A Thesis
for the Degree of Master of Science

Synergistic Mechanism of Insecticidal Activity in Basil (*Ocimum basilicum*) and Mandarin (*Citrus reticulata*) Essential Oils against the Tobacco Cutworm

담배거세미나방(*Spodoptera litura*)에 대한 바질(*Ocimum basilicum*)과 만다린(*Citrus reticulata*) 정유의 살충활성 상승 작용 기제

By
Subin Kim

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Under the Direction of Adviser Jun-Hyung Tak

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By
Subin Kim

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Department of Agricultural Biotechnology
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Approved as a Qualified Thesis of Subin Kim
for the Degree of Master of Science
By the Committee Members

Chair	Si Hyeock Lee
Vice Chair	Jun-Hyung Tak
Examiner	Yeon Ho Je

Abstract

Synergistic mechanism of insecticidal activity in basil (*Ocimum basilicum*) and mandarin (*Citrus reticulata*) essential oils against the tobacco cutworm

Subin Kim
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Owing to the complexity in the chemical composition of plant essential oils, they often display enhanced insecticidal activity when applied as a mixture. Although the insecticidal activity of plant essential oils has been gaining more attention recently, understanding in the mechanism of synergy has not been studied as much. In the present study, insecticidal activity of 28 individual essential oils and their mixtures against the third instar larvae of *Spodoptera litura* was examined. Among the oils tested, basil oil exhibited the strongest contact toxicity, and mandarin oil displayed

the greatest boosting effect when the remaining oils were mixed with basil oil. Estragole and linalool were determined as the major active constituents for the insecticidal activity of basil oil, and limonene for mandarin oil from the chemical analyses and compound elimination assay. Based on the LD_{50} values, the binary mixture of basil and mandarin oils exhibited enhanced toxicity compared to the individual application of the two oils, showing synergy ratios of 1.3 and 1.4 from two statistical models. As for the major active compounds, synergistic interaction was found in tertiary mixture of estragole, linalool, and limonene in the blending ratio of 7:2:7, displaying the same insecticidal activity of the binary mixture of basil and mandarin oils. The synergistic effect was only observed in the tertiary mixture, indicating each compound play crucial roles of the overall contact toxicity. Increased penetration through cuticular layer and amplified neurophysiological response were proposed for the mechanism of synergistic effect.

Keyword: contact toxicity; *Spodoptera litura*; cuticular penetration; central nervous system

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Introduction

Plant essential oils, mainly extracted *via* hydrodistillation, steam distillation, dry distillation, or cold pressing, are complex mixtures of phytochemicals whose constituents are mostly belonging to terpenoids, and they display a variety of bioactivity against bacteria, fungi, weeds, and toward numerous insect pests as well (Regnault-Roger et al. 2012). With a few exceptions such as nicotine or cyanogenic glycosides, most plant extracts and essential oils tend to pose relatively little threat to the environment and human health due to their low mammalian toxicity and minimal persistence in the environment, and they have been attracting a large attention in their insecticidal activity for the last couple of decades (Isman and Grieneisen 2014; Isman 2020).

The tobacco cutworm or cotton leafworm, *Spodoptera litura* Fab. (Lepidoptera: Noctuidae), is a well-known phytophagous insect pest attacking various host plants including white goosefoot, impatiens, hooked dock, white mulberry, peanut, and many other crops in Asian countries, directly resulting in the loss of yields and economic gains (Rose 1985; Xue et al. 2010; Ahmad et al. 2013). Although many previous studies have already proposed several essential oils as potential candidates for *S. litura*

control (Hummelbrunner and Isman 2001; Koul et al. 2013; Benelli et al. 2018; Murfadunnisa et al. 2019), it requires relatively high concentration of active ingredients to develop them as commercial insecticides when compared to conventional synthetic pesticides. Enhanced efficacy *via* synergistic effect can be one of the possible solutions to this limitation. Owing to the complexity in the composition of essential oils, it is often observed synergistic interactions among the major constituents of oils or the mixtures of oils in their insecticidal activity (Hummelbrunner and Isman 2001; Pavela 2015a). Nonetheless, we still understand little in the underlying mechanism of synergistic interaction of essential oils.

In the present study, we examined the insecticidal activity of twenty-eight essential oils as well as their mixtures against the third instar larvae of *S.litura*. The selected oils are either on the exemption list of US Environmental Protection Agency (US EPA 2015), have a wide range of medicinal usages (Isman et al. 2001), or were previously tested for insecticidal activities against other insect species (Pavela 2015b; Yang et al. 2020), which have potential merits for further development as botanical insecticides thanks to their safety and bioactivity. Chemical analyses and compound elimination bioassays were conducted to identify the major active constituents. Furthermore, to elucidate the synergistic interaction of selected combination, cuticular penetration of the synergistic combination as well as neurological effect in the central nervous systems were investigated.

Materials and Method

1. Test insects

Eggs of the tobacco cutworm were obtained from Crop Protection Center, Farm Hannong Co., Nonsan, South Korea, and the colony was maintained at an insectary of Seoul National University without exposure to any known insecticide. The larvae were reared on white bean and wheat bran-based artificial diets in an insect breeding dish and breeding box (100 mm diameter × 40 mm height and 200 × 100 × 180 mm cage, Figure 1), and 10% sugar solution was provided for the adults in a 300 × 300 × 300 mm cage, under 25 ± 2°C, 50 ± 5% RH, and a 14:10 h L:D photoperiod. All biological tests were conducted under the same as rearing conditions.

Figure 1. *Spodoptera litura* maintained.

2. Essential oils and standard chemicals

Twenty-eight essential oils tested in this study were purchased from Absolute Aromas (Alton, Hampshire, England), Klimtech (Dimitrovgrad, Bulgaria), Neumond (Raisting, Germany), Plant Therapy (Twin Falls, ID, USA), and Sun Essential Oils (Phoenix, AZ, USA), and information including their scientific names, family names, parts of the plant used, extraction methods and manufacturers are given in Supplementary Information Table S1. Pure standard chemicals of basil oil [4-allylanisole (98%), α-humulene (96%), linalool (97%), methyl undecanoate (99%)] and mandarin oil [limonene (97%), methyl undecanoate (99%), α-pinene (98%), β-pinene (95%), γ-terpinene (95%)] were purchased from Sigma-Aldrich, St. Louis, MO, USA, and *o*-cymene (> 99%) was purchased from Tokyo chemical industry, Tokyo, Japan. Standard chemical of deltamethrin (99.4%) was obtained from LG Chem, Seoul, South Korea, and the solvents and chemicals for buffers were of reagent grades.

3. GC-MS analyses

Major constituents of the oils were analyzed by gas chromatography–mass spectrometry with the an ISQ™ LT gas chromatograph-mass spectrometer (Thermo Scientific, Waltham, MA, USA). A VF-5ms column (60 m × 0.25 mm ID, 0.25 μm thickness) was used operating in electron ionization mode. Helium (99.999%) was used as a carrier gas at a flow rate of 1.0 mL/min, and the injection volume was 1.0 μL. The initial temperature of the oven was set at 50°C for 5 min then increased to 65, 120, 180, 210, and 310°C (each rate of 10, 5, 5, 5, and 20°C/min, respectively) with a total runtime of 100 min. To examine the *in vivo* hemolymph extracts from the third instar larvae of the tobacco cutworm, chemical analyses were conducted using the same system, but a DB-5ms column (60 m × 0.25 mm ID, 0.25 μm thickness) was used instead. The oven was set at 50°C for 2 min, then increased to 310°C with a rate of 10°C/min. Obtained data were identified using an NIST MS Search program (version 2.0) and NIST/EPA/NIH Mass Spectral Libraries.

4. Bioassays

4.1. Contact toxicity of essential oils and their chemical constituents

A topical application method was conducted to evaluate contact toxicity of the oils and the major constituents. Prior to the test, each larva was weighed and the third instar larvae ranging from 9 to 12 mg of body weight were collected for bioassays. A group of ten larvae of *S. litura* was individually treated with 0.5 μL of essential oils or test compounds dissolved in acetone using a syringe attached to a repeating dispenser, then transferred to a 90 mm diameter Petri dish. Negative control received acetone only, and no mortality was observed in the control. The treated larvae were kept in the same condition for the maintenance above, and 0.5 g of an artificial diet was provided. Mortality was recorded at 24 h post-treatment, and the larvae were considered dead if they did not show any movement or response when touched with forceps. Four to nine different doses were used to estimate LD_{50} values, and the test was repeated three times using the larvae from different cohorts.

Figure 2. Diagram of topical application assay

4.2. Interaction of binary mixtures of essential oils

To screen the synergistic effect between basil oil and the remaining oils, a series of mixtures was prepared at 1:1 (w:w) ratio. The mixtures were treated *via* a topical application method to the third instar larvae at 69.3 µg/larva of basil oil (LD_{25}) + 69.3 µg/larva of the other oils. Observed mortality of the mixtures were compared to expected mortality calculated by an equation;

$$E = O_a + O_b(1 - O_a)$$

where E is expected mortality, and O_a and O_b are observed mortality of basil and other oils. The interaction of the mixtures was determined by a chi-square comparison determined by a formula;

$$\chi^2 = \{(O_m - E)^2\}/E$$

where O_m is observed mortality of the binary mixture; χ^2 with d.f = 1 and α = 0.05 is 3.84. A pair with $\chi^2 > 3.84$ was considered to be either synergistic or antagonistic based on the comparison of observed and expected mortality, and $\chi^2 < 3.84$ as additive interaction (Trisyono and Whalon 1999; Pavela 2010).

