Toxicity and possible mechanisms of action of honokiol from *Magnolia denudata* seeds against four mosquito species

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This study was performed to determine the toxicity and possible mechanism of the larvicidal action of honokiol, extracted from *Magnolia denudata* seeds, and its 10 related compounds against third-instar larvae of insecticide-susceptible *Culex pipiens pallens*, *Aedes aegypti*, and *Aedes albopictus* and *Anopheles sinensis* resistant to deltamethrin and temephos. Honokiol (LC₅₀, 6.13–7.37 mg/L) was highly effective against larvae of all of the four mosquito species, although the toxicity of the compound was lower than that of the synthetic larvicide temephos. Structure–activity relationship analyses indicated that electron donor and/or bulky groups at the ortho or para positions of the phenol were required for toxicity. Honokiol moderately inhibited acetylcholinesterase and caused a considerable increase in cyclic AMP levels, indicating that it might act on both acetylcholinesterase and octopaminergic receptors. Microscopy analysis clearly indicated that honokiol was mainly targeted to the midgut epithelium and anal gills, resulting in variably dramatic degenerative responses of the midgut through sequential epithelial disorganization. Honokiol did not affect the AeCS1 mRNA expression level in *Ae. aegypti* larvae, but did enhance expression of the genes encoding vacuolar-type H⁺-ATPase and aquaporin 4, indicating that it may disturb the Na⁺, Cl⁻ and K⁺ co-transport systems. These results demonstrate that honokiol merits further study as a potential larvicide, with a specific target site, and as a lead molecule for the control of mosquito populations.

The yellow fever mosquito *Aedes aegypti* and Asian tiger mosquito *Aedes albopictus* are both found in tropical and subtropical regions around the world; by contrast, the common Oriental mosquito *Anopheles sinensis* and common house mosquito *Culex pipiens pallens* are found in South East Asia. Various diseases caused by these mosquito species represent several of the most serious global public health problems. The prevalence and severity of these clinical conditions are distinctly increasing and closely associated with global warming, tainted fresh water pools, and increased international travel. An estimated 198 million cases of malaria occurred globally in 2013 with at least 584,000 deaths, primarily among children under 5 years of age. A recent study determined that almost 4 billion people are at risk of infection with dengue viruses in 125 countries worldwide. Estimates of the number of annual symptomatic dengue infections range from 50 million to 100 million, including approximately 10,000–20,000 deaths annually, primarily among children. From 1999 to 2015, 43,937 West Nile virus disease cases (including 20,265 neuroinvasive disease cases) were reported in the United States (US), resulting in 1,911 deaths. Widespread insecticide resistance has been one of the major obstacles to cost-effective integrated mosquito management (IMM) programs. In addition, the number of approved insecticides will probably be reduced soon in the US and the European Union as reregistration occurs. The removal of conventional mosquitocidal products from markets due to increases in insecticide resistance or other concerns will seriously affect the proliferation of mosquitoes. Therefore, there is a pressing need to develop new improved mosquito...
control alternatives with novel target sites to establish a biorational management strategy and tactics because vaccines have limited effectiveness in controlling malaria and dengue.

Plants may provide potential sources of mosquito control products largely because they are sources of bioactive secondary metabolites (SMs) that are perceived by the general public to be relatively safe and pose less risk to the environment as well as to have minimal impact on human and animal health. SMs act at multiple, novel target sites, thereby reducing the potential for resistance. Efforts to evaluate these benefits of botanical insecticides have resulted in numerous papers being published annually. However, the mechanisms of their larvicidal action have not been clearly defined or understood. Histopathological studies have revealed that the midgut of insects is one of the main target organs for many xenobiotics, including SMs. Previous studies have shown that a hydrodistillate from the seeds of the yulan magnolia (lily tree) plant Magnolia denudata Desr. (Magnoliaceae) exhibited potent toxicity against third-instar Culex pipiens pallens and Aedes aegypti larvae. Approximately 230 species of the genus Magnolia, which is one of the most primitive angiosperms, are widely distributed throughout the temperate Northern Hemisphere. Approximately two-thirds of this species are currently distributed in temperate and tropical regions of eastern and southeastern Asia. This plant species contains a variety of SMs, such as alkaloids, lignans, neolignans, phenylpropanoids, and terpenoids. Magnolia denudata is native to eastern and southern China, and its dried flower buds have been used for the treatment of emphysema, nasal congestion, sinusitis, and allergic rhinitis. However, no previous studies have investigated the potential use of M. denudata for managing mosquitoes, particularly insecticide-resistant mosquitoes, for future commercialization, as well as the mechanisms of larvicidal action of the plant constituents. Traditional and folk medicinal uses of Magnolia species have been well-documented by Kelm and Nair and Li et al. Noted that the most promising botanical anti-mosquito products are plants in the families Asteraceae, Cladophoraceae, Lamiaceae, Meliaceae, Ooeyctaceae, and Rutaceae, although the anti-mosquito activity can vary significantly according to plant species, chemotypes, plant tissue type, plant age, geographic conditions, solvent used for extraction, and mosquito species.

The aim of this study was to assess whether the neolignan honokiol; two fatty acids, linoleic acid and palmitic acid, that were extracted from M. denudata seeds; and magnolol, a structural isomer of honokiol, showed contact toxicity against third-instar larvae of insecticide-susceptible laboratory colonies of Culex pipiens pallens, Ae. aegypti, and Ae. albopictus and laboratory colonies of An. sinensis resistant to deltamethrin and temephos. The results were compared with those of the organophosphate insecticide (OP) temephos, which is a currently used mosquito larvicide, to assess their potential for use as future commercial mosquito larvicides. Quantitative structure–activity relationship (QSAR) analysis of honokiol and 10 of its structurally-related phenolic compounds is discussed. The morphological changes in the midgut and anal gills of larval Ae. aegypti were also examined using light microscopy and transmission electron microscopy (TEM). In addition, gene expression analyses of larval Ae. aegypti chitin synthase (AecS), vacuolar-type H^+ -ATPase (AaV-type H^+ -ATPase), and aquaporin (AAQP4) genes, which act as a water channel in anal gills, after treatment with honokiol and magnolol were used to determine the possible target sites of the two neolignans. Finally, the possible mechanism underlying the larvicidal actions of the two neolignans against Ae. aegypti was elucidated using biochemical, histologic, and molecular analyses.

## Results

### Contact mortality bioassay-guided fractionation and identification.

The fractions obtained from solvent partitioning of the methanol extract of M. denudata seeds were tested for larvicidal activity against third-instar larvae of insecticide-susceptible Culex pipiens pallens and Aedes aegypti (Table 1). Significant differences in toxicity were observed among the fractions and were used to identify the peak activity fractions for the next step in purification. As judged by the 24 h LC_{50} values, the hexane-soluble fraction was the most potent larvicide, whereas no toxicity was observed using the chloroform-, ethyl acetate-, butanol-, or water-soluble fractions. Mortality in the acetone-Triton X-100-water-treated controls for all species in this study was less than 2%.

Contact mortality bioassay-guided fractionation of the M. denudata seeds led to the identification of three active compounds through spectroscopic analyses, including electron ionized mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR) spectroscopy. The three larvicidal constituents were palmitic acid (hexadecanoic acid), linoleic acid [(9Z,12Z)-9,12-octadecadienoic acid], and honokiol (2-(4-hydroxy-3-prop-2-etyl-phenyl)-4-prop-2-etyl-phenol). Palmatic acid (1) was identified based on the following characteristics: a white crystal; ultraviolet (UV) (ethanol): λ_{max} nm = 254; EI-MS (70 eV), m/z (% relative intensity): 256 [M^+] (100), 213 (49), 185 (29), 171 (26), 157 (26), 143 (12), 129 (65), 115 (23), 97 (33), 83 (36), 73 (91), 60 (69) (see Supplementary Fig. S1); 1H NMR (CDCl_3, 600 MHz): δ 0.89 (3 H, t), 1.30 (17 H, m), 1.30 (2 H, m),...
Figure 1. Structures of palmitic acid, linoleic acid, and honokiol. These compounds were identified in the seeds of Magnolia denudata in this study. The chemical formula of the saturated fatty acid palmitic acid (1) is C_{16}H_{32}O_{2}, with a molar mass of 280.45 g/mol. The chemical formula of the unsaturated fatty acid linoleic acid (2) is C_{18}H_{32}O_{2}, with a molar mass of 256.43 g/mol. The chemical formula of the neolignan honokiol (3) is C_{16}H_{18}O_{2}, with a molar mass of 266.33 g/mol.

