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Emodin Isolated from *Cassia obtusifolia* (Leguminosae) Seed Shows Larvicidal Activity against Three Mosquito Species

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Mosquito larvicidal activity of *Cassia obtusifolia* (Leguminosae) seed-derived materials against the fourth-instar larvae of *Aedes aegypti*, *Aedes togoi*, and *Culex pipiens pallens* was examined. The chloroform fraction of *C. obtusifolia* extract showed a strong larvicidal activity of 100% mortality at 25 mg/L. The biologically active component of *C. obtusifolia* seeds was characterized as emodin by spectroscopic analyses. The LC_{50} values of emodin were 1.4, 1.9, and 2.2 mg/L against *C. pipiens pallens*, *A. aegypti*, and *A. togoi*, respectively. Pirimiphos-methyl acts as a positive control directly compared to emodin. Pirimiphos-methyl was a much more potent mosquito larvicide than emodin. Nonetheless, emodin may be useful as a lead compound and new agent for a naturally occurring mosquito larvicidal agent. In tests with hydroxyanthraquinones, no activity was observed with alizarin, danthron, and quinizarin, but purpurin has an apparent LC_{50} value of ∼19.6 mg/L against *A. aegypti*.

KEYWORDS: ***Aedes aegypti*; *Cassia obtusifolia*; *Culex pipiens pallens*; emodin; mosquito larvicidal activity**

INTRODUCTION

The yellow fever mosquitoes, *Aedes aegypti* (L.) and *Aedes togoi* (Theobald), and the northern house mosquito, *Culex pipiens pallens* (Coquillett), are widespread and serious primary medical insect pests. Control of these mosquito larvae is frequently dependent on continued applications of organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene (*1*). Although effective, their repeated use has disrupted natural biological control systems and led to outbreaks of insect species, sometimes resulted in the widespread development of resistance, had undesirable effects on nontarget organisms, and fostered environmental and human health concerns (*2*−*6*). These problems have highlighted the need for the development of new strategies for selective mosquito larval control.

Plants may be an alternative source of mosquito larval agents because they constitute a rich source of bioactive chemicals (*7*, *8*). Much effort has, therefore, been focused on plant extracts or phytochemicals as potential sources of commercial mosquito-control agents or as bioactive chemical compounds (*9*, *10*). The current authors have already reported and confirmed that among 25 leguminous seeds, the methanol extract of *Cassia obtusifolia* and *Cassia tora* seeds exhibits a potent larvicidal activity against *A. aegypti* and *Cu. pipiens pallens* (*11*). These plant species are not only important as insecticides but also considered to possess some medicinal properties, such as an antiseptic, antioxidant, and antimutagen (*9*, *12*−*15*). However, despite of all these studied activities, relatively little work has been carried out on the larvicidal effects of *Ca. obtusifolia* seed-derived materials against mosquito larvae. In this paper, we assessed the larvicidal activity of the active component isolated from *Ca. obtusifolia* seeds, the synthetic larvicide pirimiphos-methyl, and four commercially available hydroxyanthraquinones against early fourth-instar larvae of *A. aegypti*, *A. togoi*, and *Cu. pipiens pallens*.

MATERIALS AND METHODS

Chemicals. Alizarin, danthron, emodin, purpurin, quinizarin, and Triton X-100 were provided from Fluka Chemical Corp. (Milwaukee, WI). All other chemicals were of reagent grade.

Insects. Laboratory F21 strain of *A. aegypti* was obtained in 2000 from the National Institute of Health, Seoul, South Korea. *Cu. pipiens pallens* and *A. togoi* were collected at Seoho stream, Suwon (Kyunggi Province), South Korea. Adult mosquitoes were maintained on a 10% aqueous sucrose solution and blood from a live mouse, whereas larvae were reared in a plastic butt (24 × 35 × 5 cm) and fed a sterilized diet (80:20 mix of chick chow powder/yeast). Mosquitoes were held at 28 ± 2 °C and 70 ± 5 °C relative humidity under a photoregime of 16:8 h (L/D).

