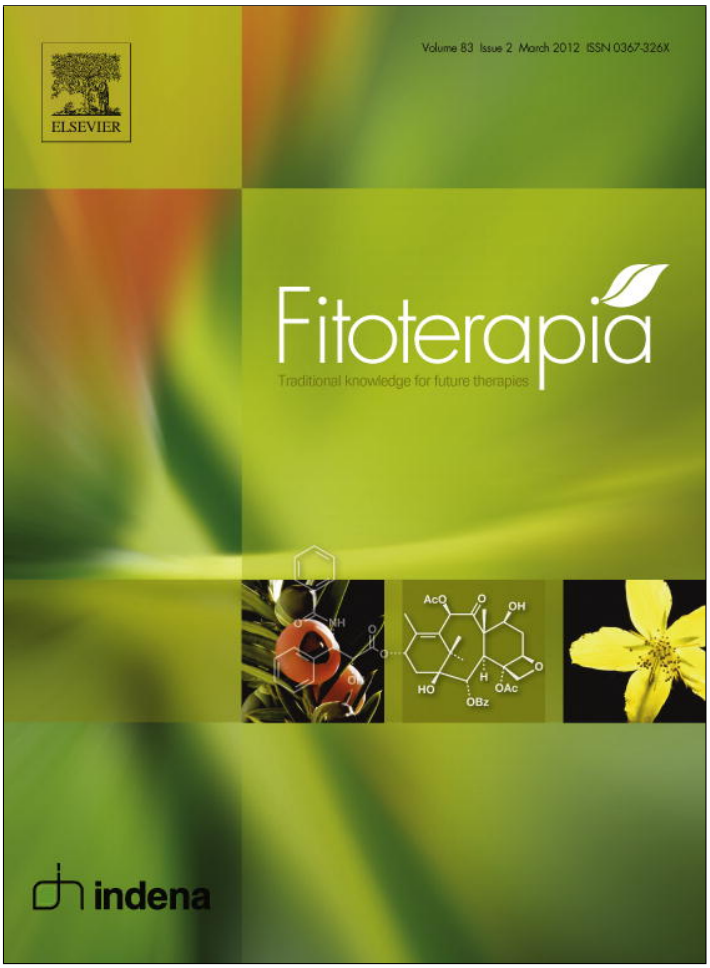

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Molecular response of *Musca domestica* L. to *Mintostachys verticillata* essential oil, (4*R*)(+)-pulegone and menthone

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ABSTRACT

Intense applications of synthetic insecticides for the control of adult *Musca domestica* have led to the insects developing resistance to most of them. In consequence, there is interest in new active ingredients as alternatives to conventional insecticides. Essential oils (EO) are potential tools for controlling *M. domestica* because of their effectiveness and their minimal environmental effects. In a fumigant assay, *M. domestica* adults treated with *Minthostachys verticillata* EO [LC_{50} = 0.5 mg/dm^3; majority components by SPME-GC: (4*R*)(+)-pulegone (67.5%), menthone (22.3%) and (4*R*)(+)-limonene (3.8%)], 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), menthone (LC_{50} = 1.9 mg/dm^3), (4*R*)(+)-pulegone (LC_{50} = 1.7 mg/dm^3) and a new component, menthofuran (LC_{50} = 0.3 mg/dm^3), in a relative proportion of 12.4, 6.5, 35.9 and 44.2% respectively. Menthofuran was formed by oxidation of either (4*R*)(+)-pulegone or menthone mediated by cytochrome P450, as demonstrated by a fumigation assay on flies previously treated with piperonyl butoxide, a P450 inhibitor, which showed a decrease in toxicity of the EO, (4*R*)(+)-pulegone and of menthone, supporting the participation of the P450 oxidizing system in the formation of menthofuran. The enzymatic reaction of isolated fly microsomes with the EO or the (4*R*)(+)-pulegone produced menthofuran in both cases. Contrary to expectations, the insect detoxification system contributed to enhance the toxicity of the *M. verticillata* EO. Consequently, resistant strains overexpressing P450 genes will be more susceptible to either *M. verticillata* EO or (4*R*)(+)-pulegone and menthone.

