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Insecticidal toxicity of *Eucalyptus cinerea* essential oil and 1,8-cineole against *Musca domestica* and possible uses according to the metabolic response of flies

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ABSTRACT

In a fumigant assay, *Musca domestica* adults treated with *Eucalyptus cinerea* EO [LC_{50} = 5.5 mg/dm^3; majority component by SPME–GC: 1,8-cineole (88.5%), α-pinene (2%), α-terpineol (9%)], died within 15 min or less. The terpenes absorbed by the flies and their metabolites, analyzed using SPME fiber, were 1,8-cineole (LC_{50} = 3.3 mg/dm^3), α-pinene (LC_{50} = 11.5 mg/dm^3), α-terpineol (LC_{50} = 36.8 mg/dm^3) and a new component 2,3-dehydro-1,8-cineole, in proportions of 74%, 0.1%, 24.7% and 1.2% respectively. 2,3-Dehydro-1,8-cineole and α-terpineol were formed by oxidation of 1,8-cineole mediated by cytochrome P450, as demonstrated by a fumigation assay on flies previously treated with piperonyl butoxide, a P450 inhibitor, which showed an increase in toxicity of the EO and 1,8-cineole, supporting its participation in the formation of metabolites. We also demonstrated that 1,8-cineole acts as a P450 inhibitor-like compound, improving the toxicity of other xenobiotics such as deltamethrin.

1. Introduction

The *Eucalyptus* genus (family Myrtaceae) is native to Australia and comprises about 900 species (Pereira et al., 2014) which has been successfully introduced and extensively cultivated in wide variety of ecological zones as one of the main biomass sources. *Eucalyptus* is one of the world's most important plantation genera representing the 8% of all planted forests (González-García et al., 2012). All over the world the *Eucalyptus* species have been harvested and traded for oil, gum, pulp, timber and medicines (Manika et al., 2013). *Eucalyptus* essential oil possess antibacterial, antifungicidal, antiseptic and pest management properties (Batish et al., 2008). In particular, *Eucalyptus cinerea* essential oil (EO) was reported as an effective insecticide against *Musca domestica* (Kumar et al., 2012a; Palacios et al., 2009a), requiring doses of 5.5 mg/dm^3 (equivalent to 6.1 μL/L) to induce 50% mortality in *M. domestica* adults (Kumar et al., 2012a; Palacios et al., 2009a) in a short period of time (30 min). The most abundant terpene of this EO, 1,8-cineole, showed an LC_{50} of 3.3 mg/dm^3. The toxicity of *E. cinerea* EO and of 1,8-cineole against house flies was 11 and 6.6 times, respectively, less active than the toxicity of the organophosphorus insecticide DDVP (LC_{50} = 0.5 mg/dm^3), indicating that both products potentially offer a natural approach to the control of houseflies.

M. domestica (L.) is the most common fly that invades households, and its presence is considered a sign of an unhygienic environment (Srinivasan et al., 2008). The feeding habitats of this fly, combined with its structural morphology, make it ideally suited to carry and disseminate pathogenic microorganisms (Fotedar, 2001), contaminating food and household utensils and leading to the development of human and animal illnesses (Förster et al., 2007). Chemical management is a widely used pest control method for houseflies. However, this is not only harmful to the environment and has toxicological effects on human health but its long time use also leads to the development of resistant fly strains (Batish et al., 2008). Considering the synanthropic nature of house flies, insecticides at least compatible with human surroundings are needed, which is not possible with chemical insecticides (Batish et al., 2008). Natural pesticides derived from edible or medicinal plants are more compatible with human habitats.

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

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In this work we analyze the scope of the *E. cinerea* EO as insecticide against *M. domestica*, through the study of the fly metabolic response to the EO and its components.

2. Materials and methods

2.1. Plant material

E. cinerea F. v. Muell. (eucalyptus) leaves were obtained from domestic organic gardens. A voucher specimen (UCCOR 384) 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. 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, 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). The mass spectrum was obtained at an ionization voltage of 70 eV. Identification of the components was based on comparison of their relative retention times and mass spectra with those obtained from authentic samples and/or the NIST MS 05 version 2.0. C_7–C_{30} saturated 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

1,8-Cineole, α-terpineol, deltamethrin and piperonyl butoxide (PBO) used as material for bioassays, were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetone was purchased from Merck (Darmstadt, Germany).

