

Isolation of Triterpenoids from the Stem Bark of *Albizia julibrissin* and Their Inhibition Activity on ACAT-1 and ACAT-2

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The dried stem bark of *Albizia julibrissin* was extracted in 70% aqueous EtOH, and concentrated extracts were partitioned with EtOAc, *n*-BuOH, and H₂O, successively. From the EtOAc fraction, three triterpenoids were isolated through repeated silica gel and octadecyl silica gel column chromatography. Based on nuclear magnetic resonance spectrometry (NMR), mass spectrometry, and infrared spectroscopy spectroscopic data, the chemical structures of the compounds were determined to be lupeol (1), betulinic acid (2), and oleanolic acid (3). This was the first report in which compounds 1, 2, and 3 were isolated from the stem bark of *A. julibrissin*. Since the NMR data for some compounds have been incorrectly or incompletely identified in previous literature, the NMR were revised on the basis of 2-D NMR experiments. Compounds 1, 2, and 3 showed inhibition activity on ACAT-1 with values of 89.3±3.7%, 61.2±3.4%, and 52.5±0.7%, respectively, at a concentration of 50 µg/mL and on ACAT-2 with values of 44.3±2.1%, 55.5±0.3%, and 22.0±2.6%, respectively, at a concentration 50 µg/mL.

Key words: ACAT-1, ACAT-2, *Albizia julibrissin*, betulinic acid, lupeol, NMR, oleanolic acid

Albizia julibrissin Durazz (Leguminosae), is a small and spreading tree with smooth, gray-brown bark and doubly pinnate leaves. Clusters of pink flower heads are borne in summer [Ikeda *et al.*, 1997; Kang *et al.*, 2000]. This tree grows throughout China and can also be found in Africa, mid-Asia, east Asia and North America [Wei *et al.*, 2004]. The stem bark (*Albizia cortex*) is used in Chinese herbal medicine to treat insomnia, diuresis, sthenia, ascaricide, and confusion [Namba, 1980]. It is recorded in the Chinese Pharmacopoeia as a sedative and anti-inflammatory agent, and specified to treat injuries from falls and remove carbuncles [The Pharmacopoeia Committee of People's Republic of China (ed.), 1995]. The main compounds of the plant are reported to be lignans [Kinjo *et al.*, 1991a; 1991b], saponins [You *et al.*, 1982; Kang and Woo, 1983; Zou *et al.*, 2005], phenolic

glycosides [Higuchi *et al.*, 1992; Jeong *et al.*, 2004], and flavonoids [Chamsuksai *et al.*, 1981]. In continuing to search for other active components from the stem bark of *A. julibrissin*, we isolated three triterpenoids, all of which were isolated from this plant for the first time. This paper describes the procedure for the isolation of the three triterpenoids using silica gel (SiO₂) and octadecyl silica gel (ODS) column chromatography. The structures were determined using spectroscopic methods such as nuclear magnetic resonance spectrometry (NMR), infrared spectroscopy (IR), and mass spectrometry (MS). The triterpenoids were also evaluated for inhibition activity on ACAT-1 and ACAT-2. Acyl-CoA: cholesterol acyltransferase (ACAT), including ACAT-1 and ACAT-2 which differ in their tissue distribution and membrane topology [Brown *et al.*, 1975], catalyzes the acylation of cholesterol to cholesteryl esters with long chain fatty acids. ACAT-1 plays a critical role in foam cell formation in macrophages, whereas ACAT-2 regulates the cholesterol absorption process in intestinal mucosal cells [Rudel *et al.*, 2001].