1. Introduction

The house fly, *Musca domestica* (L.), is a significant public health pest for humans and domesticated animals. It is a mechanical carrier of more than 100 human and animal intestinal diseases and is responsible for protozoan, bacterial, helminth, and viral infections [1,2].

The pest management of *M. domestica* is often aimed at the adult stage and based on chemical control. Intense applications of a variety of synthetic insecticides have led to the development of resistance to most of these around the world [3–9]. In consequence, there is a constant search for new active ingredients to be used as alternatives to conventional insecticides.

Essential oils (EOs) and their components, the terpenes (Ts), are potential tools for controlling *M. domestica* because of their selectivity (high toxicity for insects but not for other organisms) and their minimal environmental effects [10]. The toxicity of Ts against *M. domestica* has been studied extensively by Coats et al. [11–13], who demonstrated their insecticidal activity against several insect species via topical

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

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and fumigant application at 24 h. Recently, the fumigant toxicity of 23 EOs has been studied in a 30 minute exposure period at 26 ± 1 °C [14–16], showing *Minthostachys verticillata* EO as the most active, requiring doses of 0.5 mg/dm^3 (equivalent to 0.6 μl/l) to induce 50% mortality in *M. domestica* adults [15]. The toxicity of *M. verticillata* EO against house flies was of the same order of magnitude as the toxicity of the organophosphorus insecticide DDVP (LD_{50} = 0.5 mg/dm^3) [15], although the most abundant terpene of *M. verticillata* EO, (4*R*)(+)-pulegone, showed an LC_{50} of 1.7 mg/dm^3. Both natural products have the potential to offer a natural approach to the pest control of *M. domestica*.

Medicinal uses of *M. verticillata* date back to the native peoples of Andean South America [17]; nowadays, the aerial parts of this plant are used for the preparation of liquor and "amargos," a nonalcoholic beverage [17], and as aromatizing additives to the daily "mate" (*Ilex paraguarensis*) infusion [18], a social beverage of South America.

The aim of this work was to determine the terpenes absorbed by flies when they were exposed to *M. verticillata* EO vapors. This determination could bring together the detection of metabolites of the absorbed terpenes, and the understanding of the mechanism of toxicity exerted by the EO.

2. Materials and methods

2.1. Plant material

M. verticillata (Griseb.) Epling (peperina) leaves were collected in Traslasierra Valley, Córdoba, Argentina in 2009. A voucher specimen (UCCOR 125) has been deposited at the Herbarium Marcelino Sayago of the Faculty of Agricultural Science, Universidad Católica de Córdoba and was identified by the agronomist, Gustavo Ruiz.

2.2. Essential oil extraction and analysis

The essential oil 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 component analysis was performed by direct injection in a gas chromatography/mass spectroscopy detector (GC–MS) on a Hewlett-Packard 5890 GC interfaced with a Hewlett-Packard 5970 Series II mass spectrometer fitted with a column (HP-5MS, 15 m × 0.25 mm inner diameter, temperature range 50 °C to 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 °C to 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 comparisons of their relative retention times and mass spectra with those obtained from authentic samples and/or the NIST version 3.0 library. C7–C30 saturated alkanes (Supelco, from Sigma-Aldrich St. Louis, MO, USA) were used as reference points in the calculation of relative retention indices (RI).

Samples analyzed using solid phase microextraction (SPME, see Section 2.6) were run in 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 °C to 240 °C at 5 °C/min). Quantitative data were obtained electronically from FID area percent data without the use of correction factors. The products of enzymatic reaction were analyzed by direct injection into GC–FID under the same chromatographic conditions and using camphor as internal standard.

