Acute and synergistic effects of monoterpenoid essential oil compounds on the larvae *of Spodoptera littoralis*

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ABSTRACT

Acute toxicity and mutual synergistic effect of six monoterpenoids on *Spodoptera littoralis* larvae was recorded. Out of the essential oils compounds tested for topical acute toxicity, the most potent were thymol and carvacrol, with LD_{90} values < 100 ìg per larva (weight 25 - 30 mg). In comparison for fumigant acute toxicity, the most potent were ã-terpinene, p-cymene and 1,8-cineole, with LD_{90} values < 100 ìg per cm^3. Nine binary mixtures out of 15 tested couples showed the synergistic effect in topical application. The most significant synergism was found in p-cymene in the mixture with ã-terpinene or carvacrol. In the fumigant application, 9 binary mixtures showed a synergistic effect; 5 mixtures showed an additive effect, and one mixture showed an antagonistic effect. The most profound synergistic effect was found in ã - terpinene in the mixture with carvacrol and in p-cymene in the mixture with thymol or carvacrol.

Key words : *Spodoptera littoralis*, essential oils, acute toxicity, synergistic effects, monoterpenoids.

INTRODUCTION

Essential oils are complex and highly variable mixtures of constituents which belong, virtually exclusively, to two groups characterized by distinct biogenetic origins: the group of terpenoids, and the group, far less common, of aromatic compounds derived from phenylpropane. The essential oils can be separated from other plant tissues through steam distillation or by supercritical fluid extraction (Banthorpe, 1991). Most are mixtures, some quite complex, of monoterpenoid alcohols (e.g., a terpineol, menthol, linalool), ketones (e.g., tagetone, menthone, camphor), aldehydes (e.g., neral, cinnamaldehyde and citronellal), esters (e.g. linalyl acetate, menthyl, citonellyl acetate), ethers (e.g. 1,8-cineole) peroxide (e.g. ascaridol) and phenols (e.g. thymol, carvacrol) (Bruneton, 1999). They are often quite volatile and are commonly used as fragrances and as flavoring agent food additives or in medicine (e.g., aromatherapy). More recently, they have become the focus of interest in developing ecologically sensitive pesticides (Isman, 2000; Pavela, 2007a).

Various essential oils and their compounds are documented to exhibit acute toxic effects against insects (Rice and Coast, 1994; Lee *et al.*, 1997; Pavela, 2005, 2006 and 2008; Upadhyay, 2010). Compounds lacking acute insecticidal toxicity may still confer protection to crops by reducing fitness of insect herbivores via inhibition of larval growth, disruption of larval development, or failure in pupal eclosion (Hummelbrunner and Isman, 2001; Pavela, 2007b). Deterrence of feeding and repellency can also fulfill a protective role (Karr and Coats, 1992; Trongtokit *et al.*, 2005).

Essential oils are generally mixtures of several up to dozens of mono-, di-, sesqui-terpenes. Every plant species contains a different, unique mixture, the composition of which may vary depending on the growth stage, climatic-pedological conditions and other conditions (Hay *et al.*, 1988; Svoboda and Hay, 1990). Every plant species contains a different, unique mixture, the composition of which may vary depending on the growth stage, climatic-pedological conditions and other conditions (Hay *et al.*, 1988). Consequently, the biological activity of the essential oils will also vary. The biological activity of essential oils depends not only on qualitative content of substances contained therein, but also on the ratio of the major components. Individual substances contained in oils can namely show significant mutual synergistic support of their biological effect (Hummelbrunner and Isman, 2001, Pavela, 2008b). Although this synergistic effect is very important from the viewpoint of normalizing botanical insecticides based on essential oils and optimizing their biological efficiency, knowledge of synergistic effects of monoterpenes on insects is insufficient.

