Larvicidal activity of lignans and alkaloid identified in *Zanthoxylum piperitum* bark toward insecticide-susceptible and wild *Culex pipiens pallens* and *Aedes aegypti*

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**Abstract**

**Background:** The yellow fever mosquito, *Aedes aegypti*, and the common house mosquito, *Culex pipiens pallens*, transmit dengue fever and West Nile virus diseases, respectively. This study was conducted to determine the toxicity of the three lignans \((-\)asarinin, sesamin and \(+)\)-xanthoxylol-\(\gamma\)\(\gamma\)-dimethylallylether (XDA), and the alkaloid pellitorine from *Zanthoxylum piperitum* (Rutaceae) bark to third-instar larvae from insecticide-susceptible *C. pipiens pallens* and *Ae. aegypti* as well as wild *C. pipiens pallens* resistant to deltamethrin, cyfluthrin, fenthion, and temephos.

**Methods:** The toxicities of all isolates were compared with those of mosquito larvicide temephos. LC\(_{50}\) values for each species and their treatments were significantly different from one another when their 95% confidence intervals did not overlap.

**Results:** XDA was isolated from *Z. piperitum* as a new larvicidal principle. XDA (LC\(_{50}\), 0.27 and 0.24 mg/l) was 4, 53, and 144 times and 4, 100, and 117 times more toxic than pellitorine, sesamin, and assarinin toward larvae from susceptible *C. pipiens pallens* and *Ae. aegypti*, respectively. Overall, all the isolates were less toxic than temephos (LC\(_{50}\), 0.006 and 0.009 mg/l). These constituents did not differ in toxicity to larvae from the two *Culex* strains. The present finding indicates that the lignans and alkaloid and the insecticides do not share a common mode of larvicidal action or elicit cross-resistance.

**Conclusion:** Naturally occurring *Z. piperitum* bark-derived compounds, particularly XDA, merit further study as potential mosquito larval control agents or as lead compounds for the control of insecticide-resistant mosquito populations.

**Keywords:** Botanical mosquito larvicide, *Zanthoxylum piperitum*, Rutaceae, Lignans, Xanthoxylol-\(\gamma\)\(\gamma\)-dimethylallylether, Alkaloid, Insecticide resistance

**Background**

The yellow fever mosquito, *Aedes aegypti* (Linnaeus, 1762) [1], and the common house mosquito, *Culex pipiens pallens* (Coquillett, 1898) [2], are found in tropical and subtropical regions of the world [3] and Eastern Asia [4], respectively, and are serious disease vectoring insect pests [5, 6]. A recent study calculated that more than 2.5 billion people are at risk of dengue infection over 100 countries worldwide, and there may be 50–100 million dengue infections annually, including 22,000 deaths every year, mostly among children [7]. From 1999 to 2015, 43,937 cases of human West Nile virus disease (including 20,265 neuroinvasive disease cases) were reported in the United States (US), which resulted in 1,911 deaths [8]. The most serious problem with the mosquito species is their ability to evolve resistance to insecticides rapidly [9]. Increasing levels of resistance to the conventional insecticides have resulted in multiple treatments and excessive doses, raising serious environmental and human health concerns. Widespread insecticide resistance...
has been one of the major obstacles in the cost-effective integrated vector management program. In addition, the number of approved insecticides may be reduced soon in the US by the US Environmental Protection Agency as reregistration occurs [10]. Reregistration requirement is also a concern in other regions including in the European Union, where it is under the control of the Commission Regulation (EC) No 1048/2005 [11]. Therefore, there is a high need for the development of selective control alternatives with novel target sites to establish a biorational resistance management strategy based on all available information on the extent and nature of resistance in mosquitoes because vaccines have limited effectiveness in controlling dengue [12].

