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Nematicidal and Antifungal Activities of Annonaceous Acetogenins from *Annona squamosa* against Various Plant Pathogens

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Supporting Information

ABSTRACT: The methanol extract of *Annona squamosa* seeds was highly active against two phytoparasitic nematodes, *Bursaphelenchus xylophilus* and *Meloidogyne incognita*. It efficiently suppressed plant diseases, caused by *Phytophthora infestans* and *Puccinia recondita*. Ten annonaceous acetogenins (AAs) were isolated, and their chemical structures were identified by mass and nuclear magnetic resonance spectral data. Out of 10 substances, eight displayed strong in vitro nematicidal activity against *B. xylophilus* with LD_{50} values ranging 0.006 to 0.048 μg/mL. Squamocin-G showed potent nematicidal activity against *M. incognita*. Squamocin, squamocin-G, and squamostatin-A also displayed potent in vitro and in vivo antifungal activities against *P. infestans* causing tomato late blight. In addition, squamostatin-A effectively controlled the development of wheat leaf rust caused by *P. recondita*. Our findings suggested that *A. squamosa* seeds and its bioactive AAs can be an alternative resource of a promising botanical nematicide and fungicide to control various plant diseases.

KEYWORDS: *Annona squamosa*, antifungal activity, nematicidal activity, phytoparasitic nematode, phytopathogenic fungi

INTRODUCTION

Diseases and damages caused by plant pathogens including fungi, nematodes, bacteria, and viruses lead to yield reductions in food and crops worldwide.[1,2] The root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid et White) Chitw., and the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, are phytoparasitic nematodes that cause serious damage in agricultural crops and forestry, respectively. RKN, typically a soil-borne nematode, is a root parasite of various crop species[2,3] that induces root-knot and disrupts the physiology of the host plants through their reproduction and feeding within plant roots. This leads to a significant reduction in crop yield and deterioration of product quality. Crop loss due to RKN ranged from 30 to 60% on aubergine and 50% on cantaloupe and watermelon.[3] The annual global loss in agriculture caused by phytoparasitic nematodes has been estimated to be U.S. $100 billion worldwide.[2] In Japan, Korea, China, Taiwan, and Portugal, PWN is also listed as a harmful organism to plants or plant products.[4] Infection of PWN is an epidemic disease that heavily damages pine forests.[5]

There are more than 8000 known fungal species that cause disease in plants.[1] Most (85%) plant diseases are caused by fungi, which include *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Septoria* spp., *Rhizoctonia solani*, *Pseudoperonospora* spp., *Blumeria* spp., *Puccinia* spp., *Phytophthora* spp., *Pythium* spp., *Colletotrichum* spp., *Fusarium* spp., and *Magnaporthe oryzae*. Damage caused by these fungi leads to worldwide economic losses in crops.[1] Crop growers have mainly used synthetic agrochemicals to control plant diseases. However, side effects of these chemicals such as residual toxicity, environmental pollution, and the development of resistance in the target pathogens led scientists to research the integrated method to control plant diseases, which include biocontrols employing antagonistic microorganisms and environmental benign agrochemicals such as microbial pesticides and plant-derived pesticides.[1,2,6] Botanical extracts, essential oils, and phytochemicals have been known to be natural sources responsible for biological activities against fungi, insects, and nematodes. These materials provide advantages: environmental friendliness, specificity toward target species, selective modes of action, and little toxicity to humans.[1,6,7]

During the search for botanical pesticides from tropical plants, we found that the methanol (MeOH) extract of *Annona squamosa* seeds is highly active against two phytoparasitic nematodes, RKN and PWN, and two plant diseases, tomato late blight (TLB) (*Phytophthora infestans*) and wheat leaf rust (WLR) (*Puccinia recondita*). *A. squamosa* is widely distributed in tropical and subtropical regions. Annonaceous acetogenins (AAs), a common secondary metabolite of *A. squamosa*, is characterized by branched C_{32} and C_{34} fatty acids ending in a γ-lactone and exhibits a broad range of biological properties such as cytotoxic, antitumor, antiparasitic, pesticidal, antimicrobial, and immunosuppresive activities.[8,9] However, the biological activities of AAs from *A. squamosa* against various plant pathogens such as RKN, PWN, and phytopathogenic fungi have not yet been reported.

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The objectives of this study are to isolate the bioactive AAs from *A. squamosa* under the guidance of bioassays, to determine their chemical structures, and to evaluate their nematicidal and antifungal activities against various plant pathogens.

MATERIALS AND METHODS

General Experimental Procedures and Apparatus. Chemical structures of the purified compounds were determined mainly by mass spectrometry and nuclear magnetic resonance (NMR) analyses. Electron impact (EI) and chemical ionization (CI)−mass spectrometry (MS) spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JMS-DX303; JEOL Ltd., Tokyo, Japan). Electrospray ionization (ESI)-MS spectra were recorded on a single quadrupole mass spectrometer equipped with an ESI (MSD1100, Hewlett-Packard Co., CA). One- and two-dimensional and NMR spectra were recorded on a Bruker AMX-500 (500 MHz) spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) at 500 MHz for ^{1}H NMR spectra and 125 MHz for ^{13}C NMR spectra, using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal standard. Preparative high-performance liquid chromatography (HPLC) (Shimadzu LC-6AD) using a Capcell Pak C18 UG120 Å (5 μm, 20 mm × 250 mm; Shiseido Co. Ltd., Tokyo, Japan) column was purchased from Shimadzu Co. (Kyoto, Japan). Reverse phase C18 resin was Cosmosil 75C18-OPN grade from Nacalai Tesque Inc. (Kyoto, Japan). Sep-Pak C18 (Sep-Pak Vac35 cm^3,10 g) was from Waters Co. (Ireland). Sephadex LH20 bead size 25−100 μm was from Sigma (United States). Silica gel 60 Å grade (230−400 and 70−230 mesh) was purchased from E. Merck (Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on precoated silicagel 60 F245 plate (Kiesel gel60, code 7544; E. Merck), and spots were detected first under UV light and then by spraying with *p*-anisaldehyde regent. All solvents were purchased from Merck and SK Chemicals Co. (Seoul, Korea).

