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Larvicidal Activity of Isobutylamides Identified in *Piper nigrum* Fruits against Three Mosquito Species

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The insecticidal activity of materials derived from the fruits of *Piper nigrum* against third instar larvae of *Culex pipiens pallens, Aedes aegypti*, and *A. togoi* was examined and compared with that of commercially available piperine, a known insecticidal compound from *Piper* species. The biologically active constituents of *P. nigrum* fruits were characterized as the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A by spectroscopic analysis. Retrofractamide A was isolated from *P. nigrum* fruits as a new insecticidal principle. On the basis of 48-h LC_{50} values, the compound most toxic to *C. pipiens pallens* larvae was pipercide (0.004 ppm) followed by retrofractamide A (0.028 ppm), guineensine (0.17 ppm), and pellitorine (0.86 ppm). Piperine (3.21 ppm) was least toxic. Against *A. aegypti* larvae, larvicidal activity was more pronounced in retrofractamide A (0.039 ppm) than in pipercide (0.1 ppm), guineensine (0.89 ppm), and pellitorine (0.92 ppm). Piperine (5.1 ppm) was relatively ineffective. Against *A. togoi* larvae, retrofractamide A (0.01 ppm) was much more effective, compared with pipercide (0.26 ppm), pellitorine (0.71 ppm), and guineensine (0.75 ppm). Again, very low activity was observed with piperine (4.6 ppm). Structure−activity relationships indicate that the *N*-isobutylamine moiety might play a crucial role in the larvicidal activity, but the methylenedioxyphenyl moiety does not appear essential for toxicity. Naturally occurring *Piper* fruit-derived compounds merit further study as potential mosquito larval control agents or as lead compounds.

KEYWORDS: Natural insecticides; mosquito larvicides; mosquito; *Piper nigrum*; isobutylamide; pipercide; retrofractamide A

INTRODUCTION

The northern house mosquito, *Culex pipiens pallens* (Coquillett), and the yellow fever mosquitoes, *Aedes aegypti* (L.) and *A. 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 biological control systems 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*−*7*). 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. Recently, plants in the family Piperaceae have drawn attention because they contain insecticidal principles (*8*, *9*). The fruits of *Piper nigrum* L. are not only important as a spice or flavoring, but have also been prescribed for cholera, dyspepsia, flatulence, diarrhea, various gastric ailments, and paralytic and arthritic disorders (*10*, *11*). Little work has been done with respect to managing mosquito larvae, although *P. nigrum* fruits have been reported to repel *Heliothis zea* (Boddie) adults (*12*), to protect stored products against *Acanthoscelides obtectus* (Say) adults (*13*), and to be toxic to adults of *Musca domestica* (L.) (*14*, *15*), *Sitophilus oryzae* (L.), *Callosobruchus maculatus* (F.) (*16*), *Anthonomus grandis* (Boheman) (*17*), *Callosobruchus chinensis* (Lucas), and larvae of *C. pipiens pallens* (*18*).

This paper describes a laboratory study aimed at isolating insecticidal constituents from *P. nigrum* which are active against third instar larvae of *C. pipiens pallens*, *A. aegypti*, and *A. togoi*. Structure−larvicidal activity relationships are also discussed.

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MATERIALS AND METHODS

Chemicals. Piperine and Triton X-100 were purchased from Sigma (St. Louis, MO) and Shinyo Pure Chemicals (Osaka), respectively. All other chemicals were of reagent grade.

Insects. Cultures of *C. pipiens pallens*, *A. aegypti*, and *A. togoi* were maintained in the laboratory for six years 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 trays (24 × 35 × 5 cm) containing sterilized diet (40-mesh chick chow powder/yeast, 4:1). They were held at 27 ± 1 °C and 70−80% RH under a 14:10 h light/dark cycle.

Isolation and Identification. Dried fruits (3.6 kg) of *P. nigrum* were purchased from Boeun medicinal herb shop, Kyungdong Market, Seoul, Korea. They were finely powdered, extracted with 20 L of methanol 2× at room temperature for 2 days and filtered. The combined filtrate was concentrated in vacuo at 40 °C to yield ~11% as a dark brownish tar (based on the weight of the dried fruits). The extract (20 g) was sequentially partitioned into hexane (3.2 g), chloroform (12.6 g), ethyl acetate (1.1 g), butanol (0.7 g), and water-soluble (2.4 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 40 °C, and the water portion was freeze-dried.