Biocides derived from plants have been suggested as potential alternatives for mosquito control largely because plants constitute a potential source of bioactive secondary metabolites that are perceived by the public as relatively safe and with less risk to the environment, and with minimal impacts to human and animal health [13–19]. Phytochemicals act at multiple, novel target sites [14, 16–21], thereby reducing the potential for resistance [17–19, 22, 23]. Based on these benefits of botanical insecticides, numerous papers are published annually [19, 24]. Phytochemicals are regarded as potential sources to develop commercial mosquito larvicides as products derived from certain plants and their constituents meet the criteria as reduced risk insecticides [16–19, 25]. Recently, Zanthoxylum plants (Rutaceae) have drawn attention because they contain insecticidal constituents toward the cowpea aphid, Aphis craccivora Koch, 1854 [26, 27], the maize weevil, Sitophilus zeamais (Motschulsky, 1855) [28, 29], and larvae of various mosquito vectors [17, 30]. However, no previous studies have investigated the potential use of Japanese pepper, Zanthoxylum piperitum (L.) DC., for managing mosquitoes, particularly insecticide-resistant mosquitoes, despite its repellency to Ae. aegypti [31] and the stable fly, Stomoxys calcitrans (Linnaeus, 1758) [32, 33].

In this study, our aim was to assess whether the three lignans, asarinin, xanthoxylol-γ,δ-dimethylallyl-lether (XDA) and sesamin, and the isobutylamide alkaloid pellitorine, extracted from the bark of Z. piperitum, had the toxicity to third-instar larvae from insecticide-susceptible C. pipiens pallens and Ae. aegypti, as well as wild colonies of C. pipiens pallens resistant to various insecticides [23]. The toxicity of the bark constituents was compared with that of the currently available mosquito larvicide temephos to assess their use as future commercial mosquito larvicides because it is registered as a larvicide for the control of mosquitoes in South Korea [34]. Also, the quantitative structure-activity relationship (QSAR) of the test compounds is discussed.

Methods

Instrumental analysis

The 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded in CDC13 on Varian NMR system spectrometers (Varian, Palo Alto, CA, USA), using tetramethylsilane as an internal standard. The chemical shifts are given in δ (ppm). The ultraviolet (UV) spectra were obtained in methanol on a UVICON 933/934 spectrophotometer (Kontron, Milan, Italy) and the mass spectra on a GSX 400 spectrometer (Jeol, Tokyo, Japan). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) were used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 F254) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 series high-performance liquid chromatography (Agilent, Santa Clara, CA, USA) was used to isolate the active constituents.

Materials

The organophosphorus (OP) insecticide temephos (97.3%) was purchased from Riedel (Seelze, Lower Saxony, Germany). Triton X-100 was purchased from Coseal (Seoul, South Korea). All of the other chemicals used in this study were of reagent-grade quality and are available commercially.

Mosquitoes

The stock cultures of C. pipiens pallens (susceptible KS-CP strain) and Ae. aegypti have been maintained in the laboratory without exposure to any known insecticide, as described previously [35]. Larvae from YS-CP colony of C. pipiens pallens, originally collected near rice paddy fields and cowsheds in Yusung (Daejeon, South Korea) in September 2010, showed extremely high levels of resistance to fenthion (resistance ratio (RR), 390) and deltamethrin (RR, 164) and moderate levels of resistance to cyfluthrin (RR, 14) and temephos (RR, 14) [23]. Adult mosquitoes were maintained on a 10% sucrose solution and blood fed on live mice. Larvae were reared in plastic trays (24 × 35 × 5 cm) containing 0.5 g of sterilised diet (40-mesh chick chow powder/yeast, 4/1 by weight). All stages were held at 27 ± 1 °C, 65–75% relative humidity, and a 14:10 h light:dark cycle.

Plant material

Fresh bark of Z. piperitum was collected from the Southern Forest Resources Research Center (Jinju, Gyeongnam, South Korea), National Institute of Forest Science, in mid-August 2009. A certified botanical taxonomist was used to identify the plant. A voucher specimen (ZP-01) was deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.
Extraction and isolation

Air-dried bark (550 g) of *Z. piperitum* was pulverised, extracted with methanol (3.3 L) two times at room temperature for 2 days, and filtered. The combined filtrate was concentrated to dryness by rotary evaporation at 40 °C to yield approximately 70 g of a dark brownish sticky solid. The extract (20 g) was sequentially partitioned into hexane- (6.4 g), chloroform- (1.36 g), ethyl acetate- (0.46 g), and water-soluble (11.78 g) portions for the subsequent bioassays. This fractionation procedure was repeated three times. The organic solvent-soluble portions were concentrated under vacuum at 35 °C, and the water-soluble portion was freeze-dried. To isolate the active constituents, 10–50 mg/l of each *Z. piperitum* bark-derived fraction was tested in a mortality bioassay, as described by Perumalsamy et al. [22].