2.3. Chemicals

Camphor, deltamethrin, (4*R*)(+)-limonene, (4*S*)(−)-limonene, (+)-menthofuran, menthone, piperonyl butoxide (PBO), (4*R*)(+)-pulegone and (4*S*)(−)-pulegone used as standard and/or material for bioassays, were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetone was purchased from Merck (Darmstadt, Germany). Other reagents such as phenylmethyl-sulfonyl fluoride (PMSF), Na_2EDTA, dithiotreitol (DTT), glucose-6-phosphate dehydrogenase, glucose-6-phosphate, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. House flies

The colonies of *M. domestica* originated from adults collected from the experimental farm of the Universidad Católica of Córdoba, in Córdoba, Argentina, using a sweep net. The flies were transferred to a small cage and then reared in entomological cages (30 × 30 × 30 cm) at 26 ± 1 °C under a 12:12 light–dark cycle and 70% humidity. Adult flies were provided with water and fed a granulated mixture of sugar and powdered milk (approximately 1:1 v/v). Bran and milk were prepared at a weight ratio of 1:3 and 100 g of this mixture was placed on a plastic plate as an oviposition site.

2.5. Bioassay

The bioassay against *M. domestica* was performed as previously reported [14,15]. Briefly, 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 of *M. verticillata* EO, (4*R*)(+)-pulegone, menthone and menthofuran (dissolved in 20 μl acetone) were applied to the yarn. The jars were sealed tightly and kept in a room at 26 ± 1 °C for 30 min. Each test was replicated three times. The control vessel had only acetone on the cotton yarn. Mortality in each group was assessed after 30 min of exposure by softly stimulating each fly with the tip of a pen. Flies that did not respond were considered dead. The mortality determined was used to calculate the LC_{50} of the corresponding compound.

2.6. Determination of terpenes absorbed by house flies

After a fumigation bioassay was performed (n = 50), dead flies were collected in a vial (10 ml volume) with a septum, and then washed with hexane. The vial was placed in a bath at 60 °C for 10 min. Ts desorbed from *M. domestica* in the headspace of the vial were captured using a SPME micro

fiber (Supelco, Bellefonte, PA, USA; with polydimethylsiloxane, thickness 30 μm, length 1 cm), identified by GC–MS and quantified by GC–FID chromatography. Prior to these determinations, the optimal conditions of temperature, time of exposure and desorption temperature of the SPME fiber were established.

2.7. Determination of the synergistic effects of PBO

Four concentrations of PBO were tested on the viability of the flies in order to determine the effect of PBO by itself. The concentrations used were 1, 5, 10, and 20 μg/fly with three replicates each (n = 10). Based on the results of these tests, a dose of 10 μg/fly was chosen. To assay *M. verticillata* EO, (4*R*)(+)-pulegone, menthone and deltamethrin in combination with PBO, insects were anesthetized with a CO_2 current, and then 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, 1 h before the insecticide treatment. Then, a fumigation bioassay of *M. verticillata* EO, menthone or (4*R*)(+)-pulegone (in doses from 0.2 mg/dm^3 to 8 mg/dm^3), was performed with the PBO-treated flies, as described above. The dead flies were collected in a vial for GC-analysis. Deltamethrin, in doses from 1 to 14 μg/fly, was applied topically to the thoracic notum of the house fly, 1 h after application of PBO with three replicates each, with n = 10. Control groups received acetone alone.

2.8. Microsomal preparation

The microsomal preparation was performed as previously reported [19]. Briefly, 200 thoraces and abdomens of flies (3.8 mg of total protein) were homogenized on ice in 50 mM phosphate buffer, pH 7.2, containing 0.4 mM PMSF, 1 mM Na_2EDTA and 0.1 mM DTT. Homogenates were then centrifuged at 10,000 × *g* for 15 min at 4 °C. The resulting supernatant was subjected to ultracentrifugation at 100,000 × *g* (60 min, 4 °C), using a Beckman TLA-120.1 rotor (Beckman Coulter Inc., CA, USA). The pellet containing the microsomal fraction as a source of enzyme was suspended in the phosphate buffer containing 25% glycerol (v:v) and then analyzed for protein quantification using bovine serum albumin as standard [20]. When necessary, microsomes were stored at −80 °C to be used in the assays.