Isolation procedures for constituents of *Piper* fruits active against third instar larvae of the three vector mosquito species were performed as previously described (*19*). The chloroform fraction (10 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 600 g, 5.5 i.d. × 70 cm), and successively eluted with a stepwise gradient of chloroform/methanol (95:5, 90:10, and 0:100, by volume). Column fractions were analyzed by TLC (silica gel 60 F_{254}), and fractions with similar TLC patterns were pooled. The bioactive fraction (5.3 g) was successively rechromatographed on a silica gel column, using hexane/ethyl acetate. Preparative HPLC (Waters Delta Prep 600) was used for further separation of the constituents. The column was a Prodigy ODS (21.2 i.d. × 250 mm, Phenomenex) using acetonitrile/water (8:2, v/v) at a flow rate of 10 mL/min, and detected at 254 nm. Finally, four potent active principles were isolated: **1** (1.8 mg), **2** (2.1 mg), **3** (1.0 mg), and **4** (1.7 mg).

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, FT-IR spectra were obtained on a Nicolet Magna 550 spectrophotometer, and mass spectra were obtained on a JEOL GSX 400 spectrometer.

Bioassay. 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 *A. togoi* were separately put into paper cups (270 mL) containing each test solution (250 mL) using a pipet. The toxicity of each test compound was determined with four to 10 concentrations ranging from 0.001 to 100 ppm. Controls received acetone−Triton X-100 solution.

Treated and control larvae were held at the same conditions used for colony maintenance. Larvicidal activity was evaluated 48 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 (*20*).

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 $P = 0.05$ (*21*). Means (±SE) of untransformed data are reported.

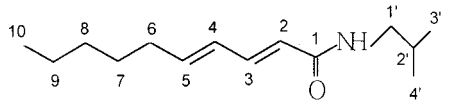
RESULTS

Identification. When fractions obtained from the methanol extract of the *Piper* fruits were bioassayed, significant differences were observed in toxicity to mosquito larvae (**Table 1**). At a concentration of 100 ppm, hexane and chloroform fractions showed potent larvicidal activity against *C. pipiens pallens*, *A. aegypti*, and *A. togoi*. There was no mortality in the untreated controls.

Table 1. Insecticidal Activity of *P. nigrum* Fruit-Derived Materials against Third Instar Larvae of *C. pipiens pallens*, *A. aegypti*, and *A. togoi*

material[a]	mortality, % (mean ± SE)[b]		
	C. pipiens pallens	*A. aegypti*	*A. togoi*
methanol ext.	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a
hexane fr.	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a
chloroform fr.	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a
ethyl acetate fr.	23 ± 3.3b	17 ± 3.3b	13 ± 3.3b
butanol fr.	3 ± 3.3c	0 ± 0.0c	0 ± 0.0c
water fr.	0 ± 0.0c	0 ± 0.0c	3 ± 3.3c

[a] Exposed for 48 h at a concentration of 100 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.



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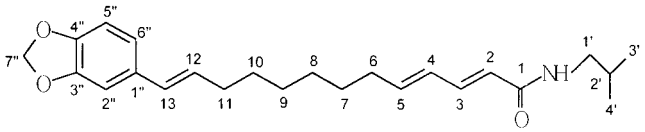

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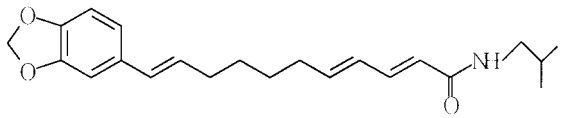

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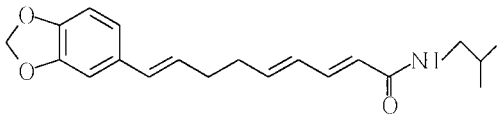

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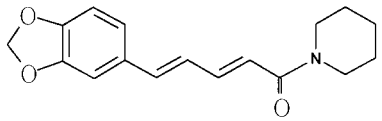

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Figure 1. Structures of *N*-isobutylamide alkaloids pellitorine (**1**), guineensine (**2**), pipercide (**3**), and retrofractamide A (**4**), and the piperidine alkaloid piperine (**5**), larvicidal constituents from *Piper nigrum* fruits against mosquitoes.

