SUPPLEMENTARY MATERIAL

Insecticidal activity of isolated phenylpropanoids from *Alpinia galanga* rhizomes against *Spodoptera litura*

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Abstract

Botanical insecticides as a means of controlling insects present an alternative approach that is safer than the use of synthetic insecticides. The present study identified the insecticidal activity of extracts of the rhizomes of *Alpinia galanga* (L.) Willd. and seven isolated phenylpropanoids against the second instar of *Spodoptera litura* Fab. by topical application. The ethyl acetate extract had the highest toxicity on this insect with LD_{50} values of 1.68 and 1.25 μg/larva after 24 and 48 h posttreatment, respectively. Among the seven phenylpropanoids separated from the ethyl acetate extract, 1'*S*-1'-acetoxychavicol acetate was identified as the most active compound with LD_{50} values of 1.63 and 1.40 μg/larva after 24 and 48 h posttreatment, respectively, followed by *p*-coumaryl diacetate. In addition, the two active compounds decreased glutathione *S*-transferase activity and increased acetylcholinesterase activity. *p*-Coumaryl diacetate also decreased carboxylesterase activity.

Keywords: *Alpinia galanga*, Detoxification enzymes, Insecticidal activity, Phenylpropanoids, *Spodoptera litura*

Experimental

Insect Rearing

S. litura were reared on an artificial diet consisting of green bean (360 g), agar (37.5 g), yeast (30 g), ascorbic acid (7.5 g), methylparaben (7.5 g), sorbic acid (4.5 g), water (2.12 L), mixed vitamin solution (60 mL), amoxicillin solution (60 mL) and 40% formalin (6 mL) (Yooboon et al. 2019). The larvae were kept at 27°C, 65 ± 5% RH and L16:D8 photoperiod before testing for insecticidal activity.

Plant Material

Alpinia galanga rhizomes were obtained from Prachin Buri province, Thailand, in August 2018. A voucher specimen (BK No. 070911) was preserved in Bangkok Herbarium, Plant Varieties Protection Office, Department of Agriculture, Bangkok, Thailand.

Extraction

A sample (1.6 kg) of dried powder of *A. galanga* rhizomes was produced using sequential extraction over seven days with either hexane, ethyl acetate or ethanol by maceration at room temperature. Each solvent extract was filtered and dried using a rotary evaporator (Heidolph, Germany) and stored at 4°C.

Isolation

A sample (9 g) of the ethyl acetate extract was fractionated into twelve fractions using silica gel column chromatography (Kiesel gel 60G, Merck, Thailand) with gradients of hexane/ethyl acetate and ethyl acetate/methanol, respectively. At first, 1'*S*-1'-acetoxychavicol acetate (**1**, 1.06 g) was isolated from fraction 4 (2.16 g) using silica gel column chromatography and eluting with hexane/ethyl acetate (3/1). Fraction 6 (0.40 g) was subjected to silica gel column chromatography using hexane/ethyl acetate (8/1) to provide subfraction, 6-1 (0.21 g). This subfraction was purified using preparative thin layer chromatography (PTLC) with hexane/ethyl acetate (5/1) to give *p*-coumaryl diacetate (**2**, 0.14 g) and *p*-coumaryl alcohol ethyl ether (**3**, 0.02 g). Fraction 8 (0.63 g) was further fractionated using silica gel column chromatography with hexane/ethyl acetate (4/1) to obtain four subfractions. Hydroxychavicol acetate (**4**, 0.16 g) was isolated from subfraction 8-3 (0.54 g) using PTLC and eluting with hexane/ethyl acetate (3/1). The purification of fraction 9 (0.22 g) produced *trans*-*p*-hydroxycinnamaldehyde (**5**, 0.02 g) using PTLC with hexane/ethyl acetate (1/1). Fraction 10 (1.07 g) was fractionated using column chromatography with

hexane/ethyl acetate (1/3) to obtain five subfractions. Subfraction 10-3 was identified as *trans-p*-acetoxycinnamyl alcohol (**6**, 0.38 g). Subfraction 10-4 was further purified using PTLC and eluting with hexane/ethyl acetate (1/1) to produce *p*-coumaryl alcohol (**7**, 0.005 g).

Identification

The chemical structures of all isolated phenylpropanoids were identified using ^{1}H and ^{13}C NMR on a Bruker 400 MHz AVANCE III HD (Karlsruhe, Germany) and high resolution mass spectrometry (HRMS) on a Maxis Bruker spectrometer (Karlsruhe, Germany).