2.9. Enzyme assay

The standard assay for microsomal cytochrome P450 monoterpene hydroxylases has been previously described [21] and was adapted directly for use with solubilized fly homogenate. The reaction mixture, in a final volume of 1 ml, contained 50 mM Tris–HCl (pH 7.4), 1 mM Na_2EDTA, 0.1 mM DTT, 0.8 units glucose-6-phosphate dehydrogenase, 2 mM glucose-6-phosphate, 5 mM FAD, 5 mM FMN, 1 mM NADPH, and 68 μl of microsomal fraction (600 μg of protein). Substrate (4*R*)(+)-pulegone or *M. verticillata* EO (12.3 μmol and 4 μl respectively, in 10 μl hexane; the reaction was not influenced by the solvent) was added to start the reaction, which was allowed to proceed for 2 h at 30 °C with gentle shaking. Control incubations containing no cofactor (NADPH), no substrate and no microsomal fraction, were included in these experiments. The enzymatic reaction was stopped by chilling the mixture on ice. After the addition of 3.8 mg camphor as internal standard for capillary GC analysis, the reaction mixture was subjected to three steps of extraction using 1.0-ml portions of diethyl ether. The combined extract was passed through a short column of activated silica gel to remove highly polar materials, dried by the addition of anhydrous $MgSO_4$ and then the solution was analyzed by GC–MS for identification of possible new metabolites and GC–FID for quantitation.

2.10. Statistical analysis

The mean mortality data of the three replicates per dose (4–5 doses per EO or T) was used to calculate the LC_{50}. Probit analysis (Harvard Programming; Hg1, 2) was used to analyze the dose–mortality response.

3. Result and discussion

3.1. Determination of terpenes absorbed by house flies

The *M. verticillata* EO used in this study was composed of the terpenes reported in Table 1, which were determined by either direct injection in GC–MS or the SPME–GC–MS technique. The most abundant terpenes were menthone and (4*R*)(+)-pulegone at 17.8 and 66.3% respectively for the direct injection, and 22.3 and 67.5% respectively, detected by SPME–GC–MS (Table 1). The latter technique indicates the relative amount of the EO component in the vapor phase which is somewhat different from the EO composition. The vapor composition is possibly the best representation of the mixture of terpenes to which the insects were exposed.

Adults of *M. domestica* treated with *M. verticillata* EO at a level of 1.5 mg/dm^3, died in less than 15 min. These dead flies were transferred to a GC-vial, sealed and the head space composition was determined using a SPME fiber, to detect the terpenes absorbed by the flies as well as their possible metabolites. The assay detected three EO components, (4*R*)(+)-limonene, menthone and (4*R*)(+)-pulegone, and a new component, identified as menthofuran. No minor EO components were detected in our quantification system at a limit of detection of 1 μg of terpene/fly.

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

M. verticillata	Components (%)		
	RI	By direct injection	By SPME
Heptane	701	3.0	0.4
Myrcene	842	0.3	2.6
α-Pinene	933	0.8	1.7
(4*R*)(+)-Limonene	1028	3.0	3.8
δ-(3)-Carene	1031	3.5	
β-Ocimene	1051	0.3	
Terpinolene	1081	0.3	
Menthone	1155	17.8	22.3
Neomenthol	1157	0.2	
(4*R*)(+)-Pulegone	1243	66.3	67.5
Piperitone	1350	4.7	1.7

Table 2
Relative amount of (4*R*)(+)-limonene, menthone, (4*R*)(+)-pulegone and menthofuran recovery from dead flies by treatment with *Minthostachys verticillata* EO, (4*R*)(+)-pulegone or menthone, with or without piperonylbutoxide.

