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Enhanced cuticular penetration as the mechanism of synergy for the major constituents of thyme essential oil in the cabbage looper, *Trichoplusia ni*

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ABSTRACT

For the past two decades, the insecticidal activity of botanicals, particularly plant essential oils has been gaining broad research interest. Due to the diverse chemical composition of essential oils, they often exhibit complex interactions among their major constituents, for example, synergistic or antagonistic relationships. Several hypotheses have been proposed for synergistic toxicity, including multiple modes-of-action and inhibition of metabolism, but the mechanism underlying synergy has not been fully elucidated. In the present study, interactions among the four major constituents of thyme (*Thymus vulgaris*) essential oil against the larvae of the cabbage looper were examined following topical administration. Two statistical models were used to determine the interactions, and several synergistic relationships were identified in natural proportional and equivalent blending ratios, but no antagonistic effects were observed. Among binary mixtures, thymol and *p*-cymene was synergistic in a topical application assay. GC–MS analyses showed increased recovery of thymol in the mixture both *in vivo* and *in vitro* from hemolymph extracts and receiver solutions, respectively, indicating enhanced penetration of thymol through the integument by *p*-cymene. An injection assay in fifth instar larvae and an *in vitro* cytotoxicity assay in an ovarian cell line of the cabbage looper showed no internal boosting effect of thymol by *p*-cymene, suggesting *p*-cymene facilitates the penetration of thymol, but does not directly contribute to increased toxicity of the mixture. A divided application method confirmed that the increased toxicity of the mixture can be produced only if the two compounds were applied as a mixture, proving *p*-cymene's role as a penetration enhancer. Contact angle measurement on a waxy surface showed lowered surface tension of thymol in the mixture, suggesting the surface tension can directly influence the penetration and efficacy of a toxicant in pesticide formulation design.

1. Introduction

Throughout the long history of insect-plant interactions, plants have evolved to develop defensive strategies for their survival. Plants produce secondary metabolites for defense either as distress signal to lure predators, or to directly deter or repel herbivores. Plant essential oils, usually obtained *via* steam distillation, exhibit many types of biological activity against insect pests including insecticidal, repellent, feeding deterrent, oviposition, and ovicidal activities (Isman, 2006; Pavela, 2015; Seo et al., 2015). Due to the complex nature of plant essential oils, mostly consisting of terpenoids (monoterpenes and/or sesquiterpenes) and sometimes phenylpropanoids (Regnault-Roger et al., 2012), the bioactivity of essential oils can vary based on their chemical composition, even within a plant species (Isman et al., 2008; Zambonelli et al., 2004), and the major constituents sometimes show synergistic interactions providing superior activity of the intact oil relative to that of its individual constituents (Akhtar et al., 2012; Bekele and Hassanali, 2001; Jiang et al., 2009; Miresmailli et al., 2006; Tak et al., 2016a).

Although some synergistic interactions among major constituents of plant essential oils have been reported, we still know little about how these enhanced toxicities are produced (Pavela, 2014). Suggested hypotheses include multiple modes-of-action, inhibition of detoxifying enzyme activity, modification of membrane protein functions (*e.g.*, ion channels), or increased cytotoxicity *via* enhanced cell membrane permeability as the potential mechanism of synergy. However, most of these are either unproven, or only partially supported hypotheses (Fields et al.,

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2010; Passreiter et al., 2004; Savelev et al., 2003; Tak and Isman, 2016; Taylor et al., 2004).

In a previous study, we showed synergistic interaction between 1,8-cineole and camphor, the two most abundant constituents of rosemary (*Rosmarinus officinalis*) essential oil against the cabbage looper (Tak et al., 2016a), and we proposed increased penetration through the insect's integument as one possible mechanism of synergy between them (Tak and Isman, 2015). As this effect was reported only for a single synergistic combination, further research is required to determine whether enhanced cuticular penetration is a general mechanism for other synergistic combinations in plant essential oils. Moreover, the previous study measured internal concentrations of those compounds through *in vivo* analysis, which could not preclude concurrent excretion of the compounds following metabolism. This indicated the necessity to develop an *in vitro* analysis method, that can demonstrate changes in penetration alone. Furthermore, there are a vast number of synergistic combinations already reported, and we need a decisive and convenient method to measure penetration-related synergistic effects.

