JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 10001–10006 10001
DOI:10.1021/jf102193k

Larvicidal Activity of *Asarum heterotropoides* Root Constituents against Insecticide-Susceptible and -Resistant *Culex pipiens pallens* and *Aedes aegypti* and *Ochlerotatus togoi*

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We investigated the toxicity of (−)-asarinin, α-asarone, methyleugenol, pellitorine, and pentadecane identified in *Asarum heterotropoides* root to third instar larvae from insecticide-susceptible *Culex pipiens pallens* (KS-CP strain), *Aedes aegypti*, and *Ochlerotatus togoi* as well as field-collected *C. p. pallens* (DJ-CP colony), identified by polymerase chain reaction. Results were compared with those of two conventional mosquito larvicides: fenthion and temephos. Pellitorine (LC_{50}, 2.08, 2.33, and 2.38 ppm) was 5.5, 10.8, and 25.6 times, 4.5, 11.6, and 24.7 times, and 6.9, 11.1, and 24.6 times more toxic than (−)-asarinin, α-asarone, and methyleugenol against susceptible *C. p. pallens*, *A. aegypti*, and *O. togoi* larvae, respectively. Pentadecane was least toxic. Overall, all the compounds were less toxic than either fenthion or temephos. However, these compounds did not differ in toxicity against larvae from the two *Culex* strains, even though the DJ-CP larvae exhibited high levels of resistance to fenthion (resistance ratio (RR), 1179), chlorpyrifos (RR, 1174), fenitrothion (RR, 428), deltamethrin (RR, 316), chlorfenapyr (RR, 225), and α-cypermethrin (RR, 94). This finding indicates that the isolated compounds and the pyrethroid, organophosphorus, and pyrrole insecticides do not share a common mode of action or elicit cross-resistance. *A. heterotropoides* root-derived materials, particularly (−)-asarinin and pellitorine, merit further study as potential mosquito larvicides for the control of insecticide-resistant mosquito populations in light of global efforts to reduce the level of highly toxic synthetic insecticides in the aquatic environment.

KEYWORDS: Botanical mosquitocide; natural mosquito larvicide; *Asarum heterotropoides*; pellitorine; (−)-asarinin; insecticide resistance

INTRODUCTION

Mosquitoes are prevalent worldwide and common and serious disease vectoring insect pests (*1*). In 2004, it was estimated that ∼3.2 billion people in some 107 countries or territories are at risk of malaria infection and that some 350−500 million people contract malaria each year, with at least one million deaths annually (*2*). Approximately 925 million people in most countries of the tropics are at risk of dengue infection (*3*). Mosquito larval abatement has been achieved principally by the use of organophosphorus (OP) insecticides such as chlorpyrifos and temephos, insect growth regulators such as diflubenzuron and methoprene, and bacterial larvicides such as *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* (*1*), which continue to be effective larvicides. Continued and repeated use of these larvicides has disrupted natural biological control systems and led to resurgences of mosquitoes (*4*), often resulting in the widespread development of resistance (*5*), and has undesirable effects on aquatic nontarget organisms (*1*, *6*). In particular, increasing levels of resistance to the most commonly used mosquitocides have caused multiple treatments and excessive doses, fostering serious environmental and human health concerns. The use of OP and carbamate insecticides will likely be reduced in the near future in the United States (U.S.) by the U.S. Environmental Protection Agency (EPA) as reregistration under the 1996 Food Quality and Protection Act (*7*) occurs. Reregistration also occurs under the Commission Regulation (EC) no. 1048/2005 in the European Union (*8*) and under the Agrochemicals Control Act in South Korea (*9*). These problems substantiate the need for the development of selective mosquito control alternatives to establish an efficient resistance management strategy based on all available information on the extent and nature of resistance.

