

Lignanamides and other constituents from the seeds of *Annona squamosa* L. and their cytotoxic activity

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Abstract

Objectives: The aim of the project was the isolation, structure elucidation, and cytotoxic evaluation of compounds from *Annona squamosa* L. seeds.

Methods: Chromatographic techniques were used to isolate cytotoxic compounds from the methanol extract of *A. squamosa* seeds. The structures of compounds were elucidated by analyses of mass spectra and nuclear magnetic resonance data. Compounds were evaluated using cytotoxic assays.

Results: One new lignanamide, squamosamide, and seven known compounds, cannabisin M (**2**), *trans*-*N*-caffeoyltyramine (**3**), cyclo(L-Ala-L-Ile-L-Pro-L-Met-L-Tyr-L-Thr-L-Val) (**4**), annosquacin B (**5**), squamocin M (**6**), 34,5-trimethoxyphenyl- β -glucopyranoside (**7**), and 34,5-trimethoxyphenyl-1-*O*- β -apiofuranosyl-(1'' $\rightarrow$ 6')- β -glucopyranoside (**8**) were isolated from the methanol extract of *A. squamosa* seeds. Compounds **5** and **6** displayed significant cytotoxic activity on both KB and PC9 cancer cell lines with IC₅₀ values ranging from 6.8 to 8.7 μ M. Compounds **1–3** exhibited significant cytotoxicity on PC9 cancer cell line with IC₅₀ values ranging from 6.9 to 9.4 μ M and moderate activity on KB cancer cell line with IC₅₀ values ranging from 11.2 to 16.7 μ M. Mechanistic assays in PC9 cells showed that compounds **1**, **2**, **3**, **5**, and **6** increased caspase-3 activity (2.41–3.40-fold) and PARP1 cleavage (2.01–3.40-fold), indicating involvement of the intrinsic apoptotic pathway.

Conclusions: The seeds of *A. squamosa* yielded one new lignanamide and seven known compounds, several of which demonstrate potent cytotoxicity associated with apoptosis. Compounds **5** and **6** emerge as promising leads for further investigation.

Keywords

Annona squamosa L., Annonaceae, lignanamide, squamosamide, cytotoxic activity

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Introduction

The genus *Annona* (Annonaceae) comprises shrubs and small to large trees distributed in tropical and subtropical regions.¹ The genus comprises approximately 120 species distributed in Central and South America, Africa, Asia, and Australia.² Four species have been recorded and described in Vietnam, namely *A. muricata*, *A. reticulata*, *A. glabra*, and *A. squamosa*.³ *Annona squamosa* L., commonly known as sugar-apple or sweetsop, is a small tropical-subtropical tree cultivated widely in Central and South America, India, Mexico, Taiwan, Vietnam, and other regions. It is typically 3–8 m in height, has a branched habit, and begins fruiting approximately in its third year.⁴ In folk practices, the seeds are ground into powder and applied externally to eradicate head lice and other ectoparasites; seed paste is used for skin ulcers and abscesses; and seed extracts are administered in small doses as

vermifuges.⁵ In some regions, crushed seeds are also employed as insecticides. Phytochemical investigations of *A. squamosa* have revealed the presence of acetogenins, diterpenes, alkaloids, and cyclic peptides. These compounds exhibit a broad spectrum