In the present study, we examined interactions among the four major constituents of thyme (*Thymus vulgaris*) essential oil in terms of their insecticidal activity against the larvae of the cabbage looper, and evaluated their cuticular penetration using both *in vivo* and *in vitro* analyses. We also used a divided application method to confirm the penetration-related synergy in a simple bioassay, showing increased cuticular penetration as a key synergy mechanism.

2. Material and methods

2.1. Chemicals

Pure standard compounds of the major constituents of thyme oil were purchased from Sigma-Aldrich (thymol, ≥99.5% and linalool, 97%, St Louis, MO, USA) and Thermo Fisher Scientific (*p*-cymene, ≥99% and caryophyllene oxide, 95%, Waltham, MA, USA). Cell line maintenance reagents were obtained from Life Technologies (AlamarBlue dye, Express Five SFM medium, gentamicin, and glutamine, Carlsbad, CA, USA).

2.2. Insect and cell line maintenance

Eggs of *T. ni* were purchased from the Great Lakes Forestry Centre (Natural Resources Canada, Sault Ste. Marie, ON, Canada). The insect colony was maintained in the insectary at the University of British Columbia on a pinto bean-based artificial diet at 25 ± 2 °C and a 16:8 h LD photoperiod. An ovarian cell line of the cabbage looper (High Five™, Life Technologies) was grown in L-glutamine (1 mM)/gentamicin (50 μg/mL)-supplemented Express Five SFM medium. The cell line was incubated at 27 °C, and subculture was performed twice a week by taking 2 mL of cell suspension to 10 mL of new growth medium (Tak et al., 2016b).

2.3. Bioassay

2.3.1. Insecticidal activity of the major constituents of thyme oil and their synergistic interactions

Test combinations of the four major constituents of thyme oil were prepared by blending the compounds based on their proportional ratio in the essential oil identified in a previous study (thymol:*p*-cymene:linalool:caryophyllene oxide = 58.6:34.5:4.6:2.4, w:w) (Tak et al., 2016b) or equivalent dosages (1:1 to 1:1:1:1). Synergistic interactions among the compounds were evaluated in 3rd instar larvae of the cabbage looper *via* topical application (Tak et al., 2016a). Briefly, each of ten 3rd instar larvae was treated with 1 μL of test compounds dissolved in acetone by using a syringe attached to a repeating dispenser, and transferred to 7 cm diameter Petri dish. A block of artificial diet was provided, and mortality was recorded at 24 h. Acetone alone served as a control, and no mortality was observed in the controls. Five to seven different doses (10.9–520.8 μg/insect) were used to calculate LD_{50} values, and the test was repeated three times.

The interactions among the four compounds were investigated by comparing observed LD_{50} values and expected LD_{50} values. Expected values of the test compounds were calculated by using two different models (Tak et al., 2016a). An expected LD_{50} value by Hewlett and Plackett's model was calculated from the equation

$$\text{Expected}LD_{50} = \left(\text{a} \times LD_{50(a)}\right) + \left(b \times LD_{50(b)}\right) + \cdots + \left(n \times LD_{50(n)}\right)$$

where a is the proportion of compound A in the mixture, and $LD_{50(\text{a})}$ is the individual LD_{50} value of the compound.

An expected LD_{50} value based on Wadley's model was determined from the equation

$$\text{Expected}LD_{50} = \frac{a + b + c + \cdots + n}{\frac{a}{LD_{50(a)}} + \frac{b}{LD_{50(b)}} + \frac{c}{LD_{50(c)}} + \cdots + \frac{n}{LD_{50(n)}}}$$

where a is ratio of compound A in the mixture, and $LD_{50(\text{a})}$ is the LD_{50} of individual compound in the combination. The synergy ratio (R) was obtained by dividing expected LD_{50} value by observed LD_{50} value, and the interaction of the compounds was defined as synergistic (when $R \geq 1.5$), additive ($1.5 > R \geq 0.5$), or antagonistic ($R < 0.5$).