SPME analysis of	Relative amount (%)			
	(4*R*)(+)-Limonene	Menthone	(4*R*)(+)-Pulegone	Menthofuran
M. verticillata EO[a]	4.1 ± 0.6	23.8 ± 3.6	72.1 ± 4.3	nd
Flies dead by action of *M. verticillata* EO	12.4 ± 1.2	6.5 ± 3.8	35.9 ± 5.3	44.2 ± 3.0
Flies dead by action of *M. verticillata* EO + PBO	15.3 ± 1.3	9.7 ± 2.9	57.5 ± 4.8	17.5 ± 5.1
(4*R*)(+)-Pulegone			99 ± 0.4	nd
Flies dead by action of (4*R*)(+)-pulegone			55.5 ± 3.8	44.5 ± 5.2
Flies dead by action of (4*R*)(+)-pulegone + PBO			65 ± 6.4	35 ± 6.4
Menthone		100 ± 0.3		nd
Flies dead by action of menthone		76.0 ± 1.7		24.0 ± 0.2
Flies dead by action of menthone + PBO		86.0 ± 1.4		14.0 ± 1.3

[a] More terpenes were detected but only (4*R*)(+)-limonene, menthone and (4*R*)(+)-pulegone were considered because these were the only three terpenes detected in flies. nd: not detected.

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, menthone and (4*R*)(+)-pulegone] in the *M. verticillata* EO as 100%, resulting in 4.1, 23.8 and 72.1%, respectively (Table 2). After treatment with *M. verticillata* EO, the dead flies showed these three terpenes plus menthofuran in a relative proportion of 12.4, 6.5, 35.9 and 44.2% respectively. This finding strongly suggested that in *M. domestica*, (4*R*)(+)-pulegone and menthone were metabolized to menthofuran.

To test this hypothesis, we assayed (4*R*)(+)-pulegone under the same conditions as *M. verticillata* EO. The SPME–GC of the fumigation experiment with (4*R*)(+)-pulegone, showed the presence of (4*R*)(+)-pulegone and menthofuran in proportions of 55.5 and 44.5%, respectively (Table 2, Fig. 1). This result demonstrated that, in *M. domestica*, (4*R*)(+)-pulegone was transformed to menthofuran, probably by the oxidative detoxification pathway. A similar experiment, but using menthone to fumigate the flies, showed the conversion of this terpene into menthofuran in a proportion of 24.0% (Table 2, Fig. 1).

(4*R*)(+)-Limonene was also assayed in the same way, recovering a high proportion of this terpene (98.5%) accompanied by carvone (1%) and carveol (0.5%) (data not shown), but the last two terpenes were not detected in the flies treated with *M. verticillata* EO.

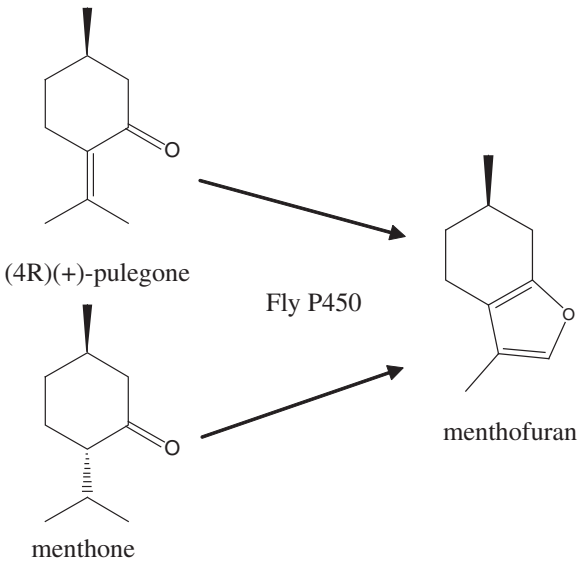

Fig. 1. Conversion of (4*R*)(+)-pulegone and menthone to menthofuran mediated by fly cytochrome P450.