4.3. Compound elimination assay

To identify the major active constituents in basil and mandarin oils, a compound elimination assay was conducted (Yeom et al. 2012; Kim et al. 2016). Artificial full mixtures of essential oils were prepared by blending the major compounds (> 1.5% in composition) of each oil as following their natural proportions, and a series of incomplete oils was prepared by omitting one compound each from the full mixture. Based on the chemical analyses, four compounds including estragole, linalool, α-humulene, and methyl undecanoate were selected for basil oil, and six compounds including limonene, β-pinene, γ-terpinene, methyl undecanoate, α-pinene, and *o*-cymene were mixed to prepare the full mixture of mandarin oil. The contact toxicity of the full mixtures and the artificial blends against the third instar larvae were tested at the doses of LD_{90} of each oil *via* a topical application method.

4.4. Interaction between the oils and the major active constituents

Based on the χ^2 comparisons in the insecticidal activity of the binary mixtures, the greatest boosting effect of mandarin oil to basil oil was observed, indicating a synergistic interaction, and compound elimination assay identified estragole and linalool as the major active components in basil oil, and limonene for mandarin oil, respectively. To further examine the interaction of the oils and active constituents, the LD_{50} values of individual oils, major active constituents, and their mixtures were determined *via* topical application. The blending ratio (w:w) of basil + mandarin oils, estragole + linalool, estragole + limonene, and estragole + linalool + limonene combinations were 1:1, 7:2, 1:1, and 7:2:7, respectively, as following their proportions in the oils.

Two statistical models were employed to calculate the expected LD_{50} values. As following to the Hewlett and Plackett's calculation (Don-Pedro 1996), the expected LD_{50} was estimated from the equation;

$$E = (a \times LD_{50}A) + (b \times LD_{50}B) + (c \times LD_{50}C) + \cdots (n \times LD_{50}N)$$

where E is expected LD_{50} value of the mixture, and a is the proportion of compound A in the mixture, and $LD_{50}A$ is the LD_{50} value of compound A. Meanwhile, Wadley's model suggested the determination of expected LD_{50} value based on the equation (Gisi et al. 1985);

$$E = \frac{a + b + c + \cdots n}{\frac{a}{LD_{50}A} + \frac{b}{LD_{50}B} + \frac{c}{LD_{50}C} + \cdots + \frac{n}{LD_{50}N}}$$

4.5. *In vivo* recovery of test compounds from hemolymph extract

The internal amount of the test compounds present in the hemolymph of *S. litura* was examined as following a previous study (Tak and Isman 2015) with a slight modification. Based on the LD_{90} value (247.9 μg/larva) of the tertiary mixture of estragole + linalool + limonene (7:2:7), individual and mixtures of estragole (108.5 μg/larva), estragole + linalool (108.5 + 31.0 μg/larva), estragole + limonene (108.5 + 108.5 μg/larva), estragole + linalool + limonene (108.5 + 31.0 + 108.5 μg/larva) were topically applied to twenty third instar larvae of *S. litura*. The treated larvae were retrieved 1h later, and the remaining compounds on the body surface were rinsed off using 10 mL of n-hexane for three times, then the larvae were ground using a tissue homogenizer in 1 mL of n-hexane. Another 1 mL of the solvent was added to rinse the homogenizer, and the supernatant was transferred into a glass vial and sealed, then the hemolymph extract was kept at 4°C for overnight. One mL of clear supernatant was carefully transferred to a clean vial, then analyzed via GC-MS using α-pinene for an internal standard. The test was repeated three times.

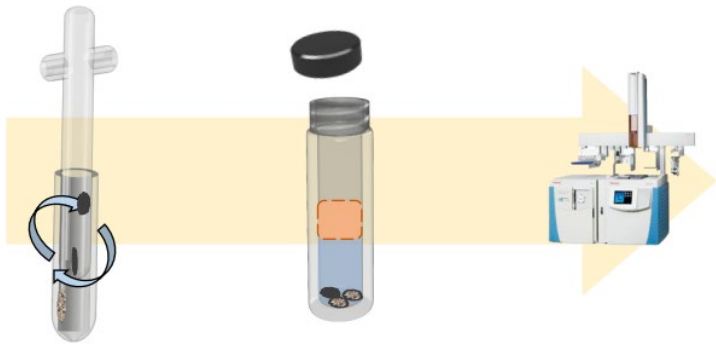

Figure 3. Diagram of *in vivo* recovery test

4.6. Neurophysiological responses of motor neurons

Neurophysiological effects of the oils and the major compounds were examined from central motor neurons of third instar larvae of *S. litura* (Wing et al. 1998). The fifth abdominal ganglion was excised and transferred to 190 μL of a saline bath containing 150 mM of NaCl, 3 mM KCl, 3 mM $CaCl_2$, and 4 mM HEPES, with a pH 6.6 in distilled water (Gammon 1980). An anterior motor nerve trunk was drawn into a recording suction electrode filled with saline (Fig. 3C). The spontaneous electrical signals descending from central interneurons were amplified (Model 3000, A-M Systems, Sequim, WA, USA), filtered (Hum Bug noise eliminator, A-M Systems) and digitized using a LabChart 8 software (Powerlab 4/26, AD Instruments, Dunedin, New Zealand) (Gross and Bloomquist 2018).

The preparation was left for 10 min to attenuate the surgical stress, and the number of neuronal events over a threshold for 3 min before each treatment was counted (Fig. 3D) and normalized as a baseline event counts (BEC). Ten μL of test compounds in DMSO/buffer complex solution was added into the bathing solution to make the final volume of 200 μL (0.1% of final concentration of DMSO), and the event counts during 0–3 min ($EC_{0\text{-}3}$) and 3–6 min post-treatment ($EC_{3\text{-}6}$) were recorded. Altered event counts (AEC) were calculated by an equation (Gaire et al. 2019);

$$AEC_{a-b}\ (\%) = \frac{EC_{a-b}}{BEC} \times 100$$

DMSO (0.1%) was used as a negative control, which exhibited no notable effect on neuronal activity (AEC_{0-3}: 101 ± 6.3 %; AEC_{3-6}: 96.3 ± 4.8 %), and different concentrations of deltamethrin (0.0001 to 10 μg/mL) was used as a positive control (Fig. 3). A new nerve setting was prepared for a single recording and then discarded, and each concentration was repeated for 8 to 12 times.

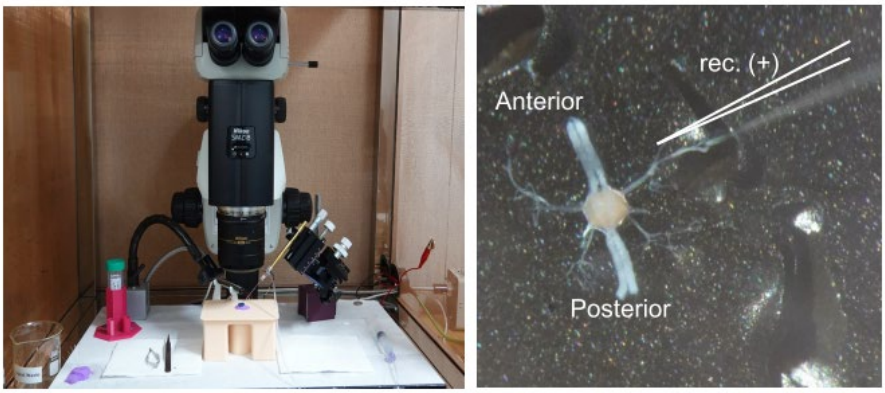

Figure 4. Experimental setup for neurophysiological recordings

4.7. Contact angle measurement

To assess the physicochemical aspect of the binary mixture of basil and mandarin oils, changes in surface tension were examined by measuring contact angles on a beeswax layer which served a surrogate of a cuticular layer of *S. litura* (Tak and Isman 2017). Individual and mixtures of the two oils and the major active compounds dissolved in acetone were prepared at 500 mg/mL, and 3 μL of a droplet was applied on the wax layer using SmartDrop Plus (Femtofab, Sungnam, Gyeonggi, South Korea), then the contact angles were analyzed using a SmartDrop software (version 5.02). Each measurement was repeated ten times.

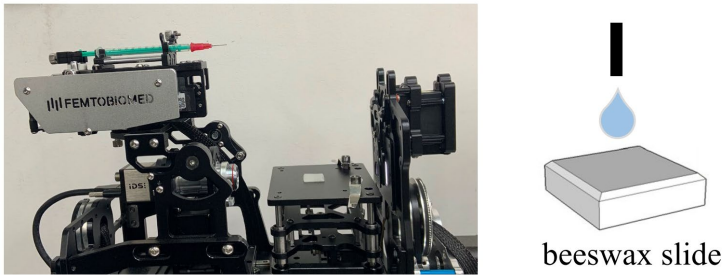

Figure 5. Measuring method of contact angle

4.8. Statistical analysis

Probit analysis was used to estimate the LD_{50} values of individual and mixtures of the oils and their major constituents. Differences in mortality, contact angles, *in vivo* hemolymph extracts, and neural effects were determined by one-way ANOVA with Tukey's HSD test post hoc. Statistical analyses were performed using a SPSS Statistics software (IBM, version 25, Armonk, NY, USA).

Results

1. Contact toxicity of individual oils and the mixtures

The insecticidal activity of 28 essential oils was examined against the third instar larvae of *S.litura* (Table 1). Based on the LD_{50} values, basil oil exhibited the greatest contact toxicity (LD_{50} of 117.4 μg/larva), followed by lemongrass, spearmint, and geranium oils, as ten oils showed < 200 μg/larva of LD_{50} values. On the other hand, cinnamon, fennel, and pine oils failed to exhibit notable activity, showing > 1000 μg/larva of their LD_{50} values.