The noctuid *Spodoptera littoralis* (Boisd.) is most important polyphagous pest, widely distributed all over the world. Larvae of this pest can feed on 90 economically important plant species belonging to 40 families and the rate of development has a strong nutritional component (Azab *et al.*, 2001), For insecticide discovery, it is a conservative model, in that this species seems to require higher doses for acute toxic effects relative to other insect species, including German cockroaches, house flies and diamondback moths. In our work, we found acute toxicity on *S. littoralis* larvae for 6 monoterpenes which often represent the majority components of many essential oils obtained from aromatic plants of the Lamiaceae family. Their mutual synergistic relationship was found for fumigant as well as topical application.

MATERIALS AND METHODS

Insects

Bioassays were conducted using larvae (weight 25-30 mg) of the tobacco cutworm, *Spodoptera littoralis* (Fab.), obtained from an established laboratory colony (>50 generations; out-crossed once). Insects were reared on an artificial diet (Instant soybean-wheat germ insect diets, Stonefly Industries, Bryan, TX, U.S.A.). The colonies were reared at the temperature of 25 ± 1°C and a 16:8 (L:D) photoperiod.

Chemicals

Pure compounds (carvacrol, 1,8-cineole, p-cymene, eugenol, ã-terpinene and thymol) were purchased from Sigma-Aldrich Chemical Co., Prague, Czech Republic. Analytical grade acetone was used as the carrier. Compounds and other test materials were dissolved in acetone.

Acute toxicity of pure compounds

Acute toxicity (measured as mortality after 24 h) of essential oil compounds was determined by topical and fumigant application to early fourth-instars (25 - 30 mg body weight). Initial screening to approximate the active dose range determined a range of five doses that were used to establish the lethal doses. Four replicates of 20 larvae were tested per dose. Larvae were individually weighed prior to treatment.

Topical application

Essential oil compounds were prepared using acetone as a carrier such that each larva received 1 ìL of oil solution per treatment, with acetone alone as the control. Doses were applied to the dorsum using a repeating topical dispenser attached to a 50 ìL syringe. All 20 treated larvae from each replicate were transferred onto a 2 cm3 block of diet placed in a 10 cm diameter plastic Petri dish (each replicate was transferred to a separate dish).

Fumigant application

At least four concentration levels were selected. Larvae were put into a cage (250 cm3) which was covered with a breather mesh (a piece of cloth). Larvae were then fed with common nutrition therein. The cage was attached to a wire and hanged in the middle of an aquarium (2.9 L). The aquarium had an inside air-circulation by means of a ventilator, which we have also placed inside the cage. In front of the ventilator, there was a small square-shaped piece of filter paper (2 cm^2) so that optimal circulation of air was maintained and so that the pure compounds would fumigate. Compounds were then poured onto the filter papers. The walls of the aquarium were secured against evaporation of the essential oils and covered with a paraffin film.

All treatment groups were then placed in sealed plastic boxes lined with moistened paper towels and held for 24 h in a growth chamber (3 x 5 x 3 m, CRI, Prague, Czech Republic) (L16 : D8, 24 ± 1 °C, RH 85 ± 5%). Mortality was recorded after 24 h. Death was recorded if larvae did not respond to prodding with forceps. Experimental tests demonstrated that more than 20% of the controlled mortality was discharged and repeated. When the controlled mortality reached 1 - 20%, the observed mortality was corrected by the Abbott’s formula (Abbott, 1925). Probit analysis of concentration-mortality data was conducted to estimate the LC_{50} and LD_{90} values and associated 90% confidence limits for topical and fumigant application (Finney, 1971).

Acute Effects of Binary Mixtures

The acute effects of binary mixtures of essential oil compounds were determined methods as in the *acute toxicity* experiments described above. Three test groups were run concurrently for each binary combination tested: the binary mixture and each of the pure compounds. The compounds were combined in a 1:1 ratio. Initially, the LD_{20} value of the compounds was calculated on the basis of the LD_{50} / LD_{90} values determined through probit analysis. The experimentation included of the dosage around this value of the active compounds. Actual mortalities were compared to expected mortalities based on the formula:

$E = Oa + Ob (1 - Oa)$

where E is expected mortality and Oa and Ob are observed mortalities of pure compounds at the given concentration (Trisyono and Whalon, 1999). The effects of mixtures were designated either antagonistic, additive, or synergistic by analysis using $÷^2$ comparisons: $÷^2 = (Om - E)^2 / E$, where Om is observed mortality from the binary mixture and E is expected mortality; $÷^2$ with df = 1 and p = 0.05 is 3.84.

