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Fumigant toxicity of *Citrus sinensis* essential oil on *Musca domestica* L. adults in the absence and presence of a P450 inhibitor

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ABSTRACT

Essential oils (EOs) are potential tools for controlling *Musca domestica* L. In a fumigant assay, *M. domestica* adults treated with *Citrus sinensis* EO (LC_{50} = 3.9 mg/dm^3), with (4*R*)(+)-limonene (95.1%) being its main component, died within 15 min or less. The terpenes absorbed by the flies and their metabolites, analyzed using SPME fiber, were (4*R*)(+)-limonene (LC_{50} = 6.2 mg/dm^3), α-pinene (LC_{50} = 11.5 mg/dm^3), β-pinene (LC_{50} = 6.4 mg/dm^3), and two new components, carveol (LC_{50} = 1122 mg/dm^3) and carvone (LC_{50} = 19 mg/dm^3), in a proportion of 50, 6.2, 12.5, 6.3 and 25%, respectively. Carveol and carvone were formed by oxidation of (4*R*)(+)-limonene mediated by cytochrome P450, as was suggested by a fumigation assay on flies previously treated with piperonyl butoxide, a P450 inhibitor. In this experiment, an increase in the toxicity of the EO and (4*R*)(+)-limonene was observed, as well as a lower production of carveol and carvone.

1. Introduction

The house fly, *Musca domestica* L., is one of the most common insects associated with vectoring etiological agents of bacterial, protozoan and viral infections, a fact that turns this insect into a threat to livestock and public health as well as a major food contaminator (Förster et al., 2007; Malik et al., 2007). Intensive applications of a variety of synthetic insecticides to control *M. domestica* have led to the development of resistant strains to most of them all over the world (Acevedo et al., 2009; Liu and Yue, 2000; Marçon et al., 2003; Pinto and Prado, 2001; Sheni and Plapp Jr., 1990; Tang et al., 2002; White et al., 2007). As a consequence, there is a continuous search for new active ingredients to be used as alternatives to conventional insecticides.

More ecofriendly methods to control *M. domestica*, such as biological control and botanical insecticides have been suggested (Sosa and Tonn, 2008; Zurek et al., 2002). Different entomopathogenic bacteria (Ruiu et al., 2007) and fungi (Anderson et al., 2011) were shown to be effective to control flies either in lab or in field conditions. The use of botanical insecticides is another control option because they are safe to the environment and leave less residue as a consequence of their biodegradability. Part of this new proposal is the use of essential oils (EOs) as fumigants because of their low toxicity to warm-blooded mammals (Isman, 2000), their high volatility and their relatively low cost (Isman, 2006).

Palacios et al. have studied the fumigant toxicity of edible EOs in a 30 min exposure period at 26 ± 1 °C, showing *Citrus sinensis* (L.) Osbeck EO as one of the most active, requiring doses of 3.9 mg/dm^3 (equivalent to 4.6 μL/L) to induce 50% mortality in *M. domestica* adults (Palacios et al., 2009a). Surprisingly, the most abundant terpene of *C. sinensis* EO, (4*R*)(+)-limonene, showed an LC_{50} of 6.2 mg/dm^3 (Palacios et al., 2009a). The toxicity of *C. sinensis* EO against house flies was in the same order of magnitude (8 times less active) as the toxicity of the organophosphorus insecticide DDVP (LC_{50} = 0.5 mg/dm^3) (Palacios et al., 2009a), becoming *C. sinensis* EO a viable alternative to harmful chemical insecticides against *M. domestica*.

Citrus fruits, which are part of the human diet, provide a variety of nutrients, including vitamin C, folic acid, potassium, flavonoids, pectin and dietary fiber together with edible essential oils used for flavoring (Manners, 2007). Insecticidal activity against various household pests such as mosquitoes (Anaso et al., 1990; Michaelakis et al., 2009) and cockroaches (Ezeonu et al., 2001) have also been reported for the *C. sinensis* EO (Ezeonu et al., 2001; Palacios et al., 2009a).