Plants, particularly higher plants, have been suggested as alternative sources for arthropod control products largely because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe

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Published on Web 08/31/2010

and pose fewer risks to the environment, with minimal impacts to animal and human health, and often act at multiple and novel target sites, thereby reducing the potential for resistance (*10*−*12*). Much effort has been focused on them as potential sources of commercial mosquito larvicides, in part, because certain plants and their constituents meet the criteria of minimum risk pesticides (*13*). In particular, we initially reported that *Asarum heterotropoides* Schmidt (Aristolochiaceae) root steam-distillate and several of its constituents had larvicidal activity against third instar larvae of *Culex pipiens pallens*, *Aedes aegypti*, and *Ochlerotatus togoi* (*14*). Historically, *A. heterotropoides* has been used as an analgesic and antitussive agent for the treatment of influenza, headache, rheumatic pain, and asthma (*15*). Very little information has been done to consider potential of *A. heterotropoides* root-derived materials to manage insecticide-resistant mosquitoes.

In the current study, we assess the mosquito larvicidal activity of the constituents that comprise *A. heterotropoides* root against third instar larvae from insecticide-susceptible *C. p. pallens*, *A. aegypti*, and *O. togoi* as well as field-collected colony of *C. p. pallens*, identified by polymerase chain reaction (PCR), resistant to various insecticides. The toxicities of the constituents were compared with those of two currently available larvicides: fenthion and temephos.

MATERIALS AND METHODS

General Instrumental Methods. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD on a Bruker AVANCE 600 spectrometer (Karlsruhe, Germany) using tetramethylsilane (TMS) as an internal standard, and chemical shifts are given in δ (ppm). UV spectra were obtained in ethanol on a UVICON 933/934 spectrophotometer (Kontron Instrument, Milan, Italy) and mass spectra on a Jeol JMS-DX 303 spectrometer (Tokyo, Japan). Optical rotation was measured with an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ). Silica gel (0.063−0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Precoated silica gel plates (Kieselgel 60 F_{254}) (Merck) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 series high-performance liquid chromatograph (HPLC) (Santa Clara, CA) was used for isolation of active principles.

Chemicals. Eleven insecticides examined in this study were as follows: bifenthrin (97.0% purity) β-cyfluthrin (98.0% purity), α-cypermethrin (97.5% purity), etofenprox (96.5% purity), fenitrothion (98.5% purity), and chlorfenapyr (99.0% purity) purchased from Sigma-Aldrich (St. Louis, MO), deltamethrin (99.0% purity), chlorpyrifos (98.9% purity), fenthion (98.4% purity), and permethrin (98.0% purity) purchased from Supelco (West Chester, PA), and temephos (97.3% purity) purchased from Riedel (Seelze, Germany). Triton X-100 was obtained from Shinyo Pure Chemicals (Osaka, Japan). All other chemicals were of reagent-grade quality and available commercially.

Mosquitoes. The stock cultures of *C. p. pallens* (susceptible KS-CP strain), *A. aegypti*, and *O. togoi* (*14*) were maintained in the laboratory without exposure to any known insecticide. Larvae of wild *C. pipiens* were field-collected near rice paddy fields and cowsheds in Daejeon (South Korea) in early August 2009. The collected larvae were immediately transferred to an insect rearing room. Larvae were reared in plastic trays (27 cm × 15 cm × 4 cm) containing 0.5 g of sterilized diet (Vivid-S:Super Terramin, 4:1 by weight). Vivid-S and Super Terramin were purchased from Sewhapet (Inchon, South Korea). Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. They were held at 27 ± 1 °C and 65−75% relative humidity (RH) under a 12:12 h light:dark cycle. Species identification based on PCR (*16*) revealed that larvae from the field-collected colony (designated DJ-CP) belong to *C. p. pallens*.

Extraction and Isolation. Air-dried root (600 g) of *A. heterotropoides* was purchased from Boeun medicinal herb shop, Kyoungdong market (Seoul, South Korea). It was pulverized and extracted with methanol (2 × 3 L) at room temperature for 1 day and filtered. The combined filtrate was concentrated under vacuum at 40 °C to yield ∼74.63 g of a dark-brownish tar. The extract (100 g) was sequentially partitioned into hexane- (14.2 g), chloroform- (23.15 g), ethyl acetate- (5.05 g), butanol- (14.0 g), and water-soluble (43.6 g) portions for subsequent bioassay. The organic solvent-soluble portions were concentrated to dryness by rotary evaporation at 40 °C, and the water-soluble portion was freeze-dried. For isolation of active principles, 50 ppm of each *A. heterotropoides* root-derived material was tested in a direct-contact mortality bioassay as described previously (*17*).

