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A Piperidine Amide Extracted from *Piper longum* L. Fruit Shows Activity against *Aedes aegypti* Mosquito Larvae

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Mosquito larvicidal activity of *Piper longum* fruit-derived materials against the fourth-instar larvae of *Aedes aegypti* was examined. A crude methanol extract of *P. longum* fruits was found to be active against the larvae, and the hexane fraction of the methanol extract showed a strong larvicidal activity of 100% mortality. The biologically active component of *P. longum* fruits was characterized as pipernonaline by spectroscopic analyses. The LC_{50} value of pipernonaline was 0.25 mg/L. The toxicity of pipernonaline is comparable to that of pirimiphos-methyl as a mosquito larvicide. In tests with available components derived from *P. longum*, no activity was observed with piperettine, piperine, or piperlongumine.

KEYWORDS: ***Aedes aegypti*; *Piper longum*; pipernonaline; mosquito larvicidal activity**

INTRODUCTION

The yellow fever mosquito *Aedes aegypti* (L.) is a widespread and serious primary medical insect pest. 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 has led to outbreaks of insect species, sometimes resulting 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 insecticidal agents because they constitute a rich source of bioactive chemicals (*7*, *8*). Much effort has been focused on plant extracts or phytochemicals as potential sources of commercial mosquito-control agents or bioactive chemical compounds (*9*, *10*). In this paper, we report isolation procedures and structural determinations of a piperidine amide active against early fourth-instar larvae of *Aedes aegypti* from a methanol extract of the dried fruit of *Piper longum* L.

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MATERIALS AND METHODS

Chemicals. Piperine and piperlongumine were purchased from Sigma Chemical Co. (St. Louis, MO). Piperettine was kindly provided by Dr. Byeoung-Soo Park, Chonbuk National University, Chonju, South Korea. All other chemicals were of reagent grade.

Insects. The laboratory F-21 strain of *A. aegypti* was obtained in 2000 from the National Institute of Health, Seoul, South Korea. Adult mosquitoes were maintained on a 10% aqueous sucrose solution and blood from a live mouse, while larvae were reared in plastic containers (24 × 35 × 5 cm) containing sterilized diet (40 mesh chick chow powder/yeast, 80:20). They were held at 28 ± 2 °C and 70 ± 5% relative humidity, with a photoregime of 16:8 (light/dark).

Extraction and Isolation. Dried fruit (2.5 kg) of *P. longum*, obtained from a traditional market in Seoul, were crushed and extracted twice with methanol (10 L) at room temperature and filtered (Toyo filter paper no. 2). The combined filtrate was concentrated in vacuo at 35 °C to yield ∼9.8% (on the basis of the weight of the dried fruit). The extract (20 g) was sequentially partitioned into hexane (3.8 g), chloroform (4.3 g), ethyl acetate (1.4 g), butanol (0.5 g), and water-soluble (10.5 g) portions for subsequent bioassay with fourth-instar of *A. aegypti*. The organic solvent portions were concentrated to dryness by rotatory evaporation at 35 °C, and the water portion was freeze-dried. The active hexane portion was chromatographed on a silica gel column (Merck 70−230 mesh, 300 g, 4.5 i.d. × 60 cm) and successively eluted with hexane/ethyl acetate, ethyl acetate, and methanol. The active fractions, eluted with hexane/ethyl acetate (3:1), were chromatographed on a silica gel column and eluted with hexane/ethyl acetate (4:1). Column fractions were collected and analyzed by thin-layer chromatography (TLC; hexane/ethyl acetate, 3:1). Fractions with a similar TLC pattern were combined. For further separation of the mosquito larvicidal substances, a Waters Delta Prep 4000 high-power liquid chromatograph

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Table 1. Mosquito Larvicidal Activity of Constituents Derived from *P. longum* L. Fruit and Organophosphorus Insecticide against Fourth-Instar Larvae of *A. aegypti*[a]

compound	mortality (%, mean ± SE) at concn of							LC_{50}[b]
	5.0 mg/L	2.5 mg/L	1.0 mg/L	0.50 mg/L	0.25 mg/L	0.12 mg/L	0.06 mg/L	
pipernonaline	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	50 ± 2.4b	17 ± 3.2c	0 ± 0.0d	0.25
piperine	0 ± 0.0d	NT[c]	NT	NT	NT	NT	NT	0
piperlongumine	0 ± 0.0d	NT	NT	NT	NT	NT	NT	0
piperettine	0 ± 0.0d	NT	NT	NT	NT	NT	NT	0
pirimiphos-methyl	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	80 ± 2.9b	46 ± 2.4c	9 ± 3.2d	0.13

[a] $P = 0.05$, Scheffe test (SAS Institute). [b] Dose expressed in mg/L; LC_{50}, median lethal concentration. [c] NT = not tested.