2.3.2. Injection assay

The internal toxicity of thymol, *p*-cymene and their binary mixture at 1:1 ratio (w:w) was examined *via* injection into fifth instar larvae of the cabbage looper as previously described (Tak and Isman, 2015). Briefly, a group of ten fifth instar larvae (269.9 ± 13.4 mg in average weight) was put into an ice-cold beaker to slow down their movement, and one μL of test solution dissolved in acetone was injected using a microneedle under a microscope into the ventral hemocoel. Mortality was recorded after 24 h and 48 h of incubation at the same conditions as above. Acetone alone served as a negative control, and the test was repeated three times.

2.3.3. Divided application

To confirm the penetration-enhancement of the thymol and *p*-cymene mixture, a divided application method was designed. The two compounds were individually dissolved in acetone, and each of a half μL of the solutions was topically applied to the dorsal and ventral abdomen individually of third instar larvae of the cabbage looper using a syringe as described above. The LD_{50} value after 24 h from the divided application was compared to those of the individual compounds as well as that of their binary mixture. To determine LD_{50} values, eight to nine different doses were used (thymol: 5–150 μg/insect, divided application: 10–300 μg/insect, and mixture: 5–300 μg/insect, respectively).

2.3.4. Cytotoxicity

The cytotoxicity of a thymol + *p*-cymene mixture was examined following a previous study (Tak et al., 2016b). In brief, after the cell suspension (50 μL, 2×10^5 cells/mL) was settled on a 96-well plate for 2 h at 27 °C, nine different concentrations (1.3–333.3 μg/mL) of the thymol + *p*-cymene mixture (1:1, w:w) dissolved in dimethyl sulfoxide/medium (1:149, v:v) was applied to each well (50 μL). Viability of cells was examined after 48 h of incubation under a florescent microscope, after staining cells with an AlarmarBlue dye solution. Fluorescence was measured using a Polarstar galaxy spectrophotometer (544 nm excitation and 590 nm emission, BMG Labtechnologies, Ortenberg, Germany).

2.4. GC–MS analyses of cuticular penetration

2.4.1. Sample preparation for in vivo and in vitro analysis

The penetration of individual thymol, *p*-cymene and the mixture thereof (1:1, w:w) through the cuticular layer of 3rd (*in vivo*) and 5th (*in vitro*) instar larvae of the cabbage looper was monitored *via* GC–MS analysis. For *in vivo* analysis, a group of thirty 3rd instar larvae was topically treated with the LD_{50} dose of the binary mixture (32.3 μg/insect) as well as the equivalent amounts of individual compounds (16.2 μg/insect) for 10 min, 1 h and 3 h observations, and at the LD_{10} dose (17.0 μg/insect) and the proportional amounts of individual compounds (8.5 μg/insect) for 8 h extraction. After incubation, twenty live larvae from each treatment was collected and rinsed with *n*-hexane twice, and transferred into a glass tube. Larvae were ground using a tissue homogenizer for 1 min with 6 mL *n*-hexane twice, and the pooled larval extracts were kept in a freezer (−20 °C) until analyzed.

For *in vitro* analysis, a 5th instar larva was used to prepare a section of integument by decapitation and removal of all the major organs and debris using deionized water. The section was mounted in a Franz cell diffuser (ID 5 mm, 5 mL of chamber volume, PermeGear, Inc., Hellertown, PA, USA, see supplementary information for details), and *n*-hexane (5 mL) was filled into the receiver chamber. One μL of individual compounds (at 250 μg/mL in acetone) and their mixture (at 500 μg/mL, 1:1, w:w) of thymol and *p*-cymene solutions were topically applied to the exposed (upper) side, and the opening was covered with aluminum foil. The receiver solution was carefully retrieved after 1 h of constant stirring with a Teflon-coated magnetic bar. Each treatment consisted of ten larvae, and the test was repeated three times from different cohorts of cabbage loopers. The solutions were kept in a freezer until analysis.

