ± 0.35	
δ-cadinene	1506	tr	0.3 ± 0.01		
hexadecanoic acid	1865	0.02 ± 0.01	0.10 ± 0.01		
citroptene	1875	0.24 ± 0.15	0.01	0.28 ± 0.07	
bergamotene	1929	0.01	0.09 ± 0.01		
ostole	1989		0.19 ± 0.01		
coumarin	2056	0.01	0.35 ± 0.47		
oxypseucedanin	2063		5.69 ± 1.82		
prangenin	2210		0.23 ± 0.01		
auraptene	2292		1.56		
unidentified	2304	0.05	1.03 ± 0.76		
monoterpene hydrocarbons		91.86	85.55	85.58	90.75
sesquiterpene hydrocarbons		1.14	1.63	4.02	1.57
alcohols		0.90	0.99	2.24	0.24
aldehydes		3.61	2.48	3.38	0.35
esters		0.69	0.18		
coumarins		0.25	1.66	0.28	0.1
total		98.57	99.86	95.12	92.09

[a]Retention index on a DB-1MS column relative to homologous series of *n*-alkanes. [b]Mean ± SE. [c]tr, trace, <0.01%.

and turgid eggs (corresponding to dead individuals) were quantified. In the controls, the filter paper was clean.

Larvae. The experimental design was similar to that used for eggs; each experimental unit consisted of 20 larvae that were placed on a black filter paper which, in this case, was placed over a pile of five pieces of white filter paper (2 cm × 3 cm) moistened with a sugar solution 10% (w/v). The extract or compound was applied on another piece of filter paper, placed at the opposite end of the Petri dish. The plate was sealed with parafilm and incubated for 24 h, the time at which larval mortality was registered (each larva was considered dead

Table 2. Fumigant Activity of the Ether Extracts of Lemon cv. Eureka and Grapefruit cv. Foster Seedless and Two Monoterpenes in Eggs and Larval Stages of *Anastrepha fraterculus*

stage	compound	n	LC_{50}[a] (95% CL[b]) $\mu L/cm^3$ air		LC_{90}[a] (95% CL[b]) $\mu L/cm^3$ air		χ^2 (d.f.)
egg	lemon ether extract	597	0.23 a[c]	(0.20−0.26)	0.36	(0.32−0.42)	0.56 (3)
	grapefruit ether extract	601	0.28 a	(0.23−0.33)	0.62	(0.50−0.87)	0.12 (2)
	limonene	599	0.16 b	(0.14−0.18)	0.27	(0.24−0.31)	1.71 (3)
	citral	599	0.04 c	(0.02−0.06)	0.16	(0.09−0.42)	2.25 (2)
larva	lemon ether extract	1060	0.07 c	(0.03−0.12)	0.34	(0.16−6.19)	8.90 (3)
	grapefruit ether extract	1017	0.08 c	(0.05−0.11)	0.40	(0.19−7.38)	3.88 (3)

[a]Lethal concentration. [b]Confidence limits. [c]Values followed by different letters are significantly different ($p < 0.05$).

Table 3. Fumigant Toxicity of the Ether Extracts of Ripe and Overripe Lemon cv. Eureka and Grapefruit cv. Foster Seedless on Eggs of *Anastrepha fraterculus*

compound	ripening stage	n	LC_{50}[a] (95% CL[b]) μL extract/cm^3 air		LC_{90}[a] (95% CL[b]) μL extract/cm^3 air		χ^2 (d.f.)
lemon ether extract	ripe	1078	0.16 ab[c]	(0.14−0.18)	0.24	(0.22−0.28)	0.30 (2)
	overripe	893	0.19 a	(0.17−0.21)	0.27	(0.24−0.34)	0.01 (1)
grapefruit ether extract	ripe	1068	0.14 b	(0.12−0.15)	0.21	(0.19−0.25)	0.06 (2)
	overripe	1074	0.16 ab	(0.14−0.17)	0.23	(0.21−0.28)	0.56 (2)

[a]Lethal concentration. [b]Confidence limits. [c]Values followed by different letters are significantly different ($p < 0.05$).

when it remained immobile even after touching it gently with a brush). The control was performed similarly but without applying any extract or compound.

Contact Toxicity Assessment. Eggs. Contact toxicity was evaluated by incubating the eggs in an emulsion of water with a sodium salt of carboxymethylcellulose containing the extract or compound.[41] The eggs were obtained and manipulated as described previously. Once the black filter paper with the 20 eggs was placed in the damp cloth, 100 μL of the emulsion was applied with a micropipette (Eppendorf). The Petri dish was sealed with parafilm and incubated at 25 °C for 5 days. The control was performed similarly, but the emulsion contained no extract or compound. Mortality was assessed as described for fumigant toxicity bioassays.