A pair with $÷^2$ values > 3.84 and having greater than expected mortality were considered to be synergistic (negativeness = antagonist effect), with $÷^2$ values < 3.84 representing additive effects (Hummelbrunner and Isman, 2001).

RESULTS

Acute toxicity of pure compounds

Of the essential oils compounds tested for topical acute toxicity, the most potent were thymol and carvacrol, with LD_{90} values < 100 µg per larva (Table 1). In comparison for fumigant acute toxicity, the most potent were γ - terpinene, p-cymene and 1,8-cineole, with LD_{90} values < 100 µg per cm^3 (Table 2).

Table 1. Acute toxicity of essential oil compounds to larvae *S. littoralis* by topical and fumigant application

Compounds	Topical application					Fumigant application				
	LD_{50}^{b}	CI_{95}^{a}	LD_{90}^{b}	CI_{95}^{a}	Chi ^{c}	LD_{50}^{b}	CI_{95}^{a}	LD_{90}^{b}	CI_{95}^{a}	Chi ^{c}
Thymol	42.1	31.1- 49.6	58.1	56.3 - 72.1	0.253	10.5	8.9 - 11.3	20.6	17.3 - 22.2	0.356
Carvacrol	65.9	48.3 - 69.5	95.1	86.2 - 103.3	0.759	16.8	15.1- 18.3	31.1	27.7 - 33.3	2.183
1,8-cineole	80.6	68.3 - 88.3	112.3	105.1 - 121.3	1.083	35.3	25.1- 39.8	54.2	48.1- 60.5	1.157
ã - terpinene	94.5	87.1 - 98.6	178.5	166.3 - 189.2	0.323	99.2	87.3 - 105.6	139.1	128.6 - 142.1	0.035
p - cymene	108.8	93.2 - 112.1	186.1	178.3 - 192.6	0.159	102.2	92.3 - 112.5	133.9	125.8 - 145.1	0.333
Eugenol	147.2	138.3 - 152.1	232.9	225.2 - 251.1	0.233	865.4	796.8-892.1	2227.4	2021.3 - 2358.6	0.456

CI_{95}^{a} – denotes confidence intervals, compound activity is considered significantly different when the 95% CI fail to overlap.
^{b} – denotes lethal doses in µg / larvae, Chi^{c} - Chi - square value, significant at $P < 0.05$ level.

Acute toxicity of binary mixtures

Sublethal doses (LD_{20}) calculated using probit analysis caused mortality in the range 2.5 - 7.5 % in pure substances in the case of topical application (Table 1). In fumigant application, pure substances caused mortality in the range 2.5 - 10.5 % (Table 3). Synergistic effect of binary mixtures applied topically is given in Table 1. 9 mixtures out of the 15 tested couples showed a synergistic effect. The most important synergism was found in p-cymene in the mixture with γ -terpinene or carvacrol. On the contrary, an additive effect was shown by eugenol in the mixture with p-cymene, γ - terpinene and 1,8-cineole, just like carvacrol in the mixture with γ -terpinene and 1,8 - cineole and 1,8 - cineole in the mixture with thymol. Synergistic effect of binary mixtures with fumigant application is presented in (Table 3). Out of the 15 mixtures tested, 9 showed a synergistic effect, as well; 5 mixtures showed an additive effect, and the mixture of carvacrol and 1,8-cineole showed even an antagonistic effect (p < 0.05). The most profound synergistic effect was found in ã-terpinene in the mixture with carvacrol and in p-cymene in the mixture with thymol or carvacrol. An additive effect was found in eugenol in the mixture with p-cymene, γ - terpinene, 1,8-cineole and carvacrol and in the mixture of p-cymene with 1,8-cineole.