2.4.2. GC–MS analyses

The hemolymph extracts for *in vivo* analysis was analyzed using an Agilent 6890A/5973N (Agilent Technologies Canada Inc., Ottawa, ON, Canada) GC–MS in a pulsed splitless mode fitted with a DB-wax column (30 m × 0.25 mm × 0.25 μm, Agilent). The oven temperature was set at 50 °C for 3 min, with an increase of 30 °C/min, to 230 °C, and the total run time was 14 min. For *in vitro* analysis, an Agilent 6890N/5975 GC–MS was used with the same column. The initial temperature was 40 °C for 3 min, and increased to 230 °C and hold for 9 min, with an increase of 25 °C/min. The mass spectra were matched against Wiley09/Nist08 MS library, and the peak areas of each compound were compared to that of the internal standard, α-pinene for quantification.

2.5. Surface tension measurement

The surface tensions of the compounds and thyme oil were examined by measuring their contact angles on a beeswax layer following our reported method (Tak and Isman, 2015). To evaluate the effect of thymol to the surface tension of thyme essential oil, an artificial full mixture of the four major compounds (*p*-cymene, linalool and caryophyllene oxide) of thyme oil excluding thymol (FM-thymol) was prepared, following proportions of the major constituents identified in a previous study (Tak et al., 2016b). A beeswax specimen was prepared by dipping glass microscope slides into melted beeswax and air-drying them overnight. Contact angles of thyme oil, thymol, *p*-cymene, thymol + *p*-cymene, and FM-thymol were measured by applying 3 μL of acetonic solution (50%, w/v) onto a beeswax surface and analyzing the angle by a drop shape analyzer (DSA 100, KRŰSS GmbH, Hamburg, Germany).

2.6. Statistics

Probit analysis was performed to determine the LD_{50} values of test compounds and their combinations. Differences in contact angles and in cuticular penetration were compared by ANOVA with Tukey's post hoc analysis using a StatPlus 2009 software (version 5.8.4, AnalystSoft, Alexandria, VA, USA). IC_{50} value of the thymol + *p*-cymene mixture was determined *via* GraphPad Prism 5 (Version 5.02, GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Insecticidal activity of thyme oil constituent combinations

The blending effect of the four major constituents of thyme oil was examined *via* topical application against 3rd instar larvae of the cabbage looper. For combinations in their natural proportions, insecticidal activities seemed to be governed by thymol, as all combinations containing thymol showed relatively similar LD_{50} values to that thymol alone (Table 1). Based on Hewlett and Plackett's calculation, all combinations including thymol were determined synergistic, whereas in Wadley's determination model, they were only synergistic when thymol constituted no more than 60% of the mixture (for thymol + *p*-cymene + linalool, R = 1.52, and for thymol + *p*-cymene + linalool + caryophyllene oxide, R = 1.57).

Among the binary mixtures with thymol at 1:1 ratio, only *p*-cymene was synergistic (R = 1.78). On the other hand, although statistically additive, caryophyllene oxide seemed to have a mildly negative effect on thymol (R = 0.82), with the thymol + caryophyllene mixture having roughly double the LD_{50} value compared to that for the thymol + *p*-cymene binary mixture (LD_{50} = 76.5 and 32.3 μg/insect, respectively). This mild negative interaction eliminated the synergistic effect between thymol and *p*-cymene, as the tertiary and full mixture of thymol, *p*-cymene and caryophyllene oxide with/without linalool failed to show synergistic interactions. Since caryophyllene oxide was synergistic when mixed with *p*-cymene (R = 3.13), the decreased toxicity of the tertiary or full mixture seemed to be caused by the decreased toxicity of thymol by caryophyllene oxide.

3.2. Cytotoxicity and insecticidal activity via injection of thymol and p-cymene mixture

To explore the possible mechanism of synergy between the two most abundant constituents of thyme oil – thymol and *p*-cymene – at a 1:1 ratio (w:w), cytotoxicity of the mixture to an ovarian cell line of the cabbage looper was examined (Table 2). In a previous study, thymol was 7.4 times more potent than *p*-cymene *via* topical application against the cabbage looper, but 29.5-fold more potent in cytotoxicity. The IC_{50} value of the binary mixture of the two compounds was 96.9 μg/mL, which was 3 times less active than the thymol alone. If Wadley's determination method is applied to the cytotoxicity of the thymol + *p*-cymene combination, the expected IC_{50} value becomes 61.1 μg/mL, with a synergy ratio of 1.93 (synergistic). On the other hand, if *p*-cymene is considered as an inert solvent, the expected IC_{50} value should be double that of thymol since half of the mixture is thymol. However, the observed IC_{50} value of the mixture was 3-fold greater than that of individual thymol (*i.e.*, antagonistic), indicating that *p*-cymene does not enhance cytotoxicity of thymol.