Table 2. Toxicity of the Magnolia denudata seed constituents, their pure organic compounds, and larvicide temephos to third-instar larvae of insecticide-susceptible Culex pipiens pallens during a 24 h exposure.

| Compound               | Slope ± SE | LC50 (mg/L) (95% CI) | LC90 (mg/L) (95% CI) | χ² | P-value |
|------------------------|------------|----------------------|----------------------|----|---------|
| Natural honokiol       | 2.4 ± 0.30 | 6.32 (5.07–7.60)    | 18.78 (15.30–25.99)  | 3.10 | 0.978   |
| Pure honokiol          | 2.1 ± 0.30 | 6.38 (5.01–7.78)    | 19.30 (15.64–27.01)  | 2.48 | 0.991   |
| Natural linoleic acid  | 4.4 ± 0.59 | 6.97 (6.21–7.68)    | 19.41 (15.94–26.34)  | 4.39 | 0.927   |
| Pure linoleic acid     | 2.5 ± 0.22 | 8.36 (6.09–10.40)   | 47.79 (33.03–61.69)  | 4.38 | 0.986   |
| Natural palmitic acid  | 3.1 ± 0.38 | 32.56 (28.22–37.19) | 112.3 (88.96–156.2) | 4.76 | 0.923   |
| Pure palmitic acid     | 2.4 ± 0.25 | 34.92 (29.48–40.76) | 119.3 (94.38–166.4) | 4.21 | 0.988   |
| Temephos               | 1.9 ± 0.21 | 0.0162 (0.0132–0.0196)| 0.0749 (0.0551–0.1177) | 3.93 | 0.991   |

1.31 (2 H, m), 1.31 (2 H, m), 1.33 (2 H, m), 1.60 (2 H, m), 2.27 (2 H, t) (see Supplementary Fig. S2); and 13C NMR (CDCl₃, 150 MHz): δ 14.5 (C-16), 23.8 (C-15), 26.7 (C-5), 27.3 (C-3, C-4), 28.3 (C-9, C-10, C-11), 30.1 (C-8), 30.4 (C-13), 30.6 (C-12), 30.9 (C-6, C-7), 35.1 (C-14), 35.2 (C-2), 177.9 (C-1) (see Supplementary Fig. S3). Linoleic acid (2) was characterized based on the following: a colorless oil; UV (methanol): λ_max nm (% relative intensity): 280 [M]⁺ (65), 137 (15), 123 (22), 110 (32), 108 (11), 96 (54), 95 (70), 82 (73), 81 (100), 67 (87), 55 (54) (see Supplementary Fig. S4); 1H NMR (CDCl₃, 600 MHz): δ 0.87 (3 H, s), 1.31 (14 H, m), 1.62 (2 H, m), 2.05 (2 H, d, J = 6.6 Hz), 2.05 (2 H, d, J = 13.3 Hz), 2.34 (2 H, t, J = 7.5 Hz), 2.78 (2 H, t, J = 5.9 Hz), 3.67 (1 H, s), 5.31 (2 H, m), 5.35 (2 H, m), (see Supplementary Fig. S5); 13C NMR (CDCl₃, 150 MHz): δ 14.1 (C-18), 22.6 (C-17), 24.6 (C-3), 25.6 (C-11), 27.2 (C-8), 27.3 (C-14), 27.8 (C-12), 29.0 (C-4), 29.1 (C-5), 29.2 (C-15), 29.3 (C-6), 29.6 (C-7), 30.2 (C-13), 31.5 (C-16), 34.0 (C-2), 128.0 (C-10), 130.0 (C-9), 180.1 (C-1) (see Supplementary Fig. S6); and distortion-less enhancement by polarization transfer (DEPT) spectra (see Supplementary Fig. S7). Honokiol (3) was characterized based on the following: a colorless oil; UV (methanol): λ_max nm = 290; EI-MS (70 eV), m/z (% relative intensity): 266 [M]⁺ (100), 237 (18), 224 (8), 197 (8), 184 (8), 152 (3), 133 (4), 105 (2), 77 (1) (see Supplementary Fig. S8); 1H NMR (CDCl₃, 600 MHz): δ 3.30 (2 H, d, J = 6 Hz, H-7), 3.37 (2 H, d, J = 6.6 Hz, H-7), 5.07 (2 H, m), 5.92 (4 H, m, H-9, H-9'), 6.78 (2 H, t, J = 5.7 Hz, H-3), 6.90 (1 H, dd, J = 2.1 and 2.2 Hz, H-3), 6.97 (1 H, d, J = 2.2 Hz, H-6), 7.19 (1 H, d, J = 2.2 Hz, H-4), 7.20 (1 H, d, J = 2.6 Hz, H-6), 7.22 (1 H, d, J = 2.1 Hz, H-2') (see Supplementary Fig. S9); 13C NMR (CDCl₃, 150 MHz): δ 35.5 (C-7), 40.6 (C-7'), 115.5 (C-6), 115.5 (C-2'), 115.6 (C-9), 115.6 (C-9'), 117.0 (C-8), 127.4 (C-4), 128.8 (C-8'), 129.3 (C-1'), 129.3 (C-5), 130.1 (C-6'), 132.2 (C-2), 132.6 (C-8), 138.7 (C-3), 139.7 (C-3'), 153.5 (C-5), 155.3 (C-1) (see Supplementary Fig. S10); and DEPT spectra (see Supplementary Fig. S11).

Larvicidal activity of the isolated compounds. The toxicity of the three isolated compounds (palmitic acid, linoleic acid, and honokiol), their corresponding pure organic compounds, and the OP temephos, which was used as a positive control, against third-instar larvae of the susceptible KS-CP strain of Cx. pipiens pallens was evaluated (Table 2). The responses varied according to the compounds that were examined. As judged by the 24 h LC₅₀ values, natural and pure organic honokiol had similar toxicities against KS-CP larvae, indicating that the activity of the methanol-extracted honokiol was purely due to honokiol. Similar results were obtained from natural and pure organic linoleic acid and palmitic acid. Natural honokiol (LC₅₀, 6.32 mg/L) and linoleic acid (6.97 mg/L) were the most toxic constituents and were 390 and 430 times less toxic than temephos, respectively. The LC₅₀ of palmitic acid was 32.56 mg/L.
val times less toxic than temephos against Ae and molecular refraction (MR)) of the 11 compounds (R² differences in the responses of third-instar larvae, respectively. The toxicities of guaiacol and caffeic acid were the lowest of all of the constituents examined. Similar results were observed with the LC₅₀ (MW, MR, and MR) analysis showed that the log R² values were only loosely negatively correlated (MW, MR, and MR) were the most toxic constituents and were 521 and 575 times less toxic than temephos, respectively.

Table 3. Toxicity of the Magnolia denudata seed constituents, their pure organic compounds, and larvicide temephos to third-instar larvae of insecticide-susceptible Aedes aegypti during a 24 h exposure.

| Compound          | Slope ± SE | LC₅₀ (mg/L) (95% CI) | LC₉₀ (mg/L) (95% CI) | χ² | P-value |
|-------------------|------------|----------------------|----------------------|----|---------|
| Natural honokiol  | 2.3 ± 0.37 | 6.51 (5.19–7.89)     | 22.59 (17.70–34.15)  | 1.47 | 0.999   |
| Pure honokiol     | 2.2 ± 0.36 | 6.48 (5.11–7.90)     | 23.84 (18.41–37.36)  | 1.09 | 0.999   |
| Natural linoleic acid | 4.4 ± 0.59 | 7.19 (6.38–7.95)     | 27.04 (20.52–43.96)  | 1.00 | 0.999   |
| Pure linoleic acid | 2.1 ± 0.29 | 7.34 (5.58–8.92)     | 29.70 (23.19–43.79)  | 4.11 | 0.917   |
| Natural palmitic acid | 3.1 ± 0.38 | 33.54 (29.06–38.38)  | 129.3 (99.59–189.2)  | 3.93 | 0.991   |
| Pure palmitic acid | 2.3 ± 0.25 | 36.49 (29.30–44.83)  | 204.1 (139.4–378.1)  | 2.17 | 0.999   |
| Temephos          | 1.7 ± 0.21 | 0.0125 (0.0098–0.0154) | 0.0658 (0.0479–0.1059) | 6.87 | 0.908   |

Table 4. Toxicity of the Magnolia denudata seed constituents and larvicide temephos to third-instar larvae of insecticide-susceptible Aedes albopictus during a 24 h exposure.

| Compound          | Slope ± SE | LC₅₀ (mg/L) (95% CI) | LC₉₀ (mg/L) (95% CI) | χ² | P-value |
|-------------------|------------|----------------------|----------------------|----|---------|
| Honokiol          | 2.9 ± 0.52 | 6.13 (5.19–7.38)     | 16.52 (13.74–21.90)  | 4.07 | 0.943   |
| Linoleic acid     | 4.4 ± 0.59 | 7.28 (6.53–8.00)     | 27.81 (20.95–46.02)  | 1.22 | 0.999   |
| Palmitic acid     | 3.1 ± 0.38 | 36.91 (31.30–41.64)  | 135.1 (104.3–196.9)  | 4.28 | 0.987   |
| Temephos          | 1.6 ± 0.21 | 0.0132 (0.0103–0.0164) | 0.0778 (0.0547–0.1343) | 4.68 | 0.912   |

Table 5. Toxicity of the Magnolia denudata seed constituents and larvicide temephos to third-instar larvae of insecticide-resistant Anopheles sinensis during a 24 h exposure.

| Compound          | Slope ± SE | LC₅₀ (mg/L) (95% CI) | LC₉₀ (mg/L) (95% CI) | χ² | P-value |
|-------------------|------------|----------------------|----------------------|----|---------|
| Honokiol          | 2.0 ± 0.35 | 7.37 (5.45–9.04)     | 31.45 (22.90–56.82)  | 1.03 | 0.999   |
| Linoleic acid     | 3.9 ± 0.56 | 7.50 (6.80–8.29)     | 16.04 (14.05–19.06)  | 2.30 | 0.986   |
| Palmitic acid     | 2.1 ± 0.22 | 47.58 (40.24–55.68)  | 192.8 (148.1–282.2)  | 2.26 | 0.999   |
| Temephos          | 2.2 ± 0.15 | 0.40 (0.34–0.47)     | 1.39 (1.05–2.13)     | 3.04 | 0.997   |

Against third-instar larvae of insecticide-susceptible Ae aegypti (Table 3), natural and pure organic honokiol did not differ significantly in their respective toxicities by each other. Similar results were also observed with natural and pure organic linoleic acid and palmitic acid. Natural honokiol (24 h LC₅₀, 6.51 mg/L) and linoleic acid (7.19 mg/L) were the most toxic constituents and were 521 and 575 times less toxic than temephos, respectively. The toxicity of palmitic acid was the lowest of any of the constituents tested.

The toxic effects of all of the compounds on third-instar larvae of insecticide-susceptible Ae albopictus were also compared (Table 4). Natural honokiol (24 h LC₅₀, 6.13 mg/L) and linoleic acid (7.28 mg/L) were 464 and 552 times less toxic than temephos against Ae albopictus larvae, respectively. Palmitic acid was the least toxic constituent. Interestingly, the toxicity of all compounds was virtually identical against Ae albopictus and An sinensis larvae (Table 5), indicating a lack of cross-resistance in the larval An sinensis.