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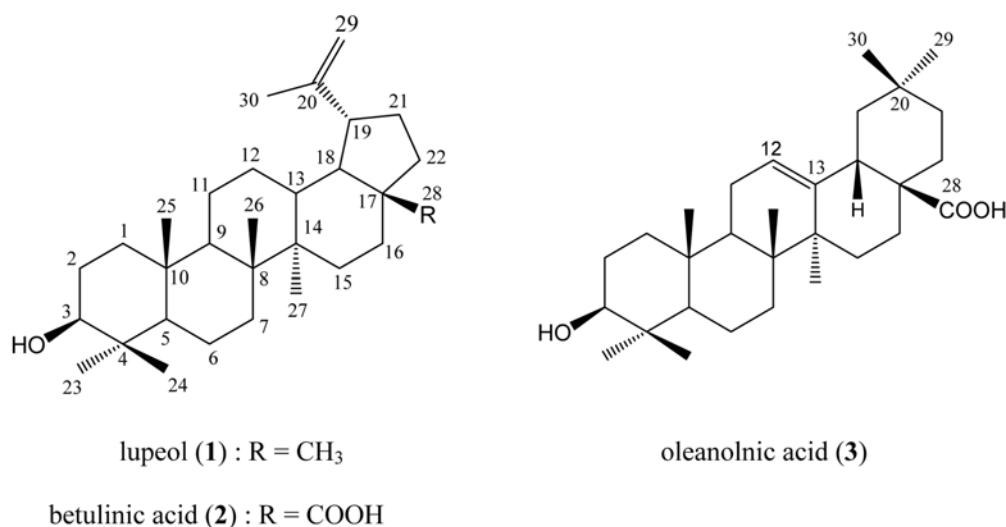


Fig. 1. Chemical structures of triterpenoids from the stem bark of *Albizia julibrissin*

Materials and Methods

Plant materials. The stem bark of *A. julibrissin* was collected at Herb Medicine Market in Daegu in 2007 and were identified by Professor Dae-Keun Kim, Woosuk University, Jeonju, Korea. A voucher specimen (KHU-07-630) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

General experimental procedures. SiO₂ resin and ODS resin used for column chromatography was Kiesel gel 60 (Merck, Darmstadt, Germany). thin layer chromatography (TLC) analysis was carried out using Kiesel gel 60 F₂₅₄ and RP-18 F_{254S} (Merck) and detected using a UV lamp, Spectroline Model ENF-240 C/F (Spectronics Corporation, New York, NY) and a 10% H₂SO₄ solution. The melting point was determined on a Fisher-John's apparatus and was not corrected. Optical rotation was measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). IR spectrum was obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). Electron ionization-mass spectrometry (EI-MS) was recorded on a JEOL JMSAX-700 (Tokyo, Japan). ¹H-NMR (400 MHz) and ¹³C-NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Palo Alto, CA).

Isolation of triterpenoids from the stem bark of *Albizia julibrissin*. The dried plant materials (3 kg) were extracted with 70% aqueous EtOH (13 L×4) at room temperature for 12 h and were then filtered through filter paper. The filtrate was evaporated *in vacuo* giving a dark brownish residue. The EtOH extract was suspended in water (1 L), and then successively partitioned with EtOAc (1 L×3) and *n*-BuOH (1 L×3) to afford the residues of EtOAc (98 g), and *n*-BuOH (69 g), respectively. The

EtOAc soluble fraction was chromatographed over a SiO₂ column (Φ 10×25 cm) using CHCl₃-MeOH (50:1→35:1→20:1→13:1→8:1→5:1) as eluents with monitoring by TLC, resulting in the production of 19 fractions (ADE-1~ADE-19). Fraction ADE-6 (8.5 g) was subjected to the SiO₂ column (Φ 7×11 cm) eluted with *n*-hexane-EtOAc (12:1→5:1→3:1) to give 14 fractions (ADE-6-1~ADE-6-14). ADE-6-10 (762 mg) was subjected to the SiO₂ column (Φ 4×13 cm) eluted with *n*-hexane-EtOAc (9:1) to give 9 fractions (ADE-6-10-1~ADE-6-10-9), yielding compound 1 [ADE-6-10-2, 18.3 mg, elution volume/total volume (Ve/Vt) 0.15-0.17, TLC (Kieselgel 60 F₂₅₄) R_f 0.2, *n*-hexan-EtOAc=10:1]. Fraction ADE-7+8 (10.3 g) was subjected to the SiO₂ column (Φ 7×13 cm) eluted with *n*-hexane-EtOAc (10:1→8:1→6:1→4:1→1:1) to give 30 fractions (ADE-7+8-1~ADE-7+8-30). ADE-7+8-12+13 (474 mg) was subjected to the SiO₂ column (Φ 3.5×15 cm) eluted with CHCl₃-MeOH (50:1→40:1→30:1→20:1→10:1→5:1) to give 10 fractions (ADE-7+8-12+13-1~ADE-7+8-12+13-10). ADE-7+8-12+13-4+5 (260 mg) was subjected to the ODS column (Φ 3.5×5.5 cm) eluted with MeOH-H₂O (4:1) to give 22 fractions (ADE-7+8-12+13-4+5-1~ADE-7+8-12+13-4+5-22), yielding compound 2 [ADE-7+8-12+13-4+5-15~19, 60 mg, Ve/Vt 0.40-0.91, TLC (RP-18 F_{254S}) R_f 0.3, MeOH-H₂O=12:1]. Fraction ADE-9+10 (3.5 g) was subjected to the SiO₂ column (Φ 6×13 cm) eluted with CH₂Cl₂-MeOH (15:1) to give 16 fractions (ADE-9+10-1~ADE-9+10-16). ADE-9+10-4 (1.6 g) was subjected to the SiO₂ column (Φ 5.5×13 cm) eluted with *n*-hexane-EtOAc (10:1→8:1→5:1→3:1) to give 24 fractions (ADE-9+10-4-1~ADE-9+10-4-24). ADE-9+10-4-6+7 (60 mg) was subjected to the ODS column (Φ 3.5×6 cm) eluted with MeOH-H₂O (2:1→4:1) to give 6 fractions (ADE-9+10-4-