Chemicals. Fungicide standards benomyl, dimethomorph, dithianon, fludioxonil, flusilazole, and validamycin were purchased from Dr. Ehrenstorfer GmbH Co. (Augsburg, Germany). Blasticidin-S was purchased from Daeil Bio Co. (Seoul, Korea). *trans*-Cinnamaldehyde used as a positive control for RKN was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Abamectin was purchased from Kyungnong Co. (Seoul, Korea).

Plant Material. The seeds of *A. squamosa* were collected in March of 2009 in Nam Dinh, Vietnam. The species was identified, and the vouched specimens (AT3122) were deposited in Luu's laboratory (Hanoi, Vietnam).

Extraction and Isolation. The MeOH extract of *A. squamosa* seeds (75 g) was dissolved in 700 mL of 80% MeOH and partitioned twice with an equal volume of *n*-hexane (Hex). The lower MeOH layer was evaporated to dryness. The residue was then suspended into 800 mL of distilled water. The aqueous solution was successively partitioned twice with ethyl acetate (EtOAc) and butanol (BuOH). The EtOAc layer showed the strongest activities against RKN, PWN, and the two plant diseases TLB and WLR caused by *P. infestans* and *P. recondita*, respectively. The EtOAc layer was therefore employed as a raw material to isolate bioactive compounds.

The EtOAc layer (23.6 g) was chromatographed on a SiO_2 column (8.0 cm × 60.0 cm; Kiesel gel 60, 250 g, 70−230 mesh) with gradient elution of dichloromethane (DCM):MeOH to give 14 fractions named ASE1−ASE14. Fraction ASE3 (2.23 g) was separated on a SiO_2 column (3.4 cm × 60.0 cm; Kiesel gel 60, 100 g, 230−400 mesh; E. Merck) with a mixture of DCM:EtOAc:MeOH (5:5:1, v/v/v) to afford two fractions, ASE3-1 and ASE3-2. Compounds **1** (28.3 mg), **2** (25 mg), **3** (23 mg), and **4** (16 mg) were isolated from fraction ASE3-1 by preparative HPLC with an isocratic elution of MeOH:water (93:7, v/v).

Fraction ASE4 (1.73 g) was subjected to a SiO_2 column (2.4 cm × 45.0 cm; Kiesel gel 60, 55 g, 230−400 mesh), eluting with a stepwise gradient of DCM:EtOAc:MeOH (5:5:0 to 5:5:1, v/v/v, 800 mL each) to yield 13 fractions, ASE4-1−ASE4-13. Fractions ASE4-6 and ASE4-7 were pooled due to the similarity of constituents in each. The combined fraction (418 mg) was repeatedly separated by preparative HPLC with an eluting solvent of MeOH:water (88:12, v/v) under isocratic conditions to afford compounds **5** (126 mg) and **6** (12 mg). ASE12 was applied onto a ODS column (2.4 cm × 45 cm; 50 g of resin), which was eluted with MeOH:water (85:15, v/v). This led to the isolation of **7** (140 mg). Fraction ASE10, containing compound **8**, was successfully chromatographed on a Sephadex LH20 (3.8 cm × 60 cm; 150 g, bead size 25−100 μm) column with an eluting solvent of DCM:MeOH (7:3, v/v). Compound **8** was further purified by Sep-Pak C18 with MeOH:water (85:15, v/v).

The Hex layer (13.1 g) was also active against the PWN nematodes. Therefore, it was further used to isolate bioactive compounds. A portion of this layer (10 g) was applied to the SiO_2 column (5.5 cm × 60 cm; Kiesel gel 60, 200 g, 230−400 mesh) and then successfully eluted with Hex:EtOAc under gradient conditions. Fractions ASH1−ASH5 were obtained on the basis of the similarity of the resulting fractions as monitored by TLC. Fraction ASH4 (6.1 g) was separated by elution on a SiO_2 column (3.8 cm × 60 cm; Kiesel gel 60, 180 g, 230−400 mesh) using mixtures of Hex:EtOAc:acetone (8:2:2 and 5:5:2, v/v/v, 800 mL each) to yield fractions ASH4-1−ASH4-5. The isolation of ASH4-1 (700 mg) by preparative HPLC with an eluent of MeOH:water (94:6, v/v) afforded **9** (16 mg) and **10** (18 mg).

Organisms and Growth Media. *M. incognita* was isolated from infected tomato roots. *B. xylophilus* was isolated from the chips of infested pine trees and grown on the mycelia of *B. cinerea* in a potato dextrose agar (PDA) Petri dish for 7 days at 28 °C. Phytopathogenic fungi such as *M. oryzae*, *R. solani*, *B. cinerea*, *P. infestans*, *P. recondita*, *Blumeria graminis* f. sp. *hordei*, and *Colletotrichum coccodes* isolated from infected tissues of each host by the authors were used as test organisms in all antifungal assays. All phytopathogenic fungi were maintained on PDA medium except *P. infestans*, for which V-8 juice agar medium was used.

Nematicidal Bioassays against RKN and PWN. Stock solutions of test materials such as AAs, fractions, and extracts obtained from *A. squamosa* were prepared in dimethyl sulfoxide (DMSO) or MeOH at a concentration of 100 times higher than the testing concentration and stored at −20 °C until use. For the nematicidal bioassays against RKN, eggs were extracted from infected tomato roots with a sodium hypochlorite aliquot, and second-stage juveniles (J2s) were hatched in modified Baermann funnels at 28 °C. All J2s hatched in the first 3 days were collected with distilled water containing 0.01% (v/v) Tween 20 and used within 24 h for the bioassay using 48-well plates. Five microliters of stock solutions was directly mixed with 495 μL of a RKN suspension (containing about 100 J2s) in 1 mL wells. Untreated controls were treated with DMSO or MeOH alone. Concentrations of DMSO and MeOH in each well never exceeded 1% volume. Abamectin and cinnamaldehyde, which are used as commercial natural nematicides and biopesticides, were used as a positive control.[10,11] The treated plates were kept under a shaking condition and incubated in the dark at 28 °C. Juveniles were observed with the aid of a light microscope (Camscope; Sometech Inc., Seoul, Korea) after 1, 2, and 3 days. The experiment was conducted twice with three replicates. The values were expressed as the percentage mortality (± standard deviation).