To examine the interaction between basil oil and the remaining oils, contact toxicity of the mixtures was evaluated at 1:1 (w:w) ratio (Table 2). Among those combinations, mandarin oil showed the greatest increase from the expected mortality when mixed with basil oil ($\chi^2 = 101.5$), followed by *E. globulus*, rosemary, marjoram, cypress, and *E. radiata* oils ($\chi^2 > 70.0$). Eighteen out of 27 combinations were determined as synergistic whereas fennel and pine oils were antagonistic to basil oil.

Table 1. Insecticidal activity of 28 essential oils against the 3rd instar larvae of the tobacco cutworm, *Spodoptera litura*

Essential oil	n[a]	LD_{25} (CL_{95})[b]	LD_{50} (CL_{95})	LD_{90} (CL_{95})	slope ± SE	χ^2
basil	210	91.0 (77.9-100.8)	117.4 (107.0-127.0)	190.3 (170.2-226.9)	6.1 ± 0.9	17.0
lemongrass	180	80.7 (51.8-102.0)	119.2 (92.2-147.1)	249.9 (193.0-422.2)	4.0 ± 0.5	37.8
spearmint	150	102.2 (65.1-121.5)	120.4 (97.0-159.2)	164.6 (134.8-408.1)	9.4 ± 1.6	52.0
geranium	150	79.0 (60.3-95.0)	123.6 (103.9-146.0)	289.9 (230.5-418.5)	3.5 ± 0.5	14.2
peppermint	240	92.8 (74.3-107.0)	130.4 (113.9-151.4)	248.7 (200.8-374.1)	4.6 ± 0.6	41.9
clove bud	240	85.8 (51.4-112.4)	137.6 (102.3-167.4)	337.7 (269.5-500.4)	3.3 ± 0.4	41.2
fennel sweet	270	92.9 (26.5-127.4)	142.5 (89.5-208.1)	321.3 (216.2-2209.2)	3.6 ± 0.5	171.4
patchouli	210	107.3 (68.7-136.9)	168.6 (130.8-203.3)	397.5 (313.7-610.7)	3.4 ± 0.5	32.7
sweet thyme	210	123.3 (103.2-139.4)	173.8 (155.9-192.0)	333.9 (288.0-418.4)	4.5 ± 0.6	12.4
citronella	330	115.7 (67.8-149.7)	183.7 (138.5-217.1)	442.1 (360.1-658.1)	3.4 ± 0.5	60.8
marjoram	150	185.1 (163.2-196.3)	201.2 (186.8-211.0)	236.0 (224.1-260.6)	18.5 ± 3.0	21.6
Eucalyptus radiata	210	184.0 (139.5-205.3)	217.3 (189.4-238.0)	297.8 (265.6-401.6)	9.4 ± 1.3	57.0
Eucalyptus globulus	300	135.2 (70.5-175.2)	219.4 (166.1-263.6)	550.5 (410.5-1181.2)	3.2 ± 0.5	72.6
frankincense	180	166.0 (95.0-209.4)	222.8 (166.2-290.2)	389.5 (296.9-875.0)	5.3 ± 0.7	64.6
lavender (Bulgarian)	180	132.0 (63.3-184.3)	242.7 (170.8-337.6)	772.0 (495.1-2423.9)	2.6 ± 0.4	35.9
rosemary	200	164.2 (133.4-189.1)	243.5 (214.9-273.5)	514.5 (430.8-680.6)	4.0 ± 0.5	21.9
mandarin	270	178.6 (138.3-206.8)	243.8 (211.2-279.2)	440.3 (363.8-640.8)	5.0 ± 0.6	55.1
orange sweet	150	144.4 (12.9-216.1)	247.1 (109.6-366.9)	685.6 (429.3-14971.5)	2.9 ± 0.6	43.1
lavender (French)	300	148.6 (78.2-201.4)	263.3 (191.1-321.1)	781.0 (593.7-1371.6)	2.7 ± 0.4	53.1

lemon	300	177.2 (123.5-210.1)	266.2 (227.6-325.6)	576.6 (426.6-1277.1)	3.8 ± 0.6	63.9
bergamot	210	183.4 (103.1-229.5)	277.2 (217.3-329.8)	607.6 (464.2-1267.7)	3.8 ± 0.6	39.2
cypress	150	229.6 (185.5-259.2)	289.1 (255.4-323.9)	448.1 (386.4-599.9)	6.7 ± 1.0	19.8
clary sage	180	157.6 (112.4-199.2)	307.6 (248.6-383.6)	>1000	2.3 ± 0.3	19.7
ylang ylang	240	253.5 (157.8-313.3)	398.1 (325.2-479.7)	938.2 (695.5-1990.3)	3.4 ± 0.6	37.6
sandal wood	180	420.0 (332.2-489.6)	648.4 (563.2-761.7)	>1000	3.6 ± 0.6	15.4
cinnamon	150	449.5 (n.d.)[c]	>1000	>1000	1.2 ± 0.4	49.5
fennel	120	873.8 (703.7-1233.3)	>1000	>1000	3.9 ± 1.0	6.2
pine	120	928.1 (727.9-1528.0)	>1000	>1000	3.5 ± 0.9	3.3
deltamethrin	210	8.0 (5.8-10.2)	15.0 (12.1-18.1)	49.0 (37.7-72.5)	2.5 ± 0.3	10.4

[a] Number of insect used to determine the LD_{50} values
[b] (μg/insect), CL denotes confidence limit
[c] Not determine

Table 2. Contact toxicity of binary mixtures of basil and the other essential oils against 3rd instar larvae of *S. litura*

Combination[a]		Larval mortality (%)				χ^2	Effect
		Pure compounds		Binary mixtures			
Oil A	Oil B	Observed A	Observed B	Expected	Observed		
basil	bergamot	20.0	1.2	21.0	33.3 ± 8.8	7.3	synergy
basil	cinnamon	20.0	4.9	23.9	20.0 ± 10.0	0.7	additive
basil	citronella	20.0	7.8	26.2	30.0 ± 5.8	0.5	additive
basil	clary sage	20.0	6.6	25.3	16.7 ± 12.0	3.0	additive
basil	clove bud	20.0	16.4	33.1	76.7 ± 6.7	57.2	synergy
basil	cypress	20.0	0.5	20.4	60.0 ± 10.0	76.7	synergy
basil	*E. globulus*	20.0	5.4	24.3	70.0 ± 5.8	85.6	synergy
basil	*E. radiata*	20.0	0.6	20.5	60.0 ± 5.8	76.5	synergy
basil	fennel	20.0	0.2	20.2	10.0 ± 5.8	5.1	antagonistic
basil	fennel sweet	20.0	12.9	30.3	73.3 ± 6.7	61.2	synergy
basil	frankincense	20.0	0.9	20.7	53.3 ± 12.0	51.5	synergy
basil	geranium	20.0	19.2	35.4	50.0 ± 11.5	6.1	synergy
basil	lavender (Bulgarian)	20.0	8.3	26.6	40.0 ± 5.8	6.7	synergy
basil	lavender (French)	20.0	5.8	24.6	26.7 ± 3.3	0.2	additive
basil	lemon	20.0	1.3	21.1	46.7 ± 3.3	31.1	synergy
basil	lemongrass	20.0	17.5	34.0	50.0 ± 5.8	7.6	synergy
basil	mandarin	20.0	1.0	20.8	66.7 ± 3.3	101.5	synergy
basil	marjoram	20.0	0.5	20.4	60.0 ± 5.8	77.1	synergy
basil	orange sweet	20.0	7.5	26.0	30.0 ± 5.8	0.6	additive
basil	patchouli	20.0	9.2	27.4	70.0 ± 10.0	66.4	synergy
basil	peppermint	20.0	10.5	28.4	66.7 ± 3.3	51.5	synergy
basil	pine	20.0	0.2	20.2	6.7 ± 6.7	9.0	antagonistic
basil	rosemary	20.0	1.6	21.3	63.3 ± 3.3	83.0	synergy
basil	sandal wood	20.0	0.5	20.4	40.0 ± 10.0	18.9	synergy
basil	spearmint	20.0	1.2	21.0	50.0 ± 0.0	40.2	synergy
basil	sweet thyme	20.0	3.6	22.9	26.7 ± 3.3	0.6	additive
basil	ylang ylang	20.0	0.8	20.7	13.3 ± 8.8	2.6	additive

[a] Mixtures were applied at LD_{25} of bail oil and the equivalent amount of the other oils (69.3 + 69.3 μg/larva).

2. Chemical analyses of the essential oils

The chemical composition of the oils was analyzed by GC-MS. As shown in Table 3, estragole (70.3%) was the most abundant compound in basil oil, followed by linalool, α-humulene, and methyl undecanoate (19.7%, 1.8%, and 1.7%, respectively). In mandarin oil, limonene (71.9%) was the most abundant, followed by β-pinene, γ-terpinene, methyl undecanoate, α-pinene, and o-cymene (7.0%, 6.2%, 3.4%, 2.7%, and 2.6%, respectively), as 96.6% and 94.9% of the constituents were identified in basil and mandarin oils, respectively. The chemical compositions of the remaining oils are presented in Supplementary Material Tables S2 – S27.

Table 3. Chemical composition of basil and mandarin oils

retention time (min)	constituent	area (%)	
		basil	mandarin
26.12	α-Pinene		2.7
33.71	β-Pinene		7.0
36.33	α-Myrcene		1.1
41.65	o-Cymene		2.6
42.34	(R)-(+)-Limonene		71.9
44.94	γ-Terpinene		6.2
48.34	Linalool	19.7	
54.08	Levomenthol	0.5	
55.83	Estragole	70.3	
59.18	Z-Citral	0.4	
61.29	E-Citral	0.6	
68.66	Methyl undecanoate	1.7	3.4
69.27	trans-Caryophyllene	0.5	
69.61	trans-α-Bergamotene	0.7	
71.50	Germacrene D	0.4	
73.17	α-Humulene	1.8	
	Total identified	96.6	94.9

3. Comparative toxicity of the major constituents of basil and mandarin oils

Four major constituents of basil oil, estragole, linalool, α-humulene, and methyl undecanoate, which comprised > 1.5% in the individual composition in the oil were selected, and their contribution to the overall toxicity was examined *via* a compound elimination assay. The full mixture showed the similar mortality of the original oil (P = 0.764), and the exclusion of α-humulene (FM-α-humulene) and methyl undecanoate (FM-methyl undecanoate) failed to show any statistical difference to basil oil (P = 0.076 and 0.398, respectively). Interestingly, not only estragole but also linalool contributed significantly to the overall contact toxicity of basil oil, as the elimination of each compound showed 10.0 ± 5.8% and 23.3 ± 6.7% of mortality, respectively, showing statistical difference to that of original oil (P = 0.001 and 0.004, respectively). Those two compounds were determined as the major active constituents in basil oil (Fig. 1A).