1'*S*-1'-Acetoxychavicol acetate (**1**): Colorless oil; $[\alpha]_D^{20}$ -50 (*c*=0.5, CH_2Cl_2): Colorless solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36 (2H, d, *J* = 8.6 Hz), 7.07 (2H, d, *J* = 8.6 Hz), 6.26 (1H, d, *J* = 5.8 Hz), 5.98 (1H, ddd, *J* = 17.1, 10.4, 5.8 Hz), 5.30 (1H, dt, *J* = 17.2, 1.3 Hz), 5.25 (1H, dt, *J* = 10.5, 1.2 Hz), 2.29 (3H, s), 2.10 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.8, 169.3, 150.4, 136.4, 136.0, 128.4, 121.7, 117.0, 75.5, 21.1, 21.0. HRMS (ESI) Calculated for $C_{13}H_{14}NaO_4$ 257.0790 ($[M+Na]^+$), Found 257.0825. NMR data were identical to the literature values (Noro et al. 1988).

p-Coumaryl diacetate (**2**): Pale yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39 (2H, d, *J* = 8.6 Hz), 7.05 (2H, d, *J* = 8.6 Hz), 6.62 (1H, d, *J* = 16.1 Hz), 6.22 (1H, dt, *J* = 15.9, 6.4 Hz), 4.75 (2H, dd, *J* = 6.4, 1.3 Hz), 2.30 (3H, s), 2.10 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8, 169.4, 150.4, 134.0, 133.1, 127.6, 123.4, 121.7, 64.9, 21.1, 21.0. HRMS (ESI) Calculated for $C_{13}H_{14}NaO_4$ 257.0790 ($[M+Na]^+$), Found 257.0796. NMR data were identical to the literature values (Noro et al. 1988).

p-Coumaryl alcohol ethyl ether (**3**)**:** Yellow oil; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.25 (2H, d, *J* = 8.6 Hz), 6.75 (2H, d, *J* = 8.6 Hz), 6.52 (1H, d, *J* = 15.9 Hz), 6.12 (1H, dt, *J* = 15.9, 6.3 Hz), 4.09 (2H, dd, *J* = 6.3, 1.3 Hz), 3.54 (2H, q, *J* = 7.0 Hz), 1.21 (3H, t, *J* = 7.0 Hz) ; ^{13}C NMR (MeOD, 100 MHz) δ 158.4, 133.9, 129.7, 128.8, 123.7, 116.3, 72.5, 66.4, 15.4. HRMS (ESI) Calculated for $C_{11}H_{14}NaO_2$ 201.0891 ($[M+Na]^+$), Found 201.0886. NMR data were identical to the literature values (Sukhirun et al. 2011).

Hydroxychavicol acetate (**4**): Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37 (2H, d, *J* = 8.5 Hz), 7.06 (2H, d, *J* = 8.6 Hz), 6.00 (1H, ddd, *J* = 17.1, 10.3, 6.1 Hz), 5.33 (1H, dt, *J* = 17.1, 1.4 Hz), 5.20-5.17 (2H, m), 2.28 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.6, 150.0, 140.2, 140.0, 127.5, 121.6, 115.4, 74.7, 21.1. HRMS (ESI) Calculated for $C_{11}H_{12}NaO_3$ 215.0684 ($[M+Na]^+$), Found 215.0679. NMR data were identical to the literature values (Janssen and Scheffer 1985).

trans-p-Hydroxycinnamaldehyde (**5**): Yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.67 (1H, d, *J* = 7.8 Hz), 7.51 (2H, d, *J* = 8.7 Hz), 7.44 (1H, d, *J* = 15.8 Hz), 6.92 (2H, d, *J* = 8.7 Hz), 6.63 (1H, dd, *J* = 15.8, 7.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 194.1, 158.8, 153.1, 130.8, 127.0, 126.6, 116.3. HRMS (ESI) Calculated for $C_9H_8NaO_2$ 171.0422 ($[M+Na]^+$), Found 171.0417. NMR data were identical to the literature values (Stange et al. 1999).

trans-p-Acetoxycinnamyl alcohol (**6**): Pale yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36 (2H, d, *J* = 8.6 Hz), 7.03 (2H, d, *J* = 8.6 Hz), 6.57 (1H, d, *J* = 15.9 Hz), 6.28 (1H, dt, *J* = 15.9, 5.7 Hz), 4.29 (2H, dd, *J* = 5.7, 1.3 Hz), 2.28 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.6, 150.0, 134.4, 129.9, 128.7, 127.3, 121.6, 63.4, 21.0. HRMS (ESI) Calculated for $C_{11}H_{12}NaO_3$ 215.0684 ($[M+Na]^+$), Found 215.0681. NMR data were identical to the literature values (Zhu et al. 2013).