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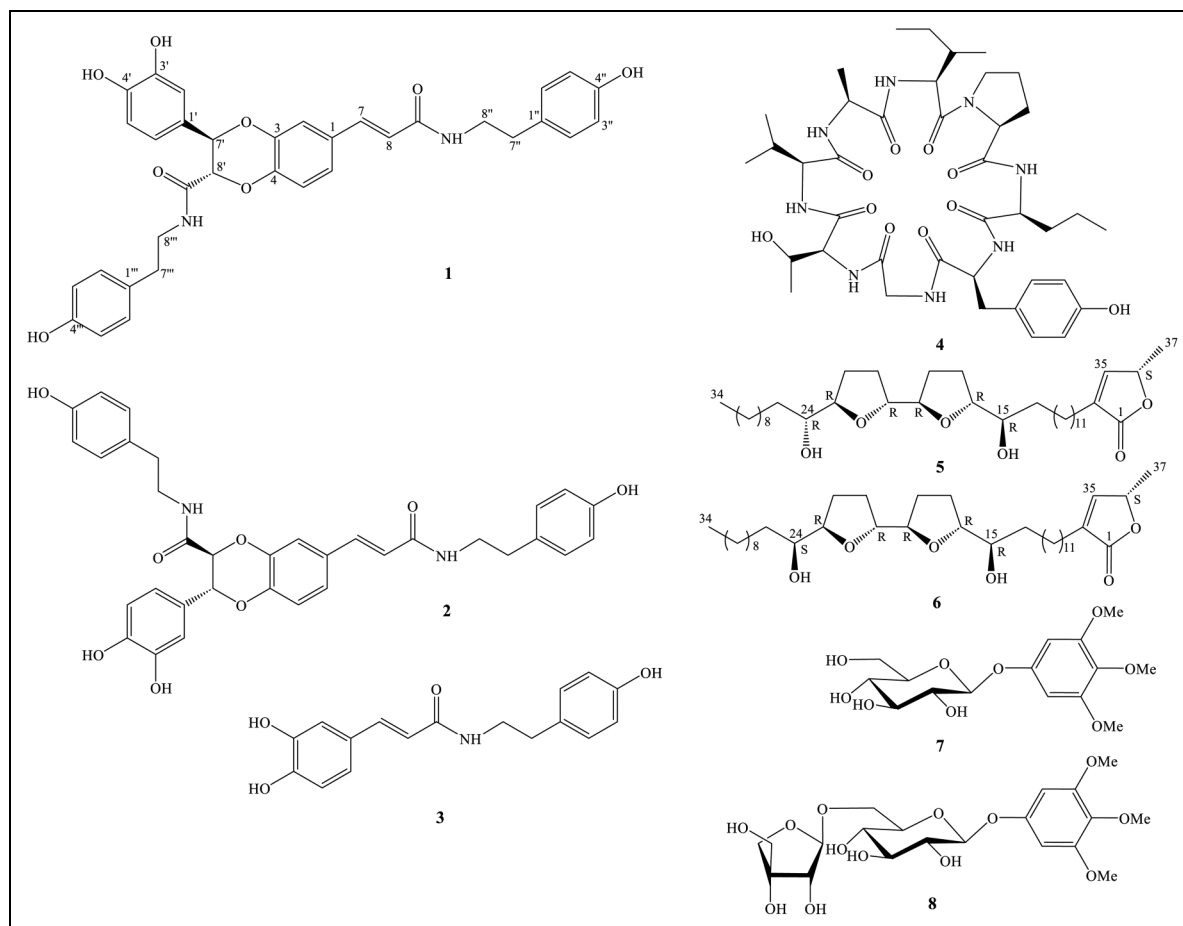


Figure 1. Chemical structures of compounds 1–8.

of biological activities, such as anticancer,^{6,7} antimicrobial,⁸ anti-inflammatory,⁹ antioxidant, antimalarial,¹⁰ and insecticidal effects.¹¹ As part of our ongoing research to discover cytotoxic compounds from this species, we herein report the isolation, structural elucidation, and evaluation of the cytotoxic activity of one new lignanamide and seven known compounds, cannabisin M (**2**) (Figures S14–S18),¹² *trans*-*N*-caffeoyltyramine (**3**),¹³ cyclo(L-Ala-L-Ile-L-Pro-L-Met-L-Tyr-L-Thr-L-Val) (**4**),¹⁴ annosquin B (**5**),¹⁵ squamocin M (**6**),¹⁶ 34,5-trimethoxyphenyl- β -glucopyranoside (**7**),¹⁷ and 34,5-trimethoxyphenyl-1-*O*- β -apiofuranosyl-(1''-6'')- β -glucopyranoside (**8**)¹⁸ (Figure 1) from the seeds of *A. squamosa*.

Material and Methods

General

See Supplemental Material.

Plant Material

The seeds of *Annona squamosa* L. (Annonaceae) were collected in Lang Son province, Vietnam, in August 2024 and identified

by one of the authors, Prof. Ninh Khac Ban, Institute of Chemistry, VAST. A voucher specimen (CDRD23) was deposited at the Institute of Chemistry, VAST.

