

Article

Chemical Constituents from the Leaves of *Annona reticulata* and Their Inhibitory Effects on NO Production

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Abstract: In the present study, the chemical investigation of the leaves of *Annona reticulata* has resulted in the identification of nine compounds, including annonaretin A, (**1**), a new triterpenoid. The purified compounds were subjected to the examination of their effects on NO inhibition in LPS-activated mouse peritoneal macrophages and most of them exhibited significant NO inhibition, with IC₅₀ values in the range of 48.6 ± 1.2 and 99.8 ± 0.4 μM.

Keywords: *Annona*; triterpenoid; diterpenoid; NO inhibition; *i*NOS; macrophage

1. Introduction

The *Annona* genus (Annonaceae) consists of about 119 species, most of which are shrubs and trees widely distributed in the tropical and subtropical regions, including the Southeast Asia countries such as Malaysia, Indonesia, Thailand, Cambodia, Laos, and Vietnam. In Indian folk medicine, various species of *Annona* have been used as vermifuges, anti-inflammatory agents, in wound healing, as antimalarial agents and in the treatment of diarrhoea and dysentery [1]. The bark of the plant *Annona reticulata* L. is a powerful astringent and given as tonic. The plant has been used as an anti-inflammatory agent in wound healing, anti-anxiety, anti-stress, anti-mutagenic, and spasmolytic agent. Leaf and stem extract shows inotropic, positive chronotropic and spasmolytic activities [2]. The plant is reported to contain acetogenins mainly *cis*- and *trans*-isomurisolenin [3], annoreticuin, bullatacin, squamosine, rolliniastatin [4], reticullacinone, rolliniastatin-2, molvizarin [5], 14-hydroxy-25-deoxy-rollinacin [6]. Reticulatacin and kaurane diterpenes were also identified from the bark of the plant [7]. Other terpenes such as spathenelol, muurolene, copaene and eudesmol were reported in the previous literature [8]. In our continued program aimed at the identification of anti-inflammatory drug leads from natural sources, the chemical composition of the leaves of *A. reticulata* was investigated to search for the bioactive constituents by assays of inhibitory effects on NO inhibition in LPS-activated mouse peritoneal macrophages. In the present study, we wished to report the characterization of nine compounds, including the structural determination of new compound **1**, as well as their NO inhibitory effects.

2. Results and Discussion

2.1. Isolation and Characterization of Compounds

Air-dried and powdered leaves of *A. reticulata* L. were soaked with methanol at room temperature, and the combined extracts were concentrated to give a deep brown syrup. The crude extract was suspended into water and partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, successively to afford *n*-hexane, ethyl acetate, *n*-butanol, and water solubles, respectively. Purification of the *n*-hexane soluble fraction by column chromatography yielded annonaretin A (**1**) (Figure 1), kaurenoic acid (**2**) [9], taraxerol (**3**) [10], β -sitosterol (**4**) [11], 16 α -hydro-19-*al-ent*-kauran-17-oic acid (**5**) [9], 6 β -hydroxystigmast-4-en-3-one (**6**) [12], and 17-acetoxy-16 β -*ent*-kauran-19-oic acid (**7**) [13]. Purification of the ethyl acetate soluble afforded 16 α -hydro-*ent*-kauran-17,19-dioic acid (**8**) [9], and (2*S*)-di-*O*-methylquiritigenin (**9**) [14]. Compounds **2–9** (Figure 2) are known compounds, and their structures were identified by comparison of their physical and spectroscopic data with those reported in the literature. Compound **1** is new.

2.2. Structural Elucidation of New Compound **1**

The purified colorless powder **1** was visualized by spraying with 1% (w/v) Ce(SO₄)₂ in 10% (v/v) aqueous H₂SO₄ followed by heating at 120 °C and displayed purplish black spots on TLC plate. It also displayed a positive response in the Liebermann–Burchard test. These results suggested that compound **1** possessed a basic triterpenoid skeleton [15]. The molecular formula of **1** was established as C₃₃H₅₆O₃ by the pseudomolecular [M+Na]⁺ ion peak at *m/z* 523.4122 in HR-ESI-MS analysis and was further

