JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Insecticidal Effect of Phthalides and Furanocoumarins from *Angelica acutiloba* against *Drosophila melanogaster*

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Insecticidal activity of *Angelica acutiloba* extract and its constituents was investigated and compared with that of rotenone. Bioassay-guided isolation of the chloroform extract of *A. acutiloba* against larvae of *Drosophila melanogaster* afforded two phthalides, *(Z)*-butylidenephthalide (**1**) and *(Z)*-ligustilide (**2**), and two furanocoumarins, xanthotoxin (**3**) and isopimpinellin (**4**). The structures of these compounds were established by spectroscopic analysis. The isolated compounds **1**, **2**, **3**, and **4** exhibited LC_{50} values of 0.94, 2.54, 3.35, and 0.82 μmol/mL of diet concentration against larvae of *D. melanogaster*, respectively. Against both sexes (males/females, 1:1) of adults (5−7 days old), compound **1** showed the most potent activity with a LD_{50} value of 0.84 μg/adult. Compound **1** is a more active insecticide than rotenone (LD_{50} = 3.68 μg/adult) and has potential as a novel insect control agent. However, compound **2** was inactive against adults. The structure−activity relationship of phthalides isolated indicated that the aromaticity appeared to play an important role in the activity of both larvae and adults. To determine the insecticide mode of action for acute adulticidal activity, acetylcholinesterase (AChE) inhibitory activity was also investigated in vitro, and the result indicated that the acute adulticidal activity of compounds **3** and **4** was due to the inhibition of AChE.

KEYWORDS: ***Angelica acutiloba*; natural insecticide; structure−activity relationship; phthalides; furanocoumarins; *Drosophila melanogaster*; acetylcholinesterase inhibition**

INTRODUCTION

Plants can provide potential alternatives to the currently used insecticides that seem to cause insecticide resistance and environmental and human health concerns; because they constitute a rich source of bioactive chemicals, such as terpenoids, alkaloids, and flavonoids against insects, they have evolved strategies to interact with other organisms for self-defense (*1*). In particular, monoterpenoids, components of essential oils in many plants, are very important to insects because they can attract beneficial insects, which can aid in pollination, and they can help plants defend against harmful insects because of its high influence with volatility (*2*). Since these often biodegrade to nontoxic products, they could be much safer insect control agents and more suitable for use in integrated pest management (IPM) (*3*). Because of the worldwide attention toward pesticide residues in agricultural products, insecticides of natural origin are very important in food safety (*4*). Moreover, these compounds also can be good leads for more effective insecticides such as pyrethroids, which have been focused on due to their strong insecticidal activity with safe use for mammals and plants by many workers (*5*). Although some naturally occurring compounds have been identified as insecticides, antifeedants, attractants, and repellents, such as azadirachtin from *Azadirachta indica* which is the most important and potent insect antifeedant in recent years, most of them have not been fully studied yet (*6*).

In our search for new naturally occurring insecticidal compounds, we used Chinese crude drugs with a history of safe use as medicines. In the course of screening for novel naturally occurring insecticides, the chloroform extract of the roots of *Angelica acutiloba* KITAGAWA var. *sugiyamae* HIKINO (Apiaceae) was found to exhibit insecticidal activity against larvae of *Drosophila melanogaster*. Using *D. melanogaster* as a test insect is helpful in searching for insecticides of natural origin, which often have a limited sample; due to its small size, the insecticidal activity can be detected very little in the sample. Moreover, *D. melanogaster* has been used to examine the insecticidal activity and insecticide mode of action because of its genetic accessibility (*7*).

A. acutiloba is widely distributed in China and Japan. The plant root has been used as a prescription in Chinese traditional medicine, and its pharmacological effects are known for treatment of obstetrical and gynecological disorders (*8*). As for the chemical components, alkylphthalide derivatives are specific for *A. acutiloba* and other Apiaceae plants. Phthalides, which

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10.1021/jf0497049 CCC: $27.50
Published on Web 06/19/2004

are components of the essential oil, are volatile and unique constituents, and ligustilide was predominantly found to be the main component of *A. acutiloba* and *Cnidium officinale* (*9*−*12*). The characteristic odor of Apiaceae plants is often due to the phthalide derivatives. Various phthalides were isolated, and their biological activities are known, such as antianginal, antispasmodic, smooth muscle relaxant, anticholinergic, and acaricidal activities (*13*−*16*). Some of the furanocoumarins are also volatile, and these are a well-defined group of compounds contained in almost all the genera of Apiaceae (*17*). Monoterpenoids and other volatile compounds have been shown to possess insecticidal and various activities against insects, but its phthalides have not been understood well.