To examine the possibility of enhanced mode-of-action as the mechanism of synergy, an injection assay using 5th instar larvae of the cabbage looper was conducted (Table 2). The LD_{50} values of the mixture were significantly greater than those of individual thymol, both at 24 h and 48 h. Similarly to the cytotoxicity assay,

Table 1
Insecticidal activity of combinations of the four major constituents of thyme oil against 3rd instar larvae of *Trichoplusia ni via* topical application.

Blending ratio (%, w/w)[a]				Obs LD_{50}[b]	Expected toxicity					
thymol	p-cymene	linalool	caryophyllene oxide		Hewlett and Plackett			Wadley		
					Exp LD_{50}[c]	χ^2	note[d]	Exp LD_{50}	R	note
63.0	37.0			33.5	110.2	53.4	S	47.9	1.43	A
92.8		7.2		27.7	43.9	5.9	S	34.6	1.25	A
96.1			3.9	34.6	70.3	18.1	S	33.8	0.98	A
	88.3	11.7		247.4	236.3	0.5	A	234.8	0.95	A
	93.6		6.4	292.4	290.9	0.01	A	254.8	0.87	A
		65.8	34.2	274.1	466.7	79.5	S	262.1	0.96	A
60.0	35.3	4.7		32.8	114.0	57.9	S	49.7	1.52	S
61.4	36.1		2.5	32.9	132.5	74.9	S	49.1	1.49	A
89.4		7.0	3.6	25.1	82.6	39.9	S	35.9	1.43	A
	83.2	11.0	5.7	308.4	279.6	3.0	A	245.8	0.80	A
58.6	34.5	4.6	2.4	32.4	135.4	78.5	S	50.8	1.57	S
50.0	50.0			32.3	137.5	80.5	S	57.4	1.78	S
50.0		50.0		40.1	111.0	45.3	S	55.6	1.39	A
50.0			50.0	76.5	516.3	374.7	S	63.1	0.82	A
	50.0	50.0		149.6	216.0	20.4	S	212.7	1.42	A
	50.0		50.0	124.6	621.2	397.1	S	390.3	3.13	S
		50.0	50.0	169.5	594.7	304.1	S	318.6	1.88	S
33.3	33.3	33.3		50.4	153.3	69.1	S	75.5	1.50	S
33.3	33.3		33.3	85.3	420.8	267.5	S	84.5	0.99	A
33.3		33.3	33.3	106.7	403.3	218.1	S	81.9	0.77	A
	33.3	33.3	33.3	164.0	472.5	201.4	S	291.3	1.78	S
25.0	25.0	25.0	25.0	108.5	366.1	181.3	S	97.4	0.90	A

[a] LD_{50} values of individual thymol, *p*-cymene, linalool and caryophyllene oxide were 32.6, 242.5, 189.5, and >1000 μg/insect, respectively (Tak et al., 2016b).
[b] Observed LD_{50} value, μg/insect.
[c] Expected LD_{50} value, μg/insect.
[d] Interactions of test compounds, S = synergistic, and A = additive interaction.

Table 2
Insecticidal activity *via* injection and cytotoxicity of thymol, *p*-cymene and their binary mixture in 5th instar larvae and an ovarian cell line of *Trichoplusia ni.*

treatment	internal insecticidal activity *via* injection						cytotoxicity	
	24 h			48 h				
	LD_{50}[a]	95% CL	slope ± SE	LD_{50}[a]	95% CL	slope ± SE	IC_{50}[b]	95% CL
thymol	244.3	186.1–357.6	1.6 ± 0.3	183.2	143.1–245.2	1.7 ± 0.3	31.6[d]	25.9–38.6
p-cymene	875.4	687.9–1293.6	2.2 ± 0.4	497.7	423.4–595.9	3.0 ± 0.4	931.7[d]	713.1–1217
mixture[c]	534.8	445.3–691.8	2.7 ± 0.5	471.8	385.7–605.4	2.5 ± 0.5	96.9	82.9–113.3

[a] μg/insect.
[b] μg/mL.
[c] Blending ratio = 1:1 (w:w).
[d] Data from a previous study (Tak et al., 2016b).

the LD_{50} value of the mixture was 2.2- and 2.6-fold greater than for individual thymol, suggesting that internally present *p*-cymene does not contribute to increased toxicity of thymol.