The most active hexane-soluble fraction (10 g) was chromatographed on a 70 cm × 5.5 cm silica gel column (600 g) and eluted with a gradient of hexane and ethyl acetate ((10:1 (2.2 L), 9:1 (2 L), 7:3 (2 L), 5:5 (1 L), and 3:7 (1 L) by volume) and finally with methanol (1 L) to provide 48 fractions (each about 250 mL). Column fractions were monitored by TLC on silica gel plates with hexane and ethyl acetate (7:3 by volume). Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 2% H_2SO_4 and then heating on a hot plate. Fractions 1−4 (2.92 g) were pooled and purified by preparative TLC (hexane:ethyl acetate (7:3) by volume) to yield compound **1** (720 mg, R_f = 0.94) and compound **2** (40 mg, R_f = 0.78). Fractions 5−8 (450 mg) were purified by preparative TLC [hexane:ethyl acetate (7:3)] to provide compound **3** (75 mg, R_f = 0.61). The active fractions 15−27 (760 mg) were pooled and recrystallized in methanol at −4 °C to afford compound **4** (2.11 mg). A preparative HPLC was used for separation of the constituents from the active fractions 28−32 (739 mg). The column was a 21.2 mm i.d. × 250 mm Prodigy ODS (Phenomenex, Torrance, CA) using a mobile phase of acetonitrile and water (8:2 by volume) at a flow rate of 1.5 mL/min. Chromatographic separations were monitored using a UV detector at 254 nm. Finally, an active principle **5** (12 mg) was isolated at a retention time of 10.89 min.

Bioassay. A direct-contact mortality bioassay (*17*) was used to evaluate the toxicity of test materials to third instar larvae from the susceptible and field-collected mosquitoes. Each compound in acetone (for (−)-asarinin) or methanol (for the other compounds) was suspended in distilled water with Triton X-100 (20 μL/L). Groups of 20 mosquito larvae were separately put into paper cups (270 mL) containing each test compound solution (250 mL). The toxicity of each test compound and insecticide was determined with four to six concentrations ranging from 0.1 to 200 ppm and 0.001 to 0.00005 ppm, respectively. Fenthion and temephos served as positive controls and were similarly prepared. Negative controls consisted of the methanol- or acetone-Triton X-100 carrier solution in distilled water. All treatments were replicated three times using 20 larvae per replicate.

Treated and control larvae were held at the same conditions used for colony maintenance. Larvae were considered dead if they did not move when prodded with fine wooden dowels 24 h posttreatment. Because all bioassays could not be conducted at the same time, treatments were blocked over time with a separate control treatment included in each block. Freshly prepared solutions were used for each block of bioassays (*18*).

Data Analysis. Concentration−mortality data were subjected to probit analysis (*19*). The LC_{50} values for each species and their treatments were considered to be significantly different from one another when their 95% confidence limits failed to overlap. A resistance ratio (RR) was calculated according to the formula, RR = LC_{50} of larvae from the DJ-CP colony of *C. p. pallens*/LC_{50} of larvae of the susceptible KS-CP strain. RR values of <10, 10−40, 40−160, and >160 were classified as low, moderate, high, and extremely high resistance, respectively (*20*). Susceptibility ratio (SR) was determined as the ratio of LC_{50} of *A. aegypti* or *O. togoi* larvae/LC_{50} of susceptible *C. p. pallens* larvae (*17*).

RESULTS

Insecticide Susceptibility. The toxicity of 11 insecticides examined against third instar larvae from the susceptible KS-CP strain of *C. p. pallens* was evaluated using the direct-contact mortality bioassay (**Table 1**). As judged by 24 h LC_{50} values, β-cyfluthrin and deltamethrin were the most toxic insecticides, followed by α-cypermethrin. Moderate toxicity was produced by bifenthrin, fenthion, and chlorpyrifos. The other five insecticides exhibited low toxicity.

