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Comparative Toxicity of Essential Oils of *Litsea pungens* and *Litsea cubeba* and Blends of Their Major Constituents against the Cabbage Looper, *Trichoplusia ni*

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Contact toxicity of essential oils of *Litsea pungens* Hemsl. and *L. cubeba* (Lour.) Pers. (Lauraceae) and of blends of their major constituents was assessed against third-instar *Trichoplusia ni* larvae via topical application. Both oils showed moderate activity against *T. ni* larvae with LD_{50} values of 87.1 and 112.5 μg/larva, respectively. 1,8-Cineole from the essential oil of *L. pungens* and γ-terpinene from the oil of *L. cubeba* accounted for much of the toxicity of the oils to *T. ni* larvae. The toxicity of blends of selected constituents indicated a synergistic effect among putatively active and inactive constituents, with the presence of all constituents necessary for full toxicity of the natural oils. The results show that essential oils of *L. pungens* and *L. cubeba* and some of their constituents have potential for development as botanical insecticides.

KEYWORDS: Cabbage looper; essential oils; *Litsea pungens*; *Litsea cubeba*; 1,8-cineole; carvone γ-terpinene; (*R*)-limonene; *p*-cymene; toxicity; topical application

INTRODUCTION

Essential oils are composed of secondary metabolites commonly concentrated in the leaves, bark, or fruit of aromatic plants. The oils are generally obtained by steam distillation and composed of complex mixtures of monoterpenes, phenols, and sesquiterpenes. Major sources of essential oils include plants of the mint (Lamiaceae), citrus (Rutaceae), myrtle (Myrtaceae), and carrot (Apiaceae) families (*1*). Widely used as fragrances and flavoring agents in foods and beverages, essential oils are generally considered safe by the U.S. Food and Drug Administration owing to their minimal adverse effects on humans and the environment. They have recently received much attention due to their multiple functions as antimicrobial, antifungal, antitumor, and insecticidal agents (*1*). Considerable effort has been focused on using plant essential oils as potential sources of commercial botanical insecticides and repellents as the U.S. Environmental Protection Agency (EPA) permits the use of certain active ingredients that pose minimum risk to users exempt from registration. A number of essential oils, including oils of rosemary, cedar, cinnamon, citronella, wintergreen, lemon grass, oregano, citrus, clove, garlic, and mints, have been used to control a variety of insects (*1*).

Litsea pungens Hemsl. is found mostly in northwestern and eastern China (*2*). Fruits of *L. pungens* Hemsl. contain 1−4% essential oil, whereas foliage contains 0.4% and roots contain only 0.2−0.3% (*3*). *L. pungens* has long been used in traditional Chinese medicine (*4*) against influenza, stomachache, and other ailments. Research indicates that *L. pungens* essential oil has immunostimulatory, antimicrobial (*5*), and antitumor activities (*6*). Although it is believed that *L. pungens* was used to prepare a botanical pesticide in ancient China, there are no records of this in the contemporary literature.

Litsea cubeba oil (aka May Chang) is an aromatic essential oil extracted from the fresh pepper-like fruits of *L. cubeba* (Lour.) Pers. The fruits contain 3−5% of an essential oil rich in citral (75%). The leaves contain more 1,8-cineole than citral. The fruit oil is used as a flavor enhancer in foods, cosmetics, and cigarettes; as a raw material for the manufacture of citral, vitamins A, E, and K, ionone, methylionone, and perfumes; and as an antimicrobial and insecticidal agent (*7*). Oral and dermal LD_{50} values for the oils reported in mice were approximately 4000 and 5000 mg/kg of body weight, respectively.

The objective of our study was to evaluate the insecticidal activities of *L. pungens* and *L. cubeba* essential oils and their major constituents via topical application against third-instar larvae of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae) Hubner. Because *T. ni* has evolved resistance against many synthetic insecticides and the microbial insecticide *Bacillus thuringiensis* (*8*), it is important to develop new tools or materials that could be used to protect crops compatible with integrated pest management (IPM) schemes (*9*).