Extraction and Purification of Compounds

The extraction and prefractionation procedures were described in the previous work.¹⁹ Powdered seeds of *A. squamosa* (3.0 kg) were extracted with methanol (8 L) by ultrasonication (2 h $\times$ 2 at 50 °C) to afford a methanol extract (AS, 400 g), which was suspended in water (2.0 L) and successively partitioned with dichloromethane and then ethyl acetate (1:1, v/v; twice) to give the dichloromethane fraction (AS1, 216 g), the ethyl acetate fraction (AS2, 87 g), and the aqueous layer (AS3). AS2 was subjected to a silica gel column chromatography (CC) eluting with a gradient solvent of *n*-hexane/acetone (40:1, 10:1, 5:1, v/v), followed by dichloromethane/methanol (10:1, 5:1, v/v), to furnish five fractions: AS2A (16.0 g), AS2B (11.4 g), AS2C (25.2 g), AS2D (27.0 g), and AS2E (2.0 g).

AS2B was further separated by a silica gel CC using *n*-hexane/acetone (7:1, v/v) to give AS2B1 (0.5 g), AS2B2 (2.4 g), AS2B3 (1.4 g), and AS2B4 (1.7 g). AS2B2 was chromatographed on an RP-18 CC eluting with acetone/water (4:1, v/v)

to yield compound **6** (400 mg). AS2B3 was chromatographed on an RP-18 CC eluting with acetone/water (3.5:1, v/v) to yield compound **5** (560 mg). AS2D was chromatographed on a silica gel CC eluting with *n*-hexane/acetone (7:1, v/v) to afford AS2D1 (1.0 g), AS2D2 (1.9 g), AS2D3 (9.0 g), and AS2D4 (9.0 g).

AS2D2 was chromatographed on an RP-18 CC eluting with acetone/water (1:2, v/v) to give AS2D2A (130 mg) and AS2D2B (166 mg). Further purification of AS2D2A on an HPLC system (J'sphere H-80 column (250 × 20 mm), 50% methanol in water, and flow rate of 3.0 mL/min) yielded compounds **1** (7.0 mg, *t_R* 60.1 min) and **2** (5.0 mg, *t_R* 64.4 min). AS2D2B was chromatographed on an HPLC system (J'sphere H-80 column (250 × 20 mm), 30% acetonitrile in water, and flow rate of 3.0 mL/min) to yield compound **3** (12 mg, *t_R* 50.5 min).

The aqueous layer AS3 was fractionated on a Diaion HP-20 CC by stepwise elution with methanol in water (25, 50, 75, and 100%; 1 L each) to give four fractions: AS3A (2.4 g), AS3B (3.0 g), AS3C (8.0 g), and AS3D (4.2 g). AS3C was chromatographed on a silica gel CC using gradient solvent of dichloromethane/methanol (20:1, 10:1, 5:1, 2.5:1, v/v) to give AS3C1 (0.9 g), AS3C2 (1.0 g), AS3C3 (2.2 g), and AS3C4 (1.4 g). AS3C2 was chromatographed on a silica gel CC using solvent of dichloromethane/acetone (2.5:1, v/v) to give AS3C2A (360 mg), AS3C2B (1.5 g), and AS3C2C (860 mg). AS3C2B was chromatographed on an RP-18 CC eluting with methanol/water (1:1, v/v) to give AS3C2B1 (310 mg) and AS3C2B2 (736 mg). AS3C2B1 was chromatographed on an HPLC system (J'sphere H-80 column (250 × 20 mm), 16% acetonitrile in water, and flow rate of 3.0 mL/min) to afford compound **4** (28.0 mg, *t_R* 36.0 min). AS3C3 was fractionated on a silica gel CC eluting with dichloromethane/acetone/water (1:3:0.1, v/v/v), to give AS3C3A (55 mg), AS3C3B (600 mg), and AS3C3C (1.7 g). AS3C3A was chromatographed on an RP-18 CC eluting with methanol/water (1:1.5, v/v) to yield compound **7** (14.5 mg). Finally, AS3C3B was chromatographed on an RP-18 CC eluting with methanol/water (1:1.5, v/v) to yield compound **8** (30.0 mg).

Squamosamide (1)

White amorphous powder; $[\alpha]_D^{25}$: -9.0 (*c* 0.01, MeOH); HR-ESI-MS: m/z 597.2233 $[M + H]^+$ (Calcd. for $[C_{34}H_{33}N_2O_8]^+$, 597.2231); ^1H - and ^{13}C -NMR data (CD_3OD): see Table 1.