Extraction and Isolation. The seeds of *Ca. obtusifolia* were purchased from a local market in Chonju and identified by Prof. Sang-Hyun Lee (Forestry Department, Chonbuk National University, South Korea). *Ca. obtusifolia* seeds (4.9 kg) were ground in a blender, extracted twice with methanol (10 L) at room temperature for 2 days, and filtered. The combined filtrate was concentrated under vacuum at 45 °C to yield 11.8%. The extract (20 g) was sequentially partitioned into hexane (1.5 g), chloroform (3.9 g), ethyl acetate (2.5 g), butanol

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(3.4 g), and water-soluble (8.7 g) portions. The organic solvent portions were concentrated to dryness by rotary evaporation at 45 °C, whereas the water portion was freeze-dried. For isolation, *Ca. obtusifolia* seed-derived fractions were bioassayed against fourth-instar larvae of *A. aegypti*.

The chloroform (15 g) portion was chromatographed on a silica gel column (Merck 70−230 mesh, 900 g, 6.0 i.d. × 90 cm), and successively eluted with a stepwise gradient of chloroform/methanol (0, 10, 20, 30, 40, and 50%). The active 30% fraction (5.9 g) of the chloroform fraction showed a strong larvicidal activity (100% mortality) against *A. aegypti* at 25 mg/L. The active 30% fraction was chromatographed on a silica gel column and eluted with chloroform/methanol (30:1). Column fractions were analyzed by TLC (silica gel 60 F_{254}, chloroform/methanol, 30:1), and fractions with similar TLC patterns were combined. The bioactive fraction (2.8 g) showed a strong larvicidal activity (100% mortality) against *A. aegypti* at 25 mg/L. The bioactive fraction was chromatographed over a Sephadex LH-20 column (Pharmacia, 800 × 49 mm) using chloroform/acetone/methanol (50:1:2). This operation was repeated three times. Active fraction (718 mg) showed a strong larvicidal activity (100% mortality) against *A. aegypti* at 25 mg/L. The active fraction was chromatographed over a Polyclar AT column (Touzart and Matignon, 100 g) packed with chloroform/acetone (50:1, v/v) and eluted with an increasing ratio of methanol (1, 2, 5, 10, and 20%). Active fraction (205 mg) showed a strong larvicidal activity (100% mortality) against *A. aegypti* at 25 mg/L. The active fraction was finally purified successively on a Sephadex LH-20 column (Pharmacia) eluted with chloroform/methanol (6:4, v/v) and cellulose (Merck) eluted with chloroform/methanol (6:4, v/v). Finally, the active compound (79 mg) was isolated. Structural determination of the active isolate was made by spectral analysis. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer. UV spectra were obtained on a Waters 490 spectrometer, IR spectra on a Bio-Rad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Bioassay. Concentrations of *Ca. obtusifolia* extract and the fractions were prepared by serial dilution of a stock solution of the sample in ethanol. Each sample in ethanol was emulsified in distilled water with Triton X-100 added at the rate of 10 mL/L. Groups of 25 early fourth-instar larvae of *A. aegypti*, *A. togoi*, and *Cu. pipiens pallens* were placed into paper cups (270 mL) containing each test solution (250 mL), using a micropipet. The toxicity of each sample was determined at 25, 20, 10, 5, 2.5, 1.0, 0.5, 0.25, 0.125, 0.1, and 0.05 mg/L. Controls received ethanol/Triton X-100 solution only. Treated and control larvae were held at the same conditions mentioned earlier. Larvicidal activity was evaluated 24 h after treatment. Larvae were considered to be dead if appendages did not move when prodded with a wooden dowel. All treatments were replicated four times. No mortality was observed in any control group. Lethal concentration 50 (LC_{50}) is median lethal concentration.

Statistical Analyses. The percentage of mortality was determined and transformed to arcsine square-root values for analyses of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at $P = 0.05$ (*16*). Means (± SE) of untransformed data are reported. LC_{50} values were calculated by Probit analysis (*17*).

