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Differential toxicological effects of natural and synthetic sources and enantiomeric forms of limonene on mosquito larvae

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Abstract

Common fragranced consumer products, such as cleaning supplies and personal care products, emit chiral compounds such as limonene that have been associated with adverse effects on human health. However, those same compounds abound in nature, and at similar concentrations as in products, but without the same apparent adverse human health effects. We investigated whether different types of limonene may elicit different biological effects. In this study, we investigated the mortality rate of mosquito larvae in response to changes in their environment. Specifically, we tested different sources of naturally occurring R-limonene and chemically synthetized limonene, containing one of its enantiomeric forms (R-, S-) in mortality bioassays with *Aedes aegypti* mosquito larvae. We found that a natural source of limonene extracted from oranges induced lower mortality of mosquito larvae compared to synthetic sources at the same concentration. However, enantiomeric forms did not differ in their effects on mortality. Our results provide novel evidence that natural sources of a chemical can cause lower rates of mortality than synthetic sources.

Keywords Limonene · LC_{50} · Fragranced consumer products · Biological effects · Bioassay · Mosquitoes

Introduction

Fragrance compounds in consumer products have been associated with a range of adverse effects on humans and environmental systems (Steinemann 2021). What remains largely unknown, however, is why these fragrance compounds can cause problems when the same compounds abound in nature. Limonene, a chiral terpene, is the most common volatile organic compound emitted by fragranced consumer products including personal care products, air fresheners, and cleaning supplies (Nematollahi et al. 2019).

Limonene is available in nature, but it can also be chemically synthesized. Limonene has two enantiomers, R-limonene and S-limonene. Insects are especially sensitive to the chirality of molecules (Tian et al. 2016; Overmyer et al. 2007; Chen et al. 2019), making them a useful model system to examine the question of whether different sources and enantiomeric forms of a chiral fragrance molecule might exert different biological or toxicological effects. We previously demonstrated that a natural source of limonene exhibited lower repellency against adult female *Ae. aegypti* mosquitoes compared to a synthetic source (Nematollahi et al. 2021). Previous studies have shown that limonene is toxic to both the adult (Hebeish et al. 2008) and larval (Kassir et al. 1989; Pitasawat et al. 2007; Vera et al. 2014) stages of mosquitoes, but few studies have compared the effects of different sources of this compound.

The overall aim of this research is to explore whether different enantiomeric forms and sources of limonene, at the same concentrations, may elicit different biological effects. We tested the effects of different sources (natural, synthetic) and enantiomeric forms (R-, S-) of limonene on mortality in controlled laboratory experiments with *Aedes*

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Table 1 Sources and forms of limonene used in this study

Limonene types	*Limonene concentration (v/v)*	*Enantiomer*	*Source*
R-limonene	97%	R	Synthetic
S-limonene	96%	S	Synthetic
Orange essential oil	96%	R	Natural/synthetic
Natural orange oil	30%	R	Natural

aegypti mosquito larvae. This study constitutes one of the first investigations into whether natural and synthetic sources of the same molecule elicit different biological effects.

Materials and methods

Limonene sources

We used four limonene types, each containing different enantiomers of limonene from natural or synthetic sources (Table 1). In this study, "natural" limonene source is defined as limonene extracted from natural oranges and "synthetic" limonene source is defined as commercially available limonene.

We purchased R-limonene and S-limonene from Sigma-Aldrich and also used a commercially available orange essential oil. Natural orange oil was extracted manually from organic and unwaxed orange peels using a cold-press method. The oranges were purchased from organic markets in Melbourne. The colored (flavedo) portion of orange peels (flavedo) was removed from the clean oranges using a stainless steel grater. Next, grated orange peels with a small amount of deionized water were placed in a food blender to make orange peel puree. Then, the orange peel puree was filtered with a fabric netting to separate orange peel pulp from the oil-in-water emulsion. Finally, the oil-in-water emulsion moved through the centrifugation step to extract orange peel oil. The orange oil ingredients were analyzed using headspace GC/MS using the technique described in Nematollahi et al. (2018), with a final concentration of 30% R-limonene. More details on the chemical composition of each limonene type can be found in Table S1.

Mosquitoes

We used a single laboratory colony of *Aedes aegypti* mosquitoes for all experiments. Mosquitoes were collected as eggs from Cairns, Queensland, in 2015 and reared in a temperature-controlled laboratory environment at 26˚C ± 1˚C with a 12-h photoperiod according to Ross et al. (2017). Larvae were reared in trays filled with 4 L of deionized water at a controlled density of 450 larvae per tray. Larvae were fed TetraMin tropical fish food tablets (Tetra, Melle, Germany) ad libitum until pupation. Female mosquitoes were blood fed on the forearms of human volunteers. Eggs were collected on sandpaper strips that were partially submerged in larval rearing water.

Larval bioassays

We performed bioassays to determine the toxicity of the four limonene types to *Aedes aegypti* mosquito larvae. Larvae that were 96 h old were used in the bioassay experiments.

To determine the range of limonene concentrations that caused mortality in mosquito larvae, we performed pilot experiments with a broad range of limonene concentrations. Stock solutions were prepared by diluting 1 mL of each limonene type in 9 mL of 100% ethanol from Chem-Supply. The stock solution was then serially diluted in deionized water to obtain a range of test concentrations. For each limonene type, we chose seven test concentrations that caused between 5 and 95% mortality for further experiments .