The nematicidal activity of test materials against PWN was assessed by the microtiter plate assay method as previously described by Choi et al.[12] PWN was grown on the mycelia of *B. cinerea* in a PDA Petri dish for 7 days at 28 °C. The J2s of PWN were collected with distilled water containing 0.01% (v/v) Triton X-100. Untreated controls were treated with DMSO or MeOH alone. Concentrations of organic solvent in each well never exceeded 1% volume. Abamectin that is produced by *Streptomyces* spp. and used as a trunk-injection agent against PWN is used as a positive control. The experiment was conducted twice with

three replicates. The activities were expressed as LC_{50} values (concentration of the compound that causes 50% lethality).

In Vitro Bioassays against *P. infestans*. For sporangium germination test, the effects of three AAs on the germination of *P. infestans* were investigated as previously described by Kim et al.[13] Serial dilutions of each of the three compounds were made in MeOH to obtain solutions at concentrations of 12500, 6250, 3130, 1570, 783, and 391 μg/mL. Each test solution (2 μL) was mixed with 198 μL of the sporangial suspension (10^5 sporangia/mL) of *P. infestans*, and aliquots (50 μL) were loaded onto three hole-slide glasses, which were then placed onto moistened papers in a sealed case. MeOH (1%, v/v) was used as an untreated control. The sporangia of *P. infestans* were incubated for 24 h at 20 °C. Then, approximately 100 sporangia from each replicate were examined under a light microscope. The experiment was repeated, and values were expressed as the mean ± standard deviation.

For the zoospore germination test, the sporangia were incubated for 1 h at 4 °C to induce the release of zoospores. The solution was then passed through a Whatman #2 filter paper to remove the sporangia. Two microliters of each test solution was mixed with 198 μL of the zoospore suspension, and then, aliquots (50 μL) of each zoospore suspension treated with AAs were loaded onto three hole-slide glasses. MeOH (1%, v/v) was used as an untreated control. The slide glasses were placed onto moistened papers in a sealed case to maintain humidity and incubated at 20 °C for 24 h in the dark.[13] The inhibitory effects of the test chemicals on the zoospore germination of *P. infestans* were determined by observing the germ number in each hole using a light microscope (Camscope, Sometech Inc., Korea). The experiment was repeated, and values were expressed as the mean ± standard deviation.

For mycelial growth test, the inhibitory activities of the MeOH extract and the EtOAc and Hex layers against the mycelial growth of *P. infestans*, along with the three main compounds squamocin, squamocin-G, and squamostatin-A, were investigated by a poison-plate assay as previously described by Kim et al.[13] The test materials were dissolved in 100 μL of MeOH and added to 9900 μL of V8-agar at 50 °C to give a homogeneous solution. Two milliliters of this solution was pipetted into each well (3.5 cm i.d.) of sterile six-well plates. After solidification, the wells were inoculated at the center with a plug (5 mm) that was cut from the margins of the growing colonies of *P. infestans*. The inoculated plates were incubated at 20 °C for 5−7 days until the growth reached the edge of the plates in the control plates. The wells containing V8-agar treated with 1% (v/v) MeOH were used as untreated controls. The antifungal activity was expressed as the percent of inhibition of radial growth. The experiment was conducted twice with four replicates, and values were presented as the mean ± standard deviation.

In Vivo Antifungal Bioassay. The extracts of *A. squamosa* seeds and the three main AAs, squamocin, squamocin-G, and squamostatin-A, were tested for antifungal activity in vivo against seven fungal plant diseases such as rice blast (RCB) caused by *M. oryzae*, rice sheath blight (RSB) caused by *R. solani*, tomato gray mold (TGM) caused by *B. cinerea*, TLB caused by *P. infestans*, WLR caused by *P. recondita*, barley powdery mildew (BPM) caused by *B. graminis* f. sp. *hordei*, and red pepper anthracnose (RPA) caused by *C. coccodes*. The in vivo bioassays were performed as described previously.[13,14] Test materials were dissolved in 2 mL of MeOH and diluted with 38 mL of 0.025% (v/v) Tween 20. Controls were treated with 0.025% (v/v) Tween 20 containing 5% MeOH. The following compounds were applied for the positive controls: Blasticidin-S (50 and 1 μg/mL) for RCB, validamycin (50 and 5 μg/mL) for RSB, fludioxonil (50 and 5 μg/mL) for TGM, dimethomorph (10 and 2 μg/mL) for TLB, flusilazole (10 and 2 μg/mL) for WLR, benomyl (100 and 1 μg/mL) for BPM, and dithianon (50 and 10 μg/mL) for RPA. The pots were arranged in a randomized complete-block design, with three replicates per treatment. All experiments were performed in duplicate, and the six estimates for each treatment were converted into a control percentage (± standard deviation) and compared to the control treatments with the equation:

$$\%\ \text{control} = 100[(A - B)/A]$$

where A = the area of infection (%) on leaves or sheaths sprayed with a Tween 20 solution alone and B = the area of infection (%) on treated leaves or sheaths.

Statistical Analysis. Mortality values for the in vitro bioassays against the RKN and PWN were corrected by using Abbott's formulation. The inhibitory and lethality concentration (IC/LC_{50}) values were calculated by Probit analysis. The means of all multiple measurements were examined for significance by a one-way analysis of variance using the R software version 2.12.0 (http://www.r-project.org). In the case of significance, Tukey's HSD test for multiple comparisons was used to determine the statistical differences between the means at $P = 0.05$.