This paper deals with the terpenes absorbed and metabolized by flies when they were exposed to *C. sinensis* EO vapors. In addition, the incidence of fly P450 on the toxicity of the essential oil was analyzed. This determination could contribute to the understanding of the mechanism of EO toxicity on this insect.

Abbreviations: EO, essential oil; PBO, piperonyl butoxide; SPME, solid phase microextraction; T, terpene.

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2. Materials and methods

2.1. Plant material

C. sinensis (sweet orange) fruit peel was obtained from domestic organic fruit gardens in Córdoba, Argentina. A voucher specimen (UCCOR 383) was deposited at the Herbarium Marcelino Sayago of the School of Agricultural Science, Catholic University of Córdoba and was identified by the agronomist Gustavo Ruiz.

2.2. Essential oil extraction and analysis

C. sinensis EO was extracted for 2 h by hydrodistillation in a Clevenger-type apparatus with a separate extraction chamber. The EO was dried over anhydrous sodium sulfate. The EO components were identified by direct injection in a gas chromatography/mass spectroscopic detector (GC–MS) Hewlett-Packard 5890 GC interfaced with a Hewlett-Packard 5970 mass spectrometer fitted with a column (HP-5MS, 30 m × 0.25 mm inner diameter, temperature range 50–240 °C at 5 °C/min). Helium was used as the carrier gas (flow rate = 0.9 mL/min). A chiral column (SUPELCO-beta-DEX 120, 60 m × 0.25 mm inner diameter, temperature range 50–240 °C at 5 °C/min) was used to resolve enantiomers. The mass spectrum was obtained at an ionization voltage of 70 eV. Identification of the components was based on the comparisons of their relative retention times and their mass spectra with those obtained from authentic samples and/or the NIST version 3.0 library. C_7–C_{30} saturated *n*-alkanes (Supelco, from Sigma–Aldrich St. Louis, MO, USA) were used as reference points in the calculation of relative retention indices (RI) (Adams, 2007). The EO components were quantified using a GC–FID chromatograph (GC-Agilent 6890) with FID and a capillary column (Agilent with 5% phenylpolysiloxane, 0.25 mm film thickness, 30 m × 0.32 mm inner diameter, temperature range 50–240 °C at 5 °C/min). Samples analyzed using solid phase microextraction (SPME) were also run in both the GC–MS and the GC–FID, for identification and quantification of the sample, respectively.

2.3. Chemicals

Compounds (4*R*)(+)-limonene, α-pinene, β-pinene, β-myrcene, linalool, terpineol and piperonyl butoxide (PBO), were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetone was purchased from Merck (Darmstadt, Germany).

2.4. House flies

M. domestica adults were collected from the experimental farm of the Catholic University of Córdoba, Córdoba, Argentina. The flies were transferred to a small cage and then reared in entomological cages (30 cm × 30 cm × 30 cm) at 26 ± 1 °C under a 12:12 light–dark cycle and 70% humidity. Flies were allowed free access to water and to milk powder. As an oviposition site, a mixture of bran (80 g), brewer's yeast (1 g), milk powder (19 g) and water (200 mL) was placed on a plastic beaker. When the larvae emerged, they were reared in this medium.

2.5. Fumigation bioassay

The bioassay against *M. domestica* was performed as previously reported (Palacios et al., 2009a,b). Ten 4–5-day-old adult houseflies, mixed sexes, were placed in a glass jar (1.2 dm^3) fitted with a screw cap with a 7-cm length of cotton yarn suspended from the center of the internal face of the cap. Different dosages (4–5 concentrations from 1 mg/dm^3 to 10 mg/dm^3) of *C. sinensis* EO or (4*R*)(+)-limonene (dissolved in 20 μL acetone) were applied to the yarn. The control glass jar had only acetone on the cotton yarn. Each assay was replicated three times. The jars were then tightly sealed and kept in a room at 26 ± 1 °C for 30 min. After 30 min of exposure, mortality was assessed in each group by softly stimulating each fly with the tip of a pen. Flies that did not respond were considered dead. Since no mortality was observed in controls, the corrections of treatment mortalities by Abbott's formula (Abbott, 1925) were not necessary. Then, mortality values were used to calculate the lethal concentration for the death of 50% (LC_{50}) provoked by each compound.