Structure–activity relationship. Because of the potent larvicidal activity of honokiol, comparisons were made to determine the toxicity differences due to the chemical structures and structural variation of the 11 compounds (R² = 0.407 for larval Cx pipientis pelligent larvae, phenol, with a basic structure, exhibited moderate toxicity (24 h LC₅₀, 41.67 mg/L). Honokiol was the most toxic compound, followed by o-eugenol, p,p′-dibiphenol, p-ethylphenol, and methoxyeugenol (LC₅₀, 6.32–8.40 mg/L), and these compounds did not significantly differ in toxicity from each other. Magnolol (LC₅₀, 26.00 mg/L) had significantly more pronounced toxicity than phenol. The LC₅₀ of eugenol and isoeugenol was 74.35 and 79.72 mg/L, respectively. The toxicities of guaiacol and caffeic acid were the lowest of all of the constituents examined. Similar differences in the toxicities of the third-instar Cx pipientis pelligent larvae to the 11 compounds were also observed in third-instar Ae aegypti larvae (Table 7).

Multiple regression analysis of the contact toxicities of compounds against larvae of the two mosquito species was performed using their LC₅₀ values and the values of the physical parameters (molecular weight (MW), log P, and molecular refraction (MR)) of the 11 compounds (R² = 0.418 for larval Ae aegypti). Correlation coefficient (r) analysis showed that the log P was negatively correlated with the LC₅₀ for larval Cx pipientis pelligent (r = −0.584 (P = 0.059)) and the MW and MR were only loosely negatively correlated with the LC₅₀ (MW, r = −0.281 (P = 0.403); MR, r = −0.364 (P = 0.271)). Similar results were also observed in larval Ae aegypti (log P, r = −0.595 (P = 0.054); MW, r = −0.291 (P = 0.386); and MR, r = −0.375 (P = 0.256)). There were no differences in the correlation coefficients between larval Cx pipientis pelligent and Ae aegypti.
Figure 2. Structures of 10 structurally related compounds of honokiol. Phenol, p-ethylphenol (p-EP), guaiacol (GC), eugenol (EN), isoeugenol (IEN), caffeic acid (CA), o-eugenol (o-EN), magnolol (MG), methoxyeugenol (MEN), and p,p′-biphenol (p,p′-BP).

Table 6. Toxicity of honokiol, its 10 structurally related compounds, and organophosphorus insecticide dichlorvos to third-instar *Culex pipiens pallens* larvae during a 24 h exposure.

| Compound          | Slope ± SE | LC50 (mg/L) (95% CI) | LC90 (mg/L) (95% CI) | χ²   | P-value |
|-------------------|------------|----------------------|----------------------|------|---------|
| Honokiol          | 2.4 ± 0.30 | 6.32 (5.07–7.60)     | 18.78 (15.30–25.99)  | 3.10 | 0.978   |
| o-Eugenol         | 2.6 ± 0.38 | 7.46 (5.98–8.78)     | 23.13 (18.42–33.49)  | 2.94 | 0.982   |
| p,p′-Biphenol     | 2.5 ± 0.37 | 7.52 (5.99–8.88)     | 23.97 (18.97–35.16)  | 1.00 | 0.999   |
| p-Ethylphenol     | 2.5 ± 0.37 | 8.30 (6.60–9.72)     | 25.86 (20.36–38.21)  | 3.78 | 0.956   |
| Methoxyeugenol    | 2.6 ± 0.37 | 8.40 (6.91–9.80)     | 25.52 (20.27–36.92)  | 1.06 | 0.999   |
| Magnolol          | 2.6 ± 0.35 | 26.00 (22.17–30.75)  | 80.42 (63.28–117.4)  | 2.34 | 0.993   |
| Phenol            | 1.9 ± 0.24 | 41.67 (34.56–50.07)  | 185.1 (133.5–306.6)  | 1.84 | 0.999   |
| Eugenol           | 2.9 ± 0.39 | 74.35 (64.14–85.27)  | 143.3 (127.2–170.3)  | 5.40 | 0.965   |
| Isoeugenol        | 2.7 ± 0.39 | 79.72 (68.33–92.65)  | 138.1 (124.4–164.5)  | 3.67 | 0.960   |
| Guaiacol          | 4.5 ± 0.55 | 107.8 (99.62–117.6)  | 205.6 (176.3–260.9)  | 3.08 | 0.997   |
| Caffeic acid      | 4.1 ± 0.56 | 119.3 (108.5–131.9)  | 245.6 (208.1–323.3)  | 2.16 | 0.999   |
| Dichlorvos        | 2.0 ± 0.22 | 0.103 (0.077–0.128)  | 0.504 (0.380–0.761)  | 3.84 | 0.993   |

Table 7. Toxicity of honokiol, its 10 structurally related compounds, and organophosphorus insecticide dichlorvos to third-instar *Aedes aegypti* larvae during a 24 h exposure.

| Compound          | Slope ± SE | LC50 (mg/L) (95% CI) | LC90 (mg/L) (95% CI) | χ²   | P-value |
|-------------------|------------|----------------------|----------------------|------|---------|
| Honokiol          | 2.3 ± 0.30 | 6.51 (5.19–7.89)     | 20.91 (16.72–30.14)  | 1.81 | 0.997   |
| o-Eugenol         | 2.9 ± 0.38 | 8.84 (7.48–10.16)    | 24.01 (19.52–33.06)  | 2.01 | 0.995   |
| p,p′-Biphenol     | 2.4 ± 0.36 | 8.34 (6.74–9.84)     | 27.76 (21.50–42.52)  | 1.77 | 0.997   |
| p-Ethylphenol     | 2.4 ± 0.36 | 9.03 (7.38–10.64)    | 30.57 (23.29–48.51)  | 2.53 | 0.990   |
| Methoxyeugenol    | 2.5 ± 0.36 | 9.15 (7.55–10.72)    | 29.46 (22.83–44.88)  | 1.68 | 0.998   |
| Magnolol          | 4.5 ± 0.53 | 25.70 (21.80–30.50)  | 83.14 (64.68–124.4)  | 3.00 | 0.981   |
| Phenol            | 1.8 ± 0.23 | 42.22 (35.62–52.46)  | 207.1 (145.1–363.6)  | 1.12 | 0.999   |
| Eugenol           | 4.7 ± 0.52 | 75.76 (68.75–82.02)  | 141.4 (126.3–166.2)  | 4.60 | 0.982   |
| Isoeugenol        | 5.6 ± 0.87 | 81.26 (73.19–88.47)  | 137.2 (124.3–161.2)  | 3.11 | 0.978   |
| Guaiacol          | 4.4 ± 0.56 | 115.4 (106.3–127.2)  | 233.1 (188.3–292.1)  | 5.95 | 0.948   |
| Caffeic acid      | 4.6 ± 0.57 | 124.5 (115.2–134.8)  | 235.9 (204.2–296.1)  | 1.58 | 0.999   |
| Dichlorvos        | 2.0 ± 0.22 | 0.158 (0.128–0.190)  | 0.660 (0.505–0.963)  | 3.84 | 0.993   |
Acetylcholinesterase inhibition. The in vitro acetylcholinesterase (AChE) inhibitory activity of honokiol, its 10 structurally related compounds, and the OP dichlorvos, which was used as a positive control, was evaluated using AChE from third-instar *Aedes aegypti* larvae (Fig. 3). The responses varied according to the compounds that were examined. Based on the IC_{50} values, there were significant differences \((F = 866.67; \text{df} = 11, 24; P < 0.0001)\) in inhibition of AChE by the test compounds. Guaiacol and caffeic acid were the most potent AChE inhibitors (IC_{50}, 13 mM), and the inhibitory activity of these compounds and dichlorvos did not differ significantly. Eugenol and magnolol (IC_{50}, 22 and 24 mM) had significantly more pronounced inhibition of AChE than honokiol (59 mM). The IC_{50} of isoeugenol, \(\alpha\)-eugenol, \(\beta\)-ethylphenol, and \(\beta,\beta\)'-biphenol was between 75 and 95 mM. The AChE inhibitory activities of methoxyeugenol and phenol were the lowest of all of the compounds that were examined.

Effects on cyclic AMP production. The effects of the two neolignans on the cyclic AMP (cAMP) levels of whole-body homogenates from third-instar *Ae. aegypti* larvae were elucidated and compared with those induced by octopamine alone (Fig. 4). As judged by the preliminary test results, the cAMP levels induced by honokiol and magnolol were determined to be 100 \(\mu\)M, because no significant differences in the levels were observed among the three concentrations examined (50, 100, and 200 \(\mu\)M). There were significant differences \((F = 869.51; \text{df} = 3, 8; P < 0.0001)\) in the cAMP levels for the different test compounds. At a concentration of 100 \(\mu\)M, the cAMP levels induced by honokiol and magnolol were significantly higher than that induced by the control group. The cAMP levels induced by the two neolignans and octopamine did not differ significantly.

Pathological symptoms associated with the two neolignans. The normal morphology of the entire body of the untreated (control) third-instar *Ae. aegypti* larvae showed a common appearance of the typical structures, with well-developed, distinct heads, thoracic regions, and abdominal regions (Fig. 5A). However, the whole body of the larval *Ae. aegypti* treated with honokiol (6.5 mg/L) was severely damaged, with an indistinct appearance of the thorax, midgut, and abdominal regions. The honokiol-treated larvae exhibited dark spots over the entire body. In particular, the midgut region was completely ruptured, and the midgut contents oozed out from the body. Likewise, larval *Ae. aegypti* treated with magnolol (25 mg/L) showed a shrunken body with no distinct abdominal segments (from the 3rd to 7th segments). The intrinsic body fluid contents became dark, and there was an unusual elongated digestive tract with damaged interior tissues. The anal gill region of control *Ae. aegypti* larvae showed well-developed anal gills with thick cuticle-covered undamaged anal gill cells (Fig. 5B). In larvae treated with honokiol, the anal gill from one side was swollen and reduced from its normal length, and the other one was completely damaged. The anal gill region of larvae treated with magnolol showed completely damaged outer cuticle membranes with undistinguishable anal gills with no internal body fluid.