The hexane-soluble fraction (19.2 g) was the most biologically active fraction (Table 1) and was chromato-graphed on a 5.5 × 70 cm silica gel (500 g) column by elution with a gradient of chloroform and methanol [(100:0 (2 l), 95:5 (1 l), 90:10 (2 l), 80:20 (1 l), 50:50 (1 l), and 0:100 (1.5 l) by volume] to provide 34 fractions (each approximately 250 ml) (Fig. 1). The column fractions were monitored by TLC on silica gel plates developed with a chloroform and methanol (9:1 by volume) mobile phase. Column fractions with similar *R* < sub > f </sub > values on the TLC plates were pooled. The spots were detected by spraying the plate with 4% H<sub>2</sub>SO<sub>4</sub> and then heating on a hot plate. Active fractions 11–17 (H<sub>3</sub>) were pooled and rechromatographed on a 5.5 × 70 cm silica gel (500 g) column by elution with a gradient of hexane and ethyl acetate [(90:10 (1 l), 80:20 (1 l), and 0:100 (1 l) by volume] and finally with 1 l methanol to afford 16 fractions (each approximately 250 ml). The fractions were monitored by TLC on silica gel plates developed with a hexane and ethyl acetate (7:3 by volume) mobile phase. Active fractions 8–13 (H<sub>3</sub>) were pooled and crystallised during being dried by rotary evaporation at 35 °C to yield compound one (0.2 l) to afford five fractions (each approximately 200 ml). A preparative high-performance liquid chromatography (HPLC) was performed to separate the constituents from the active H332212 fraction. The column was a 3.9 mm i.d. × 300 mm bondclone ten silica (Phenomenex, Torrance, CA, USA) using a mobile phase of chloroform and ethyl acetate (95:5 by volume) at a flow rate of 1 ml/min. Chromatographic separation was monitored using a UV detector at 264 nm. The two active constituents two and three were isolated at retention times of 8.05 and 10.03 min, respectively. For separation of a constituent from another active H332 fraction (1.3 g), a preparative HPLC was performed. The column was a 21.2 mm i.d. × 250 mm Phenomenex Prodigy ODS with a mobile phase of acetonitrile and water (1:1 by volume) at a flow rate of 1 ml/min. Chromatographic separation was monitored at 287 nm. Finally, an active constituent four was isolated at a retention time of 5.35 min.

Bioassay

A mortality bioassay [36] was used to assess the toxicity of all compounds to third-instar larvae from the susceptible and wild mosquitoes. In brief, each compound in acetone was suspended in distilled water with Triton X-100 (20 μl/l). Groups of 20 mosquito larvae were separately put into paper cups (270 ml) containing each compound solution (250 ml). Temephos served as a positive control and was similarly formulated. Negative

Table 1 Toxicity of fractions obtained from solvent partitioning of methanol extract of *Zanthoxylum piperitum* bark to third-instar larvae from *Culex pipiens pallens* during a 24 h exposure

| Material                                | n<sup>a</sup> | Slope ± SE | LC<sub>50</sub> mg/l (95% CI)<sup>b</sup> | LC<sub>90</sub> mg/l (95% CI)<sup>b</sup> | *P*-value
|------------------------------------------|----------------|------------|---------------------------------|---------------------------------|---------|
| Methanol extract                         | 240            | 5.1 ± 0.57 | 5.91 (5.38–6.44)                 | 10.50 (9.28–12.54)              | 3.25    | 0.974   |
| Hexane-soluble fraction                   | 240            | 4.3 ± 0.47 | 4.18 (3.69–4.64)                 | 8.27 (7.26–9.89)                | 4.90    | 0.932   |
| Chloroform-soluble fraction              | 240            | 5.1 ± 0.54 | 5.02 (4.53–5.50)                 | 9.02 (8.04–10.58)               | 6.01    | 0.921   |
| Ethyl acetate-soluble fraction           | 60             |            | >100                            |                                 |         |         |
| Water-soluble fraction                   | 60             |            | >100                            |                                 |         |         |

<sup>a</sup>Number of larvae tested

<sup>b</sup>CI denotes confidence interval

<sup>c</sup>Pearson’s chi-square goodness-of-fit test
controls consisted of the acetone-Triton X-100 solution in distilled water. Based on the preliminary test results, the toxicity of each test compound and insecticide was determined with four to six concentrations ranging from 0.1 to 100 mg/l and 0.001 to 0.1 mg/l, respectively. All treatments were replicated three times using 20 larvae per replicate.