2.6. Determination of terpenes absorbed by house flies

After a fumigation bioassay (with $n = 100$) was performed, the dead flies (550 mg) were collected in a vial (10 mL) with a septum. With the aim of quantifing the compounds absorbed by the flies, 20 μL of a solution containing 5 mg/mL of camphor (internal standard) in acetone was added to the vial. The vial was placed in a bath at 60 °C for 15 min. The terpenes desorbed from *M. domestica* to the headspace of the vial, were captured using a SPME micro fiber (Supelco, Bellefonte, PA, USA; with polydimethylsiloxane, thickness 30 μm, length 1 cm). Then it was injected in a GC–MS and a GC–FID for identification or quantification of terpenes, respectively. Prior to these determinations, the optimal conditions of temperature, time of exposure and desorption temperature of the SPME fiber were established. The desorbed terpenes were quantified and expressed in μg of terpene per mg of fly; next, these numbers were transformed into relative percentages to simplify the comparison between treatments and the EO composition.

2.7. Determination of the synergistic effects of PBO

C. sinensis EO or (4*R*)(+)-limonene was assayed in combination with PBO, according to the method previously reported (Rossi et al., 2012). One hour before the fumigation assay, insects were anesthetized with a CO_2 current and a solution of PBO in acetone (20 mg/mL) was applied topically to the thoracic notum at a dose of 10 μg (0.5 μL) per fly. Then, a fumigation bioassay of *C. sinensis* EO or (4*R*)(+)-limonene (in doses from 1 mg/dm^3 to 8 mg/dm^3) was performed with the PBO-treated flies, as described above. Control groups only received acetone. The LC_{50} was thus determined. The dead flies were collected in a vial to identify and quantify the terpenes by GC-analysis.

2.8. Statistical analysis

Probit analysis (Harvard Programming; Hg1, 2) of concentration–mortality data was conducted to estimate the LC_{50} values and associated 90% confidence limits for each treatment.

3. Results and discussion

3.1. Determination of the terpenes absorbed by house flies

The components of *C. sinensis* EO used in this study are reported in Table 1. They were identified by GC–MS and quantified by GC–FID. The samples were transferred either by direct injection or through a SPME fiber. The most abundant terpene was (4*R*)(+)-limonene according the chiral chromatographic analysis. This terpene yielded 95% for the direct injection, and 95.1% detected by SPME-GC–FID, followed by β-pinene, β-myrcene, linalool, terpineol and α-pinene in the proportions shown in Table 1. Other components were less than 1% in total.

The *M. domestica* adults that died after treatment with *C. sinensis* EO (8 mg/dm^3) were transferred to a GC-vial and sealed. The head space composition was determined using a SPME fiber, to detect

Table 1
Chemical composition of *Citrus sinensis* essential oil, determined by GC–MS and FID, expressed as relative percentage on the total area of the chromatogram.

Peak	Compounds	RI[a]	RI[b]	Area (%)		Identification methods[c]
				By direct injection	By SPME	
1	α-Pinene	933	939	0.2	0.7	RI[a], MS, std
2	β-Pinene	970	979	2.2	2.2	RI[a], MS, std
3	β-Myrcene	983	991	0.6	0.5	RI[a], MS, std
4	(4*R*)(+)-Limonene	1028	1029	95.0	95.1	RI[a], MS, std
5	Linalool	1103	1097	0.6	0.8	RI[a], MS, std
6	γ-Terpineol	1197	1199	0.3	0.3	RI[a], MS,std
7	α-Terpineol	1188	1189	0.1	0.2	RI[a], MS, std
	Total			99.0	99.8	

[a] Retention index relative to C_7–C_{30} saturated *n*-alkanes on HP-5MS column.
[b] Retention index taken from Adams (2007).
[c] Identification based on comparison of the mass spectrum, retention index and standards.