Histopathological effects of two neolignans visualized by Carson’s staining. Histological observation of the whole bodies of third-instar *Ae. aegypti* larvae in the control group showed a typical appearance of the thoracic and midgut regions. The well-developed midgut regions of untreated larvae showed distinct, single-layered midgut epithelium (ME) and peritrophic membranes (PMs) and had rich lumen contents (LCs) in the midgut region (Fig. 6A). By contrast, the midgut region of *Ae. aegypti* larvae treated with honokiol (6.5 mg/L) showed indistinct damaged midgut epithelial layers. The PM next to the epithelial layer, which surrounded the...
Figure 4. Effects on cyclic AMP levels. A whole-body homogenate from third-instar *Aedes aegypti* larvae was tested for adenylate cyclase activity, as described in the Methods section, in the presence of honokiol (100 µM) and magnolol (100 µM). The effects of the neolignans on cyclic AMP (cAMP) levels of the homogenate were compared with those of octopamine (100 µM) alone. Data were expressed as nmol/4.12 µg protein. Each bar represents the mean ± standard error of duplicate samples from three independent experiments (***P < 0.001; ns, no significant difference, using Bonferroni’s multiple comparisons test).

Figure 5. Light micrographs of midgut, thorax, and anal gill parts of larval *Aedes aegypti*. Third-instar *Ae. aegypti* larvae were placed into paper cups containing a methanol–Triton X-100 solution in distilled water with an LC₅₀ of honokiol (6.5 mg/L) or magnolol (25 mg/L) for 24 h. The morphology of the whole body of the larvae was observed with a stereo microscope (35× magnification). (A) Untreated control larvae showed normal appearances of typical structures, with well-developed, distinguished head, thorax, and abdominal regions. Honokiol-treated larvae were completely damaged with indistinct appearances, particularly in the thorax and abdominal region segments. In particular, the midgut region was completely ruptured, and the midgut contents oozed out from the body. Magnolol-treated larvae showed shrunken bodies with no distinguishable abdominal segments. Intrinsic body fluid contents became dark, and there were unusually elongated digestive tracts with damaged interior tissues. (B) Control larvae showed well-developed anal gills and anal gill cells. In larvae treated with honokiol, the anal gill from one side was swollen and reduced in length and the other one was completely damaged. In larvae treated with magnolol, the anal gill region was completely damaged, with outer cuticle membranes with indistinct anal gills and no internal body fluid. All experiments were performed in duplicate, and 20 mosquito larvae were used in each replicate. More than 10 live larvae from control and treated groups were randomly collected and used for analysis.
LCs, was collapsed; this led to luminal debris and indistinct nuclei of epithelial cells in the midgut region (Fig. 6B). Similarly, *Ae. aegypti* larvae treated with magnolol (25 mg/L) also showed indistinct midgut epithelial layers and PMs. Both midgut epithelial layers and PM cells were indistinct, with damaged LCs. Debris of the epithelial and midgut contents without proper nuclei were observed (Fig. 6C).

**Histopathological effects of two neolignans on midguts as determined via transmission electron microscopy.** The midgut regions of third-instar *Ae. aegypti* larvae untreated and treated with honokiol (6.5 mg/L) or magnolol (25 mg/L) were observed via TEM. A PM in the midgut regions of control *Ae. aegypti* larvae enclosed the midgut LCs and consisted of numerous microvilli (MV) from the outside. The midgut regions of control larvae were composed of a well-spaced cytoplasm and numerous cell organelles that were surrounded by a plasma membrane. There was no damage to the cell organelles in the cytoplasm, and there were large, prominent central nuclei (approximately 15 μm diameter), mitochondria, and other cell organelles (Fig. 7A). However, honokiol-treated larvae showed devastated cellular material and extruded masses in the cytoplasm. In particular, mitochondria and large prominent nuclei were undistinguishable from damaged cellular contents. There was no distinct nuclear material inside the nucleus. Some midgut LCs were extruded from the cytoplasm (Fig. 7B). However, magnolol-treated larvae showed a complete disappearance of nuclei as well as cytoplasmic contents. The cellular organelles, such as the nucleus and mitochondria, completely disappeared. In addition, other cytoplasmic organelles were also severely damaged with indistinguishable appearances (Fig. 7C).

**Histopathological effects of two neolignans on anal gills as determined via transmission electron microscopy.** TEM revealed that the anal gills of control third-instar *Ae. aegypti* larvae were surrounded by thick cuticles and that the inner surface of the cytoplasm became more consistent with fluid-filled tracheoles (Fig. 8A). Histopathological observation of honokiol-treated larvae indicated cytoplasmic disruption with damaged anal gill cells (Fig. 8B). However, magnolol-treated larvae showed damaged outer membranes of thick cuticles, which led to internal cytoplasmic destruction (Fig. 8C).
Figure 7. Transmission electronic micrographs of midgut regions of larval *Aedes aegypti*. Third-instar *Ae. aegypti* larvae were placed into paper cups containing a methanol–Triton X-100 solution in distilled water with an LC₅₀ of honokiol (6.5 mg/L) or magnolol (25 mg/L) for 24 h. (A) Control larvae. A transmission electron micrograph revealed that the peritrophic membrane (PM) in the midgut regions of the larvae enclosed the midgut lumen contents (LCs) and consisted of numerous microvilli (MV) from the outside. The LC consists of the cytoplasm and numerous cell organelles surrounded by a plasma membrane. There was no damage to cell organelles in the cytoplasm, including prominent large central nuclei (approximately 15 µm diameter), mitochondria, and other cell organelles. (B) Honokiol-treated larvae. The neolignan destroyed all cellular material and extruded masses in the cytoplasm. In particular, mitochondria and large prominent nuclei were indistinguishable from damaged cellular contents. There was no distinct nuclear material inside the nucleus. (C) Magnolol-treated larvae. These showed complete disappearance of the nucleus, as well as cytoplasmic contents. Nuclei and mitochondria were completely absent. Other cytoplasmic organelles were damaged with indistinct appearances.
Effects of two neolignans on target gene expression levels. To investigate whether the two neolignans affected the transcription of AeCS, which is a midgut-specific chitin synthase gene\textsuperscript{35}, we analyzed the AeCS1 mRNA levels of third-instar Ae. aegypti larvae using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 9A). A reduction in the AeCS1 mRNA levels (82%) was observed in Ae. aegypti larvae following treatment with honokiol (6.5 mg/L) compared to the control larvae. However, the AeCS mRNA level was increased, by up to 503% of the control levels, following treatment with magnolol (25 mg/L).

The mRNA expression levels of AaV-type H\textsuperscript{+}\textsuperscript{-}ATPase, which is involved in the Na\textsuperscript{+}, Cl\textsuperscript{−} and K\textsuperscript{+} uptake co-transport process\textsuperscript{36,37}, and a putative AQP\textsuperscript{38} in the anal gills were likewise evaluated in third-instar Ae. aegypti larvae following treatment with honokiol (6.5 mg/L) or magnolol (25 mg/L). The AaV-type H\textsuperscript{+}\textsuperscript{-}ATPase mRNA expression levels in larvae treated with honokiol and magnolol, respectively, were approximately 16 and 14 fold higher than the control group (Fig. 9B). The AaAQP4 mRNA expression levels in larvae treated with honokiol were approximately 18 fold higher than the control group (Fig. 9C). By contrast, magnolol treatment did not affect the AaAQP4 mRNA expression levels.

Discussion

Plant SMs, such as alkaloids, phenols and terpenoids, either alone or in combination, contribute to mosquito larvicidal activity. Selective plant preparations can be developed into mosquito larvicidal products that are suitable for IMMI and are of great interest because they are target-specific, biodegrade to nontoxic products, have few harmful effects on nontarget organisms, and are environmentally nonpersistent\textsuperscript{19–22}. These potential larvicidal products can be applied to habitats of mosquito larvae in the same manner as conventional larvicides. Certain plant preparations are toxic to different mosquito species larvae\textsuperscript{19–22} and have therefore been proposed to be alternatives to conventional larvicides. Active larvicidal constituents (LC\textsubscript{50} < 50 mg/L)\textsuperscript{39} derived from plants include alkaloids (e.g., retrofractamide A and three isobutylamides\textsuperscript{40}, 0.004–0.86 mg/L), terpenoids (e.g., 9-oxoneoprocurcumenol and neoprocurcumenol\textsuperscript{41}, 5.81 and 13.69 mg/L), coumarins (e.g., imperatorin and eight coumarins\textsuperscript{26}, 2.88–44.39 mg/L), flavonoids (e.g., karanjin, karanjachromene, pongamol, and pongarotene\textsuperscript{42}, 14.61–37.61 mg/L), phenylpropanoids (e.g., ethyl p-methoxyccinnamate\textsuperscript{43}, 12.3–20.7 mg/L), lignans (e.g., (−)-asarinin\textsuperscript{35}, 10.49–16.49 mg/L), neolignans (e.g., burchellin\textsuperscript{34}, 15.5 mg/L), cyanogenic glycosides (e.g., dhurrin\textsuperscript{45}, 12 mg/L), lactones (e.g., butenolides 1 and 2\textsuperscript{46}, 0.41 and 0.47 mg/L), acetylenic alcohols (e.g., falcarinol and falcarindiol\textsuperscript{47}, 3.49 and 6.51 mg/L), and fatty acids (e.g., oleic acid and palmitic acid\textsuperscript{42}, 18.07–18.45 and 34.50–42.96 mg/L). Kiran et al.\textsuperscript{48} considered compounds with an LC\textsubscript{50} < 100 mg/L as having a significant larvicidal effect.

