JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 969−972 **969**

Larvicidal Activity of Lignans Identified in *Phryma leptostachya* Var. *asiatica* Roots against Three Mosquito Species

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The insecticidal activity of phytochemicals isolated from the roots of *Phryma leptostachya* var. *asiatica* against third instar larvae of *Culex pipiens pallens*, *Aedes aegypti*, and *Ocheratatos togoi* was examined. The two constituents of *P. leptostachya* var. *asiatica* roots were identified as the leptostachyol acetate (**I**) and 8′-acetoxy-2,2′,6-trimethoxy-3,4,4′,5′-dimethylenedioxyphenyl-7,7′-dioxabicyclo-[3.3.0]octane (**II**) by spectroscopic analysis. Compound **I** was lethal to *C. pipiens pallens*, *A. aegypti*, and *O. togoi* at 10 ppm. Compound **II** showed weak or no insecticidal activity against three mosquito species at 10 ppm. The LC_{50} values of **I** against *C. pipiens pallens*, *A. aegypti*, and *O. togoi* were 0.41, 2.1, and 2.3 ppm, respectively. Naturally occurring *P. leptostachya* var. *asiatica* root-derived compounds merit further study as potential mosquito larval control agents or lead compounds.

KEYWORDS: Natural insecticides; mosquito larvicides; mosquito; *Phryma leptostachya* var. asiatica, lignans

INTRODUCTION

The northern house mosquito, *Culex pipiens pallens* (Coquillett), and the yellow fever mosquitoes, *Aedes aegypti* (L.) and *Ocheratatos togoi* (Theobald), are widespread and serious disease vectoring insect pests. Mosquito abatement primarily depends on continued applications of organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene, which are still the most effective larvicides (*1*). Although effective, their repeated use has disrupted natural enemies and led to outbreaks of some insect species, resulted in the development of resistance, had undesirable effects on nontarget organisms, and fostered environmental and human health concerns (*1*−*3*). These problems have highlighted the need for the development of new strategies for selective control of mosquito larvae.

Plants may be an alternative source of mosquito larval control agents because they constitute a rich source of bioactive chemicals. Much effort has, therefore, been focused on plant extracts or phytochemicals as potential sources of commercial mosquito control agents or as lead compounds (*4*−*8*). Sukumar et al. (*6*) already pointed out that the most promising botanical mosquito control agents are in the families Asteraceae, Cladophoraceae, Labiatae, Meliaceae, Oocystaceae, and Rutaceae.

The herbaceous perennial plant *Phryma leptostachya* var. *asiatica* has been traditionally used as a natural insecticide in East Asia (*9*−*11*). Although several insecticidal lignans have been isolated from the root of this species, little works have been done with respect to control mosquito larvae. This paper describes a laboratory study aimed at isolating insecticidal constituents from *P. leptostachya* var. *asiatica* active against third instar larvae of *C. pipiens pallens*, *A. aegypti*, and *O. togoi*.

MATERIALS AND METHODS

Chemicals. Triton X-100 was purchased from Shinyo Pure Chemicals (Osaka). All other chemicals were of reagent grade.

Insects. Cultures of *C. pipiens pallens*, *A. aegypti*, and *O. togoi* were maintained in the laboratory without exposure to any insecticide. Adult mosquitoes were maintained on a 10% sucrose solution and blood from a live mouse, while larvae were reared in plastic butts (24 cm × 35 cm × 5 cm) containing sterilized diet (40 mesh chick chow powder/yeast, 4:1). They were held at 27 ± 1 °C and 70−80% relative humidity under a 14:10 h light:dark cycle.

Isolation and Identification. Dried roots (4 kg) of *P. leptostachya* var. *asiatica* were collected at JeJu Island, Korea. They were finely powdered, extracted twice with methanol at room temperature for 2 days, and filtered. The combined filtrate was concentrated in vacuo at 40 °C to yield ∼2.05% (based on the weight of the air-dried roots).

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10.1021/jf048208h CCC: $30.25
Published on Web 01/26/2005

Table 1. Insecticidal Activity of *P. leptostachya* var. *asiatica* Root-Derived Materials against Third Instar Larvae of *C. pipiens pallens*, *A. aegypti*, and *O. togoi*

	mortality (%) (mean ± SE)[b]		
solvent fractions[a]	*C. pipiens pallens*	*A. aegypti*	*O. togoi*
methanol extract	100 a	100 a	100 a
chloroform fraction	100 a	100 a	100 a
water fraction	3.3 ± 3.3 b	0 b	3.3 ± 3.3 b

[a] Exposed for 24 h at a concentration of 50 ppm. [b] Means within a column followed by the same letter are not significantly different ($P = 0.05$, Scheffe's test). Mortalities were transformed to arcsine square root before ANOVA. Means (±SE) of untransformed data are reported.