3.2. Toxicity of metabolites

In order to determine the effect of menthofuran on adult *M. domestica*, we determined its LC_{50}, which was 0.3 (0.1–0.6) mg/dm^3, being 5.7 and 6.3 times more toxic than (4*R*)(+)-pulegone and menthone [LC_{50} = 1.9 (0.6–6.3) mg/dm^3; Table 3], respectively. Altogether, these results not only show the strong toxic effect of menthofuran on adult *M. domestica*, but also suggest that menthofuran, in combination with the other terpenes, may cause the death of flies after (4*R*)(+)-pulegone and menthone were absorbed by the insects.

The oxidation of (4*R*)(+)-pulegone to yield menthofuran by cytochrome P450 has been described previously in some organisms [22] including *Spodoptera* species [23], rats [24], plants [21] and humans [25]. By contrast, there is no current evidence of such conversion in *M. domestica*.

The metabolism of pulegone has been poorly studied in insects. In this context, Gunderson [23] determined that pulegone induced the activity of P450, including its own oxidation, in the larvae of *Spodoptera eridania* and *Spodoptera frugiperda* (Lepidoptera, Noctuide). In addition, when the

Table 3
LC_{50} of *Minthostachys verticillata*, (4*R*)(+)-pulegone, menthone 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)
M. verticillata	0.5 (0.02–2.8)
M. verticillata + PBO	1.5 (0.5–4.2)
(4*R*)(+)-Pulegone	1.7 (0.6–5.0)
(4*R*)(+)-Pulegone + PBO	4.4 (1.1–18.2)
Menthone	1.9 (0.6–6.3)
Menthone + PBO	2.8 (0.9–8.6)
Deltamethrin[a]	9.2 (2.8–29.5)
Deltamethrin + PBO[a]	1.5 (0.2–11.4)

[a] Applied topically and LC_{50} expressed in μg/fly.

insects were stressed with pulegone or menthofuran, acute and chronic toxicities were observed in both species.

In rats, (4*R*)-(+)-pulegone is metabolized by hydroxylation in the 5- and 9-positions to form 5-hydroxypulegone and 9-hydroxypulegone respectively, which in turn undergo further metabolism. 9-Hydroxylation is the main pathway, and the 9-hydroxypulegone undergoes cyclization to form (*R*)(+)-menthofuran [26,27]. In the second major pathway, (4*R*)-(+)-pulegone is subjected to stereoselective hydroxylation at the C-5 position to form 5-hydroxypulegone, which in turn dehydrates to form *p*-mentha-1,4(8)-diene-3-one-piperitenone [28]. (*R*)(+)-Menthofuran is converted to an α,β-unsaturated-γ-ketoaldehyde, by studies carried out *in vivo* and *in vitro* [26,29,30].

Although our results in flies clearly showed the conversion of (4*R*)(+)-pulegone and menthone into menthofuran, no transformation of this last terpene into further metabolites could be demonstrated, at least in our range of sensitivity (1 μg of terpene/fly).

On the other hand, carvone or carveol were not detected in the experiments with the EO, probably because they were in amounts below the GC-detection limit. Rice and Coast [11,12] determined the fumigation LC_{50} of carvone and carveol as 19 and 1122 mg/dm^3 respectively, considerably less toxic than limonene (LC_{50} = 6.2 mg/dm^3) [14] and other terpenes mentioned, suggesting that if those metabolites were formed in small quantities, they would not contribute to the toxicity of *M. verticillata* EO.

The other compounds present in the vapor fraction of *M. verticillata* EO (such as heptane, myrcene, α-pinene and piperitone) were not detected as compounds absorbed/metabolized by *M. domestica*. In the literature, these compounds do not appear as a possible source of menthofuran. For instance, it is known that myrcene and α-pinene are converted by the insect P450 to pheromones or polar metabolites [31], but not to menthofuran or its precursors. There is little information about the metabolism of piperitone by insects, or for neomenthol. The latter terpene is a diasteroisomer of menthol; when menthol was consumed by *Pieris brassicae* (Lepidoptera, Pieridae), it was excreted without biotransformation, suggesting that the same might occur with neomenthol [32].