6+7-1~ADE-9+10-4-6+7-6), yielding compound **3** [ADE-9+10-4-6+7-4, 9.5 mg, Ve/Vt 0.94-0.96, TLC (RP-18 F_{254S}) R_f 0.2, MeOH-H₂O=20:1].

Compound 1 (lupeol) white powder; m.p. 210; [α]_D²⁵ +23.0° (*c*=0.50, EtOH); IR (KBr, cm⁻¹) 3510, 1680; EI-MS *m/z* 426 [M]⁺, 409, 218, 207, 189; ¹H and ¹³C-NMR see Table 2 and 3.

Compound 2 (betulinic acid) white powder; m.p. 282; [α]_D²⁰ +12.4° (*c*=0.15, CHCl₃); IR (KBr, cm⁻¹) 3413, 1725, 1653; EI-MS *m/z* 456 [M]⁺, 438, 411, 248, 207, 203, 189; ¹H and ¹³C-NMR see Table 2 and 3.

Compound 3 (oleanolic acid) white powder; m.p. 196-198; [α]_D +64.6° (*c*=0.40, CHCl₃); IR (KBr, cm⁻¹) 3400, 1680, 1630; EI-MS *m/z* 456 [M]⁺, 410, 248, 208, 203, 190; ¹H and ¹³C-NMR see Table 2 and 3.

ACAT activity assay. Microsomal fractions of Hi5 cells harboring baculovirally-expressed hACAT-1 or hACAT-2, and rat liver microsomes were used as enzyme sources. The activities of hACAT-1 and hACAT-2 were determined via the method developed by Brecher and Chan [1980] with slight modifications [Lee *et al.*, 2001; Jeong *et al.*, 2004].

The reaction mixture, which contained 4 μ L of microsomes (8 mg/mL protein) and 20 μ L of 0.5 M potassium phosphate buffer (pH 7.4) with 10 mM dithiothreitol, 15 μ L of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μ L of cholesterol on acetone (20 μ g/mL, added last), 41°C of water and 10 μ L of the test sample, in a total volume of 92 μ L, was preincubated for 20 min at 37°C, with brief vortexing and sonication. The reaction was initiated by the addition of 8 μ L of [1-¹⁴C] oleoyl-CoA solution (0.05 μ Ci, final conc. 10 μ M). After 25 min of incubation at 37°C, the reaction was halted via the addition of 0.1 mL isopropanol-heptane (4:1; v/v). A mixture of 0.6 mL heptane and 0.4 mL of 0.1 M potassium phosphate buffer (pH 7.4), also containing 2 mM dithiothreitol, was subsequently added. This solution was mixed, allowed to phase separate under gravity for 2 min and cholesterol oleate recovered from the upper heptane phase (total volume 0.9-1.0 mL). The radioactivity in 100 μ L of the upper phase was then measured in a liquid scintillation vial containing 3 mL of a scintillation cocktail (Lumac Co., Upoluma, Netherland), using a liquid scintillation counter (1450 Microbeta Trilux Wallac Cy, Turku, Finland). The background values were determined via the preparation of heat-inactivated or normal insect cell lysate microsomes. The background value normally fell within 200-250 cpm, at 8,000 cpm for the ACAT reaction. The ACAT activity was expressed in units, as defined by: cholesteryl oleate pmol/min/rng protein.