Bioassay-guided fractionation of the *Piper* fruit extract afforded four active constituents identified by spectroscopic analyses, including MS and NMR, and by comparison with published data (*16*, *22*, *23*). NMR data are given in **Table 2**. The active constituents were characterized as the isobutylamide alkaloids pellitorine (**1**), guineensine (**2**), pipercide (**3**), and retrofractamide A (**4**) (**Figure 1**). Retrofractamide A, known

Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data of Compounds **1**–**4**[a]

	pellitorine (**1**)		guineensine (**2**)		pipercide (**3**)		retrofractamide A (**4**)	
position	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1	166.40	-	166.35	-	166.31	-	166.27	-
2	121.76	5.76 *d* (15)[b]	121.74	5.74 *d* (15)	121.87	5.75 *d* (14.8)	122.23	5.76 *d* (14.8)
3	141.27	7.19 *d* (15)	141.29	7.19 *dd* (15, 5)	141.20	7.19 *dd* (14.8, 15)	141.00	7.19 *dd* (14.8, 15)
4	128.20	6.09 *m*	129.35[c]	6.12 *d* (15)	128.40	6.14 *d* (15)	128.80	6.16 *d* (15)
5	128.20	6.09 *m*	129.35[c]	6.07 *d* (15)	142.77	6.07 *d* (15)	141.75	6.01 *m*
6	32.92	2.13 *dd* (7, 13.8)	32.85[d]	2.15 *m*	32.78	2.17 *m*	32.85	2.30 *d* (3.1)
7	28.49	1.37 *m*	29.00	1.42 *m*	28.31[c]	1.46 *m*	32.18	2.31 *d* (3.1)
8	31.37	1.28 *m*	28.72	1.32 *m*	28.94[c]	1.46 *m*	127.70	5.97 *m*
9	22.47	1.28 *m*	28.94	1.32 *m*	32.67	2.17 *m*	130.18	6.30 *d* (15)
10	14.01	0.88 *s*	29.33	1.42 *m*	128.95	5.98 *d* (15)	-	-
11	-	-	32.90[d]	2.15 *m*	129.55	6.28 *d* (15)	-	-
12	-	-	143.10	6.02 *d* (15)	-	-	-	-
13	-	-	129.31[c]	6.27 *d* (15)	-	-	-	-
1′	46.92	3.16 *t* (6.4, 12.9)	46.91	3.16 *t* (6.4, 12.9)	46.91	3.16 *t* (6.4, 12.9)	46.93	3.16 *t* (6.4, 13)
2′	28.63	1.76 *m*	28.63	1.79 *m*	28.63	1.78 *m*	28.63	1.79 *m*
3′	20.13	0.91 *s*	20.13	0.91[c] *s*	20.12	0.91[c] *s*	20.13	0.91[c] *s*
4′	20.13	0.93 *s*	20.13	0.93[c] *s*	20.12	0.93[c] *s*	20.13	0.93[c] *s*
	-	5.60 (NH, *br s*)	-	5.50 (NH, *br s*)	-	5.49 (NH, *br s*)	-	5.52 (NH, *br s*)
1″	-	-	132.45	-	132.34	-	132.08	-
2″	-	-	105.37	6.89 *s*	105.38	6.89 *d* (1)	105.43	6.87 s
3″	-	-	146.52	-	147.92	-	147.94	-
4″	-	-	149.90	-	146.58	-	146.74	-
5″	-	-	108.22	6.74 *m*	108.22	6.74 *m*	108.23	6.74 *m*
6″	-	-	120.19	6.74 *m*	120.23	6.74 *m*	120.39	6.74 *m*
7″	-	-	100.90	5.93 *s*	100.91	5.93 *s*	100.95	5.93 *s*

[a] In $CDCl_3$; TMS was used as internal standard. [b] Coupling constants are in parentheses and given in Hz. [c,d] Assignments were interchangeable.