As for mandarin oil, six compounds including limonene, β-pinene, γ-terpinene, methyl undecanoate, α-pinene, and o-cymene (> 1.5% in composition each) were subjected to the compound elimination assay. The result showed that limonene was solely responsible for the overall toxicity of mandarin oil. When limonene was excluded from the full mixture, no mortality was observed, whereas the other incomplete oils and full mixture did not differ to that of corresponding mandarin oil (P = 0.185, Fig.

1B).

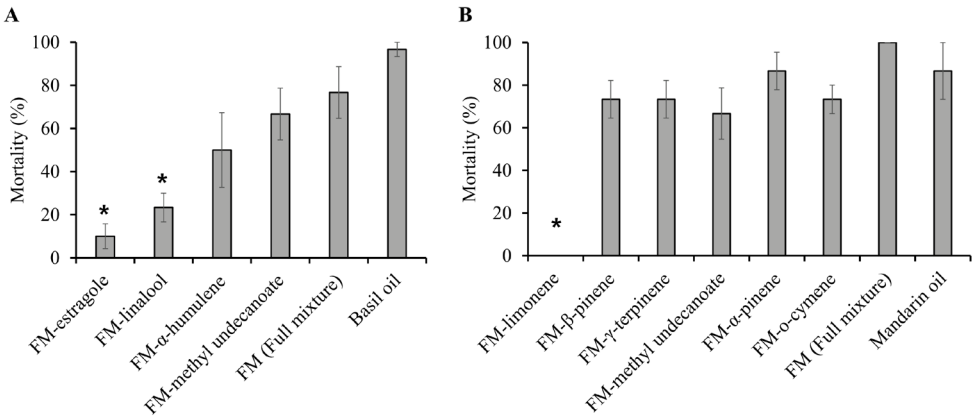

Figure 6. Compound elimination assay of (A) basil oil and (B) mandarin oils *via* topical administration. Asterisks denote significant difference at P = 0.05 in one-way ANOVA followed by Tukey's multiple comparisons test.

4. Interaction between basil and mandarin oils as well as their major active constituents

The LD_{50} values of basil and mandarin oils as well as the major active compounds were determined in topical application (Table 4). In the binary mixture of basil and mandarin oils, the observed LD_{50} value (125.1 μg/larva) was lower than those of the expected LD_{50} values calculated by both statistical models (1.3 and 1.4 of synergy ratio based on Wadley's as well as Hewlett and Plackett's models, respectively), indicating enhanced insecticidal activity of the mixture. As for the major constituents of the oils, although the individual LD_{50} values of estragole and linalool were not comparable to that of basil oil, the binary mixture of the two compound exhibited similar contact toxicity to the oil, suggesting their positive interaction constitutes the insecticidal activity of the oil. As for the mandarin oil, whereas the compound elimination assay indicated that limonene was singly responsible for the insecticidal activity of mandarin oil, it failed to produce the same degree of toxicity to the larvae of the tobacco cutworm, as the LD_{50} values of limonene and mandarin oil were 395.6 and 243.8 μg/larva, respectively, suggesting the remaining compounds in the oil may contribute the full toxicity of the oil.

Interestingly, whereas the binary mixture of estragole and limonene, which were the two of the most abundant compounds in those oils, did not exhibit a prominent positive relationship as the LD_{50} of the mixture was

180.9 μg/larva (R = 1.2 and 1.1, respectively), the tertiary mixture of estragole + linalool + limonene exhibited strong synergistic effect (LD_{50} = 118.1 μg/larva, R = 1.7 and 2.2), and the LD_{50} value was comparable to that of basil + mandarin oils (LD_{50} = 125.1 μg/larva), indicating that the toxicity of the oil mixture was produced by the combination effect of the tertiary mixture, as they interact to each other synergistically.

Table 4. LD_{50} and LD_{90} values of basil and mandarin oils as well as their major constituents against 3rd instar larvae of the tobacco cutworm

test oil/compound (w:w)	LD_{50} (95% CL)	LD_{90} (95% CL)	slope ± SE	expected LD_{50} (μg/larva)			
				Wadley	R[a]	H&P[b]	R
basil oil	117.4 (107.0-127.0)	190.3 (170.2-226.9)	6.1 ± 0.9				
mandarin oil	243.8 (211.2-279.2)	440.3 (363.8-640.8)	5.0 ± 0.6				
basil + mandarin oils (1:1)	125.1 (110.9-143.0)	324.2 (256.7-467.7)	3.1 ± 0.3	158.5	1.3	180.6	1.4
estragole	142.4 (128.3-155.6)	256.0 (227.0-305.5)	5.0 ± 0.6				
linalool	178.6 (161.7-197.9)	314.5 (272.2-389.7)	5.2 ± 0.6				
estragole + linalool (7:2)	128.8 (115.8-141.0)	230.0 (202.2-281.0)	5.1 ± 0.7	149.0	1.2	150.4	1.2
limonene	395.6 (312.0-501.2)	752.3 (562.4-1517.9)	4.7 ± 0.6				
estragole + limonene (1:1)	180.9 (167.1-194.9)	314.4 (283.4-360.8)	5.3 ± 0.5	209.4	1.2	198.1	1.1
estragole + linalool + limonene (7:2:7)	118.1 (84.7-150.0)	247.9 (185.4-538.9)	4.0 ± 0.5	202.8	1.7	259.9	2.2

[a] Synergy ratio was determined by (expected LD_{50}) ÷ (observed LD_{50}).
[b] Hewlett and Plackett's calculation of expected LD_{50} value.

5. Recovery of test compounds from hemolymph extract

To elucidate the synergistic mechanism of the tertiary mixture of estragole, linalool, and limonene, the internal concentration of the compounds in the hemolymph extracts was examined. As shown in Fig. 2, linalool significantly enhanced estragole content in the hemolymph of *S. litura* when they were applied as a binary mixture ($P < 0.001$), that the estragole concentration in the estragole + linalool mixture increased 65.9% compared to the individual application of estragole (peak area ratio of 12.18 ± 1.49 to 20.20 ± 1.01). On the other hand, the addition of limonene to estragole did not change the estragole concentration in the hemolymph, rather a slight decrease in the peak area ratio was found (12.18 ± 1.49 to 9.30 ± 0.66), although it was not statistically different ($P = 0.235$). In the tertiary mixture of estragole, linalool, and limonene, whereas the internal concentration of estragole did not show any difference to the individual application of the compound ($P = 1.000$), the limonene content from the hemolymph extracts substantially increased in the tertiary mixture ($P < 0.001$), as the interaction between linalool and limonene might be much stronger than the interaction between linalool and estragole in basil oil.

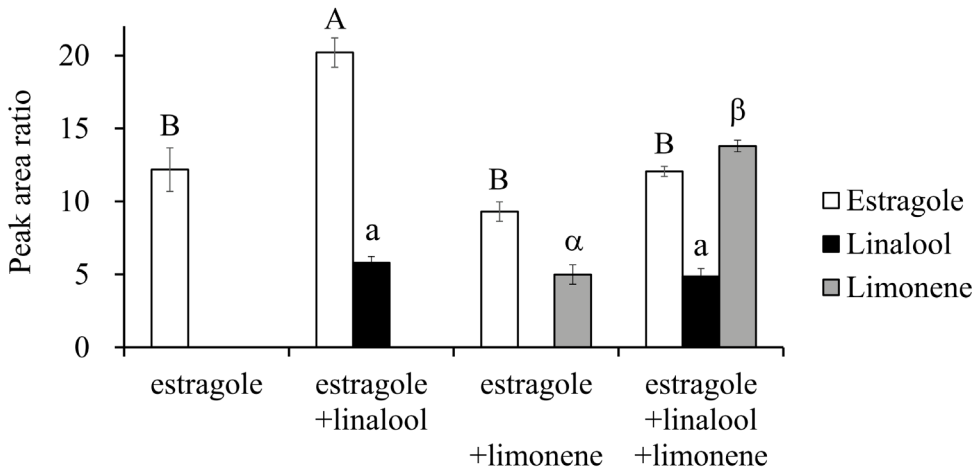

Figure 7. Comparison of peak area ratios of the compound in the mixtures from the hemolymph extracts in the 3rd instar larvae of the tobacco cutworm. Different letters indicate significant difference ($P < 0.05$).