In the current study, we used a contact mortality bioassay to identify mosquito larvicidal constituents from M. demudata seed extracts. The active constituents were determined to be the saturated fatty acid palmitic acid (1), the unsaturated fatty acid linoleic acid (2), and the neolignan honokiol (3). Interpretations of the proton and carbon signals of compounds 1, 2, and 3 were largely consistent with the findings of Perumalsamy et al.\textsuperscript{42}. Ragona

![Control](image1.png) ![Honokiol](image2.png) ![Magnolol](image3.png)

**Figure 8.** Transmission electronic micrographs of the anal gill regions of larval Aeodes aegypti. Third-instar Ae. aegypti larvae were placed into paper cups containing a methanol–Triton X-100 solution in distilled water with an LC\textsubscript{50} of honokiol (6.5 mg/L) or magnolol (25 mg/L) for 24 h. (A) Control larvae. Transmission electronic micrography (TEM) analysis revealed that the anal gills of the control larva were surrounded by thick cuticles and that the inner surfaces of the cytoplasm became fuller, with anal gill cells and fluid-filled tracheoles. (B) Honokiol-treated larvae. Histopathological observation of the larvae indicated cytoplasmic disruption in anal gills with damaged anal gill cells. (C) Magnolol-treated larvae. TEM showed damaged outer membranes of thick cuticles, which led to internal cytoplasmic destruction.
Figures 9. Effects of two neolignans on the expression levels of AeCS, AaV-type H^+ ATPase, and AaAQP4 mRNA. Third-instar Ae. aegypti larvae were placed into paper cups containing a methanol–Triton X-100 solution in distilled water with an LC_{50} of honokiol (6.5 mg/L) or magnolol (25 mg/L) for 24 h. Total RNA was extracted from the anal gills (for AaAQP4 and AaV-type H^+ ATPase) and midguts (for AeCS1) of 50 larvae. Real-time quantitative reverse transcription polymerase chain reaction was performed to determine the levels of AeCS1, AaV-type H^+ ATPase, and AaAQP4 mRNA. Specific AeCS1, AaV-type H^+ ATPase, and AaAQP4, and Aarps7 coding sequence primers were used to amplify AeCS1, AaV-type H^+ ATPase, AaAQP4, and Aarps7 DNA, as described in the Methods section. (A) Midgut specific chitin synthase AeCS1 gene expression was slightly inhibited in honokiol-treated larvae, whereas the gene expression level was significantly increased in magnolol-treated larvae. (B) AaV-type H^+ ATPase gene expression was increased in honokiol- and magnolol-treated larvae compared to control larvae. (C) The AaAQP4 gene expression level was significantly increased in honokiol-treated larve Ae. aegypti compared to control larvae. Magnolol did not affect the AaAQP4 mRNA expression level. The mRNA expression levels were normalized to constitutive expression of mRNA of the housekeeping gene Aarps7 and analyzed by the 2^(-ΔΔCt) method. Each bar represents the mean ± SE of duplicate samples run in three independent experiments (**P < 0.001; ***P < 0.01; *P < 0.05; ns, no significant difference, using Bonferroni’s multiple comparisons test).

et al.49, and Agrawal & Thakur50, respectively. The LC_{50} values of honokiol, linoleic acid, and palmitic acid were between 6.13 and 7.37 mg/L, between 6.97 and 7.50 mg/L, and between 32.56 and 47.58 mg/L, respectively, against larvae of four mosquito species (Cx. pipiens pallens, Ae. aegypti, Ae. albopictus, and An. sinensis). The LC_{50} values of the natural compounds described above were between 0.004 and 42.96 mg/L. Honokiol and linoleic acid were highly effective against three insecticide-susceptible Cx. pipiens pallens, Ae. aegypti, and Ae. albopictus larvae as well as An. sinensis larvae resistant to deltamethrin, temephos, and fenithion51. This current finding indicates that materials derived from M. denudata seeds could be promising naturally occurring mosquito larvicides that are novel and effective against wild mosquito populations in the field. Honokiol shows no toxicity or serious adverse effects in animal models52. Moreover, a honokiol microemulsion has been shown to be nontoxic up to 500μg/kg body weight, although it was associated with irritation to the vasculature of the injection site52.

QSAR analyses of phytochemicals against larval mosquitoes are common26,31,40,42,43,53,54. Not only can QSAR information contribute to the search for additional compounds with higher activities but it can also promote greater understanding of the mechanism of larvicidal action of phytochemicals as described by Dias and Moraes43. The presence of lipophilic groups in aromatic rings or in hydroxyls results in increased toxicity to larval Ae. aegypti, whereas the presence of hydroxyls in aromatic or aliphatic rings results in decreased toxicity41. For example, phenol (LC_{50} 194 mg/L) was less toxic than compounds with lipophilic groups, such as CH chains outside a phenyl ring (e.g., carvacrol and thymol, LC_{50} 69 and 81 mg/L), against larval Ae. aegypti. Wang et al.26 studied the toxicity of six simple coumarins and seven furanocoumarins against third-instar Cx. pipiens pallens and Ae. aegypti larvae. They reported that the chemical structure, alkoxy substitution, and length of the alkoxy side chain at the C8 position of the coumarin nucleus were required for toxicity. However, the MW, hydrophobicity, and MR parameters were negatively correlated with the observed coumarin toxicity. The N-isobutylamino moiety in isobutylamide alkaloids (pellitorine, guineensine, pipercide, and retrofractamide A) was found to play a crucial role in the mosquito larvicidal activity49.

In the current study, p-ethylphenol, o-eugenol, methoxyeugenol, p,p’-biphenol, and honokiol were the most toxic compounds against Cx. pipiens pallens and Ae. aegypti larvae. These compounds did not differ in toxicity from each other. Introduction of a functional group, such as ethyl, to phenol significantly increased the toxicity of the compounds. o-Eugenol and methoxyeugenol were significantly more toxic than either eugenol or isoeugenol. Introduction of a functional group, such as allyl or propenyl, to guaiacol significantly increased its toxicity. Eugenol or isoeugenol did not differ in toxicity and were more toxic than guaiacol. Honokiol was approximately 4 times more toxic than the structural isomer magnolol, which differs from honokiol only in the position of one hydroxyl group. Our findings indicate that phenolic compounds with an electron donor and/or bulky groups at the ortho or para positions were more toxic than phenolic compounds with an electron-withdrawing group or group at the meta position. The MW, hydrophobicity, and MR parameters appear not to be related to the observed phenol toxicity.

An investigation of the mechanisms of action and resistance mechanisms of botanical larvicides can provide useful information to develop efficient mosquito control alternatives with novel target sites and lower toxicities, as well as for determining the most appropriate formulations and delivery means to be adopted for their future commercialization and future resistance management20,21,25. The target sites and mechanisms underlying
the insecticidal actions of several plant SMs have been well-documented by Isman23 and Pavela22. AChE is the main target site of mosquito larvicidal action of flavonoids (e.g., karanjin and pongarotene) and fatty acids (e.g., oleic acid and palmitic acid)42. The mechanism of the larvicidal action of the fatty acids elaidic acid and arachidic acid may involve interfering with the octopaminergic system, whereas linoleic acid and linolenic acid might act on both AChE and octopaminergic receptors42. Certain terpenoids inhibit AChE from houseflies, Madagascar roaches55, and head lice56. A relationship between insect toxicity and electric eel AChE inhibition by terpenoids has been reported55, whereas no direct correlation between the insecticidal and AChE inhibitory activities of terpenoids has been reported55,56,58. In addition, the major mechanisms of resistance to insecticides currently used to control mosquitoes include target site insensitivity, which reduces the sensitivity of sodium channels of the nervous system to pyrethroid insecticides, or sensitivity of the key enzyme AChE to OP and carbamate insecticides, as well as enhanced metabolic detoxification in various groups of insecticides56. Certain phytochemicals have been found to be highly effective against insecticide-resistant mosquitoes55,56, and these are likely to be useful in resistance management strategies. For example, α-asarone, (−)-asarin, and pellitorine are effective against larvae of wild Cx. pipiens pallens and An. sinensis, which have high levels of resistance to the AChE inhibitors chlorpyrifos, fenitrothion, and fenthion; axonic nerve poisons α-cypermethrin and deltamethrin; and mitochondrial uncoupler chlorfenapyr55.

In the current study, a negative correlation (r = −0.609, P = 0.05) between Ae. aegypti larval toxicity and AChE inhibition by the 11 examined phenol compounds was observed. For example, guaiacol and caffeic acid were the most potent AChE inhibitors and were the least toxic larvicides. o-Eugenol, p,p'-biphenol, and p-ethylphenol were also highly toxic to Ae. aegypti larvae and were relatively weak AChE inhibitors. In addition, the isolated neolignan honokiol and its structural isomer magnolol moderately and strongly inhibited AChE, respectively, and caused a significant increase in cAMP levels. Our findings indicate that both AChE and octopaminergic receptors, although which target site is more important remains to be elucidated. Furthermore, honokiol and magnolol are virtually equivalent in toxicity to both insecticide-susceptible Ae. aegypti and insecticide-resistant An. sinensis larvae, suggesting that these neolignans and the pyrethroid or OP do not share a common mode of action or eliciting cross-resistance. Detailed tests are needed to understand the exact larvicidal mechanisms of honokiol and magnolol. The octopaminergic and gamma aminobutyric acid receptors have also been suggested to be novel target sites for several monoterpenoid essential oil constituents in Helicoverpa armigera24 and Drosophila melanogaster34, respectively.