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 reared in entomological cages (30 cm × 30 cm × 30 cm). The rearing conditions were as follows: 26 ± 1 °C, 70% humidity and 12:12 light–dark cycle. The diet was powdered milk and water. 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 (Kumar et al., 2012b; Palacios et al., 2009a,b). Briefly, ten 4–5-day-old adult houseflies, both 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 *E. cinerea* EO and 1,8-cineole (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 was determined and used to calculate the LC_{50} of the corresponding compound.

2.6. Determination of terpenes absorbed by house flies

After a fumigation bioassay (with $n = 100$) was performed, the dead flies (~500 mg) were collected in a vial (10 mL volume) with a septum. In order to quantify 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, which was then placed in a bath at 60 °C for 15 min. The terpenes desorbed from the *M. domestica* to the headspace of the vial were captured using a SPME microfiber (Supelco, Bellefonte, PA, USA; with polydimethylsiloxane, thickness 30 μm, length 1 cm). This was then injected into a GC–MS and a GC–FID for identification and quantification of terpenes, respectively. Prior to this, the optimal temperature, exposure time and desorption temperature conditions of the SPME fiber were established. The desorbed terpenes were quantified and expressed in μg of terpene per mg of fly, and these numbers were transformed into relative percentages to simplify the comparison between treatments and the EO composition.

2,3-Dehydro-1,8-cineole, retention time 8.396 min, RI = 1010 (HP-5MS column; for chromatographic conditions, see above) (Adams, 2007); mass spectrum (relative intensity) *m/z* 152 (3), 124 (7), 109 (100), 94 (9), 79 (30), 43(16) (Ayorinde et al., 1984).

2.7. Determination of the synergistic effects of PBO

E. cinerea EO or 1,8-cineole was assayed in combination with PBO, according to the method previously reported (Rossi et al., 2012). A fumigation bioassay of *E. cinerea* EO or 1,8-cineole (in doses from 1 mg/dm^3 to 7 mg/dm^3) was then performed with the PBO-treated flies, as described above. Control groups received only acetone. The LC_{50} was determined. The dead flies were collected in a vial for the identification and quantification of terpenes by SPME–GC-analysis.

2.8. Determination of the LC_{50} of deltamethrin and 1,8-cineole and synergic effect

Different doses of deltamethrin or 1,8-cineol, each dissolved in acetone, were applied using a micropipette (1 μL) in the pronotum of flies previously anesthetized with CO_2 current. Control groups received only acetone. The number of dead insects at each dose was recorded at 30 min. Three replicates of 10 flies each were run for each dose. The mortality values were used to calculate the LC_{50}. For the synergism study, a solution of deltamethrin/1,8-cineol in a proportion of 1/4 (w/w) was topically assayed on *M. domestica* previously anesthetized with CO_2 current. The assayed doses ranged from 9/36 mg/dm^3 to 0.25/1 mg/dm^3. Control groups received only acetone. The LC_{50} of deltamethrin (combined with 1,8-cineole) was determined.

2.9. Statistical analysis

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

Table 1
Main components of *Eucalyptus cinerea* essential oil, determined by GC–MS and expressed as relative percentage on total area in the chromatogram.

E. cinerea	Components (%)		
	RI	By direct injection	By SPME
α-Pinene	933	4.8	2.0
β-Pinene	970	0.3	0.1
1,8-Cineole	1036	90.7	88.5
β-Terpineol	1112	0.2	0.1
Geranial	1116	0.5	0.1
α-Terpineol	1190	2.4	9.0
Camphene	1150	1.1	0.2

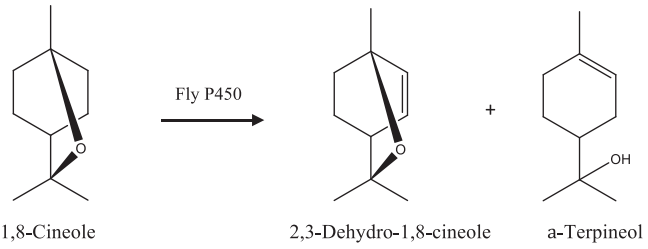

Fig. 1. Conversion of 1,8-cineole to 2,3-dehydro-1,8-cineole and α-terpineol mediated by fly cytochrome P450.