6. Neurophysiological responses of motor neurons to the mixture

In another approach to understand the synergy mechanism between basil and mandarin oils, neurophysiological effect of the oils and their major active constituents were examined using the central motor neurons of *S. litura*. The signal exhibited a biphasic response to individual basil oil depending on the concentrations. Inhibitory activity in the number of firing was observed at the highest concentration of 100 μg/mL ($P < 0.001$ and $P = 0.011$ for $AEC_{0\text{-}3}$ and $AEC_{3\text{-}6}$, respectively, compared to control AEC, Fig. 3A and 3B), while stimulating effect was occurred at the lower concentrations at 0.03, 1, 3, and 10 μg/mL ($df = 14$, $P = 0.046$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) in $AEC_{0\text{-}3}$ and 10 μg/mL ($df = 14$, $P = 0.021$) in $AEC_{3\text{-}6}$. Meanwhile, mandarin oil failed to exhibit any excitation or inhibitory activity in all concentration tested except the highest concentration, 100 μg/mL, which displayed significant inhibitory effect in $AEC_{3\text{-}6}$ ($P < 0.001$). In the binary mixture of basil and mandarin oils, it seemed that the biphasic response of basil oil was substantially amplified by the addition of mandarin oil in AEC, that 297.7 ± 19.1% and 320.0 ± 38.0% of increase in average event counts were recorded at 0–3 and 3–6 min of observation, respectively, at 1.0 μg/mL of application ($P = 0.001$ and $P < 0.001$, respectively). The excitatory effect reached its peak at 1 μg/mL in $AEC_{0\text{-}3}$ and decreased dose dependently as similarly to basil oil did, but the

decrease started in lower doses, and the same pattern was also observable in AEC_{3-6}.

When 1 ng/mL of basil oil and the corresponding concentrations of estragole, linalool, and the binary mixture of the two compounds were applied, no excitation effect was observed (Fig. 4). However, the addition of mandarin oil and limonene caused significant neuroexcitation, as showing 154.1 ± 5.6 % (P = 0.019) and 150.4 ± 16.9% (P = 0.004) increase in AEC, respectively, indicating the increased neuroexcitation as the synergistic mechanism in the mixture of basil and mandarin oils.

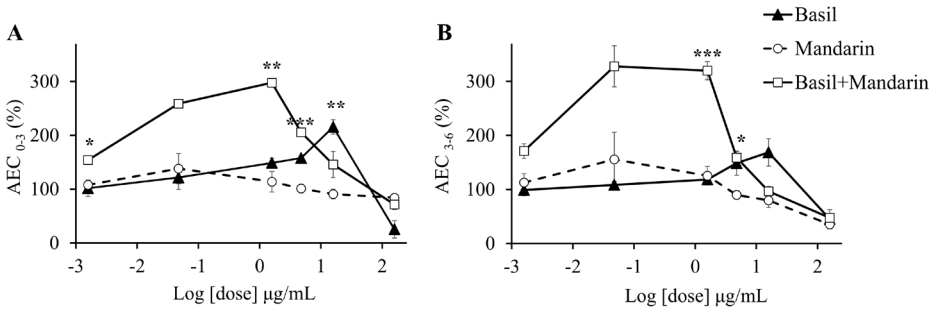

Figure 8. Neurophysiological effects of essential oils and their binary mixture on *S. liutra* larvae central neurons in (A) 0–3 min and (B) 3–6 min post-treatment. Asterisks denote statistical difference at P = 0.05 (*), 0.01 (**), and 0.001 (***) in one-way ANOVA test. Positive control (deltamethrin) was excluded in statistical

analysis due to the different concentrations tested.

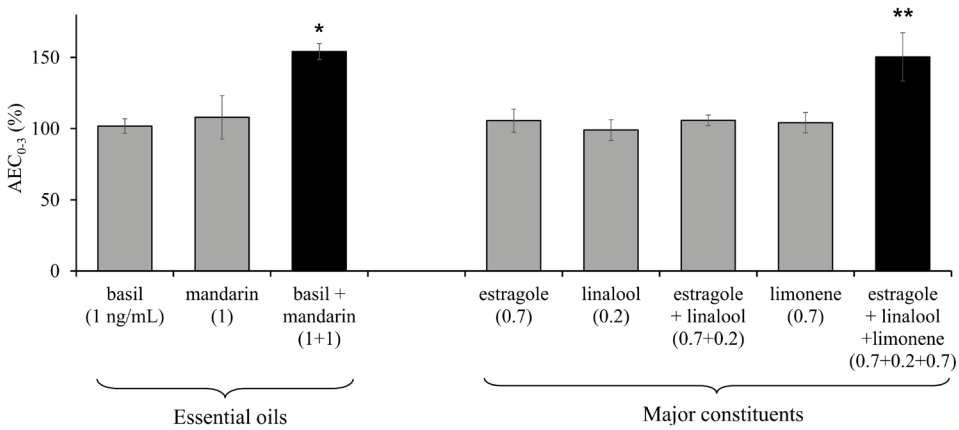

Figure 9. Comparison of altered event counts of essential oils and their major active constituents during 0–3 min. Asterisks indicate statistical differences at P = 0.05 (*) and 0.01 (**).

7. Contact angles of test oils and compounds

Contact angle of basil oil (34.9 ± 0.8°) was statistically different to those of the major active compounds of the oil, estragole and linalool (37.9 ± 0.8° and 28.5 ± 0.3°, respectively, $P < 0.05$), but the mixture of the two compounds showed similar angle to the original oil (32.9 ± 0.7°, $P = 0.332$), indicating that the surface tension of estragole is decreased by linalool to achieve that of basil oil. On the other hand, the major active compound of mandarin oil, limonene, showed significantly lower contact angle (14.5 ± 0.8°) than that of the oil (19.0 ± 0.3°, $P < 0.01$).

Between the two oils, the surface tension of mandarin oil was significantly lower than that of basil oil. A similar pattern was observed among the major active constituents, that the contact angle of the major active compounds of basil oil was decreased by the addition of limonene, resulting in the final contact angle of 24.5 ± 0.7° in the tertiary mixture of estragole, linalool, and limonene, indicating that mandarin oil and its major active compound, limonene, can lower the surface tension of basil oil and the major active constituents of the oil when they are blended.

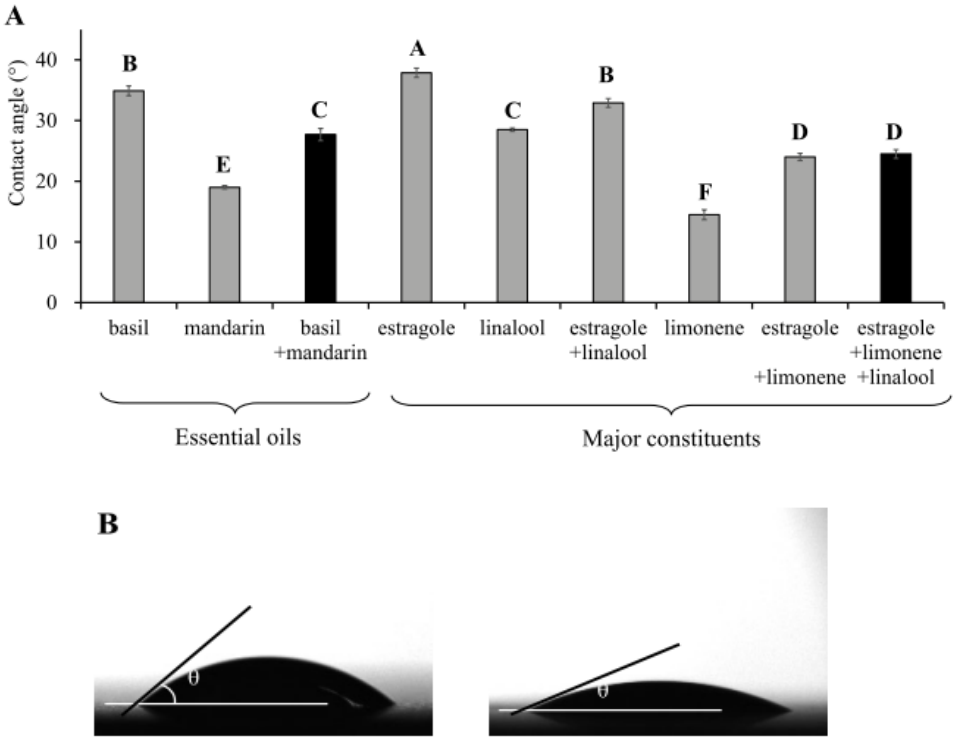

Figure 10. Comparison of surface tension on a beeswax layer. (A) Contact angles of basil, mandarin oils, and the active constituents, as well as their mixtures. Different letters indicate statistical difference at $P = 0.05$. (B) Shapes of droplets on a beeswax layer of estragole (left) and limonene (right).

Discussion

Plant essential oil-based insecticides keep getting more attention for gardens and human dwellings thanks to their nature-origin and low mammalian toxicity, which merits to be relatively easily accepted by the consumers (Isman 2015; Pavela 2016). Most of the 'usual suspects' of botanicals frequently explored for their insecticidal activity and further successfully commercialized tend to display low or no mammalian toxicity, reduced effects on non-target organisms, and minimal presence on environment (Isman 2006). Nonetheless, their efficacy is limited compared to the synthetic insecticides, and particularly, *S. litura* seems to be more tolerant than the other 'typical' insect species in which requires higher doses to achieve acute toxic effect, similarly to other tenacious insect pests such as house flies, German cockroaches, and diamondback moths (Hummelbrunner and Isman 2001). This seemingly inadequate efficacy compared to conventional insecticides can be replenished by sublethal effect of plant essential oils, many previous studies indicated their sublethal activities including feeding deterrence, repellency, and reduced fecundity/fertility of insect pests (Pavela 2015b; Liu et al. 2018). Recently, our laboratory has initiated a research on feeding deterrent activity of the essential oils on the sublethal levels as well.