In short, the other terpenes present in the volatile fraction of the EO produce virtually no menthofuran.

3.3. Determination of the synergistic effects of PBO

We performed indirect and direct assays in order to demonstrate the participation of the P450 oxidizing system in the metabolism of *M. domestica*.

In the indirect assays, we determined the LC_{50} of *M. verticillata* EO against flies previously treated with 10 μg of piperonyl butoxide (PBO), a recognized P450 inhibitor [33]. In the presence of this inhibitor, the toxicity of *M. verticillata* EO diminished three times (LC_{50} = 1.5 mg/dm^3) (Table 3) whereas the LC_{50} of (4*R*)(+)-pulegone changed from 1.7 to 4.4 mg/dm^3. The toxicity of menthone was also reduced by the presence of PBO, which showed an LC_{50} = 2.8 mg/dm^3 (Table 3). Taken together, these results indicate that the P450 system enhances the toxicity of *M. verticillata* EO or (4*R*)(+)-pulegone and menthone.

In insects, the cytochromes P450 are known for their role in hormone synthesis, energy metabolism or xenobiotic degradation. They are also involved in the mechanism of insecticide resistance [34]. In houseflies, P450 are well known to metabolize pyrethroids [33,35,36]. Inhibitors of P450 such as PBO make insects more susceptible to insecticides, such as pyrethroids. As a positive control, we also determined the LC_{50} (topically) of deltamethrin against flies previously treated with PBO. The flies were more susceptible when deltamethrin was applied in combination with PBO (LC_{50} = 1.5 μg/insect) than when treated with deltamethrin alone (LC_{50} = 9.2 μg/insect) (Table 3). While the synthetic insecticide is synergized by PBO, *M. verticillata* EO, (4*R*)(+)-pulegone and menthone showed less toxicity by action of this P450 inhibitor. In other words, contrary to what is expected, the insect detoxification system contributed to enhance the toxicity of the *M. verticillata* EO. Consequently, resistant strains overexpressing P450 genes will be more susceptible to either *M. verticillata* EO or its major terpenes, (4*R*)(+)-pulegone and menthone.

The SPME analysis of the flies that died by the action of *M. verticillata* EO plus PBO showed the presence of (4*R*)(+)-limonene, menthone and (4*R*)(+)-pulegone at 15.3, 9.7 and 57.5% respectively, and 17.5% of menthofuran (Table 2). The decrease in the formation of menthofuran (from 44.2 to 17.5%) as well as the increase of LC_{50} (from 0.5 to 1.5 mg/dm^3), are in line with a participation of P450 in the metabolism of the *M. verticillata* EO, which results in the formation of a more toxic terpene that favors the death of flies. In the experiments of (4*R*)(+)-pulegone or menthone with PBO, the relative amounts of menthofuran were reduced, ranging from 44.5 to 35 and from 24 to 14%, respectively (Table 2). Thus, these results confirmed that the changes in LC_{50} registered for these compounds were due to a low production of toxic menthofuran.

3.4. Enzyme assay

The direct approach to showing the participation of the P450 in the metabolism of *M. domestica* involved enzymatic assays, which contained the microsomal fraction isolated from *M. domestica* as a source of P450, and *M. verticillata* EO or (4*R*)(+)-pulegone as substrates.

The reaction of EO yielded menthofuran at 23% after 1 h of reaction at 30 °C (Table 4) and, when the substrate was (4*R*)(+)-pulegone, 35% of menthofuran was formed. This experiment was analyzed also by direct injection (no SPME) on GC–MS (detection limit: 100 ng/μl) and no metabolites such as α,β-unsaturated-γ-ketoaldehyde (8-pulegone aldehyde) were detected. Altogether, these results suggest that (4*R*)(+)-pulegone is converted to menthofuran, which is not subject to further significant metabolism.

Table 4
Menthofuran formation by the reaction of *Minthostachys verticillata* EO or (4*R*)(+)-pulegone with P450 microsomes.