3. Result and discussion

3.1. Determination of terpenes absorbed by house flies

The main components of *E. cinerea* EO used in this study were determined by either direct injection in GC–MS or the SPME–GC–MS techniques. The most abundant terpene was 1,8-cineole, at 90.7% by direct injection and 88.5% by SPME–GC–MS (Table 1), together with α-pinene, β-pinene, β-terpineol, geranial, α-terpineol, and camphene in the proportions reported in Table 1. The SPME 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.

M. domestica adults treated with *E. cinerea* EO at 10 mg/dm^3 died in less than 15 min. These dead flies were transferred to a GC-vial, which was 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 four EO components, 1,8-cineole, α-pinene, α-terpineol and a new component, identified as 2,3-dehydro-1,8-cineole in proportions of 74, 0.1, 24.7 and 1.2%, respectively (Table 2). The increased proportion of α-terpineol could be attributed to selective absorption of this compound or to its formation by the transformation of some of the absorbed terpenes. The reduced proportion of 1,8-cineole and the appearance of 2,3-dehydro-1,8-cineole and possibly the additional α-terpineol suggest that *M. domestica* metabolized 1,8-cineole to these terpenes. To test this hypothesis, we assayed 1,8-cineole under the same conditions as the *E. cinerea* EO. The SPME-GC analysis of the fumigation experiment with 1,8-cineole showed the presence of 1,8-cineole, 2,3-dehydro-1,8-cineole and α-terpineol in proportions of 79.5, 9.1 and 11.4%, respectively (Table 2, Fig. 1), corroborating our hypothesis and indicating that this transformation probably occurs by the oxidative detoxification pathway of the insect.

In the dead flies, α-pinene was recovered in a smaller proportion than in the EO. As no metabolites attributable to this terpene were found and in view of the recovery of α-pinene in the experiments with PBO (see Section 3.3), it seems that α-pinene is less absorbed by the flies.

3.2. Toxicity of metabolites

As mentioned above, the LC_{50} of 1,8-cineole was 3.3 mg/dm^3, while those of α-pinene and α-terpineol were 11.5 and 36.8 mg/dm^3, respectively (Kumar et al., 2012a; Palacios et al., 2009a). Although we could not determine the toxicity of 2,3-dehydro-1,8-cineole (because this chemical is not commercially available), taking into account the LC_{50} and the proportions of the terpenes absorbed and produced by the flies, we suggest that the intoxication process was due mostly to the action of 1,8-cineole.

The formation of the metabolites 2,3-dehydro-1,8-cineole and α-terpineol could be the result of the reaction of 1,8-cineole with the P450 oxidative system. The latter is involved in a wide range of metabolic processes of insects (Feyereisen, 1999), from hormone synthesis to activation (Rossi et al., 2012; Tillman et al., 1999) or degradation of xenobiotics (Rossi and Palacios, 2013).