Treated and control (acetone-Triton X-100 solution only) larvae were held under the same conditions as those used for colony maintenance without providing food. Larval mortalities were determined 24 h post-treatment. A larva was considered dead if it did not move when prodded with a fine wooden dowel [22].

Data analysis
Data were corrected for control mortality using Abbott’s formula [37]. Concentration-mortality data were subjected to probit analysis [38]. A compound having LC$_{50}$ > 100 mg/l was ineffective as described by Kiran et al. [39]. The LC$_{50}$ values for each species and their treatments were significantly different from one another when their 95% confidence intervals did not overlap.

Results
Bioassay-guided fractionation and isolation
The fractions obtained from the solvent partitioning of the methanol extract of the *Z. piperitum* bark were bioassayed toward third-inst ar larvae from insecticide-susceptible *C. pipiens pallens* (Table 1) and *Ae. aegypti* (Table 2). Significant differences in toxicity were observed among the fractions and were used to identify the peak activity fractions for the next step of purification. Based on the 24 h LC$_{50}$ values, the hexane-soluble fraction was the most toxic material, followed by the
chloroform-soluble fraction. No toxicity was obtained using the ethyl acetate- or water-soluble fractions. Mortality in the acetone-Triton X-100-water-treated controls for any of the species in this study was less than 2%.

Bioassay-guided fractionation of the *Z. piperitum* bark extract afforded four active compounds that were identified by spectroscopic analyses, including electron ionized mass spectrometry (EI-MS) and NMR spectroscopy. The four active compounds were (−)-asarinin (5-[3-(1,3-benzodioxol-5-yl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-6-yl]-1,3-benzodioxole) (1), (+)-xanthoxylol-γ,γ-dimethylallylether (XDA) (2), pellitorine [(2E,4E)-N-(2-methylpropyl)deca-2,4-dienamide] (3), and sesamin [5,5′-[(1S,3aR,4S,6aR)-tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diylibis(1,3-benzodioxole)] (4) (Fig. 2). (−)-Asarinin (1) was identified based on the following evidence: white powder. EI-MS (70 eV), m/z (% relative intensity): 354 [M]+, 336, 203, 161, 149, 135, 69 (Additional file 1). 1H NMR (CDCl3, 500 MHz): δ 2.85 (1H, dd, J = 7.0, 14.0 Hz), 3.30 (1H, m), 3.83 (2H, m), 3.83–4.09 (2H, m), 4.40 (1H, d, J = 9.5 Hz), 4.82 (1H, d, J = 7.0 Hz), 5.95 (4H, d, J = 6.0 Hz), 6.79 (4H, m), 6.86 (2H, s) (Additional file 2). 13C NMR (CDCl3, 125 MHz): δ 50.1 t, 54.6 t, 69.6 d, 70.9 d, 82.0 d, 87.6 d, 100.9 t, 101.0 t, 106.3 d, 106.5 d, 108.1 d, 118.6 d, 119.5 d, 132.2 s, 135.1 s, 146.5 s, 147.1 s, 147.6 s, 147.9 s (Additional file 3). (+)-Xanthoxylol-γ,γ-dimethylallylether (2) was characterized as follows: viscous solid. EI-MS (70 eV), m/z (% relative intensity): 424 [M]+, 356 (100), 325, 205, 178, 149, 135, 69 (Additional file 4). 1H NMR (CDCl3, 600 MHz): δ 1.74 (3H, s), 1.78 (3H, s), 2.92 (1H, q), 3.34 (1H, m), 3.86 (3H, m), 4.12 (1H, d, J = 6.0 Hz), 4.42 (1H, d, J = 9.0 Hz), 4.85 (1H, d, J = 5.4 Hz), 5.53 (1H, m), 5.97 (2H, s), 6.78 (1H, m), 6.81 (1H, m), 6.84 (1H, m), 6.86 (1H, m), 6.87 (1H, m), 6.92 (1H, d, J = 1.2 Hz) (Additional file 5). 13C NMR (CDCl3, 150 MHz): δ 18.4 q, 26.0 q, 50.4 d, 54.7 d, 56.1 t, 66.1 t, 69.9 d, 71.2 d, 82.3 d, 87.9 d, 101.2 q, 106.6 d, 108.4 d, 109.6 d, 113.2 d, 118.6 d, 118.9 d, 120.2 d, 132.5 s, 133.8 s, 137.8 s, 146.8 s, 147.5 s, 147.9 s, 149.9 s (Additional file 6). Pellitorine (3) was characterized as follows: viscous oil. EI-MS (70 eV), m/z (% relative intensity): 223 [M]+, 208, 180, 167, 152 (100), 113, 96, 72 (Additional file 7). 1H NMR (CDCl3, 400 MHz): δ 0.88 (3H, s), 0.91 (3H, s), 0.93 (3H, s), 1.28 (4H, m), 1.76 (1H, m), 2.13 (2H, dd, J = 7.0, 13.8 Hz), 3.16 (2H, t, J = 6.4, 12.9 Hz), 5.60 (1H, br s), 5.76 (1H, d, J = 15.0 Hz), 6.09 (2H, m), 7.19 (1H, d, J = 15.0 Hz) (Additional file 8). 13C NMR (CDCl3, 100 MHz): δ 14.0 q, 20.1 q, 22.5 t, 28.5 t, 28.6 d, 31.4 t, 32.9 t, 46.9 t, 121.7 d, 128.2 d, 128.2 d, 141.2 d, 166.4 s (Additional file 9).