MATERIALS AND METHODS

Test Insect. Bioassays were conducted using third-instar larvae, obtained from an established laboratory colony (> 50 generations; out-crossed

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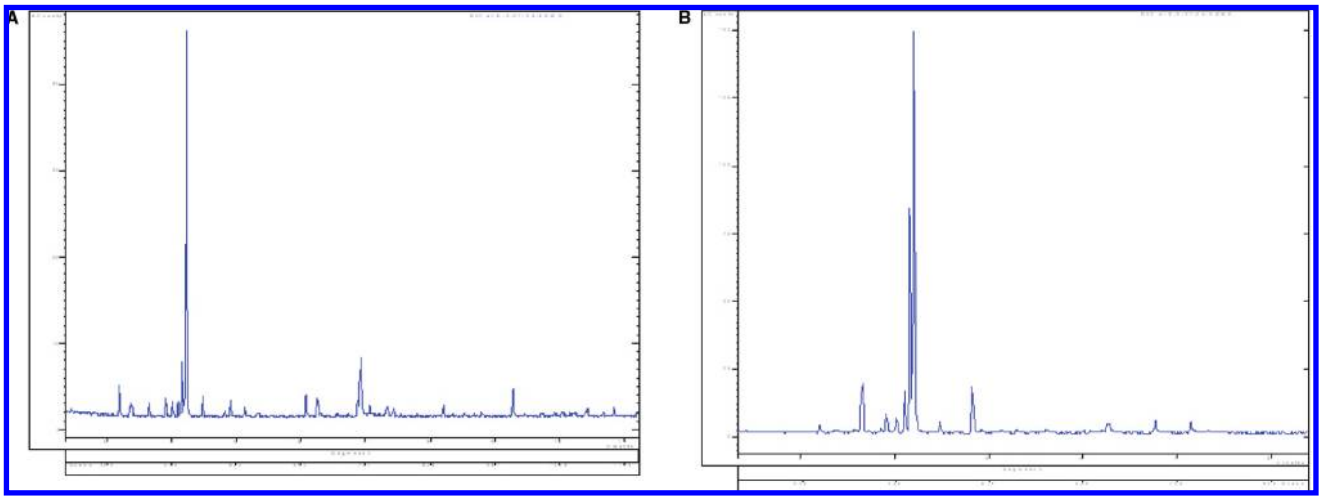

Figure 1. Chromatograms of essential oils of (**A**) *Litsea pungens* (Hemsl.) and (**B**) *L. cubeba*.

once). Insects were reared on Velvetbean Caterpillar Diet F9796 (Bio-Serv Inc., Frenchtown, NJ.) in the insectary of the University of British Columbia (UBC). The diet was supplemented with finely ground alfalfa, to improve acceptability, and a no. 8045 standard vitamin mixture (Bioserv Inc.). The colony was reared at room temperature (19−24 °C) under a 16:8 light/dark photoperiod.

Test Materials (Essential Oils and Individual Compounds). *L. pungens* essential oil was obtained through steam distillation of leaves and branches of *L. pungens* in the Research and Development Center of Biorational Pesticide, Northwest Agriculture and Forestry University, China (*2*). Aerial parts of plants were collected at Taibai mountain, Shaanxi province, China, in August 2007 and identified by H. L. Zhang (*2*). Leaves and branches weighing 25 kg yielded a total of 85.7 g of *L. pungens* oil at the rate of 3.43%.

Commercial *L. cubeba* essential oil was obtained from Chongqing Jiandun Co., Chongqing, China. The essential oils were kept at room temperature (≤25 °C) until use.

Pure compounds were purchased from Sigma Chemical Co., St. Louis, MO [α-pinene, (*R*)-limonene, 1,8-cineole, carvone, linalool, camphene, 3-carene, geraniol, *β*-caryophyllene, *β*-pinene, *p*-cymene, *γ*-terpinene, α-terpinene], Acros Organics, Fair Lawn, NJ (terpinen-4-ol), and Eco-SMART Technologies Inc., Franklin, TN (2-carene). Purities of compounds varied from 95 to 99%.