RESULTS AND DISCUSSION

Nematicidal and Antifungal Activities of Crude Extracts of *A. squamosa* Seeds. The MeOH extract of *A. squamosa* seeds displayed strong nematicidal activities against RKN and PWN with mortality values of 93% (Figure S2-1 in the Supporting Information) and 100% (data not shown), respectively, at a concentration of 250 μg/mL. The dried leaf powder of *A. squamosa* was known to significantly reduce the number of root-knot galls caused by *M. incognita* on tomato plants.[15] Methanol and other organic solvent extracts from *A. squamosa* and other species of the Annonaceae family also displayed anthelmintic properties against *Hemonchus contortus* and *Caenorhabditis elegans*.[9,16] The nematicidal activities of the *A. squamosa* seed extracts against RKN and PWN were reported for the first time in this study.

To isolate bioactive AAs, the MeOH extract was partitioned with various organic solvents such as Hex, EtOAc, and BuOH to yield four solvent-soluble fractions. Nematicidal activities of the four layers, including an aqueous layer, were tested at concentrations of 250, 500, and 1000 μg/mL against *M. incognita*. Among the four layers, EtOAc layer was the most active against RKN (Figure S2-1 in the Supporting Information), followed by BuOH, aqueous, and Hex layers. EtOAc layer showed the strongest nematicidal activity against PWN with a LC_{50} value of 0.016 μg/mL. The LC_{50} values of Hex, BuOH, and aqueous layers were 0.146, 1.967, and 274.0 μg/mL, respectively.

In vivo antifungal activities of the MeOH extract from *A. squamosa* and the four solvent layers were evaluated against RCB, RSB, TGM, TLB, WLR, BPM, and RPA. Among the seven plant diseases tested, TLB, WLR, and BPM were significantly suppressed by MeOH extract (Table 1). The EtOAc layer displayed the highest in vivo antifungal activity against *P. infestans*, followed by Hex, BuOH, and aqueous layers (Figure S2-2 in the Supporting Information). The aqueous layer was mostly inactive to the fungus. Therefore, the separation of bioactive AAs from both EtOAc and Hex layers was further conducted. This is the first report on the antifungal activity of *A. squamosa* against phytopathogenic fungi.

Isolation and Identification of AAs from *A. squamosa*. Compound **8** (Figure 1) was identical to squamocin,[17,18] with a molecular weight of 622 and a molecular formula of $C_{37}H_{66}O_7$ (m/z 604 $[M - 18]^+$ in EI-MS). The existence of two adjacent bis-tetrahydrofuran (THF) rings between C-15 and C-24 was deduced from the fragmentation pattern, presenting the cleavages of C-15/C-16 [m/z 295 (100)], C-19/C-20 [m/z 365 (10), 347 (60)], and C-23/C-24 [m/z 436 (6), 417(10)] bonds in the EI-MS spectrum. Further evidence was observed from the

Table 1. In Vivo Antifungal Activity of the Methanol Extract Obtained from *A. squamosa* Seeds and Four Solvent Layers Therefrom against Various Phytopathogenic Fungi[a]

		control value (%)[b]						
material	concn (μg/mL)	RCB	RSB	TGM	TLB	WLR	BPM	RPA
Hex	1000	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0c	95.0 ± 3.0a	73.3 ± 9.4b	41.7 ± 11.8b	8.3 ± 11.8b
	500	0.0 ± 0.0c	6.3 ± 8.8b	42.9 ± 0.0b	28.6 ± 0.0b	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0b
EtOAc	1000	5.0[b] ± 7.1c	6.3 ± 8.8b	32.1 ± 5.1bc	98.6 ± 0.0a	100.0 ± 0.0a	58.3 ± 11.8b	0.0 ± 0.0b
	500	0.0 ± 0.0b	6.3 ± 8.8b	28.6 ± 0.0bc	98.6 ± 0.0a	93.3 ± 0.0a	58.3 ± 11.8b	0.0 ± 0.0b
BuOH	1000	0.0 ± 0.0b	0.0 ± 0.0b	35.7 ± 10.1bc	7.1 ± 10.1bc	0.0 ± 0.0d	0.0 ± 0.0c	8.3 ± 11.8b
	500	15.0 ± 7.1c	6.3 ± 8.8b	7.1 ± 10.1c	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0b
aqueous	1000	0.0 ± 0.0c	0.0 ± 0.0b	21.4 ± 10.1bc	0.0 ± 0.0c	0.0 ± 0.0d	8.3 ± 11.8c	0.0 ± 0.0b
	500	0.0 ± 0.0c	6.3 ± 8.8b	21.4 ± 10.1bc	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0b
MeOH	1000	0.0 ± 0.0c	12.5 ± 0.0b	21.4 ± 10.1bc	96.4 ± 1.0a	83.3 ± 4.7b	50.0 ± 0.0b	16.7 ± 0.0b
	500	0.0 ± 0.0c	6.3 ± 8.8b	14.3 ± 0.0bc	97.9 ± 1.0a	53.3 ± 0.0c	50.0 ± 0.0b	8.3 ± 11.8b
blasticidin-S	50	100 ± 0.0a						
	1	65.5 ± 10.6b						
validamycin	50		100 ± 0.0a					
	5		70 ± 14.1a					
fludioxonil	50			100 ± 0.0a				
	5			83 ± 16.9a				
dimethomorph	10				100 ± 0.0a			
	2				85 ± 9.9a			
flusilazole	10					100 ± 0.0a		
	2					78 ± 7.1b		
benomyl	100						100 ± 0.0a	
	1						75 ± 11.3ab	
dithianon	50							100 ± 0.0a
	10							20 ± 7.1b

[a] The plant seedlings were inoculated with spores or a mycelial suspension of the test organism 1 day after the chemical solution was sprayed to runoff on the leaves. The disease rating was carried out 3−7 days after inoculation. [b] Means (±SDs) followed by the same letters within the same column are not significantly different in a Tukey's HSD test ($P = 0.05$).