Larvae. Contact toxicity also involved the use of an emulsion which, in this case, contained sugar to provide food. Larvae were obtained and manipulated as described in the fumigant toxicity section. After applying the emulsion (100 μL), the Petri dish was sealed with parafilm. For the controls, the larvae were imbibed in the emulsion that lacked any extract or compound. Larval mortality was registered after 24 h of exposure. Both bioassays were performed in compliance with the appropriate laws and institutional guidelines to meet security standards and adequate animal handling.

Experiments and Treatments. *Experiment 1. Fumigant Toxicity of Lemon and Grapefruit Ether Extracts and Limonene and Citral on Egg and Larval Stages.* For the egg stage, the concentrations used were 0.07, 0.14, 0.28, 0.56, 1.11, and 2.22 μL extract/cm^3 air for the lemon and grapefruit extracts; 0.11, 0.21, 0.42, 0.83, and 1.11 $\mu L/cm^3$ air for limonene and 0.01, 0.11, 0.22, 0.44, and 0.89 $\mu L/cm^3$ air for citral. The number of individuals evaluated for each concentration ranged between 60 and 100, involving three to five independent repetitions. For the larval stage, the concentrations used were 0.014, 0.028, 0.056, 0.111, and 0.222 μL extract/cm^3 air. The number of individuals evaluated for each concentration ranged between 100 and 200, involving five to ten independent repetitions.

Experiment 2. Fumigant Toxicity of Extracts Obtained at Different Ripening Stages from Lemon and Grapefruit on Egg Stage. The concentrations used were 0.07, 0.14, and 0.28 μL extract/cm^3 air for the extracts of ripe lemon and grapefruit. The number of individuals evaluated for each concentration in each extract ranged between 60 and 100, involving three and five independent repetitions. The concentrations for overripe fruit were 0.07, 0.14, 0.28, and 0.55 μL extract/cm^3 air. The number of individuals evaluated for each concentration in each extract ranged between 60 and 100, involving three and five independent repetitions.

Experiment 3. Comparative Analysis between A. fraterculus and C. capitata. The comparative analysis between *A. fraterculus* and *C. capitata* involved peel extracts of lemon and grapefruit and limonene and citral. A contact assay for eggs and larvae was performed. For eggs, dilutions were performed at 50%, starting from an emulsion containing 100 μL of fruit extract or limonene, while for citral, they were performed starting from an emulsion containing 20 μL per mL. Eight concentrations were used: 1.56, 5, 6.25, 12.5, 25, 50, 75, and 100 μL per mL for extracts or compound in eggs of *A. fraterculus*, seven concentrations of lemon or grapefruit extract were evaluated on *C. capitata*: 6.25, 12.5, 25, 50, 100, 200, and 400 μL per mL for extracts. Dilutions for limonene started from an emulsion containing 100 μL per mL, and seven concentrations were used. One to four replicates were performed for each extract. For the larvae, dilutions were also performed at 50%, starting with an emulsion containing 20 μL of extract or compound per mL of solution. A total of nine concentrations were applied: 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 6.25, 12.5, and 20 μL per mL.

Data Analysis. Data were analyzed by a probit analysis to obtain LC_{50} and LC_{90} values. Mortality was corrected in those cases in which the control cages had dead individuals (mortality attributable to a reason not related to the toxicity of the essential oil). The significance of the model was determined by a goodness of fit χ-square test estimated with maximum likelihood. In all cases, the statistical package POLO Plus V1 (Software LeOra 2002−2003)[42] was used. Differences between LC_{50} values were considered significant when the respective Confidence Intervals 95% (CI95%) did not overlap.

RESULTS

Chemical Characterization of the Ether Extracts from Lemon and Grapefruit. The chemical characterization of the different extracts is presented in Table 1. For all extracts, the major chemical group was monoterpene hydrocarbons, and within this group, the major compound was D-limonene (higher than 70%), except in the extract from overripe lemon. The remaining chemicals ranged from 10.96% to 0.01%. The main constituents (more than 1% of the total area) of the extract of ripe lemon were D-limonene, γ-terpinene, β-pinene, geranial, myrcene, camphene, neral, and α-pinene. In the case of grapefruit extract, the major compounds were limonene, oxypseucedanin, auraptene, and myrcene. The extract of the overripe lemon was characterized by D-limonene, γ-terpinene, β-pinene, β-bisabolene, geranial, neryl acetate, α-*trans*-berga-