Although 1,8-cineole has been recognized as the major and most toxic component of the leaf oils in many *Eucalyptus* species (Batish et al., 2008; Elaissi et al., 2011; Kumar et al., 2012a; Palacios et al., 2009a) as far as we know there are no metabolic studies in insects susceptible to this compound. However, it is known that insects from different taxonomic orders, *Paropsisterna tigrina*, *Faex nigroconspersa*, *Chrysophtharta bimaculata* and *Oxyops vitiosa*, when fed 1,8-cineole in their leaf diets, metabolize 1,8-cineole into a range of non-toxic forms by the oxidative pathway. This terpene undergoes different hydroxylations, for instance, *F. nigroconspersa* adults excreted predominantly 9-hydroxy-1,8-cineole with some 2α-hydroxy-1,8-cineole. In contrast, larvae excreted mostly 2α-hydroxy-1,8-cineole, with smaller quantities of 9-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole. *C. bimaculata* adults excreted predominantly 3α-hydroxy-1,8-cineole. *O. vitiosa* adults, on a lower 1,8-cineole diet, metabolize it to 2α,9-dihydroxy-1,8-cineole and 2α-hydroxy-1,8-cineole, with smaller proportions of 3α-hydroxy-1,8-cineole and 9-hydroxy-1,8-cineole (Southwell et al., 2003). Schmidt et al., investigating detoxification mechanisms in two pergine species (*Pergagrapta polita* and *Pergagrapta* sp.) and two from the subfamily Pterygophorinae (*Lophyrotoma interrupta* (Klug) and *Pterygophorus insignis* Kirby), observed that 1,8-cineole is metabolized to hydroxyl-cineole also in these insects (Schmidt et al., 2010).

3.3. Determination of the synergistic effects of PBO

In order to demonstrate the participation of the P450 oxidizing system in the metabolism of *M. domestica*, the LC_{50} of *E. cinerea* EO against flies treated with 10 μg of piperonyl butoxide (PBO), a recognized P450 inhibitor (Kasai and Scott, 2000), was determined. In the presence of this inhibitor, the toxicity of *E. cinerea* EO diminished about 2.5 times (LC_{50} = 2.2 mg/dm^3) (Table 3) while the LC_{50} of 1,8-cineole changed from 3.3 to 1.6 mg/dm^3.

SPME-GC analysis of the PBO-treated flies that died as a consequence of fumigation with *E. cinerea* EO showed the presence of 1,8-cineole in a proportion of 90.8% and the lack of formation of 2,3-dehydro-1,8-cineole and extra α-terpineol (Table 2). Beside, α-pinene was recovered without change (Table 2). These indicate that 1,8-cineole was not transformed by flies in this experiment, and consequently the mortality is caused by 1,8-cineole. This, together with the diminished LC_{50} in the presence of a P450 inhibitor, seems to indicate that 2,3-dehydro-1,8-cineole as well as α-terpineol are metabolites that help to detoxify the presence of the toxicant 1,8-cineole.

In houseflies, P450 are well known to metabolize pyrethroids (Kasai and Scott, 2000; Seifert and Scott, 2002; Tomita and Scott,

Table 2
Amount of 1,8-cineole, 2,3-dehydro-1,8-cineole, α-pinene and α-terpineol, recovery from dead flies by treatment with *Eucalyptus cinerea* EO, 1,8-cineole with or without piperonylbutoxide (PBO).

SPME analysis of	Relative amount (%)			
	1,8-Cineole	α-Pinene	α-Terpineol	2,3-Dehydro-1,8-cineole
E. cinerea EO	88.9 ± 1.5	2 ± 0.2	9.1 ± 0.1	nd[a]
Flies dead by action of E. cinerea EO	74 ± 1	0.1 ± 0.3	24.7 ± 0.2	1.2 ± 0.1
Flies dead by action of E. cinerea EO + PBO	90.8 ± 1	0.1 ± 0.01	9.1 ± 0.1	nd[a]
1,8-cineole	99 ± 0.5			
Flies dead by action of 1,8-cineole	79.5 ± 1.4		11.4 ± 0.2	9.1 ± 0.1
Flies dead by action of 1,8-cineole + PBO	100 ± 0.2		nd[a]	nd[a]

[a] nd: not detected with a limit of quantification of 0.3 μg.

Table 3
LC_{50} of *Eucalytus cinerea*, 1,8-cineole, and deltamethrin[a] with or without piperonylbutoxide (PBO) against *Musca domestica* in fumigant bioassay.