signals of six protons in the ^{1}H NMR at δ 3.35 (1H, m, H-15), 3.76 (1H, m, H-16), 3.89 (2H, m, H-19, 23), 3.84 (1H, m, H-20), and 3.76 (1H, m, H-24) and from the signals in the ^{13}C NMR for six oxygenated carbons at δ 74.11 (C-15), 83.33 (C-16), 82.13 (C-19), 82.47 (C-20), 82.77 (C-23), and 71.23 (C-24). An α,β-unsaturated γ-lactone subunit was suggested by proton resonances at δ 6.95 (1H, d, H-35), 4.94 (1H, qd, H36), 2.20 (2H, tt, H-3), and 1.35 (3H, d, H-37) and five carbon resonances at δ 173.84 (C-1), 148.84 (C-35), 134.2 (C-2), 77.34 (C-36), and 19.13 (C-37). The stereochemical configuration of adjacent bis-THF rings was determined to be *threo*/*trans*/*threo*/*trans*/*erythro* based on a comparison of ^{1}H and ^{13}C NMR data with the synthetic models published by Cortes.[19] The presence of an OH− group at C-28 was assigned based on fragment ions of m/z 519 (6), 501 (6), and 483 (5) in the EI-MS spectrum, along with a proton signal at δ 3.54 (1H, m) and an oxygenated carbon signal at δ 71.63.

Similarly, squamocin-G (**5**) and squamocin-H (**6**) ($C_{37}H_{66}O_7Na$; m/z 645.4 [M + Na]$^+$, ESI-MS) were determined to possess an adjacent bis-THF ring system located between C-15 and C-24, as evidenced from MS and NMR data (see Figure 1 and Table S1 in the Supporting Information). The occurrence of an α,β-unsaturated γ-lactone with a hydroxyl group at the C-4 moiety was suggested by the fragment ion at m/z 141 (10) in the EI-MS spectrum, proton resonances at δ 7.19 (1H, d, H-35), 5.05 (1H, qd, H-36), 3.84 (1H, m, H-4), 2.51 (1H, m, H-3a), 2.41 (1H, m, H-3b), and 1.45 (3H, d, H-37) and seven carbon resonances at δ 174.3 (C-1), 151.74 (C-35), 131.13 (C-2), 77.92 (C-36), 69.91 (C-4), 33.26 (C-3), and 19.06 (C-37). The relative stereochemistry of the THF ring system of **5** resembled that of **8**, while that of **6** was assigned as *threo*/*trans*/*threo*/*trans*/*threo*.[19−21]

Compounds **1** (squamocin-L) and **2** (squamocin-M) have characteristics of an adjacent bis-THF acetogenin ($C_{37}H_{66}O_6$, m/z 607 [M + H]$^+$ in CI-MS). Chemical structures and the stereochemical relationship at the adjacent bis-THF rings of **1** and **2** were deduced from a comparison of their ^{1}H and ^{13}C NMR data with those of **5** and **6** (Figure 1 and Table S1 in the Supporting Information). However, their EI-MS spectra revealed the absence of a hydroxyl group at C-4 in their structures.[21]

Squamocin-J (**3**) and squamocin-K (**4**)[21] were determined as C_{35}-adjacent bis-THF acetogenins with the same molecular formula of $C_{35}H_{62}O_6$ (ESI-MS, m/z 579.4 [M + H]$^+$, 596.5 [M + NH$_4$]$^+$, 601.5 [M + Na]$^+$). In the CI-MS spectra, the fragment ions rising from C-13/C-14 [m/z 267 (20)], C-17/C-18 [m/z 337 (5), 319 (8)], and C-21/C-22 [m/z 408 (5), 389 (5)] suggested an existence of an adjacent bis-THF ring moiety with two flanking hydroxyl groups between C-13 and C-22. The bis-THF moiety of **3** and **4** appeared to have a symmetrical *threo*/*trans*/*threo*/*trans*/*erythro* and *threo*/*trans*/*threo*/*trans*/*threo* structure as evidenced by the comparison of their NMR data with those of **5** and **6** (Figure 1 and Table S1 in the Supporting Information).

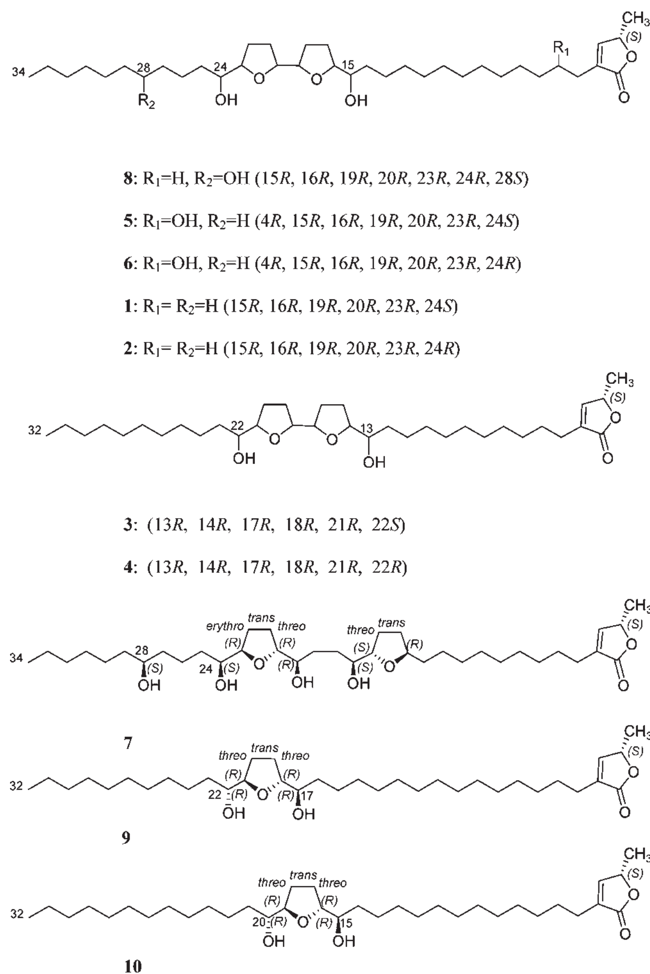

Figure 1. Chemical structures of AAs **1**−**10**. Key: **1**, squamocin-L; **2**, squamocin-M; **3**, squamocin-J; **4**, squamocin-K; **5**, squamocin-G; **6**, squamocin-H; **7**, squamostatin-A; **8**, squamocin; **9**, annotemoyin-1; and **10**, solamin.