Table 4. Contact Toxicity of the Lemon Ether Extract cv. Eureka and Grapefruit Ether Extract cv. Foster Seedless, and Compounds Limonene and Citral in *Anastrepha fraterculus* and *Ceratitis capitata* Eggs

compound	species	n	LC_{50}[a] (95% CL[b]) μL/mL		LC_{90} (95% CL) μL/mL		χ^2 (df)
lemon ether extract	*A. fraterculus*	520	18.12 ac[c]	(6.11−26.03)	36.87	(25.53−58.46)	9.95 (6)
	C. capitata	177	113.46 b	(68.15−158.75)	323.94	(231.62−596.73)	2.43 (4)
grapefruit ether extract	*A. fraterculus*	802	26.25 c	(17.83−35.59)	57.64	(41.93−97.99)	8.21 (4)
	C. capitata	264	72.94 bd	(41.13−111.50)	240.43	(150.60−632.98)	9.16 (5)
limonene	*A. fraterculus*	258	34.04 cd	(25.38−42.74)	80.37	(60.30−146.11)	5.68 (6)
	C. capitata	140	77.06 b	(55.35−95.27)	119.64	(96.47−241.28)	3.33 (5)
citral	*A. fraterculus*	717	12.82 a	(11.26−14.36)	16.79	(14.95−19.75)	4.25 (5)
	C. capitata	712	22.44 c	(19.37−34.21)	41.76	(29.77−193.58)	1.46 (5)

[a]Lethal concentration. [b]Confidence limits. [c]Values followed by different letter are significantly different ($p < 0.05$).

Table 5. Contact Toxicity of the Lemon Ether Extract cv. Eureka and Grapefruit Ether Extract cv. Foster Seedless, and Compounds Limonene and Citral in *Anastrepha fraterculus* and *Ceratitis capitata* Larvae

compound	species	n	LC_{50}[a] (95% CL[b]) μL/mL		LC_{90} (95% CL) μL/mL		χ^2 (df)
lemon ether extract	*A. fraterculus*	234	0.43 a[c]	(0.14−0.73)	2.07	(1.20−7.92)	0.789 (4)
	C. capitata	148	0.44 a	(0.34−0.55)	0.84	(0.65−1.42)	2.867 (4)
grapefruit ether extract	*A. fraterculus*	253	0.61 ab	(0.25−1.06)	3.51	(1.94−12.09)	6.084 (5)
	C. capitata	155	0.60 a	(0.40−0.76)	1.16	(0.91−1.92)	0.272 (5)
limonene	*A. fraterculus*	171	0.84 abc	(0.04−2.57)	23.93	(6.30−9012.32)	6.419 (4)
	C. capitata	451	2.30 bc	(0.88−4.80)	2.28	(1.54−4.40)	21.726 (5)
citral	*A. fraterculus*	258	1.62 abc	(0.28−2.77)	4.98	(2.93−21.32)	4.538 (4)
	C. capitata	453	3.18 c	(2.29−4.01)	7.69	(4.96−35.86)	6.315 (5)

[a]Lethal concentration. [b]Confidence limits. [c]Values followed by different letters are significantly different ($p < 0.05$).

motene, neral, sabinene, and myrcene. Overripe grapefruit contained D-limonene, NI compound, myrcene, and β-caryophyllene.

Fumigant Toxicity of Different Citrus Ether Extracts and Pure Compounds. The volatile phases from lemon and grapefruit extracts were equally toxic to eggs of *A. fraterculus* ($p > 0.05$) (Table 2). Citral was four times more toxic than limonene, with their LC_{50} values (0.04 and 0.16 μL/cm^3 air, respectively) being significantly different ($p < 0.05$). Additionally, limonene was significantly more toxic than both lemon and grapefruit extracts ($p < 0.05$). In agreement with what was found in eggs, larval mortality was equal for lemon and grapefruit extracts ($p > 0.05$) (Table 2).

Fumigant Toxicity of Ether Extracts Obtained at Different Ripening Stages from Lemon and Grapefruit. The extract from overripe lemon was slightly less toxic than the extract from ripe lemon, but the difference between their LC_{50} values was not significant ($p > 0.05$). The extracts of ripe and overripe grapefruit were equally toxic ($p > 0.05$) (Table 3).

Comparative Analysis between *A. fraterculus* and *C. capitata*. *Eggs.* Lemon extract was six times more toxic to eggs of *A. fraterculus* than to eggs of *C. capitata* with their LC_{50} values (18.12 and 113.46, respectively) being significantly different ($p < 0.05$) (Table 4). Grapefruit extract was also significantly more toxic to eggs of *A. fraterculus* than to eggs of *C. capitata* (LC_{50} 26.25 and 72.94, respectively) (Table 4). Eggs of *A. fraterculus* were significantly more susceptible to limonene and citral than those of *C. capitata* ($p < 0.05$) (Table 4).