The extract (20 g) was sequentially partitioned into chloroform (13 g) and water soluble (7 g) layers for subsequent bioassay. The organic solvent layers were concentrated to dryness by rotary evaporation at 40 °C. The active chloroform fraction (10 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 600 g, 5.5 i.d. cm × 70 cm) and successively eluted with a stepwise gradient of chloroform/methanol (50:1, 25:1, 10:1, 5:1, and 0:100, by volume). Column fractions were analyzed by thin-layer chromatography (TLC) (silica gel 60 F_{254}), and fractions with similar TLC patterns were pooled.

The bioactive fraction was successively rechromatographed on a silica gel column, using chloroform/ethyl acetate (12:1, by volume) and hexane/ethyl acetate (2:1, by volume). Preparative high-performance liquid chromatography (Waters Delta Prep 600) was used for further separation of the constituents. The column was a Prodigy ODS (21.2 i.d. mm × 250 mm, Phenomenex) using acetonitrile/water (8:2, v/v) at a flow rate of 8 mL/min and detected at 254 nm. Finally, two potent active principles, **I** (12 mg) and **II** (14 mg), were isolated.

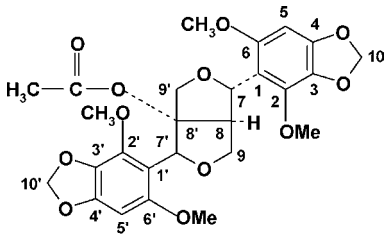
The structures of the active isolates were determined by spectroscopic analysis. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer at 400 and 100 MHz, respectively. UV spectra were obtained in methanol with a JASCO V-550 spectrometer, and mass spectra were obtained on a JEOL GSX 400 spectrometer.

Bioassay. The bioassay used in this experiment was well-described by Park et al. (*8*). In brief, concentrations of test compounds were prepared by serial dilution of a stock solution of the compounds in acetone. Each compound in acetone was suspended in distilled water with Triton X-100 (5 mL/L). Batches of 20 early third instar larvae of *C. pipiens pallens*, *A. aegypti*, and *O. togoi* were separately put into paper cups (270 mL) containing each test solution (250 mL) using a pipet. Treated and control larvae were held at the same conditions used for colony maintenance. Larvicidal activity was evaluated 24 h after treatment. The larvae were considered dead if appendages did not move when prodded with a wooden dowel. All treatments were replicated three times. The LC_{50} values were calculated by probit analysis (*12*).

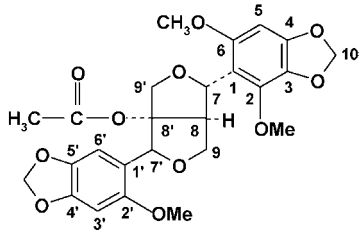
Statistical Analysis. The percentage of mortality was determined and transformed to arcsine square root values for analysis of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at $P = 0.05$ (*13*). Means (±SE) of untransformed data are reported.

RESULTS

Identification. When fractions obtained from the methanol extract of *P. leptostachya* var. *asiatica* roots were bioassayed, significant differences were observed in toxicity to mosquito larvae (**Table 1**). At a concentration of 50 ppm, the chloroform fraction showed potent larvicidal activity against *C. pipiens pallens*, *A. aegypti*, and *O. togoi*. There was no mortality in the untreated controls. Bioassay-guided fractionation of the *P. leptostachya* var. *asiatica* roots extract afforded two constituents identified by spectroscopic analyses, including MS and NMR, and by comparison with published data (*14*). The constituents were characterized as leptostachyol acetate (**I**) and 8′-acetoxy-2,2′,6-trimethoxy-3,4,4′,5′-dimethylenedioxyphenyl-7,7′-dioxabicyclo[3.3.0]octane (**II**) (**Figure 1**).



I



II

Figure 1. Structures of leptostachyol acetate (**I**) and 8′-acetoxy-2,2′,6-trimethoxy-3,4,4′,5′-dimethylenedioxyphenyl-7,7′-dioxabicyclo[3.3.0]-octane (**II**) isolated from *P. leptostachya* var. *asiatica* roots.