The biological activity of *A. acutiloba* against insects has not been previously reported. In this paper, we investigated the bioassay-guided isolation and identification of active principles, structure−activity relationships in relation to their insecticidal activity against *D. melanogaster*, and insecticide mode of action.

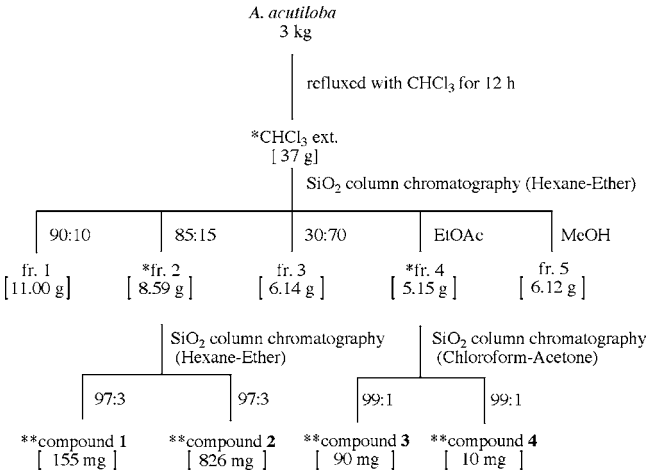

Figure 1. Isolation scheme for insecticidal compounds from *A. acutiloba* (*, active fraction; **, active compound).

MATERIALS AND METHODS

Chemical Analysis. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL FX-500 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in $CDCl_3$. This solvent was also used for ^{13}C NMR spectra. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC-MS on a Hewlett-Packard 5972 Series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m × 0.25 mm i.d., temperature 140 °C, 4 °C/min). IR spectra were determined with an FT/IR-470 Plus Fourier transform infrared spectrometer.

Plant Material. Commercially available air-dried roots of *A. acutiloba* were obtained from Takasago Yakugyou Co. (Osaka). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Kasei Kougyou Co. (Tokyo). Acetylthiocholine iodide (ATC) was purchased from Kanto Chemical Co. (Tokyo). Rotenone was purchased from Sigma Chemical Co. (St. Louis, MO).

Insects. *D. melanogaste*r used in the bioassay for insecticidal activities against larvae and adults was distributed by Professor Ishikawa of the University of Tokyo. The colony of *D. melanogaster* has been maintained without exposure to any insecticides and insects at 25 °C and RH > 60% and 12-h light:12-h dark cycle. The egg and larval periods are 12−36 h and 5−6 days, respectively, and adult longevity is about 60 days in the rearing condition.

Bioassay for Larvicidal Activity of Test Compounds. The bioassay for larvicidal activity against larvae of *D. melanogaster* was carried out as follows (*18*). The LC_{50} values of the isolates and insecticide were determined at nine concentrations (8.0, 4.0, 2.0, 1.0, 0.8, 0.6, 0.5, 0.4, and 0.2 μmol/mL of diet). Test compounds were dissolved in 50 μL of ethanol and mixed in 1 mL of artificial diet [Brewer's yeast (60 g), glucose (80 g), agar (12 g), and propionic acid (8 mL) in water (1000 mL)]. A control diet was treated with 50 μL of ethanol.

The artificial diet was poured into Petri dishes and placed on the bottom of the culture bottles to collect newly emerged eggs on the diet. About 100 adults from colonies of *D. melanogaster* were introduced into the culture bottle and allowed to oviposit for 3 h. After 3 h, the artificial diets of Petri dishes were taken out of the bottle, and 10 new eggs were collected and transferred onto the diets containing the test compounds, insecticides, and solvent in 1.8 mL glass tubes and both treated and control (solvent only) insects were reared at 25 °C and RH > 90% for 8 days. The developmental stages were observed, and the number of pupae were recorded and compared with those of a control. Ten new eggs were used in each of three replicates. LC_{50} is the lethal concentration for 50% mortality and was determined by log−probit analysis (*19*).