Larvae. Toxicity values of lemon and grapefruit extracts as well as of limonene and citral revealed no differences in sensitivity between *A. fraterculus* and *C. capitata* ($p > 0.05$) (Table 5). Contrary to what was found for eggs, citral was equally as toxic as limonene ($p > 0.05$).

DISCUSSION

The presence of toxic compounds in the citrus essential oils from the citrus peel has been proposed as the main resistance mechanism of citrus species against fruit flies. Our integrative approach allowed us to evaluate the toxicity of lemon and grapefruit peel extracts in *A. fraterculus* eggs and larvae, the toxicity of limonene (the major compound of both citrus species essential oils) and citral (one highly toxic oxygenated monoterpene), and the effect of fruit ripening on the toxicity from the extracts of the citrus peel. Moreover, with this approach we were able to compare *A. fraterculus* susceptibility with that from *C. capitata*, from which host records in citrus and resistance mechanisms are better known.

Extracts from the peels of lemon and grapefruit proved toxic to *A. fraterculus* eggs and larvae. This is in agreement with what has been found for *A. suspensa*[22] and *C. capitata*[26,31] and confirms, by contact and fumigant bioassays, the occurrence of chemical resistance mechanisms. Other works extended the detrimental effects of several essential oils to the adult stage as an attempt to find organic insecticides.[7,30] Evaluations involved essential oils from citrus species such as *C. arantium* L. against *C. capitata* and the olive fly, *Bactrocera oleae* (Gmelin),[27] and from other plants such as *Hyptis suaveolens* (L.) Poiteau, *Rosmarinus officinalis* L., *Lavandula angustifolia* Miller and *Thuja occidentalis* L.,[41] and *Tagetes* spp.[43] in *C. capitata*. There is even one case of development of a novel packaging system controlling the release of essential oils from *Eugenia caryophyllata* Thumb against *C. capitata*.[44] Here, we did not explore if the extracts were toxic to the adult stage given that our main objective was to study insect−plant interaction and the role of chemical resistance mechanisms present in the fruit as a determinant of its status as a host of *A. fraterculus*. Our

results provide good evidence to sustain that lemon has chemically mediated resistance mechanisms.

Lemon and grapefruit extracts showed the same levels of toxicity within and between fruit fly species. This was confirmed in the fumigant (*A. fraterculus* only) and contact (*A. fraterculus* and *C. capitata*) bioassays and at both the egg and larval stages. Comparative studies between citrus species in *A. suspensa* larvae showed the same trend; grapefruit, orange, and lemon oils were equally toxic.[22] In contrast, studies conducted in *C. capitata* larvae showed that lemon oil from the variety Maglini was less toxic than the oil of three varieties of sweet orange (Merlin, Artas, and New Hall) and the oil of bitter orange (*Citrus aurantium L.* (Rootstock)).[31] The authors attributed this difference to the presence of α- and β-pinene (less toxic than limonene) and to the low concentration of limonene in the lemon oil (less than 75%), while in the orange varieties evaluated, limonene was always above 95%. In our study, even when the amount of limonene in the extract from Eureka lemon (71%) was also lower when compared with the extract of Foster Seedless grapefruit (83%), the toxicity was similar. The chemical profiles of Maglini (Table 1)[31] and Eureka (this study, Table 1) lemons presented similar amounts of monoterpene hydrocarbons (91.7% and 91.9% for Papachristos et al.[31] and for our work, respectively) and oxygenated monoterpenes (6.4% and 5.2% for Papachristos et al.[31] and for our work, respectively). However, we did not evaluate extracts with limonene levels higher than 95%, whereas Papachristos et al.[31] evaluated different orange varieties that contained limonene levels higher than 95%. On the other hand, the work presented by Greany et al.[22] lacks information on oils chemical composition. In conclusion, more studies involving more citrus species are needed in order to assess different susceptibility among fruit fly species attributable to different limonene amounts.

The toxicities of limonene and citral were different and depended on the stage evaluated. For eggs, limonene was less toxic than citral, for both *A. fraterculus* (fumigant and contacts bioassays) and *C. capitata* (contact bioassays). For larvae, the confidence intervals of the LC_{50} obtained for *A. fraterculus* and *C. capitata* had a great overlap, suggesting that limonene and citral were equally toxic. This is not in agreement with what was found by Papachristos et al.[31] for *C. capitata* larvae. One possible explanation could be the interaction of some compounds with the media in which they are presented. In the case of Papachristos et al.,[31] compounds were dispensed in the larval diet while in our case they were applied in a sugar solution.