Leptostachyol Acetate (I). EI-MS *m*/*z*: 532 (M^+), 472, 375, 322, 262 (base ion), 195. ^{1}H NMR (500 MHz, CD_3OD): δ 1.77 (3H, *s*, alc-$COCH_3$), 3.57 (1H, *m*, H-8), 3.75 (1H, *dd*, $J = 2.0$, 8.0 Hz, H-9α), 3.78 (3H, *s*, OMe-2), 3.79 (3H, *s*, OMe-2′), 3.98 (3H, *s*, OMe-6), 4.00 (3H, *s*, OMe-6′), 4.24 (1H, *d*, $J = 9.5$ Hz, H-9′α), 4.35 (1H, *dd*, $J = 6.0$, 8.0 Hz, H-9*β*), 4.49 (1H, *d*, $J = 9.5$ Hz, H-9′*β*), 5.29 (1H, *d*, $J = 8.0$ Hz, H-7), 5.90 (1H, *m*, H-7′), 5.88 (2H, *s*, H-10), 5.93 (1H, *s*, H-10′), 6.39 (1H, *s*, H-5′), 6.44 (1H, *s*, H-5). ^{13}C NMR (125 MHz, CD_3OD): δ 19.84 (*q*, $COCH_3$), 55.93 (*q*, OMe-2), 56.10 (*q*, OMe-2′), 57.38 (*d*, C-8), 59.05 (*q*, OMe-6), 59.32 (*q*, OMe-6′), 71.08 (*t*, C-9), 75.83 (*t*, C-9′), 78.65 (*d*, C-7), 79.03 (*d*, C-7′), 89.47 (*d*, C-5), 89.69 (*d*, C-5′), 97.38 (*s*, C-8′), 101.23 (*t*, C-10), 101.30 (*t*, C-10′), 111.19 (*s*, C-1′), 111.95 (*s*, C-1), 131.21 (*s*, C-3), 131.43 (*s*, C-3′), 142.81 (*s*, C-6), 142.93 (*s*, C-6′), 149.85 (*s*, C-4), 150.05 (*s*, C-4′), 154.40 (*s*, C-2′, 2), 170.26 (*s*, *C*OCH_3) (**Figure 1**).

8′-Acetoxy-2,2′,6-trimethoxy-3,4,4′,5′-methylenedioxyphenyl-7,7′-dioxabicyclo[3.3.0]octane (II). EI-MS *m*/*z*: 502 (M^+), 442, 292, 263, 262 (base ion), 249, 211, 209, 196, 195, 191. ^{1}H NMR (500 MHz, CD_3OD): δ 1.67 (3H, *s*, alc-$COCH_3$), 3.12 (1H, *m*, H-8), 3.73 (3H, *s*, OMe-6), 3.80 (3H, *s*, OMe-2′), 3.93 (1H, *dd*, $J = 5.0$, 8.5 Hz, H-9α), 3.96 (3H, *s*, OMe-2), 4.16 (1H, *d*, $J = 10.5$ Hz, H-9′α), 4.46 (1H, *t*, $J = 8.8$ Hz, H-9*β*), 4.60 (1H, *d*, $J = 10.5$ Hz, H-9′*β*), 4.97 (1H, *d*, $J = 5.5$ Hz, H-7), 5.87 (1H, *d*, $J = 1.0$ Hz, H-7′), 5.88 (2H, *s*, H-10), 5.90 (2H, *s*, H-10′), 6.36 (1H, *s*, H-5), 6.67 (1H, *s*, H-5′), 6.93 (1H, *s*, H-2′). ^{13}C NMR (125 MHz, CD_3OD): δ 19.76 (*q*, $COCH_3$), 55.64 (*q*, OMe-6′), 55.86 (*q*, OME−2′), 60.81 (*d*, C-8), 72.35 (*t*, C-9), 78.15 (*d*, C-7′), 81.80 (*d*, C-7), 89.38 (*d*, C-5), 94.45 (*d*, C-5′), 97.66 (*d*, C-8′), 101.21 (*t*, C-10), 101.31 (*t*, C-10′), 105.52 (*d*, C-2′), 111.06 (*s*, C-1), 122.43 (*s*, C-3), 131.07 (*s*, C-1′), 141.45 (*s*, C-3′), 142.88 (*s*, C-2), 147.68 (*s*, C-4′), 149.95 (*s*, C-4), 151.70 (*s*, C-6′), 154.46 (*s*, C-6), 170.31 (*s*, *C*OCH_3) (**Figure 1**).

Larvicidal Activity. The toxicity of **I** was shown in **Table 2**. LC_{50} values of **I** on *C. pipiens pallens*, *A. aegypti*, and *O. togoi* larvae were on the basis of 24 h. Compound **I** was most toxic to *C. pipiens pallens* (0.41 ppm). LC_{50} values of **I** on *A. aegypti* and *O. togoi* larvae were 2.1 and 2.3 ppm, respectively. However, compound **II** revealed weak or no activity at 10 ppm.