Bioassay for Acute Adulticidal Activity of Test Compounds. The acute adulticidal activity was determined by topical application on the abdomen of both sexes (males/females, 1:1) of adults (5−7 days old) of *D. melanogaster* (*18*). Adults from culture bottles were iced to stop their movement and treated on their abdomens with each test compound at doses of 50, 20, 10, 5, 2.5, 1, 0.5 μg/adult in 0.5 μL of acetone with a 10 μL microsyringe. Controls were treated with 0.5 μL of acetone. Samples treated and control (solvent only) insects were held at the same conditions for colony maintenance. Thirty adults were used for each dose, and all doses were replicated three times. Thirty minutes after treatment, survival of adults was recorded. Mortality was defined as inability of adults to move and ensured whether insects that showed inability to move recovered. LD_{50} is the lethal dose for 50% mortality and determined from log−probit analysis (*19*).

Inhibition of Acetylcholinesterase in Vitro. The method of Grundy and Still (*20*) was used to determine the acetylcholinesterase (AChE) inhibitory activity. Enzyme mixture containing the AChE was extracted from both sexes (males/females, 1:1) of adult (5−7 days old) heads of *D. melanogaster*. About 1000 adults were frozen at −80 °C for 7 days. The frozen adults were shaken for 1 min with a mixer to detach heads. Separation of the heads from bodies was then accompanied by sieving through mesh (40 mesh/cm^2) so as to allow only the heads to pass. The heads were then homogenized in 5 mL of 0.1 M phosphate buffer at pH 8.0. The crude homogenate was centrifuged at 14000*g* for 30 min, and the supernatant was used as the enzyme source. ATC was dissolved in 10 mL of 0.1 M phosphate buffer at pH 7.0, and 15 mg of $NaHCO_3$ was added.

Inhibition of AChE was determined according to the colorimetric method of Ellman et al. (*21*). Both the control and test solutions employed 0.2 mL of the enzyme solution and 0.1 mL of DTNB added to 2.4 mL of 0.1 M phosphate buffer (pH 8.0). The test solutions were added to each of the test compounds dissolved in 50 μL of ethanol. The control solution was similarly prepared by the addition of 50 μL of ethanol. Both control and each of the test solutions were preincubated at 25 °C for 5 min. After preincubation, the enzyme reaction was started by the addition of 40 mL of ATC followed by incubation at 25 °C for 20 min. After 20 min, the absorbance at 412 nm was measured spectrophotometrically and compared with that of the control.

Extraction and Isolation of the Active Compounds. The isolation procedure from *A. acutiloba* is given in **Figure 1**. Air-dried roots of *A. acutiloba* (3 kg) were extracted with chloroform under reflux for 12 h. The solvent was removed under reduced pressure to give a chloroform extract (37 g). The chloroform extract showed a strong insecticidal activity (100% mortality at 2.0 mg/mL of diet) against larvae of *D. melanogaster*. To purify the active compounds, the extract and following fractions were separated that were responsible for the insecticidal activity against larvae of *D. melanogaster*. The chloroform extract was fractionated by silica gel column chromatography (Merck 200 mesh) with hexane/ether. The active fraction 2 eluted with hexane/ether was rechromatographed on silica gel with hexane/ether (97:3) repeatedly, and compounds **1** and **2** were isolated. Another active fraction, 4, was rechromatographed on silica gel with chloroform/acetone (99:1) repeatedly, and compounds **3** and **4** were isolated. Compounds

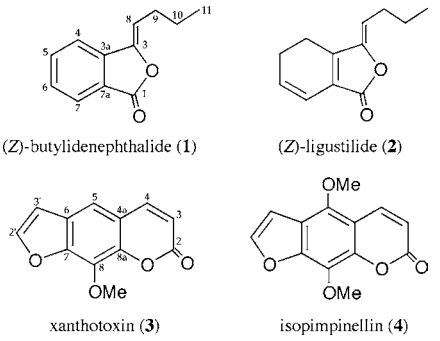

Figure 2. Structures of phthalides (**1** and **2**) and furanocoumarins (**3** and **4**) isolated from *A. acutiloba*.

1−**4** were identified as (*Z*)-butylidenephthalide (**1**), (*Z*)-ligustilide (**2**), xanthotoxin (**3**), and isopimpinellin (**4**) (**Figure 2**) by EI-MS, IR, and ^{1}H and ^{13}C NMR.