Cell Culture and Viability Assay

Human oral cancer (KB) cells: ATCC[®] (CCL-17TM); human lung cancer (PC9) cells purchased from Merck Millipore (Cat. No. 90071810). PC9 and KB cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2 . Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight.^{20,21} Compounds at

Table 1. The ^1H - and ^{13}C -NMR Spectral Data for Compounds **1** and **2** in CD_3OD .

C	1		Cannabisin M (2)		$\delta_C^\#$
	δ_C	δ_H (mult., <i>J</i> = Hz)	δ_C	δ_H (mult., <i>J</i> = Hz)	
1	130.6	-	130.3	-	128.9
2	117.2	7.18 (d, 1.8)	117.1	7.20 (d, 1.8)	115.8
3	145.0	-	143.8	-	143.6
4	145.2	-	146.3	-	142.5
5	118.5	6.99 (d, 8.4)	118.8	6.97 (d, 8.4)	117.1
6	123.1	7.12 (dd, 1.8, 8.4)	123.2	7.14 (dd, 1.8, 8.4)	121.6
7	141.1	7.44 (d, 16.2)	141.1	7.45 (d, 15.6)	139.7
8	120.5	6.46 (d, 16.2)	120.4	6.46 (d, 15.6)	119.1
9	168.8	-	168.8	-	167.4
1'	128.1	-	128.1	-	126.7
2'	115.7	6.85 (d, 1.8)	115.6	6.85 (d, 1.8)	114.2
3'	146.6	-	146.6	-	145.9
4'	147.4	-	147.4	-	145.9
5'	116.3	6.81 (d, 7.8)	116.3	6.81 (d, 7.8)	114.9
6'	120.3	6.73 (dd, 1.8, 7.8)	120.3	6.72 (dd, 1.8, 7.8)	118.9
7'	77.8	5.02 (d, 6.6)	78.0	5.05 (d, 6.6)	76.4
8'	79.6	4.52 (d, 6.6)	79.4	4.50 (d, 6.6)	78.2
9'	169.0	-	169.0	-	167.7
1''	131.3	-	131.3	-	129.9
2'', 6''	130.7	7.07 (d, 8.4)	130.7	7.07 (d, 8.4)	129.3
3'', 5''	116.3	6.73 (d, 8.4)	116.3	6.74 (d, 8.4)	114.9
4''	156.8	-	156.8	-	155.4
7''	35.8	2.77 (t, 7.2)	35.8	2.78 (t, 7.2)	34.4
8''	42.6	3.48 (t, 7.2)	42.6	3.49 (t, 7.2)	41.3
1'''	131.0	-	131.0	-	129.6
2''', 6'''	130.7	6.87 (d, 8.4)	130.7	6.87 (d, 8.4)	129.3
3''', 5'''	116.3	6.69 (d, 8.4)	116.3	6.69 (d, 8.4)	114.9
4'''	156.9	-	156.9	-	155.5
7'''	35.4	2.46 (m) 2.56 (m)	35.4	2.46 (m) 2.56 (m)	34.1
8'''	42.3	3.21 (m) 3.34 (m)	42.3	3.22 (m) 3.34 (m)	40.9

[#]) ^{13}C -NMR data of cannabisin M.¹⁵

30 μM were administered twice over a period of 48 h. Cell viability was measured using the MTS assay (CellTiter 96[®] Aqueous One Solution, Promega). Absorbance was measured at 490 nm using a microplate reader. Mitoxantrone was used as a reference compound. Compounds that reduced cell viability by more than 50% were selected for further assays.

Caspase-3 Activity Assay

PC9 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated overnight. Cells were treated with the selected compounds at 10 μM for 48 h. Caspase-3 activity was measured using a caspase-3 colorimetric assay kit (Sigma-Aldrich) following the manufacturer's instructions.²² Briefly, cells were lysed, centrifuged, and the supernatant was mixed with assay buffer and caspase-3 substrate (DEVD-pNA). Absorbance was measured at 400 nm using a microplate reader. Caspase-3 activity was calculated based on standard curves generated using known concentrations of p-nitroaniline (pNA).