Composition of Essential Oils. Essential oils used in the study were analyzed by gas chromatography−mass spectrometry (GC-MS) (**Figure 1**) using a Varian 3900 system with a Saturn 2100T ion trap mass-selective detector (Varian Inc., Walnut Creek, CA). A WCOT fused silica 30 m × 0.25 mm i.d. column was used with a CP-Sil 8 CB lowbleed MS coating (Varian Inc.), a 1 μL injection volume, and pure helium as the carrier at 1.0 mL/min. The temperature program used was 80 °C for 0.5 min, an increase of 8.0 °C/min for 8.0 min, followed by an increase of 50 °C/min for 3.2 min. Cinnamyl alcohol (Sigma) was used as an internal standard (*2*, *10*). The major constituents of the oils are listed in **Table 1**.

Acute Toxicity of Essential Oils. Acute toxicity (measured as mortality at 24 h) of essential oils and their major constituents was determined by topical application to early third-instar *T. ni* larvae (*11*). Initial screening to approximate the active concentration range determined a range of concentrations used to calculate LD_{50} values for the two oils. Each larva received 1 μL of essential oil or pure compound solution per treatment, with acetone alone as the control. Solutions of various concentrations (**Table 2**) were applied to the dorsum of each insect using a repeating topical dispenser attached to a 50 μL syringe. Larvae were then transferred to Petri dishes (90 mm × 15 mm) with a small piece of artificial diet. Each Petri dish contained 10 larvae. Three replicates of 10 larvae each were used per treatment. Treatment groups were then placed in sealed plastic boxes lined with moistened paper towels and held for 24 h in a growth chamber (16:8 light/dark photoperiod, 26 °C). Mortality was determined after 24 h. Larvae were considered to be dead if they did not respond to prodding with forceps. Boxes containing the insects were returned to the growth chamber and rechecked after 48 h to confirm mortality.

Table 1. Constituents of *Litsea pungens* and *L. cubeba* Essential Oils

	L. pungens		*L. cubeba*	
no.	constituent	composition (%, w/w)	constituent	composition (%, w/w)
1	3-carene	3.1	*β*-pinene	7.0
2	α-pinene	2.0	α-pinene	2.4
3	(*R*)-limonene	7.5	*p*-cymene	4.4
4	1,8-cineole	52.4	(*R*)-limonene	27.2
5	2-carene	2.8	*γ*-terpinene	43.6
6	linalool	2.4	α-terpinene	5.3
7	terpinen-4-ol	3.5	other compounds	10.1
8	camphene	2.3		
9	geraniol	2.2		
10	carvone	10.3		
11	*β*-caryophyllene	4.2		
12	other compounds	7.3		

Table 2. LD_{50} Values of Essential Oils against Third-Instar *Trichoplusia ni* Larvae Based on 24 h Mortality via Topical Application[a]

no.	essential oil	LD_{50} (μg/larva)	LD_{95} (μg/larva)	95% confidence limit
1	*L. pungens* oil	87.1	277.7	68.3−106.6
2	*L. cubeba* oil	112.4	486.4	85.9−164.9

[a] Four concentrations were used to calculate LD_{50} values (25, 50, 100, and 200 μg/larva for *L. pungens* essential oil and 50, 100, 200, and 400 μg/larva for *L. cubeba* essential oil). *N* = 3 replications of 10 larvae per concentration.

Statistical Analysis. Probit analysis was used to determine LD_{50} and LD_{95} values and their corresponding 95% confidence intervals using the EPA probit analysis program version 1.5. Mortality data were analyzed using the Statistics 7 program (*12*) for analysis of variance (ANOVA). Tukey's test was used to compare means. Experiments were repeated at least twice.