Table 3. Toxicity of *P. nigrum* Fruit-Derived Compounds against Third Instar Larvae of *C. pipiens pallens*

compound[a]	slope (± SE)	LC_{50} ppm	95% cl[b]	RT[c]
piperine	2.49 ± 0.36	3.21	2.57–4.02	0.3
pellitorine	2.25 ± 0.30	0.86	0.66–1.12	1.0
guineensine	1.55 ± 0.25	0.17	0.11–0.23	5.1
pipercide	1.82 ± 0.23	0.004	0.003–0.006	215.0
retrofractamide A	1.63 ± 0.19	0.028	0.020–0.038	30.7

[a] Exposed for 48 h. [b] cl denotes confidence limit. [c] Relative toxicity, LC_{50} value of pellitorine/LC_{50} value of each chemical.

Table 4. Toxicity of *P. nigrum* Fruit-Derived Compounds against Third Instar Larvae of *A. aegypti*

compound[a]	slope (± SE)	LC_{50} ppm	95% cl[b]	RT[c]
piperine	2.09 ± 0.29	5.10	3.82–6.60	0.2
pellitorine	1.85 ± 0.26	0.92	0.68–1.25	1.0
guineensine	1.78 ± 0.26	0.89	0.65–1.21	1.0
pipercide	1.37 ± 0.15	0.10	0.07–0.13	8.9
retrofractamide A	1.29 ± 0.16	0.039	0.027–0.055	23.5

[a] Exposed for 48 h. [b] cl denotes confidence limit. [c] Relative toxicity, LC_{50} value of pellitorine/LC_{50} value of each chemical.

Table 5. Toxicity of *P. nigrum* Fruit-Derived Compounds against Third Instar Larvae of *A. togoi*

compound[a]	slope (± SE)	LC_{50} ppm	95% cl[b]	RT[c]
piperine	2.07 ± 0.29	4.60	3.41–5.97	0.1
pellitorine	1.60 ± 0.25	0.71	0.48–0.98	1.0
guineensine	2.48 ± 0.34	0.75	0.58–0.96	0.9
pipercide	1.35 ± 0.21	0.26	0.15–0.38	2.7
retrofractamide A	1.36 ± 0.16	0.01	0.007–0.015	71.0

[a] Exposed for 48 h. [b] cl denotes confidence limit. [c] Relative toxicity, LC_{50} values of pellitorine/LC_{50} values of each chemical.

previously from *P. retrofractum*, was isolated from *P. nigrum* fruits for the first time.

Larvicidal Activity. The toxicity of test compounds to *C. pipiens pallens* larvae is reported in **Table 3**. The piperidine alkaloid piperine (**5**) was used because this compound has been reported to have insecticidal activity against *M. domestica* adults (*14*). A known insecticidal amide, pellitorine, served as a standard of comparison in toxicity tests. On the basis of 48-h LC_{50} values, the compound most toxic to larvae was pipercide (**3**) followed by retrofractamide A (**4**), guineensine (**2**), and pellitorine (**1**). Piperine was least toxic. Pipercide, retrofractamide A, and guineensine were 255, 31, and 5 times more toxic than pellitorine, respectively. There was no mortality in the untreated controls.

Table 4 shows the toxic effects of test compounds on *A. aegypti* larvae. Among the five test compounds, retrofractamide A was most effective. Pipercide was also highly toxic. Larvicidal activity of guineensine was comparable to that of pellitorine. Piperine was relatively ineffective. Insecticidal potency of retrofractamide A and pipercide were 24 and 9 times the activity of pellitorine, respectively.

Toxic effects of the test compounds to *A. togoi* larvae were also assessed (**Table 5**). Retrofractamide A was much more effective than pipercide, pellitorine, or guineensine. Very low activity was observed with piperine. Retrofractamide A and pipercide were 71- and 3-fold more active than pellitorine, respectively. Pellitorine and guineensine were equally toxic to *A. togoi* larvae.