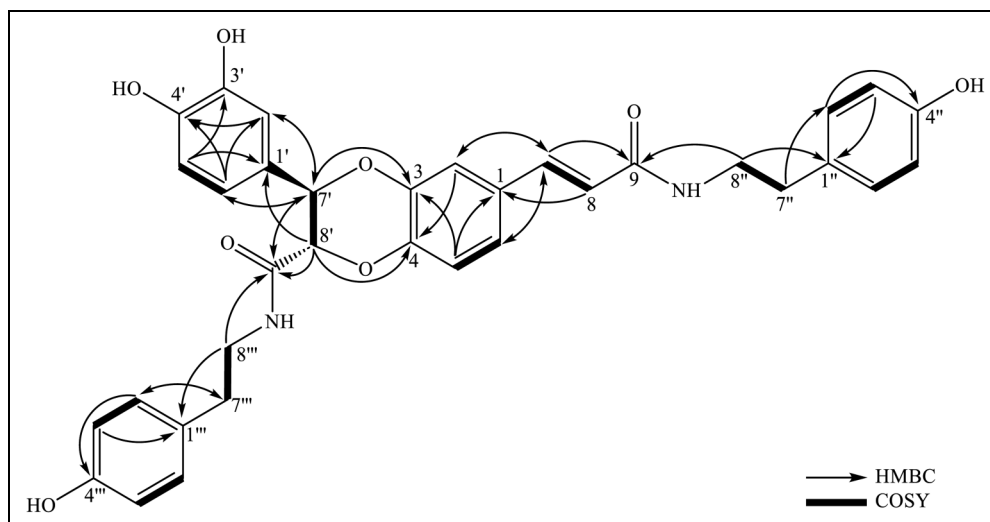


Figure 2. The key HMBC and COSY correlations of compound **1**.

Cleaved PARP1 Activity Assay

PC9 cells were seeded in 96-well plates at 1×10^4 cells/well and allowed to adhere overnight. Cells were then treated with compounds at 10 μ M for 48 h. Cleaved PARP1 activity was determined using a human cleaved PARP1 ELISA kit (Abcam) according to the manufacturer's instructions.²² Briefly, cells were lysed, centrifuged, and supernatants were collected. The cleaved PARP1 concentration was measured at 450 nm absorbance using a microplate reader.

Statistical Analysis

All experiments were conducted in triplicate and data are expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8 software. Differences between control and treated groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A *p*-value of less than 0.05 was considered statistically significant.

Results

Compound **1** was isolated as a white amorphous powder. The molecular formula of **1** was determined as $C_{34}H_{32}N_2O_8$ on the basis of HR-ESI-MS ion at m/z 597.2233 $[M+H]^+$ (Calcd. for $[C_{34}H_{33}N_2O_8]^+$, 597.2231), suggesting 20 degrees of unsaturation (Figure S1). The ^1H -NMR spectrum of **1** showed proton signals of a *trans*-substituted double bond at δ_H 6.46 (1H, $J=16.2$ Hz) and 7.44 (1H, $J=16.2$ Hz), two aliphatic oxymethines at δ_H 4.52 (1H, $J=6.6$ Hz) and 5.02 (1H, $J=6.6$ Hz), two ABX aromatic ring systems at δ_H 6.73 (1H, dd, $J=1.8, 7.8$ Hz), 6.81 (1H, d, $J=7.8$ Hz), and 6.85 (1H, d, $J=1.8$ Hz); 6.99 (1H, d, $J=8.4$ Hz), 7.12 (1H, dd, $J=1.8, 8.4$ Hz), and 7.18 (1H, d, $J=1.8$ Hz), two *para*-disubstituted