Comparative Toxicities. To determine the potential contribution of each constituent to the overall toxicity of the oils, we made blends of all major constituents for the two oils mimicking the natural oils. We also prepared a number of blends, each lacking one major constituent (**Figures 2** and **3**). Blends were based on the natural composition of the two oils as indicated by GC-MS (**Table 1**) and tested at the concentration at which the natural oil produced 95% mortality. We also compared the toxicities of the complete and incomplete blends with those of pure *L. pungens* and *L. cubeba* oils. Toxicities of individual constituents of both oils were similarly

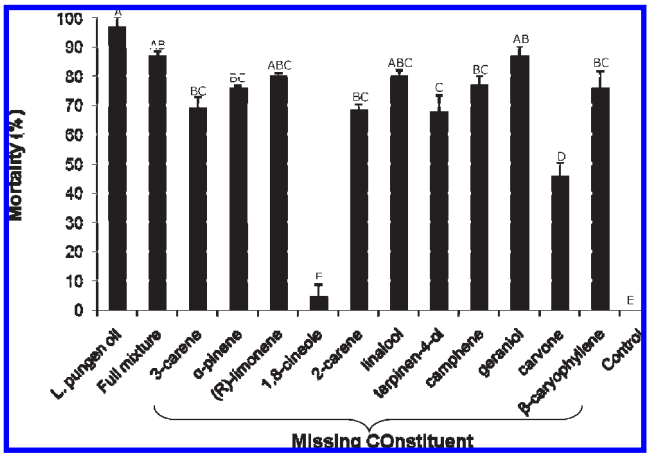

Figure 2. Mortality caused by the oil, full mixture, and selected blends of constituents of *L. pungens* oil to third-instar *T. ni* larvae applied at levels equivalent to those found in the 95% lethal concentration of the pure oil (LD_{95} = 277.7 μg/larva for *T. ni*). Error bars represent the standard error of the mean of three replicates of 10 larvae each. Means corresponding to each treatment with different letters are significantly different from each other (Tukey's test, $p < 0.05$). "Full mixture" indicates a blend of 11 constituents, whereas all others indicate full mixture missing the constituent noted.

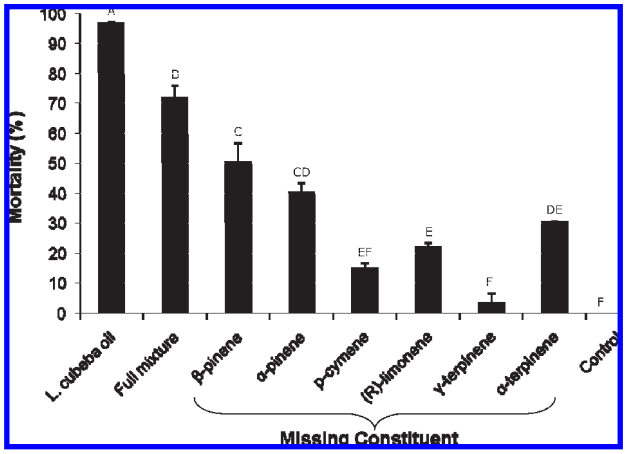

Figure 3. Mortality caused by the oil, full mixture, and selected blends of constituents of *L. cubeba* oil to third-instar *T. ni* larvae applied at levels equivalent to those found in the 95% lethal concentration of the pure oil (LD_{95} = 486.4 μg/larva for *T. ni*). Error bars represent the standard error of the mean of three replicates of 10 larvae each. Means corresponding to each treatment with different letters are significantly different from each other (Tukey's test, $p < 0.05$). "Full mixture" indicates a blend of six constituents, whereas all others indicate full mixture missing the constituent noted.

determined (**Table 3**). Finally, mixtures of the putative active constituents (those that contributed to the toxicity of the oil) were compared with mixtures of the apparent inactive constituents (those that did not affect the overall toxicity, **Figure 4**).

RESULTS AND DISCUSSION

Essential Oils Constituents and Toxicity of Oils. GC-MS analysis indicated that in the *L. pungens* oil, 11 known constituents comprised 92.7% of the oil by weight. 1,8-Cineole was the most abundant compound (52.4%), followed by carvone (10.3%) and (*R*)-limonene (7.5%) (**Table 1**). Our results corroborate an earlier finding reporting 1,8-cineole as the main constituent in the oil obtained from the leaves and fruits of *L. pungens* (*2*).