supported by its ^{13}C -NMR spectrum which showed signals for all 33 carbons of the molecule, including one set of terminal methylenes (δ_{C} 111.9, 147.4), three oxygenated carbons (δ_{C} 62.8, 71.0, 83.2), and seven methyl carbons (δ_{C} 15.1, 18.1, 19.0, 19.3, 20.7, 21.3, 25.7). In the ^1H -NMR spectrum of **1**, there were five singlets at δ 0.82 (3H, s, CH₃-18), 0.91 (3H, s, CH₃-21), 0.97 (3H, s, CH₃-22), 0.98 (3H, s, CH₃-19), and 1.60 (3H, s, CH₃-30); and two doublets at δ 0.80 (3H, d, $J = 9.5$ Hz, CH₃-32) and 0.93 (3H, d, $J = 6.0$ Hz, CH₃-33), respectively. In addition, two high field cyclopropyl proton doublets at δ 0.40 (1H, d, $J = 4.5$ Hz, H-20) and 0.62 (1H, d, $J = 4.5$ Hz, H-20) evidenced that this compound was a triterpenoid derivative with the cycloartanol skeleton. In the downfield region, four oxygenated protons at δ 3.01 (1H, d, $J = 9.5$ Hz, H-3), 3.54 (1H, dd, $J = 11.0, 6.0$ Hz, H-24), 3.64 (1H, br dd, $J = 16.0, 9.0$ Hz, H-2), and 3.70 (1H, br d, $J = 11.0$ Hz, H-24) were located at C-2, -3, and -24 determined by the 2J , 3J -HMBC correlations through H-2 to C-3 (δ 83.2), C-4 (δ 40.3); H-3 to C-1 (δ 25.7), C-2 (δ 71.0), C-4, and C-18 (δ 15.1); and H-24 to C-23 (δ 46.2), C-25 (δ 27.7), respectively. The position of terminal methylene group at δ 4.64 (1H, br s, H-29) and 4.77 (1H, br s, H-29) was also established with the HMBC analysis of correlations between H-29 and C-27 (δ 55.4), C-30 (δ 19.0). In the HMBC spectral analysis (Figure 2), correlation peaks between H-32 and C-33, C-31, C-27; H-33 and C-32, C-31, C-27, supported that one isopropyl group was attached at C-27. The high field doublets at δ 0.40 and 0.62 displayed HMBC correlations with the carbons at δ 25.7 (C-1), 39.7 (C-11), and 47.1 (C-5) also confirmed the presence of cyclopropyl functionality as C-20 connected with C-9 and C-10. The full assignments of ^1H - and ^{13}C -NMR signals were substantiated by extensive 2D NMR experimental analysis. Therefore the chemical structure of **1** was established as shown in Figure 1 and the compound was given the trivial name annonaretin A.

Figure 1. Structure and significant HMBC (\rightarrow) correlations of compound **1**.

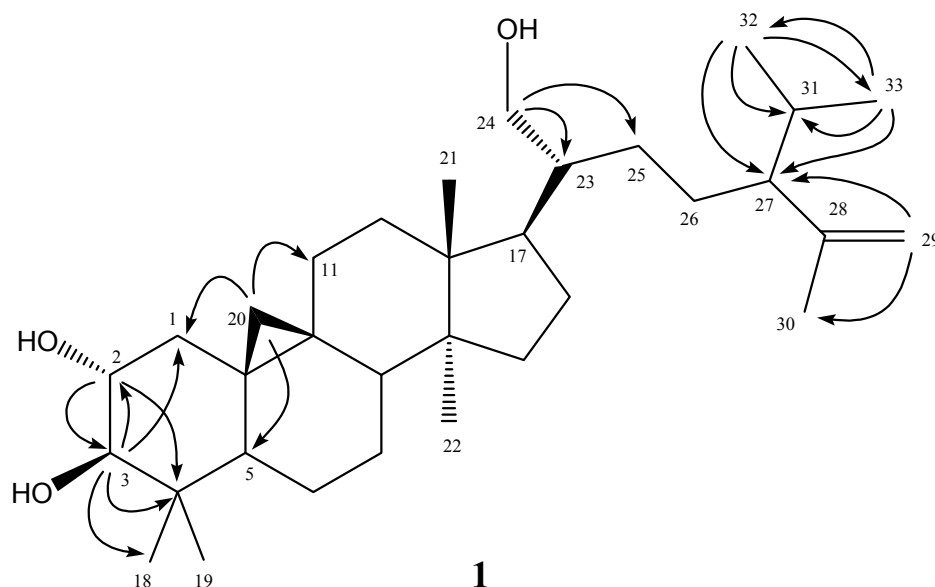
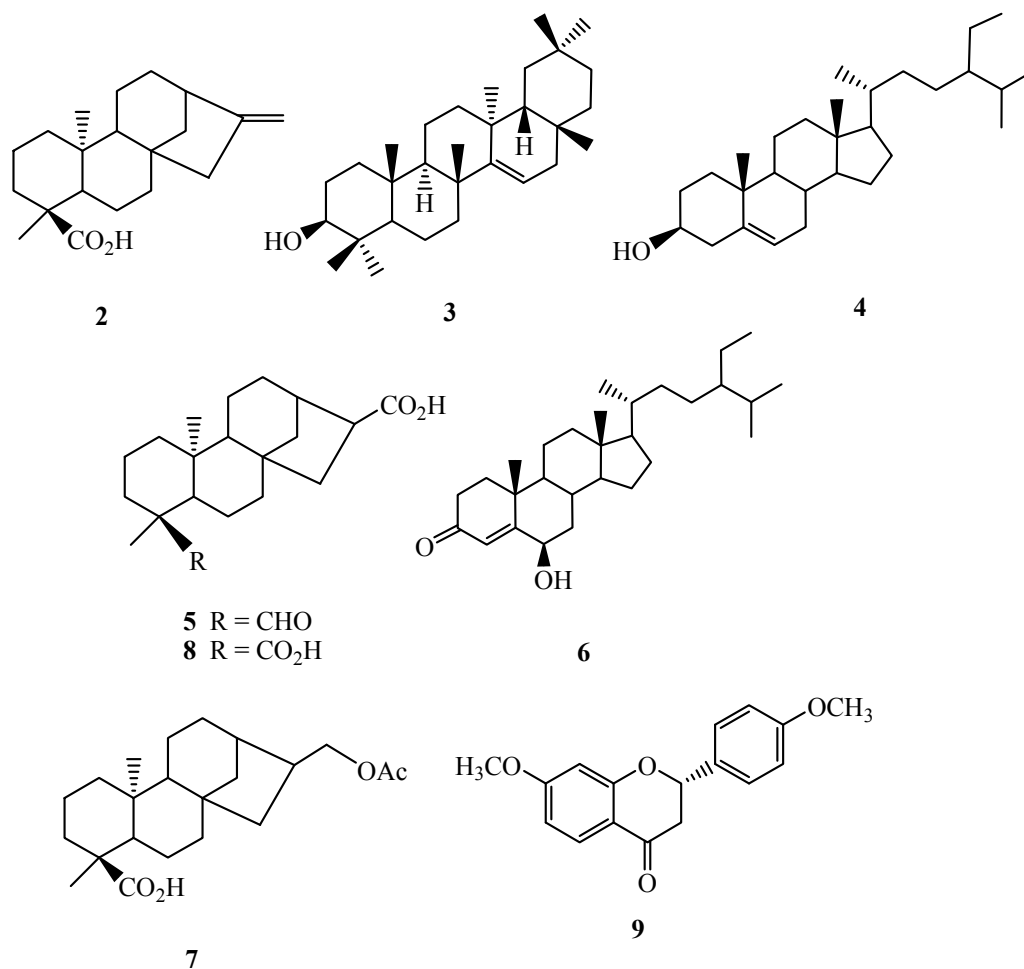