benzene rings at δ_H 6.69 (2H, d, $J=8.4$ Hz), 6.73 (2H, d, $J=8.4$ Hz), 6.87 (2H, d, $J=8.4$ Hz), and 7.07 (2H, d, $J=8.4$ Hz) (Figures S2 and S3). The ^{13}C -NMR and HSQC spectra of **1** revealed signals of 34 carbons, including two carboxyl carbons at δ_C 168.8 and 169.0, ten non-protonated carbons at δ_C 128.1, 130.6, 131.0, 131.3, 145.0, 145.2, 146.6, 147.4, 156.8, and 156.9, 18 methines at δ_C 77.8, 79.6, 115.7, 116.3 $\times$ 5, 117.2, 118.5, 120.3, 120.5, 123.1, 130.7 $\times$ 4, and 141.1, four methylenes at δ_C 35.4, 35.8, 42.3, and 42.6 (Figures S4-S7). Analysis of ^1H and ^{13}C -NMR data of **1** indicated its structure was similar to that of cannabisin M, one kind of lignanamide except for the exchanged positions of substituents at C-3' and C-4'. The large coupling constant between H-7 and H-8 ($J=16.2$ Hz) and HMBC correlations from H-7 (δ_H 7.44) to C-2 (δ_C 117.2)/C-6 (δ_C 123.1)/C-9 (δ_C 168.8), from H-2 (δ_H 7.18)/H-6 (δ_H 7.12) to C-4 (δ_C 145.2)/C-7 (δ_C 141.1), and from H-5 (δ_H 6.99) to C-1 (δ_C 130.6)/C-3 (δ_C 145.0) suggested the presence of a *trans*-caffeoyl unit (Figure 2). Two oxygenated methines were linked to C-1' and C-9' by the HMBC correlations from H-7' (δ_H 5.02) to C-1' (δ_C 128.1)/C-2' (δ_C 115.7)/C-6' (δ_C 120.3)/C-9' (δ_C 169.0) and from H-8' (δ_H 4.52) to C-1' (δ_C 128.1)/C-9' (δ_C 169.0) (Figures S8-S12). The ether groups at C-7'/C-3 and C-8'/C-4 were confirmed by HMBC correlations between H-7' (δ_H 5.02) and C-3 (δ_C 145.0) and between H-8' (δ_H 4.52) and C-4 (δ_C 145.2). A large coupling constant between H-7' and H-8' ($J=6.6$ Hz) indicated that the two protons were *trans*-oriented. HMBC correlations between H-7'' (δ_H 2.77) and C-2''/C-6'' (δ_C 130.7), between H-2''/H-6'' (δ_H 7.07) and C-4'' (δ_C 156.8)/C-7'' (δ_C 35.8), between H-8'' (δ_H 3.48)/C-1'' (δ_C 131.3), H-7''' (δ_H 2.46 and 2.56) and C-2'''/C-6''' (δ_C 130.7), between H-2'''/H-6''' (δ_H 6.87) and C-4''' (δ_C 156.9)/C-7''' (δ_C 35.4), and between H-8''' (δ_H 3.21 and 3.34) and C-1''' (δ_C 131.0) confirmed the presence of two *p*-tyramine moieties. The positions of these tyramines at C-9 and C-9' were determined by the HMBC correlations between

Table 2. The Effects of Compounds **1–8** on the Growth of PC9 and KB Cancer Cell Lines.

Compound	IC ₅₀ (μM)	
	PC9	KB
1	6.9 ± 0.3	16.7 ± 0.5
2	9.4 ± 0.4	11.2 ± 0.4
3	7.2 ± 0.4	11.3 ± 0.6
4	>100	>100
5	6.8 ± 0.2	7.6 ± 0.5
6	7.9 ± 0.3	8.7 ± 0.4
7	>100	>100
8	>100	>100
Mitoxantrone	6.7 ± 0.4	6.8 ± 0.9

Mitoxantrone was used a positive control.

Table 3. Caspase 3 and Cleaved PARP1 Activities of Selected Compounds in PC9 Cells.

Compounds	Fold Increase	
	Caspase 3	Cleaved PARP1
1	2.41 ± 0.09	2.60 ± 0.10
2	2.35 ± 0.10	2.01 ± 0.12
3	2.17 ± 0.08	2.13 ± 0.10
5	3.51 ± 0.15	3.40 ± 0.14
6	3.40 ± 0.10	3.36 ± 0.11

H-8'' (δ_{H} 3.48) and C-9 (δ_{C} 168.8) and between H-8''' (δ_{H} 3.21 and 3.34) and C-9' (δ_{C} 169.0). Thus, the structure of **1** was determined as (2,3-*trans*)-3-(3,4-dihydroxyphenyl)-N-(4-hydroxyphenethyl)-6-((E)-3-((4-hydroxyphenethyl)amino)-3-oxoprop-1-en-1-yl)-2,3-dihydrobenzo[*b*][1,4]dioxine-2-carboxamide and named squamosamide. Compound **1** was formed by combination of two *trans*-N-caffeoyltyramine units. Biosynthesis pathway of **1** from **3** was proposed (Figure S13).