RESULTS AND DISCUSSION

During the initial experiments, the larvae of *A. aegypti* were selected as reference mosquito because the current authors had already reported that the extract of *Ca. obtusifolia* and *Ca. tora* exhibits a potent larvicidal activity against early fourth-instar larvae of *A. aegypti* and *Cu. pipiens pallens* (*11*). The methanolic extract of *Ca. obtusifolia* seed possessed mosquito larvicidal activity against *A. aegypti*, as did the chloroform fraction, producing 100% mortality at 25 mg/L (not shown). However, no activity was produced from any of the other fractions even at 40 mg/L. Leguminosae plants have been known for their use as antimicrobial agents and also for their strong mosquito larvicidal materials (*11*, *12*). In our study, one active isolate from the chloroform fraction showed strong larvicidal activity against *A. aegypti* (**Table 1**), and it was characterized by spectral analyses as emodin (**Figure 1**). The compound was identified on the basis of the following evidence: orange needles from EtOH, mp 260−263 °C; IR (KBr) υ_{max} 3425 (O−H), 1677, 1627 (C═O) cm^{-1}; UV (MeOH) λ_{max} nm (log ϵ) 248 (4.11), 262 (4.12), 285 (4.18), 432 (3.92); ^{1}H NMR [$(CD_3)_2CO$] δ 2.46 (3H, s, Ar-CH_3), 6.65 (1H, d, $J = 2.5$ Hz, H-2), 7.12 (1H, br s, H-7), 7.24 (1H, d, $J = 2.5$ Hz, H-4), 7.55 (1H, br s, H-5), 12.05 (1H, s, OH), 12.18 (1H, s, OH); HREIMS, *m*/*z* 270.0515 ($C_{15}H_{10}O_5$); ^{13}C NMR [$(CD_3)_2CO$] δ 191.9, 182.2, 167.0, 165.9, 161.9, 149.3, 136.6, 134.0, 124.8, 121.2, 114.4, 110.0, 109.7, 108.6, 21.8. The isolation and spectral analyses of emodin from *Ca. obtusifolia* have already been reported for the study of anthraquinones isolated from *Heterodermia obscurata* (*18*). Our data are identical to the data of Cohen and Towers (*18*).

Table 1. Mosquito Larvicidal Activity of Constituent Derived from *Ca. obtusifolia* Seeds and Hydroxyanthraquinones against Fourth-Instar Larvae of *A. aegypti*

	mortality (%, mean[a] ± SE)					
compound	20.0 mg/L	10.0 mg/L	5.0 mg/L	2.5 mg/L	1.0 mg/L	LC_{50}[b]
alizarin	0 ± 0.0d	NT[c]	NT	NT	NT	0
danthron	0 ± 0.0d	NT	NT	NT	NT	0
emodin	100a	100a	100a	58.4 ± 2.4b	19.6 ± 3.1[c]	1.9
purpurin	51.1 ± 3.5b	14.2 ± 3.5c	0 ± 0.0d	NT	NT	19.6
quinizarin	0 ± 0.0d	NT	NT	NT	NT	0

[a] $P = 0.05$, Scheffe's test (SAS Institute). [b] Dose expressed in mg/L. [c] Not tested.

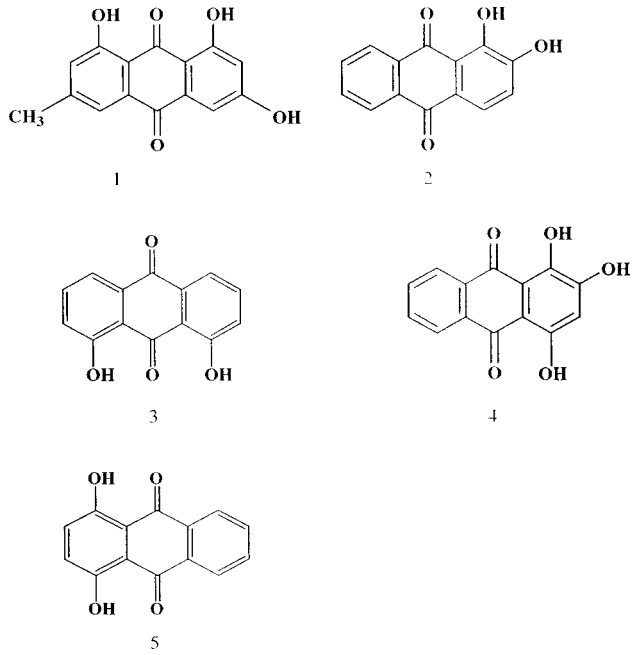

Figure 1. Structures of hydroxyanthraquinones: **1**, emodin (3-methyl-1,6,8-trihydroxyanthraquinone, C-15); **2**, alizarin (1,2-dihydroxyanthraquinone, C-14); **3**, danthron (1,8-dihydroxyanthraquinone, C-14); 4, purpurin (1,2,4-hydroxyanthraquinone, C-14); **5**, quinizarin (1,4-dihydroxyanthraquinone, C-14).