3.3. In vivo *cuticular penetration of thymol and* p-*cymene*

To examine penetration of the mixture through the cuticle as for an alternative mechanism of synergy, internal amounts of thymol and *p*-cymene after topical application were examined *via* GC–MS analysis (Table 3). In general, much more thymol was recovered compared to that of *p*-cymene in all analyses. Up to 1 h post-treatment, there was no difference in the recovery of thymol between applications of thymol alone and in the binary mixture, but at 3 h and 8 h, higher concentrations of thymol were detected when the mixture was applied, indicating enhanced penetration of thymol in the mixture. Although the applied dose at 8 h post-treatment (17.0 μg/insect) was about a half of the other treatments (32.3 μg/insect), the recovery of both compounds was significantly less than at earlier times of observation, indicating rapid metabolism of the compounds by larvae.

Table 3
In vivo GC–MS analysis of cuticular penetration of individual thymol, *p*-cymene and the mixture thereof against 3rd instar larvae of *Trichoplusia ni.*

time	treatment	Peak area ratio	
		thymol	*p*-cymene
10 min (at LD_{50})	individual	1.30	0.15
	mixture	1.24	0.14
1 h (at LD_{50})	individual	1.39	0.02
	mixture	1.39	0.11
3 h (at LD_{50})	individual	1.81	0.11
	mixture	2.64	0.19
8 h (at LD_{10})	individual	0.06	0.01
	mixture	0.13	0.01

The blending ratio of the compounds was 1:1 (w:w), and applied dosage was 32.3 (at LD_{50}) and 17.0 μg/insect (at LD_{10}), respectively.

3.4. *Toxicity of thymol,* p-*cymene and the mixture thereof* via *different application methods*

To confirm the enhanced cuticular penetration as the mechanism of synergy for the thymol + *p*-cymene combination, a divided application method was designed (Table 4). Although the same amounts of each compound were applied to 3rd instar larvae of

Table 4
Boosting effect of *p*-cymene on the insecticidal activity of thymol *via* different application methods against 3rd instar larvae of *Trichoplusia ni*.

application	n[a]	LD_{50}[b]	95% CL	slope ± SE	χ^2	thymol amount (μg)[c]
thymol	240	24.6	20.7–29.0	3.1 ± 0.3	2.6	24.6
thymol + *p*-cymene mixture	270	24.3	19.4–29.7	2.3 ± 0.2	3.6	12.2
thymol + *p*-cymene divided	240	44.4	36.8–53.0	2.8 ± 0.3	4.2	22.2

[a] Number of larvae tested.
[b] μg/insect.
[c] Amount of thymol in each LD_{50} value.

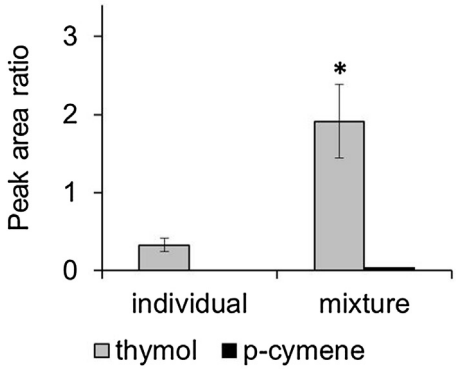

Fig. 1. *In vitro* GC–MS analysis of cuticular penetration of individual thymol, *p*-cymene and the mixture thereof. At 1 h post-treatment, the penetration of thymol in the mixture significantly increased when compared to the individual application ($P < 0.05$).