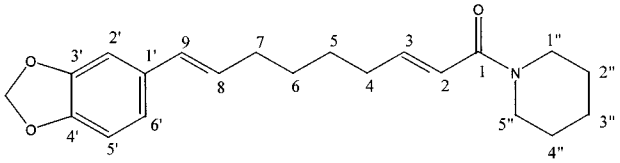

Figure 1. Structure of pipernonaline isolated from *P. longum* L.

was used. The column was a 29 i.d. × 300 mm Bondapak C_{18} (Waters) using methanol/water (3:7) at a flow rate of 7 mL/min and detection at 260 nm. One active compound (28 mg) was isolated.

Structural determination of the active isolate was based on spectral analysis. ^{1}H (400 MHz) and ^{13}C nuclear magnetic resonance (NMR; 100 MHz) spectra were measured in dimethyl-*d* sulfoxide at room temperature on a Bruker AMX-400 with tetramethylsilane (TMS) as an internal standard. Mass spectra were done on a JEOL JMS-DX30 spectrometer.

Bioassay. Concentrations of the extracts were prepared by serial dilution of a stock solution of the extracts in ethanol. Each sample extract 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* were placed into paper cups (270 mL) containing each test solution (250 mL), using a micropipet. The toxicity of each sample extract was determined with various concentrations ranging from 0.06 to 200 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 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.

Statistical Analysis. The percentage 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 $P = 0.05$ (*11*).

RESULTS AND DISCUSSION

During the initial experiments, we observed that the methanolic extract of *P. longum* (Piperaceae) dried fruit possessed mosquito larvicidal activity against *A. aegypti*, as did the hexane fraction, producing 100% mortality at 25 mg/L (not shown). However, no activity was produced from any of the other fractions even at 200 mg/L. Piperaceae plants have been known for their use as food-flavoring agents and also for their strong insecticidal components (*12*−*14*). In our study, one active isolate from the hexane fraction showed potent larvicidal activity against *A. aegypti* (**Table 1**), and it was characterized by the spectral analyses as pipernonaline (**Figure 1**). The compound was identified on the basis of the following evidence: pale yellow needles; UV λ_{max} EtOH 364.2 nm; found $[M]^+$, *m*/*z* 341.2336; EI-MS, *m*/*z* (rel int) 341 (45), 273 (18), 228 (37), 206 (61), 193 (20), 166 (100), 153 (30), 131 (75), 103 (75), 84 (45); ^{1}H NMR, **Table 2**; ^{13}C NMR, **Table 2**. The isolation and spectral analyses of pipernonaline from *P. longum* have already been reported for the study of anodyne and as a treatment for stomach disease (*15*). Our data are identical to the data of Tabuneng et al. (*15*).

Table 2. ^{1}H and ^{13}C NMR ($CDCl_3$) Data of Pipernonaline

no.	pipernonaline	
	^{13}C	^{1}H
1	165.2	
2	120.5	5.90 1H d (J = 15.0 Hz)
3	145.2	6.80 1H dt (J = 15.0, 4.0 Hz)
4	32.2	2.21 2H m
5	27.9	1.47 4H m
6	28.9	
7	32.6	2.17 2H m
8	128.6	6.07 1H dt (J = 6.6, 15.4 Hz)
9	129.5	6.32 1H d (J = 15.4 Hz)
1′	132.2	
2′	105.2	6.90 1H d (J = 1.6 Hz)
3′	146.5	
4′	147.9	
5′	108.0	6.71 1H d (J = 8.0 Hz)
6′	120.1	6.76 1H d (J = 1.5 8.0 Hz)
–OCH_2O–	100.8	5.92 2H s
1″	42.9	3.48 2H br s
2″	26.5	1.63 2H m
3″	24.6	1.56 4H m
4″	25.6	
5″	46.6	3.48 2H br s

Pipernonaline has an apparent LC_{50} value of ∼0.25 mg/L (**Table 1**), making it substantially more toxic than the limonoids obacunone, nomilin, and limonin (*16*). Recently, two studies have shown that an extract of *Tagetes minuta* L. had strong biocidal effects on both the larvae and adults of *A. aegypti* L. and *Anopheles stephensi* L. (*17*). The insecticidal components isolated from the plant extract were four thiophenes, 5-(but-3-ene-1-ynyl)-2,2′-bithiophene, 5-(but-3-ene-1-ynyl)-5′-methyl-2,2′-bithiophene, 2,2′,5′,5″-terthiophene, and 5-methyl-2,2′,5′,2″-terthiophene (*18*). These compounds eventually may be considered as alternatives to the currently used insecticides. Pipernonaline, which also contains thiophenes, also may be a potential candidate for a mosquito larvicidal agent.

To assess the mosquito larvicidal activity of other constituents of *P. longum*, three available compounds derived from this plant species (*19*) were tested against *A. aegypti* (**Table 1**). In our study, no activity was observed for piperine, piperlongumine, and piperettine, even at 5 mg/mL. The mosquito larvicidal activity of pipernonaline against *A. aegypti* is comparable to that of pirimiphos-methyl, a commonly used insecticide (**Table 1**). Pipernonaline may be useful as a bioactive chemical compound for developing new types of mosquito larvicides.

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