On the other hand, rapid development of resistance to the conventional insecticides are becoming an alarming problem in controlling this important pest of crop and vegetable plants worldwide (Gandhi et al. 2016; Wang et al. 2018; Shi et al. 2019). Efforts in developing new types of insecticides with novel modes-of-action and biorational approach have been made using different classes to organophosphates, insect growth regulators, and modifications of plant-derived compounds (Ahmad and Gull 2017; Liu et al. 2018; Tharamak et al. 2020). Due to the chemical complexity in the composition of plant essential oils, their several modes-of-action were proposed, which are substantially different to those of conventional insecticides. For example, plant essential oils seem to exhibit their acute toxicity as interacting with acetylcholinesterase, *nicotinic acetylcholine* receptor, octopamine receptor or $GABA_A$ receptor ion channel (Enan 2005; Tong and Coats 2010; Gross et al. 2013). Not only the novel modes-of-action of plant sources but also the chemical complexity itself can be highly beneficial in dealing with the resistance problem. When two lines of the green peach aphid, *Myzus persicae*, were exposed to neem seed extract or pure azadirachtin with the same content of the active compound, whereas azadirachtin-selected population developed the resistance in 9-fold, the extract-treated line showed no resistance compared to the control group, indicating the remaining inactive constituents can mitigate the resistance development significantly compared to an exposure of single active

ingredient alone (Feng and Isman 1995).

In the present study, contact toxicity of individual 28 essential oils as well as their mixtures was examined, and mandarin oil showed the strongest boosting effect to the contact toxicity of basil oil (Table 2). The compound elimination assay designated estragole and linalool as the major active constituents of basil oil and limonene for mandarin oil, and the successive investigations showed the same degree of LD_{50} values (Table 4) and contact angles (Fig. 5) between basil oil and estragole + linalool mixture, confirming the positive interaction between the two compounds produces the contact toxicity and physicochemical property (i.e., surface tension) of basil oil. However, the LD_{50} values and contact angles of mandarin oil and limonene were significantly different, suggesting the remaining constituents (28.1% in composition) may contribute to the overall attribute of the original oil. A similar result in rosemary (*Rosmarinus officinalis*) essential oil was found that to equal the toxicity of the original oil, all constituents including the inactive ones were required, as when only the active constituents were selectively mixed, it failed to produce the same mortality that rosemary oil did against the two spotted spider mites, *Tetranychus urticae*, whereas the full mixture including all inactive compounds showed significantly enhanced toxicity (Miresmailli et al. 2006).

Among the major active constituents, linalool exhibited notable

contribution to the overall activity. Although its composition was lower than those of the other constituents in both mixtures of estragole + linalool (7:2) and estragole + linalool + limonene (7:2:7), the addition of linalool turned out to be crucial to reconstruct the original contact toxicity of basil oil as well as the binary mixture of basil and mandarin oils. From the *in vivo* analyses of the hemolymph extracts and contact angle measurements, linalool seemed to assist the cuticular penetration of estragole by lowering the surface tension of estragole, resulting in increased toxicity of the binary mixture of estragole + linalool. In a previous study on the cabbage looper, *Trichoplusia ni*, the lowered contact angles in the mixtures of monoterpene compounds were directly correlated to the enhanced toxicity and increased penetration through cuticular layer (Tak and Isman 2017).

On the other hand, in the tertiary mixture, linalool displayed notably different interaction to estragole. Whereas the internal concentration of estragole in the hemolymph did not show any difference in the tertiary mixture, the limonene content was significantly increased instead, suggesting much stronger interaction of linalool to limonene than to estragole in the mixture. Nonetheless, although it was not statistically different, a slight decrease in estragole concentration in—estragole + limonene mixture was observed, and linalool seemed to counteract the weak adverse effect and restore the estragole content in the tertiary mixture. Further study is necessary to confirm this shifting behavior of linalool in the

tertiary mixture focusing on chemical bonding or affinity to a lipophilic surfaces.

Neurophysiological study on central motor neurons revealed an interesting result, that the addition of basil and mandarin oils exhibited significantly enhanced neuroexcitation. Since mandarin oil did not show any excitation effect even at a higher concentration, it can be assumed that mandarin oil and its major active compound, limonene, act as boosting agents of the neuroexcitation effect of basil oil. Previous studies showed that limonene can affect the cell membrane integrity and permeability in insect and bacteria (Tak et al. 2017; Han et al. 2019), and the direct cell membrane disruption and/or modulation of receptors of the major constituents of basil oil by limonene would result in the amplified neuroexcitation.

As for the mechanism of synergy effect, several hypotheses including multi-target effect, pharmacokinetic effect (i.e., increased permeability), inhibition of metabolism, and the removal of adverse effects have been proposed (Wagner and Ulrich-Merzenich 2009; Langeveld et al. 2014). Although other possibilities have not been fully explored yet, based on the results in the present study, it could be concluded that the complex and combined effects of increased cuticular penetration as well as enhanced neuroexcitation effect can be considered as potential synergy mechanisms between basil and mandarin oils against the larvae of *S. litura*. To the best of

our knowledge, this is the first neurophysiological approach to understand the synergy mechanism of plant essential oils against *S. litura*, and further studies should include the physiological target sites of amplified excitation, metabolism of the major active constituents, and route and/or mechanism of penetration in the cuticular layer.

Conclusion

Several synergistic insecticidal interactions among the mixtures of plant essential oils were identified against the third instar larvae of the tobacco cutworm in the present study, and the different mechanisms of synergy might be responsible for these effects. Presumably due to the complex nature of the major constituents of plant essential oils, different modes-of-action may enhance the toxicity of the mixture, along with increased cuticular penetration affected by the change of surface tension. Although not examined in this study, inhibition of detoxifying enzyme could be another mechanism of synergy as well (Norris et al. 2018). As shown in the present study, it could be challenging to exactly identify the cause of synergy when more than two candidates of mechanism are present and interact simultaneously, and further study should focus on identifying and separating their individual contribution to the overall effect.

Plant essential oils are complex mixtures of constituents, which show wide variation in chemical composition even within the same species based on geographical distribution, environmental conditions, chemotypes as well as the extraction methods. This can result in the different insecticidal efficacy largely affected by different toxicity of each compound, complex interactions among the constituents, and treatment conditions. A previous report showed notable positive and/or negative correlations in the LD_{50}

values of the same compounds based on the post-application temperature for the bioassay (Pavela and Sedlák 2018). As mentioned above, essential oil and plant extract-based insecticides tend to display lesser efficacy than the conventional pesticides, and understanding and utilizing the knowledge on synergistic interaction may contribute to the development of better control strategy using natural products.

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Supplementary Materials

Table S1. Information of essential oils tested

essential oil	scientific name	family name	plant part	extraction method[a]	manufacturer
basil	*Ocimum basilicum*	Lamiaceae	flower, leaf	steam dist.	Sun Essential Oils
bergamot	*Citrus bigaradia*	Rutaceae	peel	cold pres.	Klimtech
cinnamon	*Cinnamomum cassia*	Lauraceae	bark	steam dist.	Plant Therapy
citronella	*Cymbopogon nardus*	Poaceae	flower, leaf	steam dist.	Absolute Aromas
clary sage	*Salvia sclarea*	Lamiaceae	flower	steam dist.	Klimtech
clove bud	*Eugenia caryophyllata*	Myrtaceae	flower bud	steam dist.	Absolute Aromas
cypress	*Cupressus sempervirens*	Cupressaceae	leaf	steam dist.	Klimtech
Eucalyptus globulus	*Eucalyptus globulus*	Myrtaceae	leaf	steam dist.	Klimtech
Eucalyptus radiata	*Eucalyptus radiata*	Myrtaceae	leaf	steam dist.	Klimtech
fennel	*Foeniculum vulgare*	Apiaceae	seed	steam dist.	Sun Essential Oils
fennel sweet	*Foeniculum vulgare*	Apiaceae	seed	steam dist.	Klimtech

frankincense	*Boswellia carterii*	Burseraceae	resin	steam dist.	Klimtech
geranium	*Pelargonium graveolens*	Geraniaceae	flower	steam dist.	Klimtech
lavender (Bulgarian)	*Lavandula angustifolia*	Lamiaceae	flower	steam dist.	Klimtech
lavender (French)	*Lavandula angustifolia*	Lamiaceae	flower bud	steam dist.	Absolute Aromas
lemon	*Citrus limonum*	Rutaceae	peel	steam dist.	Klimtech
lemongrass	*Cymbopogon citratus*	Poaceae	leaf	steam dist.	Klimtech
mandarin	*Citrus reticulata*	Rutaceae	peel	cold pres.	Klimtech
marjoram	*Origanum majorana*	Lamiaceae	leaf	steam dist.	Klimtech
orange sweet	*Citrus aurantium*	Rutaceae	peel	cold pres.	Klimtech
patchouli	*Pogostemon cablin*	Lamiaceae	leaf	steam dist.	Klimtech
peppermint	*Mentha piperita*	Lamiaceae	leaf	steam dist.	Klimtech
pine	*Pinus* spp.	Pinaceae	needle	steam dist.	Sun Essential Oils
rosemary	*Rosmarinus officinalis*	Lamiaceae	leaf	steam dist.	Klimtech
sandal wood	*Santalum album*	Santalaceae	wood	steam dist.	Klimtech
spearmint	*Mentha spicata*	Lamiaceae	flower, leaf	steam dist.	Absolute Aromas
sweet thyme	*Thymus zygis*	Lamiaceae	flower, leaf	steam dist.	Neumond
ylang ylang	*Cananga odorata*	Annonaceae	flower	steam dist.	Klimtech

[a]All oils tested were prepared *via* steam distillation and cold pressing methods.