Table 2  Toxicity of fractions obtained from solvent partitioning of methanol extract of *Zanthoxylum piperitum* bark to third-instar larvae from *Aedes aegypti* during a 24 h exposure

| Material                | n  | Slope ± SE | LC50, mg/l (95% CI) | LC90, mg/l (95% CI) | χ² | P-value |
|-------------------------|----|------------|---------------------|---------------------|----|---------|
| Methanol extract        | 240| 4.3 ± 0.47 | 3.95 (3.47–4.40)    | 7.88 (6.92–9.40)    | 4.03 | 0.945   |
| Hexane-soluble fraction | 240| 4.0 ± 0.43 | 4.21 (3.75–4.73)    | 8.74 (7.47–11.19)   | 3.90 | 0.951   |
| Chloroform-soluble fraction | 240| 3.8 ± 0.47 | 3.68 (3.06–4.63)    | 12.30 (10.37–15.94) | 4.14 | 0.941   |
| Ethyl acetate-soluble fraction | 60 | > 100      |                     |                     |     |         |
| Water-soluble fraction  | 60 | > 100      |                     |                     |     |         |

*a Number of larvae tested  
*b CI denotes confidence interval  
*c Pearson’s chi-square goodness-of-fit test

Fig. 2 Structures of asarinin, xanthoxylol-γ,γ-dimethylallylether, pellitorine, and sesamin. These compounds were identified in the bark of *Zanthoxylum piperitum* in this study. The chemical formula of (−)-asarinin (1) is C20H18O6, with a molar mass of 354.35 g/mol; the chemical formula of (+)-xanthoxylol-γ,γ-dimethylallylether (2) is C25H28O6, with a molar mass of 424.48 g/mol; the chemical formula of pellitorine (3) is C14H25NO, with a molar mass of 223.35 g/mol; and the chemical formula of sesamin (4) is C20H18O6, with a molar mass of 354.35 g/mol.
Sesamin (4) was characterized as follows: colorless crystals. EI-MS (70 ev), m/z (% relative intensity): 354 [M]+, 323, 203, 178, 161, 149 (100), 135 (Additional file 10). 1H NMR (CDCl3, 500 MHz): δ 3.05 (2H, m), 3.86 (2H, dd, J = 3.0, 9.0 Hz), 4.23 (2H, dd, J = 6.5, 9.0 Hz), 4.71 (2H, d, J = 4.0 Hz), 5.95 (4H, s), 6.79 (4H, d, J = 8.0 Hz), 6.85 (2H, s) (Additional file 11). 13C NMR (CDCl3, 125 MHz): δ 50.1 t, 54.6 t, 69.6 d, 70.9 d, 82.0 d, 87.6 d, 100.9 t, 101.0 t, 106.3 d, 106.5 d, 108.1 d, 118.6 d, 119.5 d, 122.0 s, 132.2 s, 146.5 s, 147.1 s, 147.6 s, 147.9 s (Additional file 12).