Compounds **5** and **6** showed significant activity on both human cancer cell lines with IC₅₀ values ranging from 6.8 to 8.7 μM (Table 2). Compounds **1–3** exhibited significant cytotoxic activity on PC9 cancer cell line with the IC₅₀ values ranging from 6.9 to 9.4 μM. In addition, these compounds showed moderate cytotoxic activity on KB cancer cell line with IC₅₀ values ranging from 11.2 to 16.7 μM. The remaining compounds (**4**, **7**, and **8**) did not exhibit cytotoxic activity. To identify the mechanism underlying the cytotoxic activity, we assessed apoptosis induction via caspase-3 activation and PARP1 cleavage in PC9 cell line (Table 3). Compounds **1**, **2**, **3**, **5**, and **6** all markedly increased caspase-3 activity (2.41–3.40-fold) and cleaved PARP1 levels (2.01–3.40-fold).

Discussion

Previous studies have reported acetogenins, diterpenes, alkaloids, phenolics, and cyclic peptides from *A. squamosa*.⁴ The present study yielded one new lignanamide, squamosamide

and seven known compounds (**2–8**). The NMR data of squamosamide (**1**) were compared with those of cannabisin M, a lignanamide skeleton.^{12,23} Among known compounds, cannabisin M (**2**) and 34,5-trimethoxyphenyl-1-O-β-apiofuranosyl-(1''→6')-β-glucopyranoside (**8**) are reported the first time from the genus *Annona*; *trans*-N-caffeoyltyramine (**3**),²⁴ and 34,5-trimethoxyphenyl-β-glucopyranoside (**7**),²⁵ have been reported from genus *Annona*; cyclo(L-Ala-L-Ile-L-Pro-L-Met-L-Tyr-L-Thr-L-Val) (**4**),¹⁴ annosquacin B (**5**),¹⁵ and squamosin M (**6**)¹⁶ were reported from *A. squamosa*.

All compounds were evaluated for cytotoxic activity on PC9 and KB cancer cell lines. Mitoxantrone was used as a positive control and showed cytotoxic activity with IC₅₀ values of 6.7 ± 0.4 μM (PC9) and 6.8 ± 0.9 μM (KB). Compounds **5** and **6** displayed significant cytotoxic activity on PC9 and KB cancer cell lines with IC₅₀ values ranging from 6.8 to 8.7 μM. Compounds **1–3** exhibited significant cytotoxicity on PC9 cancer cell line (IC₅₀ values ranging from 6.9 to 9.4 μM) and moderate activity on KB cancer cell line (IC₅₀ values ranging from 11.2 to 16.7 μM). Case series also document ocular toxicity after accidental eye exposure to crushed *A. squamosa* seeds traditionally used against head lice, underscoring topical hazards. Seeds of *Annona* species are known to contain acetogenins, potent complex I inhibitors that have raised safety concerns. Our studies also reported cytotoxic activity of acetogenins (**5** and **6**). Mechanistic assays in PC9 cells showed that compounds **1**, **2**, **3**, **5**, and **6** increased caspase-3 activity (2.41–3.40-fold) and PARP1 cleavage (2.01–3.40-fold), indicating involvement of the intrinsic apoptotic pathway. While our data support intrinsic apoptosis *in vitro*, the evidence comes only from two cell lines (PC9, KB) and two endpoints (caspase-3 activity, PARP1 cleavage). Confirmation in additional models with complementary mechanistic assays is needed to substantiate translational relevance.

Conclusions

In summary, phytochemical investigation on the methanol extract of *A. squamosa* seeds led to the isolation of one new lignanamide, squamosamide and seven known compounds, **2–8**. Compounds **5** and **6** displayed significant cytotoxic activity on PC9 and KB cancer cell lines with IC₅₀ values ranging from 6.8 to 8.7 μM. Compounds **1–3** exhibited significant cytotoxicity on PC9 cancer cell line (IC₅₀ values ranging from 6.9 to 9.4 μM) and moderate activity on KB cancer cell line (IC₅₀ values ranging from 11.2 to 16.7 μM). Compounds **1**, **2**, **3**, **5**, and **6** all markedly increased caspase-3 activity (2.41–3.40-fold) and cleaved PARP1 levels (2.01–3.40-fold). These findings indicate that apoptosis is a key contributor to their cytotoxic mechanism.

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Ethical Approval

Ethical approval is not applicable to this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplemental Material

Supplemental material for this article is available online.

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