Table 2
Porcentage of (4*R*)(+)-limonene, α-pinene, β-pinene, carveol and carvone recovery from dead flies by treatment with *Citrus sinensis* EO, (4*R*)(+)-limonene with or without piperonylbutoxide.

SPME analysis of	Relative amount (%)[a,b]				
	(4*R*)(+)-Limonene	α-Pinene	β-Pinene	Carveol	Carvone
C. sinensis EO	97.0 ± 1.5	0.7 ± 0.2	2.2 ± 0.1	nd	nd
Flies dead by action of *C. sinensis* EO	50 ± 1	6.2 ± 0.1	12.5 ± 0.3	6.3 ± 0.1	25 ± 0.5
Flies dead by action of *C. sinensis* EO + PBO	62.5 ± 1	6.3 ± 0.1	12.5 ± 0.3	nd	18.7 ± 0.5
(4*R*)(+)-Limonene	99 ± 0.5				
Flies dead by action of (4*R*)(+)-limonene	46.2 ± 1.4			15.3 ± 0.1	38.5 ± 0.2
Flies dead by action of (4*R*)(+)-limonene + PBO	66.6 ± 2.1			6.7 ± 0.1	26.7 ± 0.2

[a] Percentages were calculated by a standard internal method.
[b] nd: undetected with a limit of quantification of 0.3 μg/vial.

both the terpenes absorbed by the flies and any compound formed as a result of the insect metabolism. The chromatographic analysis detected five terpenes, three EO components, (4*R*)(+)-limonene, α-pinene, β-pinene and two new compounds identified as carveol and carvone. No minor EO components were detected in our quantification system at a limit of detection of 1 μg of terpene/fly. In order to compare the yield of the metabolite with the amounts of its precursors in the EO, we considered the sum of the relative amounts of the three terpenes [(4*R*)(+)-limonene, α-pinene and β-pinene] in the *C. sinenesis* EO as 100%, resulting in 97%, 0.7% and 2.2%, respectively (Table 2). After treatment with *C. sinensis* EO, the dead flies showed these three terpenes plus carveol and carvone in a relative proportion of 50%, 6.2%, 12.5%, 6.3% and 25%, respectively (Table 2). This finding strongly suggests that in *M. domestica* (4*R*)(+)-limonene was metabolized to carveol and carvone.

In order to prove this hypothesis, we assayed (4*R*)(+)-limonene under the same conditions as *C. sinensis* EO. The SPME-GC analysis of the fumigation experiment with (4*R*)(+)-limonene showed the presence of (4*R*)(+)-limonene, carveol and carvone in proportions of 46.2%, 15.3% and 38.5%, respectively (Table 2 and Fig. 1). This result demonstrates that in *M. domestica* (4*R*)(+)-limonene is transformed to carveol and carvone, probably by an oxidative detoxification pathway.

Such compounds as α-pinene and β-pinene were detected in the flies in a larger proportion than in EO, suggesting a selective absorption of these terpenes (Table 2) and also indicate that they are not metabolized.

3.2. Toxicity of metabolites

The LC_{50} of (4*R*)(+)-limonene was equal to 6.2 mg/dm^3 (Palacios et al., 2009a) while the LC_{50} of carveol and carvone was 1122 and 19 mg/dm^3, respectively (Rice and Coats, 1994). This means that carveol and carvone are 181 and 3 times, respectively, less toxic against *M. domestica* adults than (4*R*)(+)-limonene (Table 3) (Rice and Coats, 1994). Compounds α- and β-pinene were also toxic against *M. domestica* with LC_{50} of 11.5 and 6.4, respectively (Palacios et al., 2009a). In agreement with the LC_{50} values of the terpenes absorbed and produced by the fly metabolism and with the proportion of each of them in the insect, (4*R*)(+)-limonene would be the principal toxicant, followed by β- and α-pinene. Carveol and carvone, however contribute to decrease the toxicity of (4*R*)(+)-limonene rather than to increase the intoxication process.