Insecticide Resistance. The comparative toxicity of the 11 test insecticides to third instar larvae from the field-collected DJ-CP

Table 1. Toxicity of 11 Insecticides to Third Instar Larvae from Insecticide-Susceptible KS-CP Strain and Field-Collected DJ-CP Colony of *Culex pipiens pallens* Using Direct-Contact Mortality Bioassay During a 24 h Exposure

	KS-CP larvae		DJ-CP larvae		
insecticide	slope ± SE	LC_{50}, ppm (95% CL[a])	slope ± SE	LC_{50}, ppm (95% CL[a])	RR[b]
β-cyfluthrin	1.9 ± 0.17	0.00020 (0.00016−0.00024)	1.6 ± 0.17	0.00118 (0.00091−0.00149)	5.9
deltamethrin	1.4 ± 0.13	0.00029 (0.00023−0.00038)	0.7 ± 0.09	0.09163 (0.05768−0.14535)	316.0
α-cypermethrin	3.5 ± 0.43	0.00047 (0.00041−0.00052)	0.9 ± 0.08	0.04435 (0.02895−0.06735)	94.4
bifenthrin	2.2 ± 0.23	0.00125 (0.00104−0.00149)	0.8 ± 0.08	0.01120 (0.00658−0.01819)	9.0
fenthion	1.9 ± 0.16	0.0031 (0.0025−0.0037)	1.9 ± 0.19	3.6549 (3.0720−4.4051)	1179.0
chlorpyrifos	2.5 ± 0.26	0.0034 (0.0028−0.0040)	1.5 ± 0.12	3.9898 (3.1956−5.0173)	1173.5
fenitrothion	1.9 ± 0.19	0.0091 (0.0074−0.0113)	1.3 ± 0.18	3.8930 (2.8366−5.0950)	427.8
permethrin	1.5 ± 0.13	0.0079 (0.0062−0.0102)	1.1 ± 0.09	0.0541 (0.0398−0.0740)	6.8
chlorfenapyr	1.6 ± 0.14	0.0108 (0.0086−0.0136)	0.7 ± 0.09	2.4312 (1.5035−3.9500)	225.1
etofenprox	2.0 ± 0.18	0.0186 (0.0153−0.0226)	1.0 ± 0.09	0.1183 (0.0833−0.1676)	6.4
temephos	1.1 ± 0.16	0.016 (0.011−0.022)	1.9 ± 0.29	0.068 (0.054−0.096)	4.3

[a] CL denotes confidence limit. [b] Resistance ratio.

Table 2. Toxicity of Fractions from Solvent Partition of the Methanol Extract of *Asarum heterotropoides* Root to Third Instar Larvae from Three Mosquito Species Using Direct-Contact Mortality Bioassay During a 24 h Exposure

	LC_{50}, ppm (95% CL[a])		
material	*Culex pipiens pallens*	*Aedes aegypti*	*Ochlerotatus togoi*
methanol extract	15.87 (12.91−20.06)	17.68 (14.28−21.72)	17.54 (14.73−21.49)
hexane-soluble fr	7.12 (5.57−10.55)	8.56 (6.19−10.79)	9.06 (7.32−11.55)
chloroform-soluble fr	14.47 (11.67−19.97)	13.68 (11.48−16.30)	16.49 (13.51−20.79)
ethyl acetate-soluble fr	16.87 (13.40−22.40)	17.54 (14.73−21.49)	19.15 (15.43−25.50)
butanol-soluble fr	33.51 (29.48−38.84)	52.42 (40.93−77.50)	45.19 (40.54−53.23)
water-soluble fr	>200	>200	>200

[a] CL denotes confidence limit.

colony of *C. p. pallens* was evaluated as stated above (**Table 1**). DJ-CP larvae exhibited extremely high levels of resistance to fenthion (RR, 1179), chlorpyrifos (RR, 1174), fenitrothion (RR, 428), deltamethrin (RR, 316), and chlorfenapyr (RR, 225), high level of resistance to α-cypermethrin (RR, 94), and low levels of resistance to bifenthrin, permethrin, etofenprox, β-cyfluthrin, and temephos (RR, < 9) compared to KS-CP larvae.