Substrate of P450 microsomes	% of menthofuran formation
M. verticillata EO	23 ± 1.8
(4*R*)(+)-Pulegone	35 ± 2.6

3.5. Potential use of *M. verticillata* EO as an insecticide

M. verticillata EO showed toxicities that are 3.4 and 3.8 times higher than (4*R*)(+)-pulegone and menthone respectively, which means that the use of the EO as a fumigant against house flies is rather more attractive than the use of EO components.

As far as we know, there is no determination of the oral toxicity of *M. verticillata* EO in rats, or of its inhalation toxicity, which would be the most appropriate parameter to consider in order to evaluate the risk of using *M. verticillata* EO as a fumigant.

Considering the EO components, pulegone is generally recognized as a toxic compound although, to our knowledge, no acute oral toxicity study of this terpene has been reported in the literature. The European Scientific Committee on Food reported an acute oral LD_{50} in rats equal to 470 mg/kg [37], while menthone presented an $LD_{50} = 500$ mg/kg [38]. Ingestion of pennyroyal essential oil, composed of more than 70% pulegone, has been associated with toxic effects [39]. In a study where rats were administered 160 mg/kg b.w./day of pulegone during 4 weeks, the dosed animals appeared depressed and a decrease in plasma glucose, creatinine and alkaline phosphatase levels was observed, indicating an adverse effect on the liver [40]. However, in that study, no significant histopathology of the liver was seen in rats given pulegone orally [40], but in another study a hepatic centrilobular necrosis was observed in mice following i.p. administration [41]. Recently, a study demonstrated that (4*R*)(+)-pulegone decreased myocardial contractility and markedly reduced both the intracellular Ca^{2+} transient and L-type Ca^{2+} current in electrically stimulated guinea pig atria; this suggests that (4*R*)(+)-pulegone may exert a negative inotropic effect on mammalian heart, mainly by decreasing the L-type Ca^{2+} current and the global intracellular Ca^{2+} transient [42]. The activity of (4*R*)(+)-pulegone exerted at 3.4 mM was similar to that of nifedipine at 40 μM [42].

Rats administered menthone, at dose levels up to 800 mg/kg b.w./day for 28 days [43], showed similar effects to those reported for animals dosed with pulegone [40], concluding that the no-effect level for menthone was lower than 200 mg/kg b.w./day [43]. Another study in which the cytotoxicity of *M. verticillata* EO, pulegone and menthone was tested on larvae of the crustacean *Artemia salina*, found LD_{50} of 2.10, 0.30 and 1.12 mg/ml respectively [44].

These values may indicate that *M. verticillata* EO might be less toxic than pulegone and menthone, although more studies are needed on the acute and inhalatory toxicity of *M. verticillata* EO and (4*R*)(+)-pulegone. In short, since the EO is more effective against *M. domestica* than (4*R*)(+)-pulegone or menthone, and could involve lower toxicity to mammals because it contains approximately 60% of (4*R*)(+)-pulegone or 20% of menthone, *M. verticillata* EO could be considered as a promising candidate for pest control of adult houseflies.

4. Conclusions

The present results indicate that (4*R*)(+)-limonene, menthone and (4*R*)(+)-pulegone are the terpenes preferentially absorbed by flies exposed to *M. verticillata* EO; this oil acts as a potent fumigant against *M. domestica* by the action of these terpenes. Flies metabolized (4*R*)(+)-pulegone and menthone to menthofuran by the P450 oxidizing system pathway, which in turn showed strong toxicity in *M. domestica*. The results suggest that flies resistant to insecticides by increased P450 expression will be more susceptible to *M. verticillata* EO (or (4*R*)(+)-pulegone or menthone), in clear contrast to the expected response to synthetic insecticides such as pyrethroids. Finally, despite all these positive characteristics of *M. verticillata* EO, a detailed study has to be made of the toxicological risk levels of its inhalation before deciding on the use of *M. verticillata* EO as a fumigant against *M. domestica*.

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