The stronger toxicity of *C. sinensis* EO compared with that of (4*R*)(+)-limonene is possibly explained by the presence of α-pinene and β-pinene which contribute to (4*R*)(+)-limonene toxicity.

The carveol and carvone formation could be the result of the reaction of (4*R*)(+)-limonene with P450 oxidative system. In insects, cytochromes P450 are involved in a wide range of metabolic processes, from hormone syntheses to activation or degradation of xenobiotics (Feyereisen, 1999).

Few studies have been made about the insects' metabolism of (4*R*)(+)-limonene. When this terpene was mixed with an artificial diet of *Spodoptera litura* larvae at a concentration of 1 mg/g, (4*R*)(+)-limonene was transformed by *S. litura* mainly into uroterpenol (52%) and perillic acid (43%) (Miyazawa et al., 1998). The oxidation of (4*R*)(+)-limonene by cytochrome P450 has been

Table 3
LC_{50} of *Citrus sinensis*, (4*R*)(+)-limonene, and deltamethrin[a] with or without PBO against *Musca domestica* in fumigant bioassay.

Essential oil or terpene	Mean LC_{50} in mg/dm^3 (95% CI)	Slope	X^2
C. sinensis	3.9 (1.2–13)	2.5	6.532
C. sinensis + PBO	2.4 (0.9–6.6)	3.1	1.746
(4*R*)(+)-Limonene	6.2 (1.7–23)	6.4	0.404
(4*R*)(+)-Limonene+PBO	3.6 (1.4–9.8)	2.2	1.432
Deltamethrin[a,b]	9.2 (2.8–29.5)	1.1	0.920
Deltamethrin + PBO[a,b]	1.5 (0.2–11.4)	0.7	0.168

X^2: chi-square value, significant at $P < 0.05$ level.
[a] Applied topically and LC_{50} expressed in μg/fly.
[b] Taken from Rossi et al. (2012).

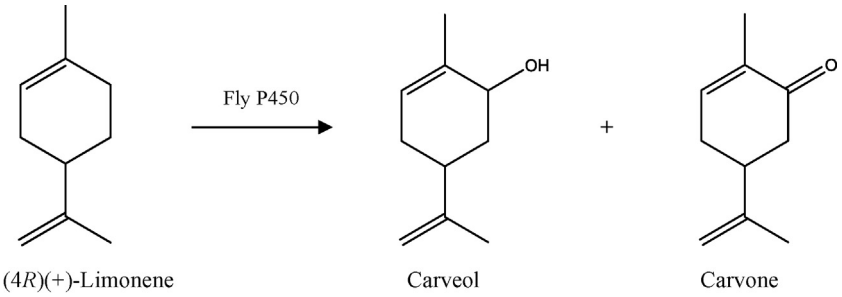

Fig. 1. Conversion of (4*R*)(+)-limonene to carveol and carvone mediated by fly cytochrome P450.

previously described for some organisms, including microbials (Lerin et al., 2010), plants (Wüst et al., 2001) and rats (Miyazawa et al., 2001). Microalga *Oocystis pusilla* transforms (4*R*)(+)-limonene into carveol, carvona and limonene oxide (Ghasemi et al., 2009). In rats, (+)- and (−)-limonene were found to be oxidized to their respective carveol and perillyl alcohol derivatives (Miyazawa et al., 2001).

3.3. Determination of PBO synergistic effect

In order to demonstrate the participation of *M. domestica* P450 oxidizing system in the metabolism of *C. sinensis* and of (4*R*)(+)-limonene, the LC_{50} of the EO and of the terpene were determined in flies previously treated with PBO, a recognized P450 inhibitor (Kasai and Scott, 2000). In the presence of this inhibitor, the toxicity of *C. sinensis* EO increased twice as much (LC_{50} (with PBO) = 2.4 mg/dm^3) (Table 3), whereas the LC_{50} of (4*R*)(+)-limonene diminished from 6.2 to 3.6 mg/dm^3, raising its toxicity level nearly twice. This result shows the same tendency demonstrated for the toxicity (topical) of deltamethrin against flies treated with PBO, where deltamethrin was 6 times more toxic to PBO treated flies than to untreated ones (Rossi et al., 2012).