Bioassay-Guided Fractionation and Isolation. Fractions obtained from the methanol extract of *A. heterotropoides* root were bioassayed against third instar larvae from *C. p. pallens*, *A. aegypti*, and *O. togoi* by the direct contact application (**Table 2**). Significant differences in larvicidal activity in fractions of the extract were observed, and they were used to identify peak activity fractions for the next step in the purification. After 24 h of exposure, the hexane-soluble fraction was significantly toxic than the chloroform- and ethyl acetate-soluble fractions. Weak and no toxicity were produced by the butanol- and water-soluble fractions, respectively. There was no mortality in controls for any the species in this study.

Direct-contact mortality bioassay-guided fractionation of *A. heterotropoides* root extract afforded five active principles identified by spectroscopic analyses, including MS and NMR. The five active principles were methyleugenol (**1**), pentadecane (**2**), α-asarone (**3**), (−)-asarinin (**4**), and pellitorine (**5**) (**Figure 1**). α-Asarone (**3**) was identified on the basis of the following evidence: viscous oil; UV (EtOH) λ_{max} = 254. EI-MS (70 eV), *m*/*z* (rel int): 208 $[M]^+$ (100, base peak), 193, 177, 165, 134, 77, 69. ^{1}H NMR (CD_3OD, 600 MHz) δ 3.32 (2H, d, *J* = 5.4 Hz), 3.73 (1H, s), 3.75 (1H, s), 3.78 (1H, s), 3.80 (3H, s), 4.83 (3H, s), 5.03 (1H, s), 5.93 (1H, m), 6.48 (2H, s), 6.72 (1H, s). ^{13}C NMR (CD_3OD, 150 MHz) δ 34.8 q, 56.7 q, 56.9 q, 57.0 q, 99.7 d, 115.3 s, 116.1 d, 121.6 d, 137.8 s, 144.4 d, 149.9 s, 154.5 s. (−)-Asarinin (**4**): white powder or needle; $[\alpha]_D^{15.2}$: −152° (*c* +003; chloroform). UV (EtOH) λ_{max} = 241. EI-MS (70 eV), *m*/*z* (rel int) 354 $[M]^+$ (100, base peak), 323, 203, 178, 161, 149, 135, 122. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.85 (1H, dd, *J* = 4.2 and 13.2 Hz), 3.30 (2H, m), 3.83 (2H, m), 4.10 (1H, d, *J* = 11.4 Hz), 4.40 (1H, d, *J* = 6.6 Hz), 4.82 (1H, d, *J* = 7.2 Hz), 5.95 (2H, d, *J* = 5.4 Hz), 5.97 (2H, d, *J* = 10.2 Hz), 6.79 (4H, m), 6.86 (1H, s), 6.88 (1H, s). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 50.3 t, 54.8 t, 69.8 d, 71.1 d, 82.2 d, 87.8 d, 101.1 t, 101.3 t, 106.6 d, 106.8 d, 108.3 d, 108.4 d, 118.8 d, 119.7 d, 132.4 s, 135.3 s, 146.7 s, 147.4 s, 147.8 s, 148.1 s. Pellitorine (**5**): viscous oil; UV (EtOH) λ_{max} = 220. EI-MS (70 eV), *m*/*z* (rel int) 223 $[M]^+$ (100, base peak), 208, 180, 167, 152, 113, 96, 72. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 0.88 (3H, s), 0.91 (3H, s), 0.93 (3H, s), 1.28 (4H, m), 1.37 (2H, m), 1.76 (1H, m), 2.13 (2H, dd, *J* = 7.0 and 13.8 Hz), 3.16 (2H, t, *J* = 6.4 and 12.9 Hz), 5.60 (1H, br s), 5.76 (1H, d, *J* = 15.0 Hz), 6.09 (2H, m), 7.19 (1H, d, *J* = 15.0 Hz). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 14.0 q, 2 × 20.1 q, 22.5 t, 28.5 t, 28.6 d, 31.4 t, 32.9 t, 46.9 t, 121.7 d, 2 × 128.2 d, 141.2 d, 166.4 s. Structures of methyleugenol (**1**) and pentadecane (**2**) were similarly determined. The interpretations of proton and carbon signals of compounds **1**, **2**, **3**, **4**, and **5** were largely consistent with those of Kelm et al. (*21*), Siddiqui et al. (*22*), Siergiejczyk et al. (*23*), Ju et al. (*24*), and Park et al. (*25*), respectively.