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*, *24*, *25*). Certain plant-derived compounds were found to be highly effective against insecticide-resistant insect pests (*18*, *26*, *27*). For example, *P. nigrum* fruit-derived guineensine exhibits remarkable lethal activity against a pyrethrin-resistant strain of *M. domestica* (*18*). Additionally, observation of poisoning symptoms and elucidation of modes-of-action of chemicals are of importance for practical use. The insecticidal constituents of *Piper* fruits are *N*-isobutylamide alkaloids such as dihydropipercide, guineensine, pellitorine, and pipercide. These isobutylamides have knockdown activity against adults of *C. chinensis* (*18*), *M. domestica* (*28*), and *Periplaneta americana* (*29*). Lethal activity of pellitorine against *M. domestica* adults is half of that of pyrethrins (*28*). Bioactivity against *C. chinensis* adults is high, in the order of dihydropipercide, guineensine, and pipercide (*5*). They are, on average, one-half to one-third of the activity of pyrethrins. However, the order of knockdown activity is the reverse of the order of toxicity for these three amides (*5*). Su and Horvat (*16*) also reported guineensine, pellitorine, and pipercide as insecticidal constituents from *P. nigrum* fruits against adults of *S. oryzae* and *C. maculatus*: the order of lethal effect was pipercide > guineensine > pellitorine. Electrophysiological investigations have shown that pipercide induced only repetitive discharge on the exposed central nerve cord of *P. americana* adult males, but pyrethrin I induced repetitive discharge as well as conduction blockage on the exposed central nerve cord (*29*).

In this present study, a methanol extract of *Piper* fruits exhibited potent larvicidal activity against *C. pipiens pallens*, *A. aegypti*, and *A. togoi*. The larvicidal constituents were identified as the isobutylamide alkaloids pellitorine, guineensine, pipercide, and retrofractamide A. The amide retrofractamide A was isolated from *P. nigrum* fruits for the first time. Retrofractamide A was highly effective against larvae of *C. pipiens pallens*, *A. aegypti*, and *A. togoi*, whereas pipercide was most toxic to *C. pipiens pallens*. However, piperine was nontoxic to three mosquito species, although the lethal effect of this compound against *M. domestica* adults was noted (*14*). The activity of piperine against adults of *M. domestica* and *C. chinensis* has been previously reported (*18*). The two *Aedes* species tested here were more tolerant to the test compounds than *C. pipiens pallens*. Retrofractamide A might be a good candidate for a naturally occurring mosquito control agent.

Structure−activity relationships in insects have been well studied (*5*, *30*, *31*). Miyakado et al. (*5*) studied the toxic effects of the compounds by changing the amide moiety from the isobutylamine of dihydropipercide to other branched or cyclic aliphatic amines: insecticidal activity of these synthetic amines was decreased by one-third or one-fourth compared with that of the parent dihydropipercide. Steric limitation on the size of the amine was suggested to be related with insecticidal activity. Structural modification studies on the effect of aromatic ring substituents on insecticidal activity have shown that the 3,4-methylenedioxyphenyl group does not appear essential for toxicity (*5*). Without structural modifications of *N*-isobutylamine and 3,4-methylenedioxyphenyl moieties, the suitability of conjugated olefins, chain length, and the introduction of an ether group in a straight chain has also been examined by Miyakado et al. (*5*). Compounds with a conjugated dienamide chromophore (−C═C−C═C−CONH−), such as pellitorine, are quite unstable owing to their unsaturation (*5*, *32*). However, the introduction of a phenyl ring has improved chemical stability (*5*). In our toxicological study, larvicidal activity against three vector mosquito species was much more pronounced in compounds such as guineensine, pipercide, and retrofractamide A with an isobutylamine moiety than in one such as piperine without this moiety among the methylenedioxyphenyl-containing compounds. Additionally, there was a slight difference in larvicidal activity between the isobutylamides with and without a methylenedioxyphenyl moiety: the former was more active than the latter. Our findings, along with those of earlier studies, indicate that the isobutylamine moiety appears to be essential for toxicity against mosquito larvae. Although the methylenedioxyphenyl moiety is thought to stabilize the chemical structure, it might also contribute, to some extent, to the larvicidal effect.

In conclusion, the *Piper* fruit-derived materials could be useful for managing field populations of *C. pipiens pallens*, *A. aegypti*, and *A. togoi*. Further studies on the insecticidal mode-of-action of the *Piper* fruit-derived compounds, their effects on nontarget 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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