The SPME analysis of the flies that died by the action of *C. sinensis* EO plus PBO showed the presence of (4*R*)(+)-limonene, α-pinene, β-pinene and carvone at 62.5%, 6.3%, 12.5% and 18.7%, respectively, while the metabolite carveol was not detected (Table 2). The decrease of the formation of carveol and carvone in *M. domestica*, as well as the decrease of LC_{50} (from 3.9 to 2.4 mg/dm^3), are in agreement with the participation of P450 in the metabolism of *C. sinensis* EO. Such participation leads to a smaller formation of less toxic terpenes (carveol and carvone), thus avoiding the detoxification of (4*R*)(+)-limonene and provoking the death of flies at smaller doses of EO or (4*R*)(+)-limonene. In the fumigation experiments of (4*R*)(+)-limonene against PBO treated flies, the relative amounts of carveol and carvone were reduced, ranging from 15.3% to 6.7% and from 38.5% to 26.7%, respectively (Table 2). Hence, these results confirm that the changes in LC_{50} registered for these compounds were due to a lower yield of (4*R*)(+)-limonene metabolites.

The other EO components absorbed by the flies, α- and β-pinene, were not metabolized by the P450 system, detecting them in *M. domestica* treated with and without PBO in the same proportion (Table 2). However, it has been reported that α-pinene is converted by the P450 insect into pheromones or polar metabolites (Tillman et al., 1999). This result may suggest that in *M. domestica* (4*R*)(+)-limonene reacts faster with P450, decreasing or avoiding the reaction of α- and β-pinene by this oxidation system.

The results of this and other studies (Rossi et al., 2012) suggest that once a terpene goes inside the fly, the P450 system detoxifies it. Terpenes like (4*R*)(+)-pulegone and menthone are oxidated by P450, transforming them in more toxic terpenes (Rossi et al., 2012). In contrast, (4*R*)(+)-limonene is turned into less toxic metabolites. However, when (4*R*)(+)-pulegone is mixed with (4*R*)(+)-limonene in the essential oil of *Minthostachys verticillata*, for example, the former is oxidized but the latter is not (Rossi et al., 2012). Terpenes such as α-pinene (Tillman et al., 1999) and β-pinene (Renwick et al., 1973), are known to be transformed by some insects, but they are not metabolized by *M. domestica* P450 when they go into fly together with (4*R*)(+)-limonene. These findings suggest that P450 may actually react only with the most abundant terpene, while the other terpenes present in the EO could positively contribute to the toxicity of the mix.

As a result of these findings, we suggest that if *C. sinensis* EO (or (4*R*)(+)-limonene) is to be used as a commercial insecticide against flies, it should be formulated with a P450 inhibitor, with the aim of increasing *C. sinensis* EO toxicity. Most common P450 inhibitors, including PBO, are non-volatile compounds. However, they have been used as ingredients of fumigant formulations. For example, PBO has been extensively used in residential fumigant products (Liu and Yue, 2000).

4. Conclusions

The present results indicate that the components of *C. sinensis* EO, (4*R*)(+)-limonene, α-pinene and β-pinene are absorbed by flies exposed to it; consequently, this oil acts as a potent fumigant against *M. domestica*. Flies metabolize (4*R*)(+)-limonene into carveol and carvone; these metabolites showed less toxicity against *M. domestica* and this fact suggests that the fly uses the oxidation reaction for the detoxification of (4*R*)(+)-limonene. The toxicity of the EO and of (4*R*)(+)-limonene increase when a P450 inhibitor is used in combination with either of them, suggesting that P450 monooxygenase mediated this detoxification.

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