Compound 1. Compound **1** was a yellowish oil. EI-MS: *m*/*z* (relative intensity, %) 188 (17, M^+), 159 (100), 146 (35), 131 (26), 103 (28), 77 (26). IR ($CHCl_3$, υ_{max}, cm^{-1}): 1779, 1686, 1474, 1339, 1272, 1069, 997, 978, 760, 689. ^{1}H NMR ($CDCl_3$): δ 7.89 (1H, d, J = 7.8 Hz, H-7), 7.64−7.69 (2H, m, H-4,5), 7.51 (1H, ddd, J = 11.0, 7.8, 1.4 Hz, H-6), 5.64 (1H, t, J = 7.7 Hz, H-8), 2.46 (2H, td, J = 7.2, 7.7 Hz, H-9), 1.56 (3H, tq, J = 7.2, 7.4 Hz, H-10), 0.99 (3H, t, J = 7.4 Hz, H-11). ^{13}C NMR ($CDCl_3$): δ 167.2 (C-1), 145.7 (C-3a), 139.6 (C-3), 134.2 (C-5), 129.3 (C-6), 125.2 (C-7), 124.5 (C-7a), 119.6 (C-4), 109.5 (C-8), 27.8 (C-9), 22.5 (C-10), 13.8 (C-11). The spectral data were identical to the published data of (*Z*)-butylidenephthalide (*22*).

Compound 2. Compound **2** was a yellowish oil. EI-MS: *m*/*z* (relative intensity, %) 190 (57, M^+), 161 (100), 148 (89), 134 (20), 133 (19), 106 (59), 105 (75), 91 (22), 78 (51), 77 (57). IR ($CHCl_3$, υ_{max}, cm^{-1}): 1768, 1670, 1421, 1272, 960. ^{1}H NMR ($CDCl_3$): δ 6.29 (1H, dt, J = 9.8, 2.1 Hz, H-7), 6.01 (1H, dt, J = 9.8, 4.3 Hz, H-6), 5.22 (1H, t, J = 8.0 Hz, H-8), 2.60 (2H, t, J = 8.9 Hz, H-4), 2.44−2.49 (2H, m, H-5), 2.38 (2H, dt, J = 8.0, 7.5 Hz, H-9), 1.51 (2H, tq, J = 7.5, 7.5 Hz, H-10), 0.96 (3H, t, J = 7.5 Hz, H-11). ^{13}C NMR ($CDCl_3$): δ 167.7 (C-1), 148.6 (C-3a), 147.1 (C-3), 129.9 (C-6), 124.0 (C-7a), 117.2 (C-7), 112.9 (C-8), 28.1 (C-9), 22.4 (C-5, 10), 18.6 (C-4), 13.8 (C-11). The spectral data were identical to the published data of (*Z*)-ligustilide (*22*).

Compound 3. Compound **3** was a colorless prism: mp 147 °C. EI-MS: *m*/*z* (relative intensity, %) 216 (100, M^+), 201 (29), 173 (54), 145 (19), 89 (32), 63 (25). IR (KBr, υ_{max}, cm^{-1}): 1728, 1620, 1588, 1585. ^{1}H NMR ($CDCl_3$): δ 7.76 (1H, d, J = 9.4 Hz, H-4), 7.68 (1H, d, J = 2.3 Hz, H-2′), 7.33 (1H, s, H-5), 6.81 (1H, d, J = 2.3 Hz, H-3′), 6.36 (1H, d, J = 9.4 Hz, H-3), 4.29 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$): δ 160.6 (C-2), 147.8 (C-7), 146.7 (C-2′), 144.4 (C-4), 143.1 (C-9), 132.9 (C-8), 126.2 (C-6), 116.5 (C-10), 114.8 (C-3), 113.0 (C-5), 106.8 (C-3′), 61.4 (OCH_3). The spectral data were identical to the published data of xanthotoxin (*17*, *23*).