Essential oil or terpene	Mean LC_{50} in mg/dm^3 (95% CI)
E. cinerea	5.5 (4.1–7.4)
E. cinerea + PBO	2.2 (0.9–5.2)
1,8-Cineole	3.3 (1.1–10.4)
1,8-Cineole + PBO	1.6 (0.9–3.1)
1,8-Cineole[a]	196.1 (121.5–316.5)
Deltamethrin[a]	9.2 (2.8–29.5)
Deltamethrin + PBO[a]	1.5 (0.2–11.4)
Deltamethrin + 1,8-cineole[a,b]	1.0 (0.08–12.1)

[a] Applied topically and LC_{50} expressed in μg/fly.
[b] Deltamethrin/1,8-cineole ratio: 1/13 mol/mol or 1/4 mg/mg.

1995), and inhibitors of P450 such as PBO make insects more susceptible to these compounds.

As a positive control, we also determined the LC_{50} (topically) of deltamethrin against flies previously treated with PBO. The flies were more susceptible to deltamethrin when it was applied in combination with PBO (LC_{50} = 1.5 μg/insect) than when flies were treated with deltamethrin alone (LC_{50} = 9.2 μg/insect) (Table 3). Similarly, *E. cinerea* EO and 1,8-cineole showed more toxicity when the P450 oxidation system was inhibited.

Some authors report the involvement of cytochrome P450-dependent monooxygenase in the detoxification of essential oil or monoterpenes by *M. domestica* (Rossi et al., 2012; Rossi and Palacios, 2013). The fumigant toxicities of *Eucalyptus* EO and 1,8-cineole were tested against a chlorpyrifos-methyl resistant strain and a reference strain of the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.). The resistant strain showed 1.9- and 2.2-fold higher tolerance against essential oil and 1,8-cineole fumigation toxicity, respectively, relative to the susceptible strain. The increased tolerance for the essential oil may be the result of cross-resistance. The resistance mechanism in the resistant strain was discussed in relation to elevated detoxifying enzymes such as cytochrome P450 and esterase (Lee et al., 2000). Conversely, PH_3-resistant *Tribolium castaneum*, whose resistance was conferred by antioxidant enzymes, showed the same susceptibility to 1,8-cineole as the *T. castaneum* PH_3-susceptible strain (Lee et al., 2002).

3.4. Determination of the synergistic effects of deltamethrin

The above results suggest that 1,8-cineole, as a substrate of the P450 enzymes, may compete with the oxidation of other xenobiotics absorbed by flies. In consequence, it can act as a P450 inhibitor-like compound as long as it is offered to the insect in higher proportions with respect to the xenobiotic. Otherwise, and from a kinetic point of view, the xenobiotic would likely be faster metabolized.

With this in mind, we first determined the topical LC_{50} of 1,8-cineole, which was 196.1 μg/fly (Table 3). Then, the topical LC_{50} of deltamethrin was determined using a mix of this pyrethroid with 1,8-cineole, taking care that 1,8-cineole doses were lower than its topical LC_{50} but higher than the concentration of deltamethrin. Thus, *M. domestica* adults treated with different doses of a mix of deltamethrin/1,8-cineole in a proportion of 1/4 (w/w) (1/13 M relation) (see Section 2.8) showed a deltamethrin LC_{50} of 1.0 μg/fly (Table 3), quite similar to the LC_{50} of this pyrethroid plus PBO. This indicates that 1,8-cineole can act as a P450 inhibitor-like compound and suggests that it could be used in this manner to diminish at least 10 times the doses of deltamethrin needed to control flies.

4. Conclusion

These results indicate that the components of *E. cinerea* EO, 1,8-cineole, α-pinene and α-terpineol are absorbed by flies exposed to it; consequently, this oil acts as a potent fumigant against *M. domestica*. Flies detoxified 1,8-cineole to 2,3-dehydro-1,8-cineole and α-terpineol. The toxicity of the EO and of 1,8-cineole increases when a P450 inhibitor is used in combination with either of them. 1,8-Cineole could act as a P450 inhibitor-like compound as well, thereby improving the toxicity of other xenobiotics such as deltamethrin. The use of the *E. cinerea* EO and or 1,8-cineole as fumigants or as synergistic of pyrethroid insecticides is an interesting alternative to control flies in human habitats, especially against resistant flies, being able to decrease the doses of synthetics insecticides.

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