Table 1. Inhibition activity of triterpenoids from the stem bark of *Albizia julibrissin* on ACAT-1 and ACAT-2¹⁾

compounds (50 μ g/mL)	ACAT-1 (%)	ACAT-2 (%)
	IC ₅₀ (mM) ³⁾	IC ₅₀ (mM)
1	89.3±3.7 2.6×10 ⁻²	44.3±2.1 13.8×10 ⁻²
2	61.2±3.4 8.8×10 ⁻²	55.5±0.5 10.0×10 ⁻²
3	52.5±0.7 10.5×10 ⁻²	22.0±2.6 -
oleic acid anilide ²⁾ (0.1 μ M)	45.2±1.8 1.2×10 ⁻²	55.9±0.8 -

¹⁾The data are presented as the mean±SD of three replications.

²⁾Oleic acid anilide was used as a positive control.

³⁾The IC₅₀ value is the concentration (mM) at which 50% inhibition of enzyme activity occurs.

Results and Discussion

Compounds were extracted from the stem bark of *A. julibrissin* in aqueous ethanol, and the obtained extracts were successively partitioned using EtOAc, *n*-BuOH, and H₂O. The EtOAc fraction was treated using repeated silica gel and ODS column chromatography (c.c.) to obtain three purified compounds, **1-3**. When all of the isolated compounds were developed on a SiO₂ and ODS TLC plate, they were visualized as pink by spraying with a 10% H₂SO₄ solution and heating, which indicated that they might be of a triterpenoid or a steroid.

Compound **1**, a white powder, showed absorbance bands due to the hydroxyl group (3510 cm⁻¹) and the double bond (1680 cm⁻¹) in the IR spectrum. The molecular weight was determined to be 426 from the molecular ion [M]⁺ at *m/z* 426 in the EI-MS spectrum. In the ¹H-NMR (400 MHz, CDCl₃) spectrum, an oxygenated methine proton (δ _H 3.16, dd, *J*=11.2, 5.2 Hz), a terminal methylene group of the vinylic proton signal (δ _H 4.66, 4.54, each), and seven singlet methyl signals (δ _H 1.65, 1.00, 0.94, 0.92, 0.80, 0.76, 0.73) were observed. All of these data indicated that compound **1** was a lupine-triterpenoid. The ¹³C-NMR (100 MHz, CDCl₃) spectrum showed thirty carbon signals such as one oxygenated methine (δ _C 79.0), an olefine quaternary (δ _C 150.8), one olefin methine (δ _C 109.2), seven methyls (δ _C 28.0, 19.4, 18.0, 16.2, 16.0, 14.6, 14.4), ten methylenes (δ _C 40.0, 38.7, 35.6, 34.3, 29.9, 29.7, 27.5, 25.2, 21.0, 18.4), five methines (δ _C 55.3, 50.4, 48.3, 48.0, 38.1), and five quaternary carbons (δ _C 43.0, 42.8, 40.8, 38.9, 37.2), which were determined using DEPT experiments. The *sp*²

Table 2. ^1H -NMR and ^{13}C -NMR data of compounds **1**, **2**, and **3** from the stem bark of *Albizia julibrissin*