Emodin has apparent LC_{50} values of approximately 1.4, 1.9, and 2.2 mg/L against *Cu. pipiens pallens*, *A. aegypti*, and *A. togoi* (**Table 2**). Recently, two reports showed that the extract of *Tagetes minuta* L. had strong biocidal effects on both the larvae and adults of *A. aegypti* L. and *Anopheles stephensi* L. (*19*). The insecticidal components isolated from the plant extract were four thiophenes: 5-(but-3-en-1-ynyl)-2,2′-bithiophene, 5-(but-3-en-1-ynyl)-5′-methyl-2,2′-bithiophene, 2,2′,5′,5″-ter-

Table 2. Mosquito Larvicidal Activity of Emodin Derived from *Ca. obtusifolia* Seeds and Organophosphorus Insecticide against Fourth-Instar Larvae of Various Mosquitoes

compound	mosquito species	slope ± SE	LC_{50} (mg/L)	95% CL[a]
emodin	*C. pipiens pallens*	1.95 ± 0.19	1.4	1.18–1.88
	A. aegypti	2.31 ± 0.27	1.9	1.42–2.58
	A. togoi	2.67 ± 0.35	2.2	1.69–3.07
pirimiphos-methyl	*C. pipiens pallens*	1.29 ± 0.15	0.13	0.10–0.16
	A. aegypti	1.55 ± 0.21	0.16	0.12–0.19
	A. togoi	1.78 ± 0.16	0.21	0.18–0.26

[a] Confidence limit.

thiophene, and 5-methyl-2,2′,5′,2″-terthiophene (*20*). These compounds eventually may be considered as alternatives to the currently used insecticides. Furthermore, emodin isolated from *Rhamnus alnifolia* had strong functions as a deterrent to foliage-feeding insects such as gypsy moth larvae and eastern tent caterpillar larvae (*21*). There was little attack on foliage of *R. alnifolia* in wild stands compared with associated species of woody plants. In this present study, the two *Aedes* species tested were more tolerant to emodin than *Cu. pipiens pallens*. Emodin may be useful as a lead compound and new agent for a naturally occurring mosquito larvicidal agent.

To assess mosquito larvicidal activity of hydroxyanthraquinones structurally related to alizarin, danthron, purpurin, and quinizarin (**Figure 1**), four commercially available hydroxyanthraquinones were tested against *A. aegypti* (**Table 1**). In our study, no activity was observed for alizarin, danthron, and quinizarin, even at 20 mg/L. Purpurin has an apparent LC_{50} value of ~19.6 mg/L against *A. aegypti* (**Table 1**). Emodin could be useful for managing field populations of *Cu. pipiens pallens*, *A. aegypti*, and *A. togoi*, although the mosquito larvicidal activity of emodin was lower than that of pirimiphos-methyl, a commonly used insecticide (**Table 2**). In the pioneering work on three series of analogues or derivatives of α-terthienyl as mosquito larvicides, only the methyl-substituted derivative of α-terthienyl increased phototoxicity against mosquito larvae (*22*). The methyl terthienyl is the only naturally occurring derivative, probably suggesting that evolutionary pressure by insects on plants has selected efficient structures for plant defense. In this study, the data of emodin (3-methyl-1,6,8-trihydroxyanthraquinone) in four commercially available hydroxyanthraquinones are similar to those of the methyl terthienyl. In the iodo series, activity declines rapidly in the order of increasing iodination. In the third series, substitution of the middle ring with a benzene ring slightly reduces activity, whereas pyridine substitution reduces the phototoxic effect by an order of magnitude. In this regard, further studies on the insecticidal mode of action of *Ca. obtusifolia* seed-derived compounds, their effects on nontargent organisms and the environment, and formulations for improving the insecticidal potency and stability are needed for their practical use as a naturally occurring mosquito larval control agent.

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