Histopathological investigations indicated that the ME is the action site of plant SMs in Rhodnius prolitis28 and several aquatic dipteran larvae29. The ME has diverse functions, such as ionic and osmotic regulation30, lipid and carbohydrate storage31,32,33, midgut lumen pH control, digestive enzyme secretion, and nutrient absorption34,35. The histopathological effects differ qualitatively according to the localization of organs along the midgut and quantitatively according to the concentration of the material that was examined, duration of the treatment, and taxon34. The mosquito ME is composed of a single layer of polarized epithelial cells supported by an underlying basal lamina36,37. The tetranortriterpenoid azadirachtin has been reported to cause several of the initial effects of necrosis and is particularly associated with swelling of cell and organelles, vesiculation of membranes, and dilation of the rough endoplasmic reticulum in Schistocerca gregaria and Locusta migratoria38. It has also been reported that tannic acid, which is a highly water-soluble polyphenol, causes dramatic degenerative responses of the midgut through sequential epithelial disorganization in larval Cx. pipiens39. Tannins can bind to the lipid components of ion membranes integrity and/or function39.

The current microscopy analysis clearly indicated that the neolignans honokiol and magnolol caused histopathological alterations in the thorax, midgut, and anal gill regions of third-instar Ae. aegypti larvae. Honokiol-treated larval Ae. aegypti showed complete damage with an indistinct appearance of the whole body, particularly in the thorax and abdominal region segments, whereas magnolol-treated larvae showed unusual elongated digestive tracts with damaged interior tissues. Honokiol- or magnolol-treated larvae showed impaired anal gills with damaged outer cuticle layers of anal gill cells compared to control larvae, which showed well-developed anal gills and anal gill cells. In addition, the AeCS gene expression levels in control and honokiol-treated larvae did not differ significantly, whereas the gene expression level was significantly increased in magnolol-treated larvae. This finding suggests differences in the toxicity and mode of larvicidal action between honokiol and magnolol. It has been reported that the isobutylamide alkaloid pellitorine causes dramatic degenerative responses in the thorax and anterior and posterior midgut regions of larval Ae. aegypti by targeting ion transporting cells in the gastric caeca of the thorax region and epithelial cells of the anterior and posterior midgut regions39. Osmoregulation-related machineries, such as V-ATPase40,41,42, are highly expressed in the midguts of larval Ae. aegypti.

Larval mosquitoes rapidly respond as well as restore water and ion balance following stress. In larval Ae. aegypti, four anal gills surrounding the anal opening serve as the major sites for Na⁺, Cl⁻, and K⁺ uptake by H⁺-ATPase and Na⁺/K⁺-ATPase27,29,79. The osmotic uptake of water at the anal gills is the primary external site of ion uptake, normally contributing to 33% of the body weight gain per day28. Donini and O’Donnell30 measured the ion concentration gradients adjacent to the surface of the anal papillae of larval Ae. aegypti using the self-referencing ion-selective microelectrode technique. They confirmed that the anal gills of mosquito larvae serve as the major site for Na⁺, Cl⁻, and K⁺ uptake, complementing the role of the Malpighian tubules, an excretory and osmoregulatory organ, and the rectum, an organ that resorbs ions and some water from primary urine into the hemolymph. In addition, four putative AQP homologs are expressed in the Malpighian tubules, whereas two putative AQP homologs, AaAQP1b and AaAQP4, are expressed in the anal gills. In particular, AaAQP4 plays a crucial role in mediating water transport across the anal gill epithelia of the larval Ae. aegypti31. However, very little information is available with respect to the histopathological changes and gene expression patterns of AaV-type H⁺-ATPase and AaAQP4 induced by conventional insecticides or plant SMs on the anal gills of mosquito larvae. Perumalsamy et al.30 studied the histopathological and molecular effects of natural pellitorine on the
larval ME and anal gills of Ae. aegypti. They reported that pellitorine inhibited the AaAQP4 expression levels and suggested that this compound may disturb the Na\(^{+}\), Cl\(^{-}\) and K\(^{+}\) co-transport system, mainly by degeneration of anal gill cells and damaging the outer thick permeable cuticle membranes of the mosquito larvae. The alkaloid also caused slight decreases in the expression levels of the gene encoding the AaV-type H\(^{-}\)-ATPase protein in the Na\(^{+}\), Cl\(^{-}\} and K\(^{+}\} ion co-transport system, despite significant effects on the gene expression level with pellitorine treatment. Furthermore, expression of the genes encoding AaV-type H\(^{-}\}-ATPase in the Na\(^{+}\}, Cl\(^{-}\} and K\(^{+}\} ion transport system and AQP protein in the anal gills of larve Ae. aegypti was determined in control and pellitorine-treated larvae.

Our current study revealed that honokiol and magnolol caused increases in expression of the gene encoding the AaV-type H\(^{-}\}-ATPase protein in the Na\(^{+}\}, Cl\(^{-}\} and K\(^{+}\} ion co-transport system. In addition, honokiol treatment increased the AaAQP4 expression level, whereas magnolol treatment did not affect this gene expression level. Our findings indicate that honokiol may disturb the Na\(^{+}\}, Cl\(^{-}\} and K\(^{+}\} co-transport system, mainly by degeneration of anal gill cells and damaging the outer thick permeable cuticle membranes of larve Ae. aegypti. The ion exchange effects in the anal gills due to honokiol or magnolol treatment remain to be demonstrated, even though it has been reported that it is difficult to measure ion exchange in situ due to the morphological characteristics of mosquito larvae. The finding that honokiol caused histopathological alterations and increased expression of AaV-type H\(^{-}\}-ATPase and AQP may contribute to a better understanding of the mode of larvicidal action of this neolignan against Ae. aegypti. In addition, our findings, along with previous studies, indicate that the mode of larvicidal action of honokiol might be different from that of the alkaloid pellitorine.

In conclusion, honokiol caused degenerative responses in the cell organelles of the thorax, midgut regions, and anal gills, possibly by targeting the osmoregulation system. Magnolia denudata seed-derived products may be useful as novel larvicides with specific target sites for controlling mosquito populations, particularly in light of their activity against insecticide-resistant mosquito larvae. Magnolia denudata is a fast-growing deciduous tree with the potential for efficient seed production and represents a potential source of a seed extract that can be used as an eco-product. Further research is needed regarding the practical applications of plant-derived preparations as novel mosquito larvicides to establish their safety profiles in humans, although Magnolia denudata seed products have been widely used, as a carminative and diaphoretic. In addition, the effects of these seed products on nontarget aquatic organisms, including larvivorous fishes; as biological control agents for mosquitoes; and on the aquatic environment need to be established. Additional detailed tests are needed to understand how best to improve the larvicidal potency and stability of the compounds isolated from Magnolia denudata for eventual product development.

Methods

Instrumental analysis. \(^{1}\)H and \(^{13}\)C NMR spectra were recorded in deuterated chloroform on an AVANCE 600 spectrometer (Bruker, Rheinspettum, Germany) at 600 and 150 MHz, respectively, using tetramethylsilane as an internal standard. The chemical shifts are given in \(\delta\) (ppm). The DEPT spectra were acquired using Bruker software. The UV spectra were obtained in ethanol or methanol on a UVICON 933/934 spectrophotometer (Kontron, Milan, Italy) and mass spectra on a JMS-DX 303 spectrometer (Jeol, Tokyo, Japan). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 F\(_{254}\) ) were used for analytical thin-layer chromatography (TLC). An Isolera One medium-pressure liquid chromatograph (Biotage, Uppsala, Sweden) and Agilent 1200 high-performance liquid chromatograph (Agilent, Santa Clara, CA, USA) were used to isolate the active constituents.

Materials. Three constituents, palmitic acid, linoleic acid and honokiol, were identified in this study, and their pure organic compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Magnolol, which is a structural isomer of honokiol, and nine commercially available pure organic phenol compounds are listed in Table 8 along with their purities and were used in this study for QSAR. These phenol compounds were purchased from Sigma-Aldrich. For the QSAR analysis, the values of MW, hydrophobic parameter (log P), and steric effects for these compounds were obtained from ChemDraw Ultra 10.0 (Cambridge Soft Corporation, Cambridge, MA, USA) and recorded in Table 8. The MR was used as the parameter to describe steric effects. The structures of these phenol compounds are shown in Fig. 2. The OPs temephos (97.3\% purity) and analytical dichlorvos were purchased from Sigma-Aldrich. Acetylthiocholine iodide (ATChI), 5,5\'-dithio-bis-(2-nitrobenzoate) (DTNB), and octopamine were supplied by Sigma-Aldrich. The cAMP Biotrak Enzymeimmunoassay system and bovine serum albumin (BSA) were supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Sigma-Aldrich, respectively. Horseradish peroxidase (HRP)-labeled cAMP was purchased from R&D Systems (Minneapolis, MN, USA). All of the other chemicals and reagents used in this study were of reagent-grade quality and are available commercially.

Plant material. Fresh seeds of Magnolia denudata were collected from the garden at Huazhong Agriculture University (30°34′60″N, 114°16′0″E) (Wuhan, Hubei, China) in August 2011. The plant was identified by a certified botanical taxonomist. A voucher specimen (MD-SD-01) was deposited in the Research Institute of Agriculture and Life Sciences at Seoul National University.

Mosquitoes. The stock cultures of Cx. pipiens pallens and Ae. aegypti were maintained in the laboratory without exposure to any known insecticide. Engorged Ae. albopictus and Anopheles females were collected from bamboo forest near a village in Jeonju (Jeonbuk, Republic of Korea (ROK)) and rice paddy fields and cowsheds at Osong (Chungbuk, ROK), respectively, in August 2011 using black light FL-6w traps (Shinyoung, Seoul, ROK) and a D-CELL collecting aspirator (Gemplers, Janesville, WI, USA). These mosquito species were separately maintained in temperature-controlled insect rearing rooms to prevent cross-contamination. Females were placed individually in paper cups (270 mL) lined with filter paper and filled with 150 mL distilled water (DW). Larvae
and adults were reared in plastic trays and nylon cages, respectively, under the same conditions as those described previously. All stages were maintained at 27 ± 1 °C and 65–75% relative humidity under a 16:8 h light:dark cycle. Species identification for *Anopheles* females based on PCR confirmed that females from the wild collections were *An. sinensis*. *Aedes albopictus* and *An. sinensis* mosquitoes were reared for 4–5 generations to ensure sufficient numbers for testing. Larvae of *An. sinensis* showed extremely high level of resistance to deltamethrin (relative susceptibility ratio (RSR), 611) and moderate levels of resistance to temephos (RSR, 29) and fenthion (RSR, 11) compared with *Ae. aegypti* larvae. Larvae of *Ae. albopictus* showed similar susceptibilities to deltamethrin, temephos, and fenthion as *Ae. aegypti* larvae.