Compound 4. Compound **4** was a colorless prism: mp 150 °C. EI-MS: *m*/*z* (relative intensity, %) 246 (95, M^+), 231 (100), 203 (15), 188 (25), 175 (22), 160 (20), 147 (14). IR (KBr, υ_{max}, cm^{-1}): 1720, 1590, 1478, 1352. ^{1}H NMR ($CDCl_3$): δ 8.12 (1H, d, J = 9.7 Hz, H-4), 7.63 (1H, d, J = 2.3 Hz, H-2′), 7.00 (1H, d, J = 2.3 Hz, H-3′), 6.29 (1H, d, J = 9.7 Hz, H-3), 4.17 (3H, s, OCH_3), 4.16 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$): δ 160.6 (C-2), 150.1 (C-7), 145.2 (C-2′), 144.4 (C-5), 143.8 (C-9), 139.1 (C-4), 128.3 (C-8), 114.9 (C-6), 112.9 (C-3), 107.7 (C-10), 105.2 (C-3′), 61.8 (OCH_3), 60.9 (OCH_3). The spectral data were identical to the published data of isopimpinellin (*24*).

RESULTS

Larvicidal Activity of Test Compounds. The larvicidal activity of compounds **1**−**4** against larvae of *D. melanogaster* is given in **Table 1**. These compounds had 100% mortality at a concentration of 8.0 μmol/mL of diet. Phthalide compound **1** (LC_{50} = 0.94 μmol/mL) was more active than compound **2** (LC_{50} = 2.54 μmol/mL), and furanocoumarin compound **4** (LC_{50} = 0.82 μmol/mL) was stronger than compound **3** (LC_{50} = 3.35 μmol/mL). Compound **4** was the most insecticidally active compound in isolated compounds against larvae. However, these isolated compounds were less active than rotenone (LC_{50} = 0.02 μmol/mL). Control mortality was not observed.

Acute Adulticidal Activity of Test Compounds. The acute adulticidal activity of the isolated compounds was determined by topical application on the abdomen of adults (**Table 2**). In this test a dramatic difference in the acute adulticidal activity between compounds **1** and **2** was obtained. The most active compound was revealed to be compound **1**, which illustrated 100% mortality at a dose of 5.0 μg/adult with LD_{50} = 0.84 μg/adult. Therefore, compound **1** was more active than rotenone (LD_{50} = 3.68 μg/adult). However, no mortality effect was observed with compound **2** in this test despite insecticidal activity against larvae. The effects of furanocoumarin compounds **3** and **4** toward adults were similar to that of larvae. At a dose of 50.0 μg/adult, compounds **3** and **4** showed insecticidal activities of 53% and 56%, respectively. Compound **4** (LD_{50} = 32.83 μg/adult) was more effective than compound **3** (LD_{50} = 44.42 μg/adult), whereas their effects were not comparable to that of compound **1** and rotenone. Control mortality and recovery of the insects that showed inability to move were not observed.

AChE Inhibitory Activity in Vitro. The in vitro study of AChE inhibitory activity by compounds **1**−**4** was examined to clarify the mode of action of acute adulticidal activity against adults of *D. melanogaster* (**Figure 3**). The in vitro study with two furanocoumarins had a similar result: compound **4** was more active than compound **3**. At a concentration of 1.00 μmol/mL, AChE inhibitory activities of compounds **3** and **4** were 55.8% and 80.7%, respectively. AChE inhibitory test gave IC_{50} values for compounds **3** and **4** of 0.58 and 0.32 μmol/mL, respectively. However, the effect of compound **1**, which had potent insecticidal activity against adults of *D. melanogaster* in vivo, was only slight activity. Furthermore, the result obtained on compound **2** was also less active than furanocoumarin

Table 1. Larvicidal Activity of Compounds **1**–**4** against *D. melanogaster* (Expressed as Numbers of Pupae)[a]

		concentration[b] (μmol/mL of diet)											
compd	control	8.0	4.0	2.0	1.0	0.8	0.6	0.5	0.4	0.2	LC_{50} [c]	95% confidence limit	RT[d]
(*Z*)-butylidenephthalide (**1**)	10, 10, 10	0, 0, 0	0, 0, 1	4, 5, 5	3, 5, 6	4, 6, 6	6, 7, 7	NE[e]	7, 8, 8	9, 9, 9	0.94 ± 0.13	0.78–1.20	0.023
(*Z*)-ligustilide (**2**)		0, 0, 0	5, 6, 6	6, 7, 7	6, 7, 7	8, 8, 8	8, 9, 9	NE	9, 9, 9	9, 9, 10	2.54 ± 0.19	1.95–3.55	0.009
xanthotoxin (**3**)		0, 0, 0	4, 5, 6	7, 8, 8	9, 10, 10	9, 10, 10	NE	10, 10, 10	NE	NE	3.35 ± 0.30	2.80–3.96	0.007
isopimpinellin (**4**)		0, 0, 0	0, 0, 0	0, 0, 0	3, 5, 5	5, 5, 5	NE	8, 8, 8	NE	NE	0.82 ± 0.03	0.71–0.94	0.027
rotenone											0.02 ± 0.01	0.02–0.03	1