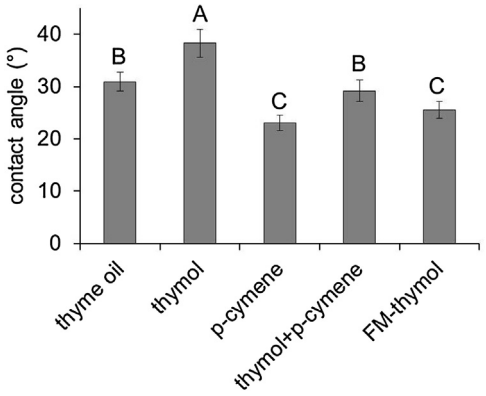

Fig. 2. Contact angle measurement of thyme oil and its major constituents on a beeswax layer. Individual thymol had the highest contact angle, and *p*-cymene lowered the angle of thymol in the mixture, *i.e.*, surface tension, in the mixture due to its relatively higher spreading activity on a waxy surface. FM = full mixture.

the cabbage looper, the divided application required almost the same amount of thymol for toxicity as that with individual thymol, whereas the mixture produced the same toxicity with only half as much thymol. This confirms that the synergy can only be observed when the compounds are applied together, indicating the physico-chemical interaction of the mixture is directly involved in the enhanced toxicity.

3.5. In vitro *cuticular penetrations of thymol and* p-*cymene*

Since the internal recovery through *in vivo* extraction represents the amount of 'penetrated amount – excreted amount *via* metabolism', an *in vitro* method only focusing on penetration was designed. As shown in Fig. 1, the penetration of thymol through the cuticle of 5th instar larvae was significantly increased in the mixture, providing direct evidence of enhanced penetration of thymol by *p*-cymene. Although the penetration of *p*-cymene also increased slightly in the mixture compared to individual application, the difference was not statistically significant. More importantly, significantly greater quantities of thymol penetrated into the receiver solution compared to *p*-cymene, a trend consistent in both *in vivo* and *in vitro* analyses.

3.6. *Contact angles on a beeswax layer*

The surface tension of thyme oil and its major constituents was examined by measuring their contact angles on a beeswax layer (Fig. 2). Compared to thyme oil (30.9 ± 1.8°), the surface tension of thymol was significantly greater (38.3 ± 2.7°, $P < 0.05$), and the full mixture of the major constituents except thymol showed a significantly lower contact angle than that of thyme oil ($P < 0.05$), indicating that thymol does not spread well on a waxy surface. Since *p*-cymene exhibited much better spreadability, the binary mixture of thymol and *p*-cymene showed lowered surface tension than individual thymol, which suggests better spreading on the integument of the cabbage looper.

4. Discussion

Generally, the chemical composition of plant essential oils can vary depending on environmental and biological factors (Burt, 2004; Ćavar et al., 2012; Tak et al., 2016a), and the chemical composition of thyme oil shows a wide range of the concentrations of thymol (22.1–75.4%) and *p*-cymene (4.0–44.9%), resulting in differential antibacterial, antifungal and antioxidant activities (Teixeira et al., 2013; Zambonelli et al., 2004).

As shown in the present study, both the types of compounds in the combination and their proportions can be factors contributing to overall bioactivity. This suggests the importance of standardization of chemical composition of essential oils used as active ingredients in bioinsecticides to maintain optimal efficacy of such products. Among the four major constituents of thyme oil, the synergy between thymol and *p*-cymene in the cabbage looper was noticeable among the binary mixtures at a 1:1 ratio. This insecticidal synergy between the two compounds was also reported in *Spodoptera littoralis* (Pavela, 2014) and the house fly, *Musca domestica* (Pavela, 2008). However, as shown in Table 1, the synergy between these two compounds disappeared in the tertiary or the full mixture including caryophyllene oxide. Since caryophyllene oxide was synergistic when individually mixed with *p*-cymene (R = 3.13), the insignificant or slightly negative interaction between thymol and caryophyllene oxide (synergy ratio of 0.82) may cause the disappearance of positive effects among those synergistic combinations.