Table S2. Chemical constituents of bergamot essential oil

RT (min)	constituent	area %
25.83	α-Thujene	0.5
27.00	α-Pinene	24.5
29.64	Camphene	0.7
34.88	β-Pinene	0.8
37.50	α-Myrcene	0.5
40.42	3-Carene	5.0
41.19	Isocineole	2.0
42.27	o-Cymene	0.4
42.80	D-Limonene	46.0
43.05	Eucalyptol	1.5
45.41	γ-Terpinene	0.4
47.43	α-Terpinolene	3.7
48.34	Linalool	3.3
55.54	α-Terpineol	0.7
60.04	Linalyl acetate	3.2
65.26	Triacetin	0.7
65.83	alpha-Terpinyl acetate	0.7
68.98	Methyl undecanoate	2.1
total		96.6

Table S3. Chemical constituents of cinnamon essential oil

RT (min)	constituent	area %
49.53	Phenylethyl Alcohol	0.3
59.80	2,3-Dihydro-benzofuran-3-ol	0.4
62.51	*trans*-Cinnamaldehyde	74.6
68.95	Methyl undecanoate	1.8
70.06	*trans*-Cinnamyl acetate	2.8
70.17	Coumarin	1.9
73.06	3-Methoxycinnamaldehyde	9.5
76.69	1,5-Dihydroxy-1,2,3,4-tetrahydronaphthalene	0.3
total		91.7

Table S4. Chemical constituents of citronella essential oil

RT (min)	constituent	area %
42.55	Limonene	3.8
48.20	Linalool	0.8
51.67	Isopulegol	1.2
51.98	Citronellal	35.7
52.47	Isopulegol	0.6
58.03	α-Citronellol	13.4
59.99	Geraniol	19.6
61.14	E-Citral	0.5
65.68	Citronellyl acetate	4.2
65.94	Eugenol	0.6
67.00	Geranyl acetate	2.6
67.78	α-elemene	1.7
68.87	Methyl undecanoate	2.9
71.40	Germacrene D	1.2
71.89	α-Muurolene	0.7
72.42	γ-Muurolene	0.6
72.53	ë-Cadinene	2.2
73.45	Elemol	2.2
74.40	Cubenol	0.5
76.24	.tau.-Muurolol	0.4
76.57	α-Cadinol	0.5
76.67	α-Eudesmol	0.6
total		96.5

Table S5. Chemical constituents of clary sage essential oil

RT (min)	constituent	area %
48.37	Linalool	32.4
55.54	α-Terpineol	3.9
55.97	2-Carene	0.4
58.06	Geraniol	1.4
60.07	Linalyl acetate	45.2
61.33	dihydro linalool	0.5
65.83	α-Terpinyl acetate	3.5
65.93	Ocimenyl acetate	0.3
66.24	Neryl acetate	3.1
67.12	Geranyl acetate	4.4
68.98	Methyl undecanoate	2.0
total		97.2

Table S6. Chemical constituents of clove bud essential oil

RT (min)	constituent	area %
65.91	Eugenol	94.4
68.69	Methyl undecanoate	1.3
68.96	β-Caryophyllene	1.5
72.04	Phenol, 2-methoxy-4-(2-propenyl)-,acetate	2.1
total		99.3

Table S7. Chemical constituents of cypress essential oil

RT (min)	constituent	area %
26.96	α-Pinene	1.7
29.62	Camphene	0.5
33.90	Sabinene	0.6
34.91	β-Pinene	48.8
37.47	α-Myrcene	2.7
40.41	3-carene	20.9
42.26	o-Cymene	1.0
42.76	Limonene	3.9
42.88	α-Phellandrene	0.4
47.42	α-Terpinolene	3.5
47.69	o-Isopropenyltoluene	0.4
48.33	Linalool	0.5
51.32	Isopinocarveol	0.4
54.37	Terpinen-4-ol	3.7
54.80	p-Cymen-8-ol	0.4
55.70	Myrtenol	0.4
65.83	α-Terpinyl acetate	3.9
65.94	Bicyclo[3.1.0]hexene, 6-isopropylo	0.4
68.98	Methyl undecanoate	2.0
total		95.8

Table S8. Chemical constituents of *Eucalyptus globulus* essential oil

RT (min)	constituent	area %
26.95	α-Pinene	2.3
40.39	3-Carene	1.9
42.25	o-Cymene	2.4
42.76	D-Limonene	5.7
43.06	Eucalyptol	75.6
45.39	γ-Terpinene	2.8
47.41	α-Terpinene	1.3
54.37	Terpinen-4-ol	0.3
67.36	α-Copaene	0.4
68.97	Methyl undecanoate	1.8
69.27	β-Caryophyllene	0.8
total		95.3

Table S9. Chemical constituents of *Eucalyptus radiata* essential oil

RT (min)	constituent	area %
26.97	α-Pinene	2.4
34.88	β-Pinene	0.5
37.49	α-Myrcene	0.3
40.41	3-carene	1.9
42.26	o-Cymene	2.8
42.77	D-Limonene	6.2
42.90	α-Phellandrene	0.4
43.07	Eucalyptol	65.1
45.40	γ-Terpinene	2.7
47.42	α-Terpinolene	1.3
54.37	Terpinen-4-ol	0.4
55.53	α-Terpineol	7.3
55.97	γ-Terpineol	1.1
67.36	α-Copaene	0.4
68.98	Methyl undecanoate	1.8
69.27	β-Caryophyllene	0.9
total		95.5

Table S10. Chemical constituents of fennel essential oil

RT (min)	constituent	area %
26.09	α-Pinene	3.2
36.32	α-Myrcene	0.7
39.22	l-Phellandrene	1.9
42.14	D-Limonene	2.6
47.17	Fenchone	1.2
62.12	Anethole	44.6
68.66	Methyl undecanoate	21.2
69.09	Undecanoic acid, 2-methyl-	1.3
total		76.7

Table S11. Chemical constituents of fennel sweet essential oil

RT (min)	constituent	area %
26.08	α-Pinene	0.8
41.60	o-Cymene	1.0
42.14	D-Limonene	4.0
47.15	L-Fenchone	1.5
55.19	Estragole	3.6
59.95	Anisaldehyde	2.1
62.28	Anethole	79.6
68.65	Methyl undecanoate	3.2
total		95.8

Table S12. Chemical constituents of frankincense essential oil

RT (min)	constituent	area %
25.08	2-Thujene	15.3
26.27	α-Pinene	44.4
32.82	Sabinene	7.5
33.71	β-Pinene	2.7
36.33	α-Myrcene	1.4
39.64	3-Carene	1.0
41.66	o-Cymene	6.1
42.22	Limonene	14.9
68.67	Methyl undecanoate	2.8
total		96.3

Table S13. Chemical constituents of geranium essential oil

RT (min)	constituent	area %
24.73	Hexylene glycol	0.4
48.36	Linalool	11.3
52.44	l-Menthone	0.9
53.16	p-Menthone	5.2
58.22	α-Citronellol	39.6
60.09	Geraniol	15.3
61.65	Citronellyl formate	11.0
63.19	Geraniol formate	4.7
65.82	Citronellyl acetate	0.8
67.13	Geranyl acetate	2.2
68.39	Diphenyl ether	0.6
68.78	α-Gurjunene	0.5
69.00	Methyl undecanoate	2.2
69.59	Diphenylmethane	0.3
total		95.2

Table S14. Chemical constituents of lavender (Bulgarian) essential oil

RT (min)	constituent	area %
27.03	α-Pinene	0.3
42.31	o-Cymene	3.2
42.80	Limonene	2.2
43.07	Eucalyptol	3.5
48.39	Linalool	33.3
53.52	3,5,5-Trimethylhexyl acetate	0.4
54.41	Terpinen-4-ol	3.7
55.58	α-Terpineol	0.7
60.09	Linalyl acetate	35.5
61.36	Dihydro linalool	0.4
65.85	α-Terpinyl acetate	0.6
66.26	Neryl acetate	1.1
67.14	Geranyl acetate	3.4
69.00	Methyl undecanoate	1.9
69.30	β-Caryophyllene	4.7
74.78	Caryophyllene oxide	1.2
total		96.1

Table S15. Chemical constituents of lavender (French) essential oil

RT (min)	constituent	area %
35.23	3-Octanone	0.8
42.94	*trans*-α-Ocimene	0.9
43.93	β-Ocimene	0.6
48.05	Linalool	33.6
48.48	1-Octen-3-yl-acetate	0.8
51.27	(-)-Camphor	0.5
59.64	Linalyl acetate	47.5
68.67	Methyl undecanoate	3.7
68.77	α-Santalene	0.7
68.94	β-Caryophyllene	3.3
69.86	α-Farnesene	1.7
74.48	Caryophyllene oxide	0.6
total		94.7

Table S16. Chemical constituents of lemon essential oil

RT (min)	constituent	area %
26.32	α-Pinene	3.1
34.01	β-Pinene	9.2
36.60	α-Myrcene	0.8
39.82	3-Carene	2.1
41.81	o-Cymene	1.2
42.53	D-Limonene	75.7
45.06	γ-Terpinene	4.5
47.11	α-Terpinolene	0.4
68.75	Methyl undecanoate	0.8
total		97.7

Table S17. Chemical constituents of lemongrass essential oil

RT (min)	constituent	area %
27.02	α-Pinene	1.1
42.80	Limonene	6.6
43.07	Eucalyptol	0.9
48.36	Linalool	3.5
54.23	Verbenol	0.4
58.20	α-Citronellol	12.5
59.24	Z-Citral	23.0
60.09	Geraniol	9.4
61.34	E-Citral	28.6
66.26	Neryl acetate	1.1
67.14	Geranyl acetate	3.7
69.00	Methyl undecanoate	2.1
69.30	β-Caryophyllene	2.8
total		95.5

Table S18. Chemical constituents of marjoram essential oil

RT (min)	constituent	area %
27.22	α-Pinene	1.4
34.38	Sabinene	2.6
35.48	β-Pinene	1.0
40.73	3-Carene	2.7
41.71	α-Terpinene	1.8
42.62	o-Cymene	9.0
43.03	Limonene	1.0
43.20	α-Phellandrene	0.5
45.61	γ-Terpinene	6.1
47.57	Terpinolene	2.2
48.52	Linalool	6.9
54.84	Terpinen-4-ol	30.4
54.99	α-Thujone	0.7
55.96	α-Terpineol	4.9
56.34	γ-Terpineol	0.5
60.09	Linalyl acetate	11.2
60.88	Piperitone	1.3
68.96	Methyl undecanoate	1.9
69.36	β-Caryophyllene	8.5
total		94.6