The molecular formula of squamostatin-A (**7**) was determined to be $C_{37}H_{68}O_8$ (m/z 639 $[M + H]^+$ in CI-MS). The presence of a nonadjacent bis-THF ring system with four flanking hydroxyls was indicated by ^1H and ^{13}C NMR data. The placement of the THF rings was established at C-12 to C-15 and C-20 to C-23 based on the observed fragmentation pattern in the CI-MS with fragment ions at m/z 451 (7), 433 (35), 415 (12), 363 (48), 345 (18), 327 (21), 293 (70), and 275 (23). The relative stereochemistry of the THF ring system was assigned as *trans*/*threo*/*threo*/*trans*/*erythro* (Figure 1) based on the comparison with synthetic models published by Fujimoto et al.[22,23]

Compounds **9** and **10** (Figure 1) had a molecular formula of $C_{35}H_{65}O_5$ (m/z 565 $[M + H]^+$ in CI-MS). The placement of the mono-THF ring between C-17 and C-22 in the structure of **9** was established based on the fragment ions rising from the cleavages of C-17/18 [m/z 323 (100), 241 (9), 223 (6)] and C-21/22 [m/z 375 (22), 171(6)] in the CI-MS. On the other hand, the THF ring in **10** is located between C-15 and C-20, as evidenced by ion peaks at m/z 295 (33) and 347 (6). The interpretation of the spectroscopic data of **9** and **10** led to their structural assignment as annotemoyin-1 (**9**) and solamin (**10**), respectively.[24−26]

Nematicidal Activity of AAs from *A. squamosa* Seeds. The 10 purified compounds were tested for in vitro nematicidal

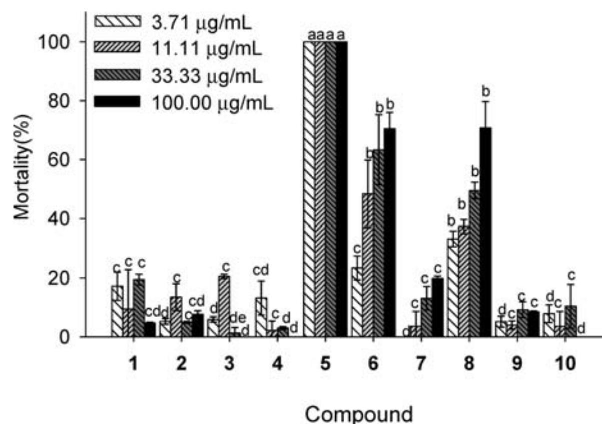

Figure 2. Nematicidal activity of AAs isolated from *A. squamosa* seeds against *M. incognita*. The mortalities were measured 3 days after treatment. Each value represents the mean ± standard deviation of two runs with three replicates each. Means (±SDs) followed by the same letters within bars are not significantly different for each treatment concentration ($P = 0.05$) in a Tukey's HSD test.

activities against J2s of RKN and PWN. Of these, the three compounds, squamocin-G (**5**), squamocin-H (**6**), and squamocin (**8**), were significantly active against RKN. The other compounds caused low mortalities for RKN at concentrations of less than 100 μg/mL (Figure 2). The nematicidal activities of compounds **5**, **6**, and **8** were compared with those of abamectin and cinnamaldehyde. The time course of nematicidal activities of the three acetogenin compounds, abamectin, and cinnamaldehyde is summarized in Table 2. Out of the test acetogenins, squamocin-G (**5**) displayed the highest mortality against RKN; its LC_{50} value was less than 1 μg/mL. Squamocin-G caused 100% mortality of RKN within a short period of 2 h at a concentration of 11 μg/mL (data not shown). Cinnamaldehyde that displayed a nematicidal activity with LC_{50} values varying from 12.24 to 15.14 μg/mL was significantly weak when compared with squamocin-G (**5**). The commercial nematicide, abamectin, showed the strongest activity against RKN with LC_{50} values from 0.012 to 0.109 μg/mL. In comparison, LC_{50} values of squamocin-G (**5**) varied from 0.287 to 0.864 μg/mL. The nematicidal activity of squamocin-H (**6**) was stronger than that of squamocin (**8**) but weaker than that of cinnamaldehyde. The nematicidal activities of the two compounds **6** and **8** dramatically increased 3 days after the treatment.

The nematicidal activities of the purified acetogenins against PWN were tested at concentrations ranging from 0.004 to 1 μg/mL. Compounds **1**−**8** were highly active, which caused 100% mortality of PWN 2 days after treatment at concentrations higher than 0.33 μg/mL (data not shown). The eight acetogenins showed LC_{50} values against PWN at concentrations ranging from 0.006 to 0.048 μg/mL, which are comparable to that of abamectin (Table 3). Compounds **9** and **10** were relatively less active against PWN with LC_{50} values of 0.947 and over 1.0 μg/mL, respectively.