**Table 3** Toxicity of Zanthoxylum piperitum bark constituents and temephos to third-instar larvae from insecticide-susceptible KS-CP strain of Culex pipiens pallens during a 24 h exposure

| Compound         | n | Slope ± SE | LC50, mg/l (95% CI)c | LC90, mg/l (95% CI)c | χ²d | P-value |
|------------------|---|------------|-----------------------|----------------------|-----|---------|
| XDA (2)\(^a\)   | 420| 1.8 ± 0.10 | 0.27 (0.24–0.30)      | 1.44 (1.21–1.79)     | 6.61| 0.980   |
| Pellitorine (3)  | 300| 2.5 ± 0.23 | 1.12 (0.95–1.35)      | 3.75 (2.95–5.20)     | 5.67| 0.957   |
| Sesamin (4)      | 240| 2.8 ± 0.31 | 14.28 (12.24–16.66)   | 40.48 (32.03–56.93)  | 1.82| 0.997   |
| Asarinin (1)     | 300| 4.4 ± 0.45 | 38.90 (35.26–42.50)   | 75.77 (67.01–89.69)  | 6.34| 0.932   |
| Temephos         | 300| 1.4 ± 0.18 | 0.006 (0.005–0.008)   | 0.049 (0.032–0.096)  | 2.49| 0.999   |

\(^{a}\)Number of larvae tested  
\(^{b}\)CI denotes confidence interval  
\(^{c}\)Pearson’s chi-square goodness-of-fit test  
\(^{d}\)Xanthoxyl-γ,γ-dimethylallyl ether
Zanthoxylum monophyllum leaf essential oil had potent larvicidal activity toward Anopheles subpictus (LC$_{50}$ 41.50 and 82.19 mg/l), Aedes albopictus (LC$_{50}$ and LC$_{90}$ 45.35 and 88.07 mg/l), and Culex tritaeniorhynchus (LC$_{50}$ 49.01 and 92.08 mg/l).

Active larvicidal constituents (LC$_{50}$ < 50 mg/l) derived from plants in-clude alkaloids (e.g. pellitori ne, guineensine, pipercide, and eupomatenoid-5, and eupomatenoid-6, LC$_{50}$ < 1 mg/l), cyanogenic glycosides (e.g. dhurrin, LC$_{50}$ 18.45 and 34.50 mg/l), phenylpropanoids (e.g. methyleu-sarin, LC$_{50}$ 12.28 mg/l), aporphine alkaloids (e.g. quassin, LC$_{50}$ 6.0 mg/l), terpenoids (e.g. germacrene D-4-ol, LC$_{50}$ 2.88 and 3.14 mg/l), flavonoids (e.g. karanjin, karanja-acetate and karanji-acetate), and fatty acids (e.g. oleic acid and palmitic acid, LC$_{50}$ 0.24–0.27 mg/l for two mosquito species) meet the stage 3 criteria (LC$_{50}$ < 1 mg/l) set by Shaalan et al. [22, 45]. In addition, these constituents were also effective toward C. pipiens pallens larvae resistant to various insecticides. The present finding indicates that Z. piperitum bark-derived preparations containing the active constituents, particularly XDA and pellitorine, hold promise for the development of novel, effective, naturally occurring mosquito larvicides even toward currently insecticide-resistant mosquito populations, because XDA (LC$_{50}$ 0.24–0.27 mg/l for two mosquito species) and pellitorine (LC$_{50}$ 0.98–1.12 mg/l for two mosquito species) meet the stage 3 criteria (LC$_{50}$ < 1 mg/l) set by Shaalan et al. [14]. The next step stage 4 involves the determination of effective field application rates of various formulations in simulated field trials and/or small-scale field trials [14].

### Table 4

| Compound          | n$^a$ | Slope ± SE | LC$_{50}$, mg/l (95% CI$^b$) | LC$_{90}$, mg/l (95% CI$^b$) | $\chi^2$ | P-value |
|-------------------|-------|------------|-----------------------------|-----------------------------|---------|---------|
| XDA (2)$^i$       | 300   | 1.9 ± 0.21 | 0.31 (0.26–0.38)            | 1.39 (1.03–2.13)            | 6.23    | 0.037   |
| Pellitorine (3)   | 300   | 2.1 ± 0.29 | 1.42 (1.17–1.80)            | 5.46 (3.71–10.29)           | 1.81    | 0.997   |
| Sesamin (4)       | 300   | 2.3 ± 0.24 | 12.64 (10.48–14.90)         | 45.73 (36.14–63.54)         | 5.96    | 0.047   |
| Asarinin (1)      | 240   | 5.5 ± 0.67 | 33.80 (31.30–36.47)         | 57.67 (51.01–69.59)         | 4.41    | 0.927   |
| Temephos          | 360   | 4.1 ± 0.34 | 0.149 (0.133–0.166)         | 0.307 (0.271–0.358)         | 6.90    | 0.075   |