Table 3. Mortality Caused by Individual Constituents of *L. pungens* and *L. cubeba* Essential Oils against Third-Instar *T. ni* Larvae Applied at Levels Equivalent to Those Found in the 95% Lethal Concentration of the Pure Oils [a]

	L. pungens		*L. cubeba*	
no.	constituent	mortality (%)	constituent	mortality (%)
1	3-carene	0	β-pinene	0
2	α-pinene	0	α-pinene	0
3	(*R*)-limonene	0	*p*-cymene	0
4	1,8-cineole	23.3	(*R*)-limonene	3.3
5	2-carene	0	γ-terpinene	70
6	linalool	0	α-terpinene	0
7	terpinen-4-ol	0		
8	camphene	0		
9	geraniol	0		
10	carvone	13.3		
11	β-caryophyllene	0		

[a] *N* = three replicates of 10 larvae each.

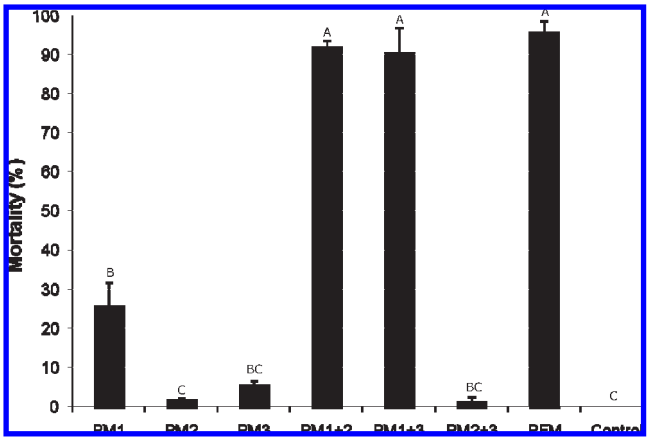

Figure 4. Mortality caused by selected blends of active and inactive constituents of *L. pungens* oil to third-instar *T. ni* larvae when applied at levels equivalent to those found in the 95% lethal concentration of the pure oil (LD_{95} = 277.7 μg/larva for *T. ni*). Error bars represent the standard error of the mean of three replicates of 10 larvae each. Means corresponding to each treatment with different letters are significantly different from each other (Tukey's test, $p < 0.05$). PM1 (very active constituents) = 1,8-cineole + carvone; PM2 (moderately active constituents); PM3 (inactive constituents); PM1 + 2 = PM1 + PM2; PM1 + 3 = PM1 + PM3; PM2 + 3 = PM2 + PM3; PFM = full mixture of all constituents. PM = *L. pungens* mixture.

Similarly, there were six known constituents in the *L. cubeba* oil, comprising 89.9% of the total weight. γ-Terpinene was the most abundant compound (43.6%), followed by (*R*)-limonene (27.2%) and β-pinene (7%) (**Table 1**). The LD_{50} and LD_{95} values for *L. pungens* oil against third-instar *T. ni* larvae were 87.1 and 277.7 μg/larva and 112.4 and 486.4 μg/larva for *L. cubeba* oil, respectively (**Table 2**). No mortality was observed in the controls.

Comparative Toxicities of Individual Constituents and Blends. Bioassays with artificial mixtures showed that a blend containing the 11 known constituents of *L. pungens* ("full mixture") was the most toxic. Mortality caused by the artificial mixture with all of the constituents present did not differ significantly (Tukey's test, $p < 0.05$) from that caused by the natural essential oil as shown in **Figure 2**. However, mortality caused by the artificial mixture of the 6 constituents of *L. cubeba* oil was significantly lower (Tukey's test, $p < 0.05$) than that caused by the natural essential oil (**Figure 3**). This suggests that unknown constituents comprising 10% of the oil make a significant contribution to the overall toxicity of the oil.