### Bioassay-guided fractionation and isolation

Seeds (1.5 kg) of *M. denudata* were pulverized, extracted with methanol (5 × 3L) at room temperature for 2 days, and filtered. The combined filtrate was concentrated by rotary evaporation at 40 °C to yield approximately 266.4 g of a dark brown tar. The extract (20 g) was sequentially partitioned into hexane- (3.11 g), chloroform- (5.10 g), ethyl acetate- (6.09 g), butanol- (1.53 g), and water-soluble (4.17 g) fractions for subsequent bioassays. This fractionation procedure was repeated 12 times. The organic solvent-soluble portions were concentrated at 40 °C as described above, and the water-soluble portion was freeze-dried. To isolate the active constituents, 10–100 mg/L of each *M. denudata* seed-derived fraction was tested in a contact mortality bioassay as described by Perumalsamy et al.

The hexane-soluble fraction (32.4 g) was the most biologically active fraction (Table 1), and medium-pressure liquid chromatography (MPLC) was performed using an Isolera apparatus equipped with a UV detector at 254 and 365 nm and SNAP column cartridge (100 g silica gel) with a column volume of 132 mL (Fig. 10). Separation was achieved with a gradient of hexane and ethyl acetate (100:0, 98:2, 90:10, 80:20, 70:30, 60:40, 50:50, 60:40, and 50:50 by volume), and then, elution with methanol (1 L) was performed at a flow rate of 30 mL/min to provide 42 fractions (each approximately 100 mL). The column fractions were monitored by TLC on silica gel plates developed with a chloroform and methanol (8:2 by volume) mobile phase. Fractions with similar Rf values on the TLC plates were pooled. The spots were detected by spraying the plate with 2% sulfuric acid and then heating the samples on a hot plate. Active fractions 3 (H2) and 14–18 (H6) were obtained. Fraction H2 was separated by MPLC with a UV detector and column cartridge (100 g silica gel) through elution with a gradient of hexane and ethyl acetate (98:2, 97:3, 96:4, 95:5, 90:10, 87:13, 83:17, 80:20, 73:17, 65:35, 60:40, and 50:50 by volume), followed by a final elution with methanol (1 L) at a flow rate of 50 mL/min to provide 233 fractions (each approximately 22 mL). The column fractions were monitored by TLC on silica gel plates as described above. Active fractions 103–125 (H22) were pooled and separated by MPLC with a UV detector at 254 and 280 nm and column cartridge (25 g silica gel) with a column volume of 33 mL by elution with a gradient of chloroform and methanol (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 87:13, 83:17, 80:20, 73:17, 65:35, 60:40, and 50:50 by volume), followed by a final elution with methanol (1 L) at a flow rate of 15 mL/min to provide 70 fractions (each approximately 20 mL). Fractions 8–45 (H22) was purified by preparative TLC (chloroform:methanol, 98:2 by volume) to yield compound 1 (21.4 mg). Another active fraction, H6, was separated by MPLC with a UV detector at 254 and 360 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and methanol (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 89:11, 60:40, and 0:100 by volume) at a flow rate of 15 mL/min to provide 70 fractions (each approximately 20 mL). Fractions 8–45 (H22) was purified by preparative TLC (chloroform:methanol, 98:2 by volume) to yield compound 1 (21.4 mg). Another active fraction, H6, was separated by MPLC with a UV detector at 254 and 360 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and methanol (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 89:11, 60:40, and 0:100 by volume) at a flow rate of 25 mL/min to provide 204 fractions (each approximately 22 mL). The column fractions were monitored by TLC on silica gel plates developed with a hexane and ethyl acetate (9:1 by volume) mobile phase. Active fractions 4–21 (H62) and 45–51 (H64) were obtained. Fraction H62 was separated by MPLC with a UV detector at 254 and 280 nm and column cartridge (100 g silica gel) by elution with a gradient of hexane and ethyl acetate (96:4, 94:6, 90:10, 85:15, 80:20, 73:27, 70:30, 60:40, and 40:60 by volume), followed by a final elution with methanol (350 mL) at a flow rate of 40 mL/min to provide 110 fractions (each approximately 22 mL). The column fractions were monitored by TLC (hexane:ethyl acetate, 8:2 by volume). Active fractions 57–77 (H64) was pooled and separated by MPLC with a UV detector at 254 and 365 nm and column cartridge (25 g silica gel) by elution with a gradient of hexane and ethyl acetate (100:0, 96:4, 90:10, 85:15, 82:18, 81:19, 75:25, 70:30, 65:35, and 60:40 by volume), followed by a final elution with methanol (80 mL) at a flow rate of 25 mL/min to afford 68 fractions (each approximately 22 mL). Preparative high-performance liquid chromatography (HPLC) was performed to separate the constituents from active fractions 8–16 (H6232) from H623 with a 7.8 mm i.d. × 300 mm μBondapak C18 column (Waters, Milford, MA, USA) and mobile phase of acetonitrile and water (93:7 by volume) at a flow rate of 1 mL/min.

### Table 8. Values of physical parameters of 11 phenol compounds examined in this study.

| Compound   | Molecular weight (g/mol) | log P | Molecular refraction | Purity (%) |
|------------|-------------------------|------|----------------------|------------|
| Phenol     | 94.11                   | 1.48 | 28.1                 | >99        |
| p-Ethylphenol | 122.17                 | 2.42 | 37.7                 | 99         |
| Guaiacol   | 124.14                  | 1.19 | 34.8                 | 98         |
| o-Eugenol  | 148.21                  | 2.96 | 46.9                 | 98         |
| Eugenol    | 164.20                  | 2.20 | 48.7                 | 99         |
| Isoeugenol | 164.20                  | 2.45 | 50.7                 | 98         |
| Caffeic acid | 180.16                 | 1.42 | 47.5                 | ≥98        |
| p,p′-Biphenol | 186.21                 | 2.42 | 54.6                 | 97         |
| Methoxyeugenol | 194.23                | 1.79 | 55.4                 | ≥98        |
| Honokiol   | 266.33                  | 4.20 | 82.4                 | ≥98        |
| Magnolol   | 266.33                  | 3.94 | 82.4                 | ≥98        |
separation was monitored using a UV detector at 210 nm. Finally, active constituent 2 (28 mg) was isolated at a retention time of 19.81 min. Fraction H64 was separated by MPLC as described above. The column fractions were monitored by TLC on silica gel plates developed with hexane and ethyl acetate (8:2 by volume). Active fractions 26–59 (H643) were pooled and separated by MPLC with a UV detector at 254 and 365 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and ethyl acetate (100:0, 99:1, 98:2, 97:3 and 96:4 by volume), followed by a final elution with methanol (300 mL) at a flow rate of 40 mL/min to afford 147 fractions (each approximately 22 mL). Fractions 60–72 (H6433) were pooled and purified by preparative TLC (chloroform:ethyl acetate, 8:2 by volume) to provide four fractions. A preparative HPLC was performed to separate the constituents from active fractions H64333 from H6433 as described above. Finally, potent active constituent 3 (43.25 mg) was isolated at a retention time of 13.57 min.

Bioassay. A contact mortality bioassay was used to evaluate the toxicity of all compounds against third-instar larvae of the four mosquito species. Each test compound in methanol was suspended in DW with Triton X-100 (20 µL/L). Groups of 20 mosquito larvae were separately placed into paper cups (270 mL) containing each compound solution (250 mL). Temephos served as a positive control and was used in a similar manner. Negative controls consisted of a methanol–Triton X-100 solution in DW. As judged by the preliminary test results, the toxicity of each test compound and insecticide was determined with four to six concentrations ranging from 0.1 to 400 mg/L and 0.001 to 0.1 mg/L, respectively.

Treated and control (methanol–Triton X-100 solution only) larvae were maintained 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 it was prodded with a fine wooden dowel. All treatments were performed three times using 20 larvae per replicate.

Acetylcholinesterase inhibition assay. The in vitro AChE inhibitory activity of the test compounds was assessed according to the method of Perumalsamy et al. Third-instar Ae. aegypti larvae were used in all experiments. Whole bodies of 50 larvae were homogenized in 1 mL of ice-cold 0.1 M phosphate buffer (pH 8.0) using a glass tissue homogenizer. After filtering through cheesecloth, the homogenate was centrifuged at 1,000 × g at 4°C for 5 min. The supernatant was used directly as the enzyme source for AChE. The protein levels were determined using the Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as the standard. The reaction mixture consisted of 50 µL of the crude enzyme preparation (4.3–4.8 µg protein equivalents), 150 µL of...
0.1 M phosphate buffer, 20 μL of 3 mM DTNB in phosphate buffer (pH 7.0), and 1 μL of various concentrations of each test compound in ethanol. The reaction mixture was incubated at 30 °C for 5 min, and 20 μL of 32 mM ATP was then added to the mixture. After incubation for 30 min, the reaction was terminated by adding 20 μL of 5 mM eserine salicylate. Absorbance was recorded at 412 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The OP dichlorvos served as a positive control and was used in a similar manner. The results are expressed as the means ± standard errors (SEs) of triplicate samples from three independent experiments.