Larvicidal Activity of Test Compounds. The toxicity of five isolated compounds to third instar larvae from KS-CP strain and DJ-CP colony of *C. p. pallens* was evaluated as stated above (**Table 3**). On the basis of 24 h LC_{50} values, pellitorine was 5.5, 10.8, 25.6, and 46.9 times more toxic than (−)-asarinin, α-asarone, methyleugenol, and pentadecane against KS-CP larvae, respectively. Overall, all the compounds were less toxic than either fenthion or temephos (**Table 2**). Interestingly, all four compounds and *A. heterotropoides* root extract were of equal toxicity against both the KS-CP and DJ-CP larvae, indicating a lack of cross-resistance in the DJ-CP.

Toxic effects on third instar larvae from *A. aegypti* of five test compounds and two larvicides were likewise compared (**Table 4**).

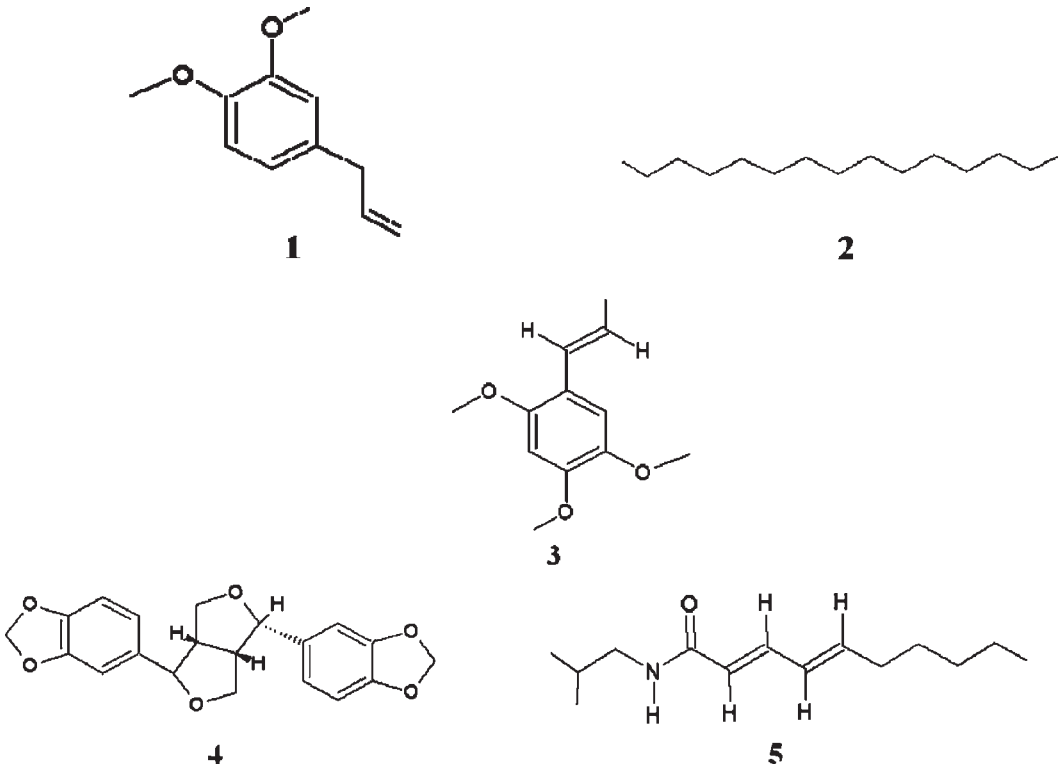

Figure 1. Structures of methyleugenol (**1**), pentadecane (**2**), α-asarone (**3**), (−)-asarinin (**4**), and pellitorine (**5**).