Table 2. Synergy, additive and antagonistic effect of binary mixtures of compounds by topical application to 4th instar larvae of *S. littoralis*

Compound a	Compound b	Dosage (µg / larvae)	Larval mortality (%)					
			Pure compounds		binary mix			
			Observed a	Observed b	Expected	Observed	χ^2	Effect
p-cymene	ã-terpinene	51+31	5.1	2.5	7.5	37.5	119.1	synergy
p-cymene	eugenol	51+87	5.1	5.0	9.9	13.3	1.2	additive
p-cymene	carvacrol	51+43	5.1	7.5	12.0	46.7	99.6	synergy
p-cymene	1,8-cineole	51+57	5.1	5.0	9.9	20.1	10.5	synergy
p-cymene	thymol	51+34	5.1	7.5	12.0	26.7	17.8	synergy
ã - terpinene	eugenol	31+87	2.5	5.0	7.2	10.0	1.0	additive
ã - terpinene	carvacrol	31+43	2.5	7.5	9.4	13.3	1.6	additive
ã - terpinene	1,8-cineole	31+57	2.5	5.0	7.2	16.7	12.2	synergy
ã - terpinene	thymol	31+34	2.5	7.5	9.4	30.0	27.3	synergy
eugenol	carvacrol	87+43	5.0	7.5	11.9	36.7	51.2	synergy
eugenol	1,8-cineole	87+57	5.0	5.0	9.8	6.6	1.0	additive
eugenol	thymol	87+34	5.0	7.5	11.9	26,7	18.2	synergy
carvacrol	1,8-cineole	43+57	7.5	5.0	11.9	17.5	2.2	additive
carvacrol	thymol	43+34	7.5	7.5	14.3	36.8	34.3	synergy
1,8-cineole	thymol	57+34	5.0	7.5	11.9	6.7	2.3	additive

Table 3. Synergy, additive and antagonistic effect of binary mixtures of compounds by fumigant application to 4th instar larvae of *S. littoralis*

Compound a	Compound b	Dosage (µg / larvae)	Larval mortality (%)					
			Pure compounds			binary mix		
			Observed a	Observed b	Expected	Observed	χ^2	Effect
p-cymene	ã-terpinene	51 + 31	5.1	2.5	7.5	37.5	119.1	synergy
p-cymene	ã-terpinene	3.9 + 3.5	2.5	5.0	7.3	16.7	12.2	synergy
p-cymene	eugenol	3.9 + 152.0	2.5	5.0	7.3	12.1	3.2	additive
p-cymene	carvacrol	3.9 + 70.1	2.5	5.5	7.7	42.5	157.3	synergy
p-cymene	1,8-cineole	3.9 + 21.6	2.5	5.3	7.5	12.5	3.4	additive
p-cymene	thymol	3.9 + 78.3	2.5	10.5	11.9	60.1	194.5	synergy
ã-terpinene	eugenol	3.5 + 152.0	5.0	5.0	9.8	10.0	0.01	additive
ã-terpinene	carvacrol	3.5 + 70.1	5.0	5.5	10.2	57.5	219.4	synergy
ã-terpinene	1,8-cineole	3.5 + 21.6	5.0	5.3	9.9	37.5	75.9	synergy
ã-terpinene	thymol	3.5 + 78.3	5.0	10.5	14.4	32.5	22.7	synergy
eugenol	carvacrol	152.0 + 70.1	5.0	5.5	10.2	15.0	2.3	additive
eugenol	1,8-cineole	152.0 + 21.6	5.0	5.3	9.9	6.7	1.1	additive
eugenol	thymol	152.0 + 78.3	5.0	10.5	14.4	33.3	24.9	synergy
carvacrol	1,8-cineole	70.1 + 21.6	5.5	5.3	10.5	3.3	4.8	
antagonistic								
carvacrol	thymol	70.1 + 78.3	5.5	10.5	14.9	43.3	54.3	synergy
1, 8-cineole	thymol	21.6 + 78.3	5.3	10.5	14.6	23.4	5.2	synergy