Overripe lemon extract was marginally less toxic to *A. fraterculus* eggs than that from ripe lemon. This can be explained by the fact that, in overripe lemon, limonene showed values below 70% of the area. It could be proposed that mixtures containing less than 70% of limonene will be less toxic than mixtures with higher values of this compound. This could be one reason for the senescence-related susceptibility of grapefruit to *A. suspensa* infestation.[37] However, our inferences are based on tests that evaluated fumigant activity. The extract from overripe lemon had equal content of citroptene compared to that of ripen fruit. This compound, being less volatile than limonene, could have not been available at the same amounts in the fumigant tests as in the contact. In addition, our asseveration needs further confirmation, since the confidence intervals had a slight overlap. As for grapefruit, the chemical profile was similar to that from the lemon, and this may be the main reason for the similar toxicity.

Larvae were more sensitive than eggs. This was shown in both the fumigant and contact evaluations. In the fumigant tests, larvae were approximately three times more sensitive while, for the contact tests, the relationship was much higher (approximately 45 times more sensitive). A major susceptibility in larvae compared to eggs has been reported for *C. capitata* after topical application of *Fueniculum vulgarem* Mill. oil.[45] In a study that evaluated the effect of egg age on fumigant toxicity of the essential oil from *Lavandula hybrid* Rev., *Rosmarinus officinalis* L., and *Eucalyptus globules* Labill in the beetle *Acanthoscelides obtectus* (Say), it was demonstrated that as the eggs aged, and consequently the embryo developed, the susceptibility to the toxic compounds from the essential oils increased.[46] The authors attributed this to the fact that monoterpenes act against insects as neurotoxins; thus, the ovicidal activity could only be apparent when the nervous system begins to develop. In our case, the exposure to toxic compounds was done to fresh eggs, so embryonic development is expected to have been at an early stage. Therefore, it would be necessary to test older eggs to confirm this hypothesis. Another possible and nonmutually exclusive explanation could be the importance of the chorion as a barrier to penetration of toxic compounds.

The comparison between *A. fraterculus* and *C. capitata* revealed differences between the two species. Eggs of *A. fraterculus* were more sensitive than eggs of *C. capitata* to the effect not only of lemon and grapefruit extracts but also of limonene and citral. In contrast, for the larvae, the toxicities in both species were similar. The differences found in eggs could be explained by differences in the permeability of the egg shell or the susceptibility to certain compounds or chemical groups. It is worth wondering whether the differences in egg sensitivity between species had an impact on the immature performance of the fruit. To express this difference, it is necessary that eggs are exposed to the essential oils, and this can happen if the females lay their eggs in the glands of the flavedo. It has been proposed that females can adjust their oviposition behavior and prefer laying their eggs in the less toxic area of the albedo to avoid the toxic flavedo. However, this can be possible only on those species in which the female has a long ovipositor. Birke et al.[36] have already proposed this as an explanation why *A. ludens* can infest different citrus species. In the same line of evidence, it has been indicated that the resistance that lemon exhibits to the attack of *A. suspensa*[22] would be related to the thickness of the flavedo, the high concentration of oxygenated terpenoids, such as linalool, and the absolute amount of oil present in the peel. Under this hypothesis, we should expect that *A. fraterculus* will not survive or will have a poor performance in citrus with thin albedo such as lemon and oranges and will be able to develop better in fruits with a thicker albedo such as grapefruit and bitter orange, since it has a long ovipositor. Field infestation data[17−19] and laboratory experiments[47] seem to support this hypothesis.

Conclusively, we showed that extracts from the citrus peel are toxic to *A. fraterculus* immature stages and this contributes to the nonhost condition of lemon for *A. fraterculus*. It is probable that this condition is attained by a combination of the presence of chemical resistance mechanisms, as shown here, and the structure of the lemon peel that enhances the chances of the eggs or the larvae to enter in contact with the essential oil. Our results and those from Augier et al.,[48] with thorough

inspections of field and packing houses, as well as those of Gastaminza et al.,[33] with fruit-infestation laboratory and field experiments, provide good evidence and follow the guidelines to determine the host condition of a given species and variety of fruit to a given fruit fly species.[49,50] The recognition of the nonhost status of lemon will surely contribute to improve trade agreements.

AUTHOR INFORMATION

Corresponding Author

*Telephone: (+54) 381 4390003. Fax: (+54) 381 4364156. E-mail: josefinaruiz2802@hotmail.com.

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Notes

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