No.	Compound 1		Compound 2		Compound 3	
	$\delta_{\text{C}}^{(1)}$	$\delta_{\text{H}}^{(1)}$	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	38.7	0.95, 1.62	39.2	0.95, 1.68	38.9	1.00, 1.52
2	29.7	1.23	28.2	1.84	28.3	1.17
3	79.0	3.16 (dd, $J=11.2$, 5.2 Hz)	78.1	3.45 (dd, $J=10.4$, 3.9 Hz)	78.1	3.44 (dd, $J=9.3$, 6.9 Hz)
4	38.9	-	39.5	-	39.4	-
5	55.3	0.65	55.9	0.80	55.8	0.84
6	18.4	1.33, 1.48	18.7	1.41, 1.55	18.8	1.38, 1.52
7	34.3	1.36	34.8	1.39	33.3	1.21, 1.33
8	40.8	-	41.1	-	39.8	-
9	50.4	1.23	50.9	1.36	48.1	1.68
10	37.2	-	37.6	-	37.4	-
11	21.0	1.33	21.1	1.41	23.8	1.22
12	25.2	1.54	26.1	1.19, 1.92	122.6	5.50 (t-like, $J=3.3$ Hz)
13	38.1	1.59	38.6	1.54	144.8	-
14	42.8	-	42.8	-	42.2	-
15	27.5	1.49	31.1	1.51, 2.23	28.1	1.80
16	35.6	1.36, 1.44	32.8	1.55, 2.62	23.8	1.92
17	43.0	-	56.6	-	46.7	-
18	48.3	2.25	47.7	3.56	42.0	3.31 (dd, $J=13.5$, 4.2 Hz)
19	48.0	1.28	49.7	3.53 (br s)	46.5	1.32, 1.80
20	150.8	-	151.3	-	31.0	-
21	29.9	1.23	30.2	1.27, 1.84	43.2	1.17, 1.41
22	40.0	1.18, 1.36	37.5	1.55, 2.23	33.2	1.80, 2.08
23	28.0	0.94 (s)	28.6	1.22 (s)	28.8	1.24 (s)
24	14.4	0.73 (s)	16.3	1.00 (s)	16.5	1.02 (s)
25	16.2	0.80 (s)	16.4	0.82 (s)	15.5	0.89 (s)
26	16.0	1.00 (s)	16.4	1.06 (s)	17.4	1.01 (s)
27	14.6	0.92 (s)	14.8	1.05 (s)	26.2	1.28 (s)
28	18.0	0.76 (s)	178.8	-	180.2	-
29	109.2	4.54 (br s), 4.66 (br s)	109.9	4.76 (br s), 4.94 (br s)	33.3	0.94 (s)
30	19.4	1.65 (s)	19.4	1.78 (s)	23.8	1.00 (s)

¹H- (400 MHz) and ¹³C-NMR (100 MHz) were measured in CDCl₃ (**1**) and pyridine-*d*₅ (**2** and **3**), respectively.

²The signals, the coupling pattern of which was not described, overlapped each other.

carbons observed at δ_{C} 150.8 and 109.2 in the ¹³C-NMR spectrum confirmed the 20, 29-functionality of the lupane skeleton. The proton signals of methines or methylenes in the high magnetic field were identified using gradient heteronuclear single quantum correlation experiments. Thus, compound **1** was identified as a lupane triterpenoid, 3*b*-hydroxy-lup-20(29)-ene (lupeol) through comparison of our spectroscopic data with published spectroscopic data [Matsunaga *et al.*, 1988; Fotie *et al.*, 2006].

Compound **2**, a white powder, showed absorbance bands due to the hydroxyl group (3413 cm⁻¹), the carboxyl group (1725 cm⁻¹) and the double bond (1653 cm⁻¹) in the IR spectrum. The molecular ion peak [M]⁺ at *m/z* 456 indicated the molecular weight to be 456 in the EI-MS spectrum. The ¹H-NMR (400 MHz, pyridine-*d*₅) and ¹³C-

NMR (100 MHz, pyridine-*d*₅) spectra of compound **2** were almost the same as those of compound **1**, with the exception of six singlet methyl signals (δ_{H} 1.78, 1.22, 1.06, 1.05, 1.00, 0.82; δ_{C} 28.6, 19.4, 16.4 (x 2), 16.3, 14.8) and of a carboxyl group (δ_{C} 178.8) in compound **2**. Thus, compound **2** was identified as a lupane-triterpenoid, 3*b*-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid) through comparison of our spectroscopic data with published spectroscopic data [Choi *et al.*, 2006; Lee *et al.*, 2009].