Table 3. Toxicity of *Asarum heterotropoides* Root Constituents to Third Instar Larvae from Insecticide-Susceptible KS-CP strain and Field-Collected DJ-CP Colony of *Culex pipiens pallens* Using Direct-Contact Mortality Bioassay During a 24 h Exposure

	KS-CP larvae		DJ-CP larvae	
material	slope ± SE	LC_{50}, ppm (95% CL[a])	slope ± SE	LC_{50}, ppm (95% CL[a])
MeOH ext	2.3 ± 0.32	14.25 (11.95−17.05)	2.2 ± 0.31	16.26 (13.64−19.75)
pellitorine	2.3 ± 0.44	2.08 (1.68−2.92)	2.2 ± 0.28	2.19 (1.81−2.68)
(−)-asarinin	1.5 ± 0.39	11.45 (8.48−16.88)	1.7 ± 0.40	12.69 (9.89−18.05)
α-asarone	2.7 ± 0.40	22.38 (19.22−26.00)	3.1 ± 0.42	23.82 (20.87−27.62)
methyleugenol	7.0 ± 1.00	53.30 (50.23−56.43)	7.8 ± 1.19	67.02 (63.75−70.87)
pentadecane	6.2 ± 1.08	97.58 (91.08−109.62)	7.1 ± 1.47	117.93 (106.00−149.00)

[a] CL denotes confidence limit.

Pellitorine was 4.5-, 11.6-, 24.7-, and 41.5-fold more toxic than (−)-asarinin, α-asarone, methyleugenol, and pentadecane, respectively. These compounds were less toxic than either fenthion or temephos.

Against third instar larvae from *O. togoi*, pellitorine was 6.9, 11.1, 24.6, and 41.7 times more toxic than (−)-asarinin, α-asarone, methyleugenol, and pentadecane, respectively (**Table 5**). These compounds were less toxic than either fenthion or temephos.

Species Susceptibility. On the basis of SR (**Tables 4** and **5**), there were no significant differences in the toxicity of each test compound among *C. p. pallens*, *A. aegypti*, and *O. togoi* larvae.

Table 4. Toxicity of Test Compounds and Two Larvicides to Third Instar Larvae from *Aedes aegyptii* Using Direct-Contact Mortality Bioassay During a 24 h Exposure[a]

compound	slope ± SE	LC_{50}, ppm (95% CL[b])	SR
pellitorine	2.0 ± 0.26	2.33 (1.89−2.93)	1.1
(−)-asarinin	2.6 ± 0.21	10.49 (9.03−12.09)	0.9
α-asarone	2.8 ± 0.42	26.99 (23.42−31.88)	1.2
methyleugenol	6.4 ± 1.00	57.65 (54.25−61.87)	1.1
pentadecane	7.9 ± 1.18	96.71(91.46−105.35)	0.9
fenthion	1.9 ± 0.28	0.0043 (0.0035−0.0054)	1.4
temephos	1.2 ± 0.17	0.017(0.013−0.023)	1.0

[a] Susceptibility ratio = LC_{50} of *A. aegypti* larvae/LC_{50} of *C. p. pallens* larvae. [b] CL denotes confidence limit.

DISCUSSION

Various compounds, including phenolics, terpenoids, and alkaloids, exist in plants (*26*). They jointly or independently contribute to behavioral efficacy such as repellence and feeding deterrence and physiological efficacy such as acute toxicity and developmental disruption against various arthropod species (*11*, *12*). Many plant preparations and their constituents manifest larvicidal activity against different mosquito species (*10*, *17*, *25*) and have been proposed as alternatives to the widely available larvicides. For example, it has been reported that the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A possess potent larvicidal activity against larvae of *C. p. pallens*, *A. aegypti*, and *O. togoi*. and *A. aegypti* and *O. togoi* larvae were more tolerant than *C. pipiens pallens* larvae to these compounds (*25*). In the current study, the larvicidal principles of *A. heterotropoides* root were identified as the phenylpropanoids methyleugenol (**1**) and α-asarone (**3**), the saturated hydrocarbon