Table 2. Toxicity of Leptostachyol Acetate against Third Instar Larvae of *C. pipiens pallens*, *A. aegypti*, and *O. togoi*

mosquito species[a]	slope (±SE)	LC_{50} (ppm)	95% CL[b]
C. pipiens pallens	2.60 ± 0.30	0.41	0.35–0.49
A. aegypti	2.39 ± 0.25	2.10	0.68–1.25
O. togoi	2.96 ± 0.28	2.30	0.65–1.21

[a] Exposed for 24 h. [b] CL denotes confidence limit.

DISCUSSION

It has been well-recognized that plant extracts and phytochemicals could be developed into products suitable for mosquito control because many of them are selective, are often biodegradable to nontoxic products, and may be applied to mosquito breeding places in the same way as conventional insecticides (*6*, *7*). Many plant extracts and essential oils possess larvicidal activity against various mosquito species (*5*−*8*, *15*, *16*). Certain plant-derived compounds were found to be highly effective against insecticide resistant insect pests (*17*−*19*).

The herbaceous perennial plant, *P. leptostachya* L., is widely distributed in the Himalayas, temperate Asia, and northern East America and is delineated as the representative of the monotypic family, Phrymaceae. The herb has been traditionally used as a natural insecticide in East Asia (*10*, *11*, *20*). From the root extract of this plant, various lignans of the 3,7-dioxabicylclo-[3.3.0]octanes (furofuran) type have been isolated (*10*, *11*, *21*). Phrymarolins I and II, which have a uniquely oxygenated 3,7-dioxabicyclo[3.3.0]octane skeleton, together with several highly oxygenated 1-acetoxy/hydroxy-2,6-diaryl-3,7-dioxabicyclo-[3.3.0]octane types of lignans such as **I**, have been isolated from a root extract of *P. leptostachya*. Phrymarolins I and II are not, by themselves, actively insecticidal to houseflies but show considerable synergystic activities to pyrethrin and a carbamate pesticide, carbarly, in preliminary tests (*10*, *11*). Another lignan isolated from *P. leptostachya* root, haedoxan A, proved active against houseflies by topical application (LD_{50} = 1.6 ng/fly with piperonyl butoxide), which was comparable to commercial synthetic pyrethroids (*22*). The larvae of some species of lepidopterous insects tested were also strongly affected by ingestion of a small amout of haedoxan A, which showed no repellency but caused early cessation of feeding and muscle relaxation and was followed by the insect's death. Flaccid paralysis of housefly intoxicated with haedoxan A was apparently similar to symptoms caused by nereistoxin, ryanodine, and reserpine.

In this present study, a methanol extract of *P. leptostachya* var. *asiatica* roots exhibited quite an effective larvicidal activity against *C. pipiens pallens*, *A. aegypti*, and *O. togoi*. The larvicidal constituents were identified as the lignans, **I** and **II**. The chemical structrue of two isolated compounds was very similar differing only by the methoxy group at C-2′ in **I**. However, there is a significant difference in insecticidal activity against three mosquito species. Compound **I** showed very strong activty against three mosquito (LC_{50} = 0.4 ppm/*C. pipiens pallens* larvae) species whereas the insecticidal activity of **II** was weak.

Structure−activity relationships in insecticidal activity of *Phryma* lignans have been well-studied by Yamauchi and Taniguchi (*23*, *24*). Several analogues modifying the 6-methoxy-2-methoxymethyl-3-(3,4-methylenedioxyphenyl)-1,4-benzodioxan-7-yl group of haedoxans and other unique *Phryma* lignans composing a series of phrymarolins were synthesized, and their insecticidal activity was examined. Among several analogues of haedoxans, analogues having the absolute configuration identical with that of natural lignans was highly insecticidal, and the 1,4-benzodioxanyl group, a series of sesquilignans of the genus *Phryma*, is an important structural requirement for their insecticidal activity, since phrymarolins, a class of dilignans deprived only of the 3-phenyl-2-hydroxymethyl-1,4-benzodioxane framework of haedoxans, are totally inactive. However, **I** was active agianst three mosquito species in our study although the chemical structure is very similar to that of phrymarolins I and II. The mode of action and target site of **I** are unknown.

In conclusion, the *P. leptostachya* var. *asiatica* root-derived materials could be useful for managing field populations of *C. pipiens pallens*, *A. aegypti*, and *O. togoi*. Further studies on the insecticidal mode of action of the *P. leptostachya* var. *asiatica* root-derived compounds, their effects on nontarget organisms and the environment, and formulations improving the insecticidal potency and stability are needed for their practical use as a naturally occurring mosquito larval control agent.

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Received for review October 27, 2004. Revised manuscript received November 22, 2004. Accepted December 3, 2004.

JF048208H