The GC–MS analyses of penetration both *in vivo* and *in vitro* of thymol and *p*-cymene revealed substantially lower peak area ratios of *p*-cymene compared to those of thymol, although the applied amounts of both compounds were the same (Table 3 and Fig. 1). Insects have evolved to produce a thin layer of lipids on the cuticular surface (*i.e.*, the epicuticle) as a primary barrier to prevent or control water loss (Gibbs, 1998). Some insect species also use these long-chain hydrocarbons as nest mate recognition signals or sex pheromones (Qiu et al., 2012). Underneath this lipophilic

layer, hydrophilicity is greater in the endocuticle, which consists predominantly of chitin and protein (Andersen, 1979). Therefore, hydrocarbon compounds such as *p*-cymene may have difficulty penetrating further due to a lack of oxygenation needed for hydrogen bonding. Due to the higher affinity to a wax layer which was observed in the contact angle measurement (Fig. 2) and the more hydrophobic nature of the hydrocarbon, *p*-cymene may have stronger bonding to the wax, limiting its penetration.

Another possible explanation for the difference in recovery of the compounds could be faster metabolism of *p*-cymene than of thymol. Based on the *in vivo* analysis as shown in Table 3, the recovery of individual thymol gradually increased from 10 min to 3 h, whereas the highest recovery of *p*-cymene was observed at 10 min, suggesting different metabolic rates. Not only the rates but also the pathways of metabolism may differ. Whereas metabolites of *p*-cymene were excreted in the urine of the koala *via* oxidation in a single metabolic step (Boyle et al., 2000), presumably involving cytochrome P450s monooxygenases (phase I detoxification), the metabolism of thymol was reported to undergo a phase II process of glucosylation in the cabbage looper (Passreiter et al., 2004), suggesting further metabolism compared to *p*-cymene. In fact, *p*-cymene can be metabolized into thymol catalyzed by cytochrome P450s (Meesters et al., 2009), suggesting that higher recovery of thymol could partially come from *p*-cymene metabolism, but judging by the significantly lesser penetration of *p*-cymene, this possibility seems unlikely. Inhibition of detoxifying enzymes such as cytochrome P450s, glutathione S-transferases, or general esterases as a mechanism for synergy is an alternative explanation, and further research is being conducted to investigate this hypothesis.

Nonetheless, *in vitro* investigation clearly shows significantly increased penetration of thymol through the cuticle when applied as a mixture (Fig. 2). Together with the results of the injection and divided applications, a penetration-enhancing effect is the strongest possible explanation for the synergy between the two compounds. Injection of the mixture did not increase toxicity compared to individual injection of thymol, suggesting that internal *p*-cymene does not directly contribute to the increased insecticidal activity of thymol, and confirmed by their cytotoxicity. Moreover, divided application provides the direct evidence of a penetration-related response since the synergy was only found when the two compounds were applied as a mixture. This simple assay technique demonstrated in the present study can be very beneficial since it can decisively determine the penetration-enhancing effect (or, alternatively, the interference of penetration) in synergistic combinations.

The penetration enhancement by some essential oil constituents has been studied in the pharmaceutical context as well. Generally, non-polar monoterpenes or hydrocarbons produce better penetration of lipophilic drugs, whereas polar ones are better for hydrophilic drugs through human or mammalian skins (Williams and Barry, 2012). The present study shows that the increased cuticular penetration of a toxicant can significantly increase toxicity in an insect, and sometimes the penetration enhancer itself can significantly contribute to overall bioactivity as an additional toxicant (Tak and Isman, 2015). The exact mechanism of the changes in penetration has not been studied especially for the cuticle of insects. The increased penetration could be produced either by impacting the integrity of the physical structure of the lipid bilayer, *i.e.*, through an increase in lipid fluidity (Narishetty and Panchagnula, 2005) or by simple expansion of the surface area of toxicant *via* better spreading (due to lowered surface tension), mitigating excessive evaporation of toxicant. Further research should focus on gaining further insight into the penetration mechanism, including the exact route of penetration, impact on lipid layers, or structure-activity relationships in terms of their impact on cuticular penetration.

5. Conclusions

In the present study, we examined the interactions among the four major constituents of thyme oil on their insecticidal activity against the cabbage looper, and identified several synergistic combinations. Thymol and *p*-cymene exhibited synergy at a 1:1 ratio, and GC–MS analyses showed increased cuticular penetration of thymol in the mixture as a possible mechanism of synergy. The *in vitro* study supported by *in vivo* bioassays clearly demonstrated increased penetration through the cuticular layer, and can be considered as conclusive evidence of a penetration-enhancing effect as the mechanism underlying this synergy. Future studies should focus on the mechanism and routes of penetration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.03.003.

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