For *L. pungens* oil, component elimination assays (**Figure 2**) indicated that the omission of 1,8-cineole, carvone, or terpinen-4-ol from the artificial mixture caused a significant decrease in toxicity of the blend (one-way ANOVA; $F_{13,41} = 63.5, p < 0.05$). 1,8-Cineole was the major contributor to the toxicity of the oil followed by carvone. Removal of most other constituents [3-carene, 2-carene, (*R*)-limonene, β-caryophyllene, linalool, camphene, geraniol, and α-pinene] from the mixture had no significant effect on the toxicity of the blend (Tukey's test; $p < 0.05$) as shown in **Figure 2**. Testing individual constituents of the oil confirmed the role of 1,8-cineole as the major contributor to the toxicity of the oil followed by carvone (**Table 3**). None of the other constituents produced mortality at the dose tested.

For *L. cubeba* oil, omission of any of the six constituents [γ-terpinene, *p*-cymene, (*R*)-limonene, α-terpinene, β-pinene, or α-pinene] from the artificial mixture caused a significant decrease in the toxicity of the blend (one-way ANOVA; $F_{8,20} = 125$, $p < 0.05$). γ-Terpinene was the major contributor to the toxicity of the oil, although all constituents contributed significantly to the toxicity of the artificial blend (Tukey's test, $p < 0.05$) (**Figure 3**). Testing individual constituents of the oil confirmed the role of γ-terpinene as the major contributor to the toxicity of the oil (**Table 3**), responsible for 70% mortality. None of the other constituents produced larval mortality except for (*R*)-limonene (3.3% mortality).

The comparison of toxicity caused by selected blends of active and inactive constituents (**Figure 4**) showed that the mixture of the most active constituents (PM1 = 1,8-cineole + carvone, whose removal from the mixture dropped the toxicity to <30% compared to the full mixture) alone of *L. pungens* essential oil caused only 25% mortality. Mixture of the moderately active constituents (PM2 = terpinen-4-ol + 3-carene + 2-carene, the removal of which from the mixture dropped the toxicity to <70% compared to the full mixture) caused no mortality, whereas mixture of the inactive constituents [PM3 = (*R*)-limonene + β-caryophyllene + linalool + camphene + geraniol + α-pinene, the removal of which from the mixture did not influence toxicity compared to the full mixture] caused only 5% mortality. Combination of the mixture of active constituents with either the moderately active (PM1 + PM2) or inactive constituents (PM1 + PM3) restored toxicity consistent with the full mixture of the 11 major compounds (**Figure 2**). The absence of PM1 from the mixture PM2 + PM3 caused a significant decrease in toxicity (one-way ANOVA; $F_{7,21} = 70.2, p < 0.05$).

Similarly, the mixture of active constituents [CM1 = γ-terpinene + (*R*)-limonene + *p*-cymene] of *L. cubeba* essential oil alone produced 40% mortality, whereas the mixture of inactive constituents (CM2 = β-pinene + α-terpinene + α-pinene) produced almost no mortality of *T. ni* larvae. Both CM1 (42%) and CM2 (2%) produced significantly less toxicity than the full mixture (CM1 + CM2; 73%) ($F_{3,7} = 176, p < 0.05$). For both *Litsea* oils, the toxicity of the mixtures became equivalent to the natural oils only when active constituents were mixed with the putatively inactive constituents.

Our results clearly indicate that essential oils of *L. pungens* and *L. cubeba* and some of their constituents have modest toxicity to the cabbage looper. The insecticidal activity of plant essential oils is often enhanced by the presence of suites of closely related compounds, acting synergistically or at the least diffusing selection by insect herbivores and thus forestalling the development of resistance (*13*) and habituation (*14*) in those insect populations (*15*). Plant defense chemicals that exhibit more than one mode of action are especially suitable for crop protection (*16*), constituting a "multichemical defense" against a variety of potential herbivores. Although the major mode of action in insects is considered to be neurotoxicity (*17*), disruption of cell membranes or blockage of the tracheal system in insects through fumigation effects cannot be ruled out. The efficacy of essential oils for the control of a variety of pests is well established, including mosquitoes (*18*), cockroaches (*19*), house flies (*20*, *21*), termites (*22*), caterpillars (*11*, *23*, *24*), aphids (*25*), beetles (*26*), whiteflies (*27*), and mites (*10*).