In previous studies, acetogenins were described as natural pesticides and feeding deterrents with great potential efficacies against mosquito larvae, spider mites, aphids, blowfly larvae, and cockroach.[9,18,27] Squamocin-H, also known as asimicin, was remarkably active (LD_{100} = 0.1 ppm) toward *Caenorhabdtis elegans*.[9,28] Other acetogenins were shown to have strong activities against rodent filarial parasites *Molinema dessetae*.[28] This study demonstrated the nematicidal activities of the AAs isolated

Table 2. Effects of Squamocin, Squamocin-G, and Squamocin-H Isolated from *A. squamosa* Seeds on the Mortality of *M. incognita*

compd	LC_{50} (95% CI)[a] (μg/mL)		
	1 DAT[b]	2 DAT	3 DAT
squamocin (**8**)	>100	>100	54.90 (34.00−75.90)
squamocin-G (**5**)	0.864 (0.515−1.022)	0.339 (0.236−0.381)	0.287 (0.193−0.386)
squamocin-H (**6**)	>100	71.21 (58.70−83.70)	24.32 (10.59 - 31.85)
cinnamaldehyde	15.14 (4.95−21.76)	13.94 (4.49−20.14)	12.24 (4.44−16.95)
abamectin	0.109 (0.061−0.131)	0.051 (0.029−0.061)	0.012 (0.009−0.013)

[a] 95% CI, 95% confidence interval. [b] DAT, days after treatment.

Table 3. Effects of AAs Isolated from *A. squamosa* Seeds on the Mortality of *B. xylophilus*

compd[a]	LC_{50} (95% CI)[b] (μg/mL)	conf.[c]
1	0.018 (0.009−0.044)	*th/t/th/t/er*
2	0.024 (0.015−0.028)	*th/t/th/t/th*
3	0.025 (0.014−0.031)	*th/t/th/t/er*
4	0.039 (0.015−0.053)	*th/t/th/t/th*
5	0.008 (0.006−0.010)	*th/t/th/t/er*
6	0.012 (0.011−0.013)	*th/t/th/t/th*
7	0.048 (0.024−0.059)	
8	0.006 (0.001−0.009)	*th/t/th/t/er*
9	0.947 (0.615−1.275)	
10	>1.000	
abamectin	0.043 (0.028−0.057)	

[a] Key: **1**, squamocin-L; **2**, squamocin-M; **3**, squamocin-J; **4**, squamocin-K; **5**, squamocin-G; **6**, squamocin-H; **7**, squamostatin-A; **8**, squamocin; **9**, annotemoyin-1; and **10**, solamin. [b] LC_{50} values (μg/mL) of the test compounds against PWN were calculated from the mortalities 2 days after treatment. 95% CI, 95% confidence interval. [c] Stereochemical configuration of the central adjacent bis-THF rings. *th*, threo; *t*, trans; and *er*, erythro.

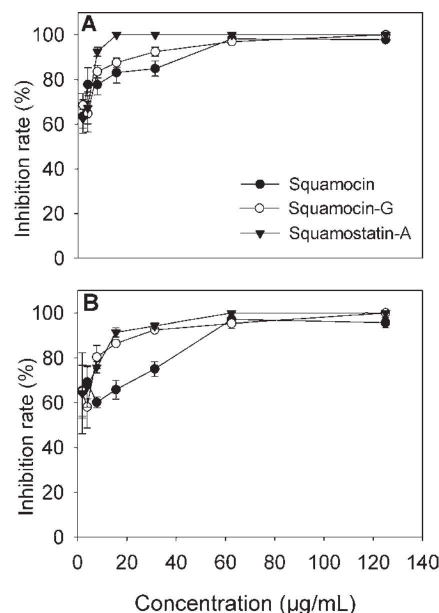

Figure 3. Inhibitory activities of squamocin, squamocin-G, and squamostatin-A isolated from *A. squamosa* seeds against the germination of sporangium (A) and zoospore (B) of *P. infestans*. The inhibitory effects of the chemicals were determined 24 h after treatment. Each value represents the mean ± standard deviation of two runs with three replicates each.

from *A. squamosa* against RKN and PWN. In comparison with squamocin-H, the nematicidal activity of squamocin-G (also known as bullatacin) against RKN was significantly higher (Tables 2 and 3). This is similar to the pesticidal properties of squamocin-G (bullatacin) and squamocin-H (asimicin) against brine shrimp and yellow fever mosquito larvae.[9] The nematicidal activity of the acetogenins may be due to the inhibitory character of the mitochondrial NADH:ubiquinone oxidoreductase (complex I).[9,29,30] Among the 10 purified AAs, the three compounds squamocin, squamocin-G, and squamocin-H, which contain a hydroxyl group at C4 and adjacent bis-THF rings, showed relatively high nematicidal activities against RKN. In addition, the nematicidal activity of squamocin-G was much higher than that of its isomer, squamocin-H. The remarkable activity of squamocin-G against RKN may be due to the similarity in their structures, which contain adjacent bis-THF rings with three hydroxyl groups and a configuration of C-24.

Among the 10 AAs isolated from *A. squamosa* seeds in this study, two compounds, containing only one THF ring in their structure, were relatively less active against PWN than those containing two rings. In addition, the nematicidal activities of the chemicals containing bis-THF rings (compounds **1**−**6** and **8**) against PWN increased in parallel to the increase in the number of hydroxyl groups (Table 3). This study also demonstrated a structure−activity relationship (SAR) for the stereochemistry of the central adjacent bis-THF rings in the stereoisomers such as **1** vs **2** and **3** vs **4** and **5** vs **6**. The compounds with a configuration of *threo*/*trans*/*threo*/*trans*/*erythro* caused slightly higher mortality values for PWN when compared to those with a configuration of *threo*/*trans*/*threo*/*trans*/*threo* (Table 3 and Figure 1). This was in good agreement with the claims of Oberlies et al.,[31] where the authors tested AAs against adriamycin-resistant human mammary adenocarcinoma (MCF-7/Adr) cells.