Table 5. Toxicity of Test Compounds and Two Larvicides to Third Instar Larvae from *Ochlerotatus togoi* Using Direct-Contact Mortality Bioassay During a 24 h Exposure[a]

compound	slope ± SE	LC_{50}, ppm (95% CL[b])	SR
pellitorine	1.8 ± 0.27	2.38 (1.91−3.08)	1.1
(−)-asarinin	1.9 ± 0.30	16.49 (13.51−20.79)	1.4
α-asarone	2.8 ± 0.40	26.38 (22.70−31.39)	1.2
methyleugenol	7.4 ± 1.18	58.52 (54.64−61.71)	1.1
pentadecane	8.4 ± 1.26	99.19 (93.81−108.10)	1.0
fenthion	1.5 ± 0.18	0.0047 (0.0039−0.0059)	1.5
temephos	1.7 ± 0.31	0.020 (0.019−0.040)	1.2

[a] Susceptibility ratio = LC_{50} of *O. togoi* larvae/LC_{50} of *C. p. pallens* larvae. [b] CL denotes confidence limit.

pentadecane (**2**), the lignan (−)-asarinin (**4**), and the isobutylamide alkaloid pellitorine (**5**). No significant difference in toxicity among three mosquito species larvae was observed. Pellitorine and (−)-asarinin were highly effective against three mosquito species larvae. In addition, these compounds were also effective against *C. p. pallens* larvae resistant to various insecticides. This original finding indicates that the *A. heterotropoides* root-derived materials may hold promise for the development of novel and effective mosquito larvicides even against currently insecticide-resistant mosquito populations.

Investigations on the modes of action and the resistance mechanisms of natural insecticidal products are of practical importance for mosquito control because it may give useful information on the most appropriate formulations and delivery means to be adapted for their future commercialization and for future resistance management. Major mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sodium channel sensitivity to pyrethroid insecticides or acetylcholinesterase sensitivity to OP and carbamate insecticides and enhanced metabolism of various groups of insecticides (*27*). Alternative control agents with novel modes of action, low mammalian toxicity, and little environmental impact are urgently needed. Additionally, certain plant-derived compounds were found to be highly effective against insecticide-resistant insect species (*28*, *29*), and they are likely to be useful in resistance management strategies. Although available information is limited in mosquitoes, the phenylpropanoids such as ethyl cinnamate and ethyl *p*-methoxycinnamate are effective against larvae from field-collected *C. p. pallens* with low levels of resistance to fenthion and temephos (*17*). Our current finding that pellitorine, (−)-asarinin, α-asarone, and methyleugenol are virtually equal in toxicity to both insecticide-susceptible and -resistant larvae of *C. p. pallens* suggests that these compounds and the pyrethroid, OP, and pyrrole insecticides do not share a common mode of action or elicit cross-resistance. Detailed tests are needed to fully understand the modes of action of the isolated compounds, although the octopaminergic and γ-aminobutyric acid (GABA) receptors have been suggested as novel target sites for some essential oil constituents by Kostyukovsky et al. (*30*) and Priestley et al. (*31*), respectively.

In conclusion, *A. heterotropoides* root-derived materials containing pellitorine and (−)-asarinin could be useful as larvicides in the control of mosquito populations, particularly due to their activity against insecticide-resistant mosquito larvae. For the practical use of these materials as novel mosquito larvicides to proceed, further research is needed to establish their safety to human health, nontarget aquatic organisms, and the aquatic environment. In addition, formulations for improving larvicidal potency and stability, thereby reducing costs, need to be developed.

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Received for review June 8, 2010. Revised manuscript received August 9, 2010. Accepted August 12, 2010. This work was supported by the Rural Development Administration (BioGreen 21 program PJ007109) and the Ministry of Education, Science and Technology of the Korean Government (World Class University program R31-10056) to Y.J.A.