**Determination of cyclic AMP levels.** The method of Perumalsamy et al. was used to assess the in vitro octopamine-sensitive adenylate cyclase activity of the test compounds. In brief, whole bodies of Ae. aegypti larvae (106.2 mg) were homogenized in 500 μL of 2 mM Tris-maleate buffer (pH 7.4) containing 0.8 mM ethylene glycol tetraacetic acid (EGTA). The adenylate cyclase activity was determined using the cAMP Biotrak Enzymeimmunoassay system according to the manufacturer’s protocol. The assay was conducted in a total volume of 100 μL containing 80 mM Tris-maleate buffer, 5 mM theophylline, 2 mM MgSO₄, 0.5 mM adenosine triphosphate (ATP), 0.2 mM EGTA, 50 μL of whole-body homogenate (equivalent to 4.12 μg protein), and 1 μL of the test compounds in Tris-maleate buffer containing 0.2% ethanol. After incubation at 20 °C for 5 min, the reaction was initiated by addition of ATP. Incubation was performed at 30 °C for 3 min in a shaking water bath. The reaction was stopped by boiling for 2 min, and then, the assay tube was cooled and centrifuged at 8,000 × g for 10 min. Fifty microliters of the supernatant were assayed for cAMP levels.

Polystyrene microplates (1 strip of 8 wells) coated with a goat anti-mouse polyclonal antibody were used. Fifty microliters of a mouse monoclonal antibody solution were added to each well except for the blank wells (or the nonspecific binding wells). The wells covered with the adhesive strip were incubated at 25 °C for 1 h in a shaking incubator (480 rpm). After washing with 400 μL of water, buffer four times, 50 μL of the two neolignan samples for cAMP determination and cAMP standard were added to wells. Control, blank (nonspecific binding), and zero standard wells were supplemented with 50 μL of a diluent RSD5–55 buffer. Fifty microliters of HRP-labeled cAMP were then added to the wells. The plate was covered with a new adhesive strip and incubated at 25 °C for 2 h on the shaker. After an additional wash as described above, 200 μL of a substrate solution (equal volumes of stabilized hydrogen peroxide and stabilized chromogen) was added to each well and the test plate was incubated at 25 °C for 30 min on the benchtop in darkness. Finally, the reaction was terminated by addition of 100 μL of 2 N sulfuric acid to each well. The optical densities at 450 and 540 nm were determined using the microplate reader described above. The readings at 540 nm were subtracted from the readings at 450 nm. The cAMP concentrations are expressed as nmol/μg protein. The results are expressed as the means ± SEs of triplicate samples from three independent experiments.

**Light microscopy analysis.** Third-instar Ae. aegypti larvae were placed into paper cups (270 mL) containing a methanol–Triton X-100 solution (250 mL) in DW with an LC₅₀ of honokiol (6.5 mg/L) or magnolol (25 mg/L) as described previously. At 24 h post-treatment, the untreated and treated larvae were placed on a microscope slide at room temperature for light microscopy. Morphological observations were made with an EZ4 HD stereo microscope (×35) equipped with an Integrated 3.0 Mega-Pixel CMOS camera (Leica Microsystems, Heerbrugg, Switzerland). All experiments were performed in duplicate, with 20 mosquito larvae used in each replicate. More than 10 live larvae from the control and treated groups were randomly collected and used for analysis.

**Histology analysis by Carson’s trichome staining.** Third-instar Ae. aegypti larvae were treated with an LC₅₀ of honokiol (6.5 mg/L) or magnolol (25 mg/L) in all experiments as described above. At 24 h post-treatment, Ae. aegypti larvae untreated and treated with honokiol or magnolol were immediately fixed in Bouin’s fluid at 4 °C for 24 h. Larvae were then dehydrated in an ethanol-tetrahydrofuran-xylene series and embedded in Paraplast X-tra (Sigma-Aldrich). The embedded preparations of the larvae were sectioned at a 5 μm thickness using a Microm HM 340E rotary microtome (Thermo Scientific, Walldorf, Germany). The sections were dried at 40 °C overnight, subsequently deparaffinized with CitriSolv (Fisher Scientific, Fair Lawn, NJ, USA), and rehydrated with a series of ethanol in phosphate-buffered saline (PBS) solutions. The rehydrated sections were stained in Weigert’s iron hematoxylin for 30 s, followed by Carson’s trichrome staining procedure. This staining protocol stained the columnar and goblet cells of the midgut blue and red, respectively. These sections were then dehydrated, cleared in xylene, and mounted in EMS Permount (Electron Microscopy Sciences, Hatfield, PA, USA). Images were observed and captured using a DMIL LED microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica MC 170 HD. Observations were taken of 15 larvae under the microscope.

**Transmission electron microscopy analysis.** Third-instar Ae. aegypti larvae were treated with an LC₅₀ of honokiol (6.5 mg/L) or magnolol (25 mg/L) in all experiments as described above. At 24 h post-treatment, the midgut and anal gills of the untreated and treated Ae. aegypti larvae were fixed in Karnovsky’s fixative (2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2)) at 4 °C in darkness for 2–4 h and were washed three times with the same buffer. The specimens were post-fixed with 1% (w/v) osmium tetroxide in the same buffer at 4 °C for 2 h and washed with DW three times. The post-fixed specimens were then dehydrated through a graded series of ethanol with increasing concentrations up to 100% for 15 min. The specimens were further treated with propylene oxide twice each for 15 min as a transitional fluid and embedded in Spurr’s resin. Ultrathin sections (approximately 50 nm thickness) were cut with a UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained in 2% aqueous uranyl acetate for 7 min at room temperature as well as with Reynolds lead citrate for 7 min. The sections were mounted on copper grids, and micrographs were obtained from a CM120 transmission electron microscope (Philips Electronics, Amsterdam, Netherlands) at 80 kV. Images were captured using a MegaView III digital camera (Olympus-SIS, Lakewood, CO, USA). Observations were taken of 12 larvae via TEM.
Table 9. Primers used for real-time quantitative reverse transcription polymerase chain reaction in this study.

| Gene               | RefSeq ID | Forward primer and reverse primer | cDNA amplicon size |
|--------------------|-----------|-----------------------------------|-------------------|
| Aarps7             | AY380336.1| 5′-CTGGAGGAGGACCTGATC-3′          | 838               |
| AeCSI              | XM_001662150| 5′-GGTGTCACTGTTTGGTCC-3′          | 4767              |
| AaAQP4             | XM_001647996| 5′-ATGGGACTGCGGCTTCACT-3′         | 786               |
| AaV-type H+ -ATPase| AF992934  | 5′-TGTTGCTCGGCTGTCGTTA-3′         | 2538              |

Real-time reverse transcription-polymerase chain reaction assay. Real-time qRT-PCR with SYBR Green dye was performed to determine whether honokiol and magnolol treatment affected the expression levels of three target genes, AaAQP4, AaV-type H+ -ATPase and AeCSI, in Ae. aegypti. Third-instar Ae. aegypti larvae were placed into paper cups (270 mL) containing a methanol–Triton X-100 solution (250 mL) in DW with an LC50 of honokiol (6.5 mg/L) or magnolol (25 mg/L) for 24 h as described previously. Total RNA was extracted from the anal gills (for AaAQP4 and AaV-type H+ -ATPase) and midgut (for AeCSI) of 50 larvae using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Residual genomic DNA was removed using RNase-Free DNase (Promega, Fitchburg, WI, USA), and 1 μg of total RNA from each sample was used for complementary DNA (cDNA) synthesis with an oligo(dT) primer (Invitrogen, Grand Island, NY, USA) according to the protocol of the SuperScript III Reverse Transcriptase Kit. Real-time qRT-PCR was performed in 96-well plates using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster, CA, USA). Each reaction mixture consisted of 10 μL of the SYBR Green PCR Master Mix Kit (Applied Biosystems, Warrington, Cheshire, UK), 2 μL of the forward and reverse primers (10 pmol each), 1 μL of cDNA (25 ng) and diethylpyrocarbonate-treated water for a final volume of 20 μL. The oligonucleotide PCR primer pairs are listed in Table 9 and were purchased from Cosmo Genetech (Seoul). The cycling program included an initial hold at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, at 58 °C for 30 s, at 72 °C for 1 min, and an extension at 72 °C for 5 min using an Applied Bioscience Thermal Cycler. The mRNA expression levels of the target genes were normalized to the mRNA expression level of the housekeeping gene Aarps7 and analyzed by the 2-ΔΔCT method using the Applied Biosystems StepOne Software v2.1 and DataAssist Software. The results are expressed as the means ± SEs of duplicate samples from three independent experiments.

Data analysis. The concentration of the test compounds required to produce 50% AChE inhibitory activity (IC50) was determined using the SAS 9.13 program (Cary: SAS Institute; 2014). Concentration-mortality data were subjected to probit analysis using the SAS 9.13 program. The LC50 values for the test phenol compounds were considered significantly different from one another when the 95% confidence intervals (CIs) did not overlap. A compound that had an LC50 > 100 mg/L was considered to be ineffective as described by Kiran et al. Correlation coefficient analysis of the toxicities of compounds to third instar Cx. pipiens pallens and Ae. aegypti larvae was performed using their LC50 values and physical parameter (MW, MR, and log P) values for the test phenol compounds. One-way analysis of variance was used as indicated in the tables and figure legends. Statistical analyses were performed using the SAS 9.13 program. Multiple linear regression and correlation analysis were performed using the SPSS 24 program (Armonk: IBM Corp.; 2016).

Data Availability Statement
All data are disclosed in the text or tables in the article. EI-MS, 1H NMR, 13C NMR, and DEPT spectra of compounds 1, 2, and 3 are provided as Additional files 1–3, 4–7, and 8–11, respectively.

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**Author Contributions**

Z.W. and Y.-J.A. conceived and designed the experiments. Z.W. and H.P. performed the experiments. Z.W., X.W. and Y.-J.A. analyzed the data. Z.W. and Y.-J.A. wrote the paper. Y.-J.A. supervised the project. Z.W., H.P. and Y.-J.A. interpreted the spectroscopic data. Z.W., H.P. and Y.-J.A. read and approved the final manuscript.

**Additional Information**

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