We performed bioassays with the following concentrations of R-limonene, S-limonene, and orange essential oil:

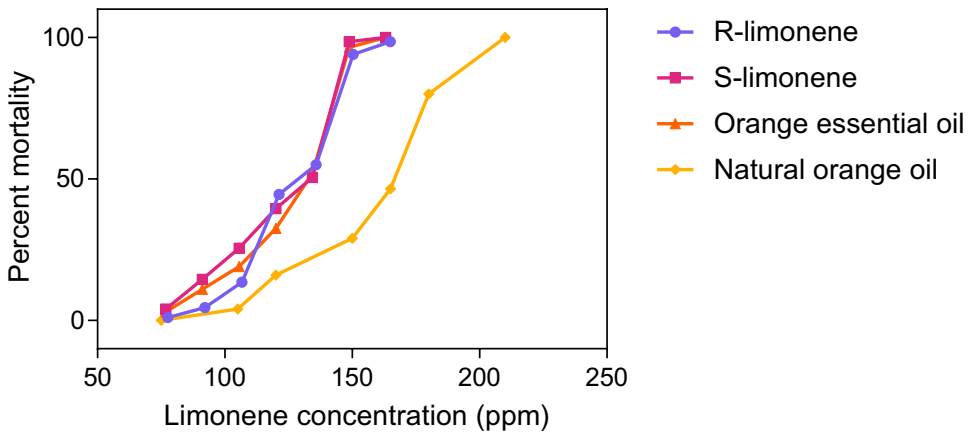

Fig. 1 Mortality of *Aedes aegypti* larvae following exposure to different types of limonene for 24 h at a range of concentrations. Medians of four replicate experiments are shown for each concentration of limonene

Table 2 Lethal concentrations (LC_{50} and LC_{90}) of different types of limonene to *Aedes aegypti* larvae

Limonene types	*LC_{50} in ppm (lower–upper 95% confidence interval)*	*LC_{90} in ppm (lower–upper 95% confidence interval)*
R-limonene	122.1 (117.1–127.3)	153.7 (145.4–166.6)
S-limonene	119.5 (109.8–130.1)	168.2 (149.9–210.3)
Orange essential oil	118.0 (111.7–124.6)	155.9 (144.7–175.0)
Natural orange oil	157.4 (151.7–163.4)	205.2 (194.1–221.8)

0 (control), 80, 95, 110, 125, 140, 155, and 170 ppm. For natural orange oil, we selected concentrations of 0 (control), 250, 350, 400, 500, 550, 600, and 700 ppm. One hundred mosquito larvae were transferred to glass jars filled with 99 mL of deionized water. Then, 1 mL of each dilution was transferred to jars of larvae using a pipette with disposable tips. For controls (0 ppm), 1 mL of ethanol was added to each jar of larvae, with separate controls for each limonene type. We performed four replicate experiments for each concentration and limonene type, with each replicate performed on a different day with a fresh stock solution and an independent set of serial dilutions. Larvae were exposed to each concentration for 24 h at 26℃, with no food provided to larvae during the experiment. Mortality was determined after 24 h by counting the number of live and dead larvae in each jar, with dead larvae being those that did not move after 5 s of physical stimulation (repeated touching with a metal skewer).

Statistical analysis

All data were analyzed using SPSS statistics version 24.0 for Windows (SPSS Inc, Chicago, IL). We used probit analysis to estimate concentrations of limonene that caused 50% mortality (LC_{50}) and 90% mortality (LC_{90}) for each limonene type. Because each type had different concentrations of limonene, concentrations (in ppm) were adjusted to reflect the percentage of limonene prior to analysis.

Results and discussion

The results of the bioassays are presented in Fig. 1. All types of limonene caused mortality in *Aedes aegypti* larvae, with higher concentrations resulting in higher larval mortality. Almost no larval mortality (1 larva or fewer) was observed in the controls (0 ppm). R-limonene, S-limonene, and orange essential oil had similar levels of mortality, with non-overlapping confidence intervals (Table 2); these results are consistent with previous studies (Santos et al. 2011; Giatropoulos et al. 2012; Seo et al. 2015). However, natural orange oil showed lower mortality compared to all the other limonene types for all concentrations tested. For instance, concentrations of 160 ppm of R-limonene, S-limonene, and orange essential oil resulted in near-complete mortality, while the same concentration of natural orange oil caused around 50% mortality. The LC_{50} of natural orange oil (157.4 ppm) was also notably higher than those for the other three synthetic limonene sources.

Conclusions

In this study, we tested whether different sources of naturally occurring R-limonene and chemically synthetized limonene, containing one of its enantiomeric forms (R-, S-), may produce varying rates of mortality in *Aedes aegypti* mosquito larvae. Natural orange oil (a natural source) showed lower toxicity at all concentrations than R-limonene, S-limonene, and the commercially available orange essential oil with synthetic origins. These experiments provide novel evidence that natural sources of a chemical can show lower toxicity than synthetic sources. However, different enantiomeric forms of the same source of limonene did not differ markedly in their effect on mortality.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Perran A. Ross and Neda Nematollahi. The first draft of the manuscript was written by Perran A. Ross and Neda Nematollahi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data are contained within the manuscript and its associated supplementary information.

Code availability Not applicable.

Declarations

Ethics approval Blood feeding of female mosquitoes on human volunteers for this research was approved by the University of Melbourne Human Ethics Committee (Approval 0723847). All adult subjects provided informed written consent (no children were involved).

Conflict of interest The authors declare no competing interests.

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