p-Coumaryl alcohol (**7**): White solid; ^{1}H NMR (Acetone-d6, 400 MHz) δ 7.26 (2H, d, *J* = 8.6 Hz), 6.80 (2H, d, *J* = 8.6 Hz), 6.52 (1H, d, *J* = 15.9 Hz), 6.21 (1H, dt, *J* = 15.9, 5.6 Hz), 4.22 (2H, dd, *J* = 5.6, 1.6 Hz); ^{13}C NMR (Acetone-d6, 100 MHz) δ 157.8, 129.6, 128.3, 116.2, 63.5. HRMS (APCI) Calculated for $C_9H_{13}O_2$ 151.0759 ($[M+H]^+$), Found 157.0754. NMR data were identical to the literature values (Ly et al. 2003).

Bioassay

Contact toxicity assays to second instars of *S. litura* were examined to determine the mortality and median lethal dose (LD_{50}) using a topical application. Sample concentrations of 0.5, 1, 2, 5, 10 and 20 µg/larva were prepared in acetone. Then, 2 µL of each sample solution was slowly applied to the thorax region of *S. litura*. Each sample was repeated in five replicates each consisting of a population of ten larvae. The treated larvae were transferred into Petri dishes (diameter 100 mm) under controlled conditions with an artificial feeding diet and then mortality was recorded after 24 and 48 h posttreatment. The LD_{50} values were determined using Probit analysis in the StatPlus Program (2018 version).

Enzyme assays

Enzyme preparation method

After 24 h posttreatment of active phenylpropanoid at the LD_{50} level, the surviving *S. litura* larvae were used to determine detoxification and neuron enzyme activities. The larvae were placed in a microcentrifuge tube and crushed in homogenised buffer (pH 7.2) prepared from 0.1 M potassium phosphate buffer and 1 mM ethylenediaminetetraacetic acid for enzyme

extraction. The homogenate was then centrifuged at 15,520 g (12,000 rpm) for 15 minutes at 4°C to produce the supernatant for analysis of the enzyme activities.

Carboxylesterase activity

The supernatant (10 µL) was examined for carboxylesterase activity by mixing with potassium phosphate buffer (190 µL, 50 mM, pH 7.4) and *p*-nitrophenyl acetate in DMSO (40 µL, 10 mM). The reaction was analysed at 410 nm and 37°C for 90 seconds with a Biotek PowerWave XS microplate spectrophotometer (Winooski, VT, USA). The extinction coefficient of *p*-nitrophenyl acetate was 176.4705 (Bullangpoti et al. 2012).

Glutathione S-transferase activity

The supernatant (100) µL was examined for glutathione *S*-transferase activity by mixing with potassium phosphate buffer (100 µL, 50 mM, pH 7.2), glutathione solution (10 µL, 10 mM GSH reduced form) and 1-chloro-2,4'-dinitrobenzene (CDNB) (100 µL, 150 mM). The reaction was analysed at 340 nm and 25°C for 3 min on the microplate spectrophotometer. The extinction coefficient of CDNB was 0.000137 (Oppenoorth F. J. et al. 1979).

Acetylcholinesterase activity

The mixture of supernatant (50 µL) and potassium phosphate buffer (50 µL, 100 mM, pH 7.2) was incubated at 30°C for 30 minutes, followed by adding a mixture of 5,5'-dithiobis(2-nitrobenzoic acid) (2µL, 100 mM), ATCI (2µL, 100 mM) and potassium phosphate buffer (46 µL, 100 mM, pH 7.2)). The reaction was analysed at 412 nm to determine the acetylcholinesterase activity on the microplate spectrophotometer. The extinction coefficient was 1.36×10^4 M^{-1} cm^{-1} (Kumrungsee et al. 2014).

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Table S1. Characteristics and amounts of extracts from *A. galanga* rhizomes

Extract	Weight (g)	Yield[a] (% wt/wt)	Characteristics
Hexane	6.83	0.43	Pale yellow gum
Ethyl acetate	19.11	1.19	Brown gum
Ethanol	82.86	5.18	Dark brown gum

[a](crude extract / weight of dried plant) × 100

Table S2. Toxicity of the rhizomes of *A. galanga* extracts against *S. litura*

Extract	Time (h)	LD_{50}	LCL	UCL	*P*-value	χ^2
Hexane	24	8.02	6.78	9.68	0.36	4.36
	48	6.87	4.40	12.29	0.04	9.78
Ethyl acetate	24	1.68	0.78	2.89	0.27	5.15
	48	1.25	1.02	1.50	0.78	1.74
Ethanol	24	NA	-	-	-	-
	48	NA	-	-	-	-

LD_{50}: Lethal Dosage that kills 50% of the exposed larvae, expressed in μg/larva.