Antifungal Activities of AAs from *A. squamosa* Seeds. Among the 10 acetogenins isolated from *A. squamosa* seeds in this study, squamocin-G (**5**), squamostatin-A (**7**), and squamocin (**8**) were found to be abundant in the plant sample, and the other compounds were only in limited quantities. Therefore, only the three abundant compounds were tested for their in vitro and in vivo antifungal activities. All of the three compounds showed dose-dependent activities against the germination of sporangium and zoospore of *P. infestans* (Figure 3A,B). All three compounds highly inhibited the germination of both sporangium and zoospore. The IC_{50} values for sporangium germination were 1.24 μg/mL for squamocin, 1.43 μg/mL for squamocin-G, and 2.09 μg/mL

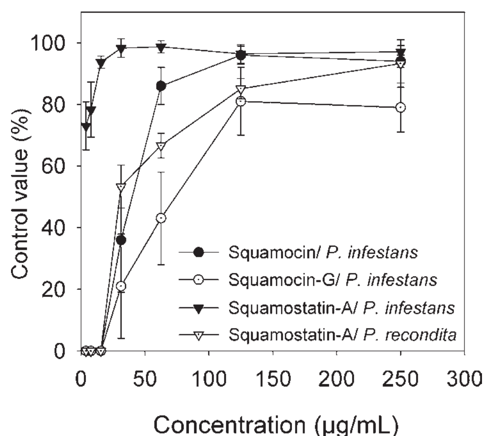

Figure 4. In vivo antifungal activities of squamocin, squamocin-G, and squamostatin-A isolated from *A. squamosa* seeds against *P. infestans* and *P. recondita*. The seedlings were inoculated with spores of the test fungi 1 day after the chemical solutions were sprayed to runoff on the leaves. Disease severity was assessed 3 days after inoculation for TLB caused by *P. infestans* and 7 days after inoculation for WLR caused by *P. recondita*.

for squamostatin-A. Zoospore germination was suppressed by the three compounds with IC_{50} values of 1.89 μg/mL for squamostatin-A, 1.92 μg/mL for squamocin-G, and 3.05 μg/mL for squamocin. The inhibitory activity of squamocin against *P. infestans* zoospore germination was slightly weaker than those of the other two compounds (Figure 3B).

The three compounds, squamocin-G (**5**), squamostatin-A (**7**), and squamocin (**8**), also inhibited 31−83% of the mycelial growth of *P. infestans* (Figure S3-1 in the Supporting Information). These three compounds also highly suppressed the development of TLB caused by *P. infestans* on tomato seedlings, showing dose-dependent responses (Figure 4 and Figure S3-2 in the Supporting Information). As compared to squamocin and squamocin-G with adjacent bis-THF rings in their structures, squamostatin-A, which contains nonadjacent bis-THF rings, displayed much stronger activity (Figure 4). Squamostatin-A showed a control value of greater than 70% even at a low concentration of 3.9 μg/mL. Additionally, *P. recondita*, which causes WLR, was found to be sensitive to squamostatin-A (Figure 4). Squamocin-G was virtually inactive against *P. recondita*. Squamocin caused a suppression of 83% for the development of rust symptoms on the wheat seedlings treated at a concentration of 500 μg/mL. However, it lost efficacies at lower concentrations of 125 and 250 μg/mL (data not shown). The in vivo antifungal activities of the three compounds were also evaluated at concentrations of 125, 250, and 500 μg/mL against the other fungi (Table S3 in the Supporting Information). Squamostatin-A displayed a moderate activity with a control value of 70% against RPA at 500 μg/mL. All three compounds were found to have little or no efficacy against the development of RCB, RSB, TGM, and BPM. These results suggested that *P. infestans*, a filamentous pseudofungus belonging to Oomycetes, is highly susceptible to AAs.

As for the antifungal activities of AAs from *A. squamosa*, Mukhlesur Rahman et al.[32] reported in vitro antifungal activities of annotemoyin-1, annotemoyin-2, and squamocin against some fungi strains such as *Aspergillus flavus*, *A. niger*, *A. fumigatus*, and *Candida albicans*. Squamocin was found to be inactive toward all of the tested fungi. In contrast, annotemoyin-1 and annotemoyin-2 caused inhibitory effects on all of the test fungi with the exception of *C. albicans*.[32] Those claims, in part, confirmed our findings on the specific antifungal activities of squamocin, squamocin-G, and squamostatin-A against *P. infestans* causing TLB. The disease control efficacies of the three compounds were evidenced through their in vitro inhibitory activities against mycelial growth and the germination of sporangium and zoospore of *P. infestans*. The mode of action for the acetogenins squamocin, squamocin-G, and squamostatin-A may be an inhibition of complex I for O_2 consumption in zoospores of *P. infestans*, similar to amoxadone and its oxazolidinone analogues, which strongly suppressed *P. infestans* by inhibition for mitochondrial electron transport.[33] Fungicides interfering with fungal respiration were described to be active against Oomycetes and the true fungi such as Ascomycetes and Basidiomycetes.[34] However, the discovery of complex I inhibitors with market relevant fungicidal properties appears to be difficult, and no broad spectrum fungicide with the complex I mode of action has been found to date.[35]

In this study, the adjacent bis-THF acetogenins that contain three OH-groups, such as squamocin and squamocin-G, were found to display both nematicidal and antifungal activities with high efficacies against RKN, PWN, and TLB. Squamostatin-A with a nonadjacent bis-THF ring system was specifically and strongly active against *P. infestans* and *P. recondita*.

In conclusion, the nematicidal and antifungal activities of acetogenins and organic layers of *A. squamosa* were important findings of this study. Squamocin, squamocin-G, and squamocin-H demonstrated high activities against RKN and PWN. In addition to potent in vitro antifungal activities, squamocin, squamocin-G, and squamostatin-A efficiently suppressed the development of TLB caused by *P. infestans* on tomato seedlings. Our results suggested that *A. squamosa* seeds and their bioactive AAs could be used as an alternative resource to produce promising botanical nematicides and fungicides for the control of various plant diseases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. NMR spectral data of the 10 isolated compounds, antifungal and nematicidal activities of purified substances squamocin, squamocin-G and -H and squamostatin-A, methanol extract, and organic solvent layers. This material is available free of charge via the Internet at http://pubs.acs.org.

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