Table S19. Chemical constituents of orange sweet essential oil

RT (min)	constituent	area %
26.99	*α*-Pinene	0.4
37.52	*α*-Myrcene	0.4
42.83	Limonene	83.8
48.35	Linalool	0.3
49.89	*trans*-*p*-Mentha-2,8-dienol	0.6
50.71	Limonene oxide	0.4
50.94	*cis*-*p*-Mentha-2,8-dien-1-ol	0.6
51.02	*trans*-Limonene oxide	0.7
56.02	Perilla alcohol	0.5
57.65	*trans*-Carveol	1.1
58.78	*cis*-Carveol	0.5
59.20	Z-Citral	0.6
59.74	Carvone	1.3
61.31	E-Citral	0.8
65.69	7-Oxabicyclo[4.1.0]heptane	1.8
68.42	*cis*-Limonene oxide	0.5
68.99	Methyl undecanoate	1.9
80.16	Cedrene	0.4
87.15	Phenylethyl salicylate	0.4
total		96.7

Table S20. Chemical constituents of patchouli essential oil

RT (min)	constituent	area %
42.29	o-Cymene	11.1
43.04	Benzyl alcohol	2.8
48.35	Linalool	9.2
67.87	α-Patchoulene	0.9
68.66	α-Gurjunene	0.4
68.77	Isoledene	7.1
68.99	Methyl undecanoate	2.0
69.18	Di-epi-α-cedrene	2.8
69.29	β-Caryophyllene	24.6
69.52	Cedrene	0.7
69.78	α-Guaiene	3.3
69.90	Thujopsene	3.7
70.50	Seychellene	1.9
70.81	Aromadendrene	3.2
70.93	α-Patchoulene	1.5
71.04	γ-Gurjunene	0.4
71.20	Azulene	1.3
71.80	Ledene	0.3
71.96	Azulene	0.7
72.17	α-Bulnesene	4.0
72.42	Benzene, 1-methyl-4-(1,2,2-trimethylcyclopentyl)-	1.3
75.47	Widdrol	0.5
75.58	Cedrol	3.2
75.89	Isoaromadendrene epoxide	0.3
76.79	Veridiflorol	0.5
77.46	Patchouli alcohol	8.0
total		95.7

Table S21. Chemical constituents of peppermint essential oil

RT (min)	constituent	area %
27.04	α-Pinene	0.4
34.97	β-Pinene	0.7
42.31	o-Cymene	6.0
42.81	D-Limonene	6.8
48.37	Linalool	11.0
51.88	Isopulegol	0.8
52.47	Isomenthone	28.1
53.17	p-Menthone	11.2
53.52	Menthol	2.2
54.14	Levomenthol	18.4
54.32	Isopulegol	0.5
60.53	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	0.4
62.81	Menthyl acetate	1.6
69.00	Methyl undecanoate	1.9
69.30	β-Caryophyllene	5.2
total		95.1

Table S22. Chemical constituents of pine essential oil

RT (min)	constituent	area %
25.90	α-Pinene	43.9
28.40	Camphene	1.0
33.36	β-Pinene	7.6
36.00	α-Myrcene	2.2
39.40	3-Carene	8.0
41.45	o-Cymene	1.0
41.98	D-Limonene	5.9
42.10	α-Phellandrene	1.4
61.94	(-)-Bornyl acetate	0.6
68.48	Longifolene	0.4
68.60	Methyl undecanoate	16.2
68.83	β-Caryophyllene	2.5
69.05	Undecanoic acid, 2-methyl	2.6
total		93.3

Table S23. Chemical constituents of rosemary essential oil

RT (min)	constituent	area %
26.98	α-Pinene	15.9
29.63	Camphene	0.4
34.89	β-Pinene	9.0
40.41	3-Carene	1.8
42.27	o-Cymene	6.8
42.77	D-Limonene	7.9
43.05	Eucalyptol	30.3
45.40	γ-Terpinene	0.8
47.42	α-Terpinolene	1.1
51.78	Camphor	19.9
54.37	Terpinen-4-ol	0.3
68.98	Methyl undecanoate	1.8
total		96.0

Table S24. Chemical constituents of sandal wood essential oil

RT (min)	constituent	area %
42.96	Benzyl alcohol	14.1
68.87	Methyl undecanoate	2.9
77.08	Norbornane	14.5
78.28	α-Santalol	5.8
80.71	Geranylgeraniol	3.9
81.02	Corymbolone	3.8
81.15	Eudesma-3,11-dien-2-one	11.1
81.27	2,6,11-Tridecatrien-10-ol, 2,6,10-trimethyl-	3.5
81.88	Longipinocarvone	4.4
82.21	Geranylgeraniol	3.4
82.43	1-Cyclohexene-1-butanal, à,2,6,6-tetramethyl-	5.0
82.61	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-ethyl]-vinyl ester	8.9
82.88	9,17-Octadecadienal, (Z)-	2.1
total		83.4

Table S25. Chemical constituents of spearmint essential oil

RT (min)	constituent	area %
26.65	*α*-Pinene	0.7
33.48	Sabinene	0.5
34.41	*β*-Pinene	0.9
37.08	Myrcene	1.6
38.34	3-Octanol	0.3
42.61	D-Limonene	21.2
42.82	Eucalyptol	2.1
53.89	Levomenthol	0.6
54.16	Terpinen-4-ol	0.6
55.60	Dihydrocarvone	1.8
59.75	(-)-Carvone	61.4
67.64	*α*-Bourbonene	0.8
68.87	Methyl undecanoate	2.4
69.15	*β*-Caryophyllene	0.6
70.06	*trans*-*β*-Farnesene	0.4
71.39	Germacrene D	0.4
total		96.1

Table S26. Chemical composition of sweet thyme essential oil

RT (min)	constituent	area %
26.11	α-Pinene	3.0
28.64	Camphene	0.8
32.76	Sabinene	1.0
36.35	á-Myrcene	7.1
40.73	à-Terpinene	3.7
41.62	o-Cymene	2.1
42.16	D-Limonene	2.7
42.28	Sabinene	0.7
42.40	Eucalyptol	0.7
44.93	gamma-Terpinene	6.1
45.83	trans Sabinene hydrate	2.5
47.01	Terpinolene	1.6
48.07	Linalool	42.6
48.20	Linalool	2.9
51.27	(-)-Camphor	0.7
53.87	Terpinen-4-ol	11.7
54.97	(-)-beta-Fenchyl alcohol	0.67
55.23	Dihydrocarvone	0.62
59.51	Linalyl acetate	0.75
68.67	Methyl undecanoate	3.62
68.93	Caryophyllene	1.32
total		96.8

Table S27. Chemical constituents of ylang ylang essential oil

RT (min)	constituent	area %
41.08	4-Methylanisole	13.0
47.54	Methyl benzoate	5.8
47.98	Linalool	18.0
52.35	Benzyl acetate	19.9
54.74	Methyl salicylate	0.5
62.11	Anethole	0.4
66.78	Geranyl acetate	6.2
68.66	Methyl undecanoate	3.0
68.93	β-Caryophyllene	4.8
69.58	Cinnamyl acetate	3.7
70.29	α-Caryophyllene	1.6
72.89	cis-α-Bisabolene	0.4
73.45	Nerolidol	0.4
79.60	Benzyl benzoate	3.8
83.34	Benzyl salicylate	14.6
total		95.9

Abstract in Korean

담배거세미나방(*Spodoptera litura*)에 대한 바질(*Ocimum basilicum*)과 만다린(*Citrus reticulata*) 정유의 살충활성 상승 작용 기제

김 수 빈
농생명공학부 곤충학 전공
서울대학교 대학원

초록

식물 에센셜 오일의 화학적 구성은 복잡하기 때문에, 혼합 처리 시 살충력이 상승하는 경우가 존재한다. 이러한 에센셜 오일의 활성은 주목을 받고 있지만, 상승 매커니즘에 대한 이해는 많은 연구가 이루어지지 않은 실정이다. 본 연구에서는 담배거세미나방(*Spodoptera litura*)의 세 번째 유충에 대해 28종류의 에센셜 오일과 그 혼합물의 살충력을 평가하였다. 이들 중 바질 오일은 가장 높은 접촉독성을 보였으며, 만다린 오일은

바질 오일과의 혼합 평가에서 가장 높은 상승 효과를 나타냈다. GC-MS와 주 성분 소거 평가(Compound elimination assay)를 통해, 바질 오일은 estragole과 linalool, 만다린 오일은 limonene이 주요한 살충 활성을 보이는 물질임을 확인하였다. 반수치사량을 기준으로, 바질 오일과 만다린 오일의 이원 혼합물은 두 오일을 개별적으로 처리하였을 때에 비해, 두 가지 통계 모델에서 1.3, 1.4 라는 값을 나타냈다. 각 오일의 주 활성물질인 estragole, linalool, limonene의 화합물의 살충력을 평가했을 때, 혼합비 7:2:7에서 시너지 효과가 나타났으며 바질 오일과 만다린 오일 이원 혼합물과 유사한 살충 활성을 보였다. 시너지 효과는 세 가지 주 물질이 모두 혼합되었을 경우 나타났으며, 이는 각 화합물이 접촉 독성에서 중요한 역할을 한다는 것을 나타낸다. 바질 오일과 에센셜 오일의 활성은 표피층에 대해 높아진 침투효과와 신경 생리학적 반응이 시너지 효과의 매커니즘으로 여겨진다.

검색어: 접촉독성, 담배 거세미나방, 표피 침투, 중추 신경계

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