LCL: Lower Confidence Limit, UCL: Upper Confidence Limit.

NA: Not Active at the highest concentration tested (20 μg/larva).

Table S3. Toxicity of 1'*S*-1'-acetoxy-chavicol acetate (**1**), *p*-coumaryl diacetate (**2**) and racemic mixture of **1** ((±)-**1**) to the second instar of *S. litura* after 24 and 48 h posttreatment

Compound	Time (h)	LD_{50}	LCL	UCL	*P*-value	χ^2
1	24	1.63	1.26	2.03	0.58	2.87
	48	1.40	1.09	1.74	0.95	0.71
2	24	2.37	1.32	3.89	0.68	2.28
	48	2.14	1.25	3.32	0.59	2.81
(±)-1	24	13.31	11.06	16.38	0.9	1.08
	48	11.41	9.59	13.79	0.85	1.36

LD_{50}: Lethal Dosage that kills 50% of the exposed larvae, expressed in µg/larva.

LCL: Lower Confidence Limit, UCL: Upper Confidence Limit.

Table S4. Effect of 1'*S*-1'-acetoxychavicol acetate (**1**) and *p*-coumaryl diacetate (**2**) on enzyme activities

Treatment	Carboxylesterase[a,b]	Glutathione *S*-transferase[a,b]	Acetylcholinesterase[a,b]
Control	1.007 ± 0.143 a	0.953 ± 0.129 a	0.119 ± 0.003 a
1	0.994 ± 0.082 ab	0.794 ± 0.035 b	0.136 ± 0.018 b
2	0.774 ± 0.096 b	0.815 ± 0.053 b	0.137 ± 0.002 b

[a]Means ± SE within a column followed by the same lowercase letter are not significantly different (P > 0.05, ANOVA).

[b]Carboxylesterase activity (nM *p*-nitrophenol/min/mg protein); Glutathione-*S*-transferase activity (CDNB conjugated product/mg protein/min); Acetylcholinesterase (acetylcholinesterase activity/mg protein/mL).

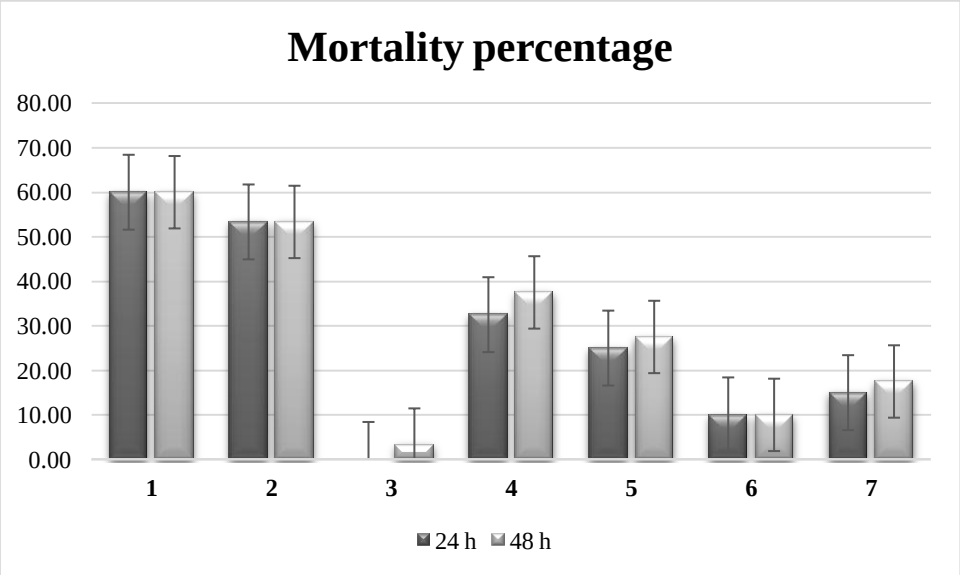

Figure S1. Toxicity of seven isolated phenylpropanoids against *S. litura* at a concentration of 2 µg/larva after 24 and 48 h posttreatment.