Our results have shown that both *L. pungens* and *L. cubeba* essential oils have potential for development as commercial insecticides. Although orders of magnitude less toxic than conventional insecticides, the LD_{50} values of *L. pungens* (87.1 μg/larva) and *L. cubeba* (112.4 μg/larva) essential oils are nonetheless 2−3 times lower than that for A4 blend (259.6 μg/larva) against third-instar cabbage looper larva (*28*). The A4 blend is currently marketed in the United States as a broad-spectrum insecticide/miticide (EcoTrol, EcoSMART Technologies Inc.) with rosemary oil as the active ingredient. Rosemary oil itself has a LD_{50} of 209.1 μg/larva in the cabbage looper (*28*). Although *L. cubeba* oil has been shown to be repellent to termites (*29*), there have been no other reports of insecticidal effects of these oils. This is the first report demonstrating insecticidal activity of *L. pungens* oil to our knowledge, although some of its constituents have been tested against a number of insect pests (*2*, *3*). Like many other essential oils, *L. pungens* and *L. cubeba* oils may have limited or no effect on nontarget organisms. Owing to their volatility, essential oils have limited persistence under field conditions; therefore, predators and parasitoids reinvading a treated crop one or more days after treatment are unlikely to be poisoned by contact with foliar residues as often occurs with conventional insecticides. Most of the essential oils themselves or pesticide products based on oils with few exceptions are nontoxic to mammals, birds, and fish (*30*).

To corroborate the role of individual constituents toward the total toxicity of *L. pungens* and *L. cubeba* essential oils to *T. ni* larvae, each individual constituent was eliminated from a synthetic mixture that simulated natural oils in toxicity. We found that the absence of some constituents (1,8-cineole from *L. pungens* oil and γ-terpinene from *L. cubeba* oil) from the artificial mixture caused a significant decrease in toxicity (82 and 46%, respectively), leading us to conclude that these constituents are the major contributors to the oils' toxicity. Because the toxicity of the artificial blend produced only 72% mortality as opposed to 97% for the natural *L. cubeba* oil, other compounds (**Table 1**), although in minor proportion (10%), must also be responsible for the total toxicity of the oil. Future studies will focus on their identification and role in the total toxicity of the oil.

1,8-Cineole, the major constituent (52.4%) of *L. pungens* oil, was the major contributor to the toxicity of the artificial blend. Our results confirm a previous report (*10*) that 1,8-cineole is responsible for the major toxicity of rosemary oil against the twospotted spider mite *Tetranychus urticae*. Another factor favoring development of botanical insecticides based on plant essential oils is the relatively low cost of the active ingredients resulting from their extensive worldwide use as fragrances and flavorings. Knowing the role of each constituent in the toxicity of the oil renders an opportunity to create artificial blends of different constituents on the basis of their activity and efficacy against different pests. Blends are more effective than individual compounds in terms of forestalling and diluting resistance and habituation for long-term use. The exemption of some common essential oils from registration in the United States (*1*) has stimulated their development for use as commercial insecticides.

One interesting aspect of our study is the difference in the role of the major constituents in a mixture as opposed to their individual toxicities in most cases. 1,8-Cineole was responsible

for 82% of the toxicity in the artificial blend but caused only 23% toxicity when tested individually. Similarly, carvone was responsible for 41% of the toxicity in the artificial blend but produced only 13% toxicity when tested individually. (*R*)-Limonene was responsible for 50% of the toxicity in the blend but caused only 3% toxicity in individual testing. However, there was a strong correlation in the toxicity of γ-terpinene (~70%) in the blend as well as in individual testing. This kind of information could be very helpful in the creation of an artificial blend to control insect pests. The toxicity of our artificial mixtures reached the level of the natural oils only when we mixed the blends of active constituents with inactive ones. This indicates that the "inactive" constituents have some synergistic effect on the "active" constituents and that, although not active individually, their presence is necessary to achieve full toxicity.

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