Compound **3**, a white powder, showed absorbance bands due to the hydroxyl group (3400 cm⁻¹), the carboxyl group (1680 cm⁻¹) and the double bond (1630 cm⁻¹) in the IR spectrum. The molecular ion peak [M]⁺ at *m/z* 456 indicated the molecular weight to be 456 in the EI-MS spectrum. In the ¹H-NMR (400 MHz, pyridine-*d*₅)

spectrum, an olefin methine (δ_{H} 5.50, t-like, $J=3.3$ Hz) and an oxygenated methine (δ_{H} 3.44, dd, $J=9.3$, 6.9 Hz) were observed. Additionally, in the high magnet field, seven singlet methyl signals (δ_{H} 1.28, 1.24, 1.02, 1.01, 1.00, 0.94, 0.89) were observed. These indicated that compound **3** could be an oleanane type triterpenoid. The ^{13}C -NMR (100 MHz, pyridine- d_5) spectrum showed thirty carbon signals including one oxygenated methine (δ_{C} 78.1), one olefin quaternary (δ_{C} 144.8), one olefin methine (δ_{C} 122.6), seven methyls (δ_{C} 33.3, 28.8, 26.2, 23.8, 17.4, 16.5, 15.5), ten methylenes (δ_{C} 46.5, 43.2, 38.9, 33.3, 33.2, 28.3, 28.1, 23.8 (x 2), 18.8), three methines (δ_{C} 55.8, 48.1, 42.0), and six quaternary carbons (δ_{C} 46.7, 42.2, 39.8, 39.4, 37.4, 31.0), which were determined using DEPT experiments. The chemical shift of a carboxyl group (δ_{C} 180.2), and two sp^2 hybridized carbons (C-12, δ_{C} 122.6; C-13, δ_{C} 144.8) indicated that compound **3** was an olean-12-en-oic acid derivative. Thus, compound **3** was identified as the pentacyclic triterpenoid of oleanane type, 3*b*-hydroxy-olean-12-en-28-oic acid (oleanolic acid) through comparison of our spectroscopic data with published spectroscopic data [Balanehru and Nagarajan, 1991; Ahmad and Rahman, 1994; Mahato and Kundu, 1994]. Compounds **1-3** were isolated for the first time from *A. julibrissin*.

The three triterpenoids isolated from the stem bark of *A. julibrissin* were evaluated for inhibition of ACAT-1 and ACAT-2. ACAT-1 plays an important role in foam cell formation in macrophages, while ACAT-2 controls the cholesterol absorption process in intestinal mucosal cells [Rudel *et al.*, 2001]. These findings are consistent with the discovery that atherosclerosis lesions are decreased in ACAT-1 mice, while ACAT-2 mice have restricted cholesterol absorption in the intestine, and decreased cholesterol ester content in the liver and plasma lipoproteins [Accad *et al.*, 2000]. Therefore, ACAT inhibitors could be developed as an effective treatment of atherosclerosis and hypercholesterolemia [Buhman *et al.*, 2000].

Compounds **1-3** showed significant inhibitory activity on ACAT-1 with 89.3±3.7%, 61.2±3.4%, and 52.5±0.7%, respectively at a concentration of 50 µg/mL, and on ACAT-2 with values of 44.3±2.1%, 55.5±0.3%, and 22.0±2.6%, respectively at a concentration of 50 µg/mL. The positive control, oleic acid anilide, inhibited ACAT-1 by 45.2±1.8% and ACAT-2 by 55.9±0.8% at 0.1 µM (Table 1) and compound **1** demonstrated inhibition activity on ACAT-1 and ACAT-2 with IC_{50} value of 0.026 mM and 0.138 mM, respectively. Although compounds **1-3** had lower inhibition activity on ACAT-1 and ACAT-2 compared to the positive control oleic acid anilide, the relative paucity of naturally occurring ACAT-1 and

ACAT-2 inhibitors makes this discovery worth further exploration. In general, lupane triterpenoids exhibit a slightly higher inhibitory activity on ACAT than the oleanane triterpenoid in this experiment. The carboxylic acid at C-28 in the lupane triterpenoid might be responsible for lowering the inhibition activity on ACAT-1. Therefore, the findings of this study suggest that the alcohol extracts or some triterpenoids from the stem bark of *A. julibrissin* may prove useful in the treatment of hypercholesterolemia and atherosclerosis.

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