$^a$Number of larvae tested  
$^b$CI denotes confidence interval  
$^i$Pearson's chi-square goodness-of-fit test  
$^x$Xanthoxyloyl-$\gamma$-dimethylallylether

### Table 5

| Compound          | n$^a$ | Slope ± SE | LC$_{50}$, mg/l (95% CI$^b$) | LC$_{90}$, mg/l (95% CI$^b$) | $\chi^2$ | P-value |
|-------------------|-------|------------|-----------------------------|-----------------------------|---------|---------|
| XDA (2)$^j$       | 300   | 1.8 ± 0.17 | 0.24 (0.20–0.30)            | 1.29 (0.95–1.97)            | 6.37    | 0.098   |
| Pellitorine (3)   | 240   | 2.6 ± 0.30 | 0.98 (0.84–1.16)            | 2.98 (2.30–4.36)            | 3.49    | 0.096   |
| Sesamin (4)       | 240   | 2.4 ± 0.25 | 23.98 (20.48–28.33)         | 82.75 (63.03–122.72)        | 2.66    | 0.098   |
| Asarinin (1)      | 300   | 7.5 ± 0.79 | 28.15 (26.25–29.95)         | 41.60 (38.51–46.18)         | 2.07    | 0.095   |
| Temephos          | 240   | 1.6 ± 0.38 | 0.009 (0.007–0.012)         | 0.062 (0.032–0.373)         | 1.48    | 0.099   |

$^a$Number of larvae tested  
$^b$CI denotes confidence interval  
$^j$Pearson's chi-square goodness-of-fit test  
$^x$Xanthoxyloyl-$\gamma$-dimethylallylether
QSARs of phytochemicals in many insects have been well noted. For example, Wang et al. [23] studied the toxicity of six linear furanocoumarins including imperatorin and six simple coumarins including osthole. They reported that the chemical structure and alkoxy substitution and length of the alkoxy side chain at the C8 position are essential for imparting toxicity. Park et al. [44] reported that the larvicidal activity toward three vector mosquito species was much more pronounced in compounds such as guineensine, pipercide, and refractamide A with an isobutylamine moiety than in one such as piperine without this moiety among the methylenedioxyphenyl (MDP)-containing compounds. In addition, the isobutylamides with an MDP moiety was more active than the ones without an MDP moiety. The MDP moiety is thought to stabilise the chemical structure [54]. In the current study, XDA with an MDP moiety was more toxic than either asarinin or sesamin with two MDP moieties. In addition, sesamin was more toxic than asarinin, 7-epimer of sesamin. Our findings, along with previous studies, indicate that other factor(s) such as chemical structure, functional group, and isomerism, as well as hydrophobic (log P) and molecular refraction parameters, may play, in part, a role in determining the lignan toxicities to mosquito larvae, although the MDP moiety might contribute, to some extent, to the larvicidal effect.

An investigation of the modes of action and the resistance mechanisms of biolarvicides may contribute to the development of selective mosquito control alternatives with novel target sites. Major mechanisms of resistance to insecticides currently available to control mosquitoes are target site insensitivity that reduces sodium channel sensitivity to pyrethroid insecticides or sensitivity of acetylcholinesterase to OP and carbamate insecticides, as well as enhanced metabolism of various groups of insecticides [55, 56]. Some phytochemicals were found to be highly effective toward insecticide-resistant mosquitoes [14, 22, 23], and they are likely to be useful in resistance management strategies. For example, imperatorin and osthole are effective toward larvae from wild C. pipiens pallens with extremely high to moderate levels of resistance to cyfluthrin, deltamethrin, fenithion, and temephos [22]. The current findings that the three furofuranoid lignans and the isobutylamide alkaloid described were of equal toxicity to both insecticide-susceptible and -resistant larvae of C. pipiens pallens imply that the phytochemicals and the pyrethroid and OP insecticides do not share a common mode of action or elicit cross-resistance. Detailed tests are needed to understand fully the exact mode of action of the furofuranoid lignans and the isobutylamide alkaloid, although the octopaminergic and γ-aminobutyric acid receptors have been suggested as novel target sites for some monoterpenoid essential oil constituents in the American cockroach [57] and the cotton bollworm [20] and the fruit fly [21], respectively. It has also been reported that tannins and pellitorine primarily affect the midgut epithelium and secondarily affect the gastric caeca and the malpigian tubules in C. pipiens larvae [58] and Ae. aegypti larvae [59], respectively.