[a] Eight days after transplantation (10 eggs newly laid, 3 replicates). [b] Test compounds of each concentration were dissolved in 50 μL of ethanol and mixed in 1 μL of artificial diet. [c] LC_{50} is the lethal concentration for 50% mortality, determined by log–probit analysis. LC_{50} values are the mean ±SE of three replicates. [d] Relative toxicity = LC_{50} value of rotenone/LC_{50} value of each test compound. [e] NE = not experiment.

Table 2. Acute Adulticidal Activity of Compounds **1**–**4** against *D. melanogaster* (Expressed as Numbers of Survival)[a]

	dose[b] (μg/adult)									
compd	50.0	20.0	10.0	5.0	2.5	1.0	0.5	LC_{50}[c] (μg/adult)	95% confidence limit	RT[d]
(*Z*)-butylidenephthalide (**1**)	0	0	0	0	7	43	73	0.84 ± 0.10	0.75–0.94	4.4
(*Z*)-ligustilide (**2**)	100	100	100	100	100	100	100	>50.0	ND[e]	<0.07
xanthotoxin (**3**)	47	77	93	100	100	100	100	44.42 ± 3.39	44.42–3.39	0.08
isopimpinellin (**4**)	44	54	83	100	100	100	100	32.83 ± 1.47	27.57–41.62	0.11
rotenone								3.68 ± 0.14	3.33–4.06	1

[a] After 30 min, survival of adults was recorded (percent relative to control). Thirty adults (males/females 1:1, 5–7 days old) were tested for each dose, and all doses were replicated three times. [b] Test compounds of each dose were dissolved in 0.5 μL of acetone and applied on the abdomen of adults with a 10 μL microsyringe. Negative controls were treated with 0.5 μL of acetone only. [c] LD_{50} is the lethal dose for 50% mortality, determined by log–probit analysis. LD_{50} values are the mean ± SE of three replicates. [d] Relative toxicity = LD_{50} value of rotenone/LD_{50} value of each test compound. [e] ND = not determined.

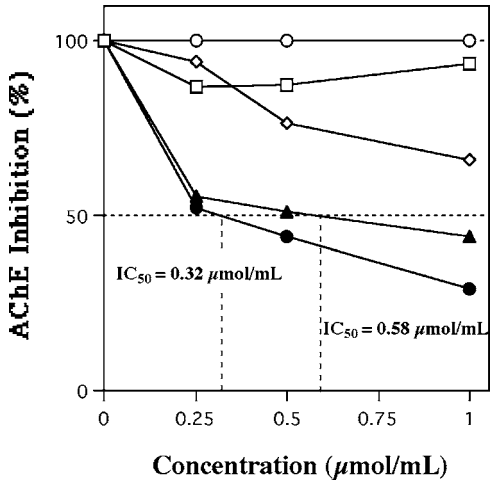

Figure 3. AChE inhibitory effect by compounds **1**–**4** and rotenone in vitro: (*Z*)-butylidenephthalide (**1**, □); (*Z*)-ligustilide (**2**, ◇); xanthotoxin (**3**, ▲); isopimpiellin (**4**, ●); rotenone (control, ○).

compounds **3** and **4**. Rotenone had no activity because the acute toxicities of rotenone to insects and mammals are attributable to the inhibition of NADH−ubiquinone oxidoreductase complex I activity (*25*).