DISCUSSION

Synergistic effects of complex mixtures are thought to be important in plant defenses against herbivore insects. Plants usually present defenses as a suite of compounds, not as individual ones, and it is thought that the minor constituents may act as synergists, enhancing the effect of the major constituents through a variety of mechanisms. It is frequently noted that the "original" complex essential oils are considerably more efficacious than the pure compounds derived from them.

In this study, 6 monoterpenes were tested which are most frequently present in essential oils of the Lamiaceae family plants (Banthorpe, 1991; Bounatirou *at al.*, 2007). We found that the efficiency of pure substances was significantly different depending on the way of their application. While upon topical application, phenols; thymol and carvacrol (LD_{50} 42 and 65 ìg/larvae, respectively) were most efficient, hydrocarbons ã-terpinene and p-cymene (LD_{50} 11 and 17ìg/cm^3) were most efficient in the case of fumigant application. Allylbenzene eugenol was least efficient in both ways of application. Similar results were obtained for house fly (*Musca domestica*) adults (Pavela, 2008b). The difference between efficiency of monoterpenic substances with topical and fumigant application is probably caused by the fumigant capability of movement of the substances in space, which corresponds directly to the different efficiency of essential oils determined earlier (Pavela, 2008a).

Effects of some monoterpenes on acute toxicity of *Spodoptera litura* larvae were studied by Hummelbrunner and Isman (2001). Only topical application was studied, using methodology identical to ours. In their work, too, thymol and carvacrol were most efficient. In eugenol, its low efficiency is caused by the generally low biological activity, confirmed also by other authors (Hummelbrunner and Isman, 2001).

Sub-lethal doses applied in binary mixtures showed a significant synergistic effect on acute toxicity in some cases. Hydrocarbons ã-terpinene and p - cymene were found to be the most significant synergists for both ways of application. Nevertheless, while in topical application, the most significant synergism between p-cymene in combination with γ - terpinene and carvacrol was found, the most significant synergistic effect found in fumigant application was shown by the combinations of ã-terpinene with carvacrol and p-cymene with thymol and carvacrol.
Although understanding the synergistic effect of terpenes on insects is necessary to normalize the formulations of new botanical insecticides, many available published results are not at disposal. The most significant contributions were published by Hummelbrunner and Isman (2001), examining the synergistic effect of 4 terpenes on acute toxicity in connection with *Spodoptera litura* larvae, while the most significant effect was found for the combination of thymol with trans - anethole.

In a previous study (Pavela, 2008b) we published the same compounds against musea domestica adults. We found that twelve binary mixtures out of 15 tested couples showed synergistic effects in topical application. The most significant synergism was found with p-cymene in combination with 1,8- cineole, γ - terpinene or carvacrol. In the fumigant application, 8 binary mixtures showed a synergistic effect. The most profound synergistic effect was found in p-cymene with γ -terpinene or with 1,8-cineole and γ - terpinene in the mixture with 1,8-cineole. Products based on essential oils are an important alternative to synthetic pesticides. Comparison of lethal doses found for acute toxicity is usually used to compare the biological efficiency of oils. Nevertheless, essential oils can have other effects on reduction of fertility, antifidancy, repellency, larvae growth inhibition (Karr and Coats, 1992; Maganga *et al.*, 1996; Hummelbrunner and Isman, 2001; Pavela, 2007b), all representing significant ways of efficiency leading to reduced population density and overall harmfulness of pests.

It follows from our work that in development of new plant insecticides based on essential oils or their efficient substances, their way of application should be considered, as well, and the mutual ratio and combination of majority efficient substances. Knowledge of the biological efficiency of terpenes and their mutual relationships shall contribute to normalizing the products and thus also to declaring their biological efficiency.

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