Conclusion
Zanthoxylum piperitum bark-derived products containing xanthoxylol-γ,γ-dimethylallyl ether and pellitorine could be useful as larvicides in the control of mosquito populations, particularly in the light of their activity toward insecticide-resistant mosquito larvae. Further research is needed on the practical applications of plant-derived preparations as novel mosquito larvicides to establish their safety profiles in humans, although Z. piperitum is commonly used as a spice and as a traditional medicinal plant [60, 61]. In addition, their effects on nontarget aquatic organisms including larvivorous fishes, biological control agents for mosquitoes [62], and the aquatic environment need to be established. Lastly, detailed tests are needed to understand how to improve the larvicidal potency and stability of the compounds isolated from Z. piperitum for eventual commercial development.

Additional files

**Additional file 1:** EI-MS spectrum of (−)-asarinin (1). (TIF 115 kb)
**Additional file 2:** $^1$H NMR (CDCl$_3$, 500 MHz) spectrum of (−)-asarinin (1). (TIF 60 kb)
**Additional file 3:** $^{13}$C NMR (CDCl$_3$, 125 MHz) spectrum of (−)-asarinin (1). (TIF 62 kb)
**Additional file 4:** EI-MS spectrum of (+)-xanthoxylol-γ,γ-dimethylallyl ether (2). (TIF 167 kb)
**Additional file 5:** $^1$H NMR (CDCl$_3$, 600 MHz) spectrum of (+)-xanthoxylol-γ,γ-dimethylallyl ether (2). (TIF 98 kb)
**Additional file 6:** $^{13}$C NMR (CDCl$_3$, 150 MHz) spectrum of (+)-xanthoxylol-γ,γ-dimethylallyl ether (2). (TIF 87 kb)
**Additional file 7:** EI-MS spectrum of pellitorine (3). (TIF 80 kb)
**Additional file 8:** $^1$H NMR (CDCl$_3$, 400 MHz) spectrum of pellitorine (3). (TIF 110 kb)
**Additional file 9:** $^{13}$C NMR (CDCl$_3$, 100 MHz) spectrum of pellitorine (3). (TIF 82 kb)
**Additional file 10:** EI-MS spectrum of sesamin (4). (TIF 63 kb)
**Additional file 11:** $^1$H NMR (CDCl$_3$, 500 MHz) spectrum of sesamin (4). (TIF 62 kb)
**Additional file 12:** $^{13}$C NMR (CDCl$_3$, 125 MHz) spectrum of sesamin (4). (TIF 42 kb)

**Abbreviations**
EI-MS: Electron ionized mass spectrometry; HPLC: High-performance liquid chromatography; MDP: Methylene dioxyphenyl; NMR: Nuclear magnetic resonance; OP: Organophosphorus; QSAR: Quantitative structure-activity relationship; RR: Resistance ratio; TLC: Thin-layer chromatography; UV: Ultra violet; XDA: (+)-xanthoxylol-γ,γ-dimethylallyl ether
Acknowledgements
The authors thank Dr Haribalan Perumalsamy for reviewing this manuscript. We also thank He Min Shin for her assistance with mosquito rearing.

Funding
This research was supported by the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University and BioGreen 21 Program (PJ007109), the Rural Development Administration, to Y.-J. Ahn.

Availability of data and materials
All data are disclosed in the text or in tables in the article. El-MS, 1H NMR, and 13C NMR spectra of compounds 1, 2, 3, and 4 are provided as Additional files 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, respectively.

Authors’ contributions
Y.-J.A conceived and designed the experiments. S-IK performed the experiments. S-IK and Y.-J.A analyzed the data. Y.-J.A wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent of publication
Not applicable.

Ethics approval and consent to participate
Ethical approval was obtained from the Institutional Animal Care and Use Committee of Seoul National University for this study.

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Received: 19 January 2017 Accepted: 22 April 2017
Published online: 04 May 2017

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