DISCUSSION

Four insecticidal principles from *A. acutiloba* against *D. melanogaster* have been identified as compounds with potential use for natural insecticides. Against larvae, both phthalide compounds **1** and **2** were active; compound **1** was more insecticidally active than compound **2**. A study of the structure−activity relationship indicated that the aromaticity in the phthalide appeared to play an important role for enhanced activity. Furanocoumarin compound **4** was the most potent of the compounds isolated against larvae, and the structure−activity relationship study of furanocoumarins indicated that the methoxy group at the C-5 position was important for enhanced activity. The major finding of this report is the potent insecticidal activity of volatile (*Z*)-butylidenephthalide (**1**) against adults of *D. melanogaster* because compound **1** was more active than rotenone. In contrast, compound **2**, which lacks aromaticity in the structure, was inactive toward adults. This result indicates that the aromaticity of phthalides also seems to be an important factor for activity similar to that of larvae. The acute adulticidal activities of compounds **3** and **4** were similar to those of larvae, and compound **4** was more active than compound **3**. We previously reported that (−)-7-*epi*-deoxynupharidine, (−)-tetrahydroberberine, and (−)-canadine were isolated as active ingredients against *D. melanogaster* with LD_{50} values of 0.9, 2.5, and 2.5 μg/adult, respectively (*18*, *26*, *27*). Compared with the insecticidal activities toward adults of *D. melanogaster* in our previous related works, (*Z*)-butylidenephthalide (**1**) has one of the most potent activities.

To clarify the mode of action of the acute adulticidal activity, AChE inhibitory activity was researched because the two phthalides were isolated as anticholinergic substances in the past and the two furanocoumarins have been known to possess AChE inhibitory activity (*15*, *28*). Furanocoumarin compounds **3** and **4** inhibited AChE of adults of *D. melanogaste*r in vitro, and their inhibitory activities were strong. Moreover, the investigation of acute adulticidal activity in vivo and AChE inhibitions in vitro had a similar result: compound **4** is slightly more active than compound **3**. Therefore, one possibility is that the acute adulticidal activity of compounds **3** and **4** is due to inhibition of AChE. However, phthalide compounds **1** and **2** showed slight activity. Therefore, AChE inhibition was not involved in the acute adulticidal activity of these two compounds. As for the insecticidal route of action, a recent investigation by Kwon et al. (*16*) reported that butylidenephthalide (**1**) was an active constituent for the acaricidal activity from the rhizome of *Cnidium officinale* against adults of *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* and acted as a fumigant. In this study, (*Z*)-butylidenephthalide (**1**) did not inhibit AChE activity. These two results suggested that butylidenephthalide acts as fumigant and not a direct contact agent. While the ligustilide (**2**) slightly inhibited AChE activity compared with (*Z*)-butylidenephthalide (**1**), this compound may be partially a direct contact agent. Some essential oil terpenoids are known as AChE inhibitors, and others are known as neurotoxic agents, such as antagonists of octopimine receptors (*29*, *30*). Therefore, octopaminergic action might be involved in the insecticidal activity of phthalides. However, the exact insecticide mode of action remains unknown.

Consequently, this result suggests that plants containing a high amount of (*Z*)-butylidenephthalide (**1**) are highly affective against insects, and one of the roles of this compound might be to prevent attack from harmful insects for plants. Although the activities of monoterpenoids and other terpenoids have been studied by many workers and a few of these compounds are currently used commercially as pesticides or repellents (D-limonene, menthol, linalool) (*2*), phthalide derivatives have not been investigated well. The result of this study proposed that butylidenephthalide and plants containing it might be used as a new tool for plant protection from harmful insects in organic agriculture. The Japanese government has established increasingly restrictive legislation regarding the maximum residue limits (MRLs) of pesticides in agricultural products (*4*). In Europe, organic production may use natural insecticides and not synthetic ones in pest control (*25*). It is concluded that further

studies about insecticides of natural origin are essential to find new plants and compounds with insecticidal effects and to find a new strategy of plant protection with environmental safety. In conclusion, the chloroform extract obtained from roots of *A. acutiloba* and its active compounds have been evaluated as potential natural insecticides using bioassay against larvae and adults of *D. melanogaster*. Further studies of the mechanism of insects and mammals in vitro and in vivo are needed to understand the pharmacological actions of (*Z*)-butylidenephthalide (**1**) and other phthalide derivatives. This is the first report on the insecticidal activity of (*Z*)-butylidenephthalide (**1**) and *A. acutuloba* against insects. We hope that this result will be used in the future to obtain safe foods for agriculture.

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Received for review February 23, 2004. Revised manuscript received May 6, 2004. Accepted May 8, 2004.

JF0497049