Figure 2. Structures of purified known compounds 2–9.



2.3. The Inhibitory Effects of Isolated Compounds on NO Production

The isolated compounds **1–9** were subjected into the examination of their effects on LPS-induced *i*NOS-dependent NO production in RAW 264.7 cells. Cells cultured with **1–9** at different concentrations used in the presence of 100 ng/mL LPS for 24 h (Table 1). Some cell toxicity was observed in cells treated with compounds **1**, **2**, **4**, and **9**, whereas other compounds had no influence on cell viability. NO production was significantly decreased by the treatment with **2**, **3**, and **5–8** in a dose-dependent manner, with IC₅₀ values in the range of 48.6 ± 1.2 and 99.8 ± 0.4 μM (Table 1). The inhibitory effects of **1**, **4** and **9** were less obvious. **2**, **3**, and **5–8** did not exhibit significant cytotoxicity in the concentration range of 12.5–100 μM (Table 1), thus the NO inhibiting effects were probably not due to cytotoxicity. Among the tested compounds, **2**, and **5–8** belonged to the *ent*-kaurane diterpenoids noted for the occurrence in the *Annona* species [16,17]. In the previous literature, kaurenoic acid (**2**) was reported to inhibit NO production, prostaglandin E₂ release, cyclooxygenase-2, and inducible nitric oxide synthase expression in LPS-induced RAW264.7 macrophages [18]. It also exhibited anti-inflammatory [19,20], cytotoxic [21,22], antiplasmodial [21], antimicrobial [16], hypoglycaemic [23], vasorelaxant [24,25], and antispasmodic [25] bioactivities. 16 α -Hydro-19-*al-ent*-kauran-17-oic acid (**5**) displayed the most significant inhibitory effect on NO production with the lowest IC₅₀ value of 48.6 ± 1.2 μM. It was also reported from the stems of *A. squamosa* to exhibit anti-platelet aggregation

activity [26]. In addition, taraxerol (**3**) could downregulate the expression of proinflammatory mediators in macrophages by interfering with the activation of TAK1 and Akt, thus preventing NF- κ B activation [27]. In our experimental results, **2**, **3**, and **5** displayed NO inhibitory effects similar to these anti-inflammatory reports.

Table 1. Inhibitory effects of purified samples from the leaves of *A. reticulata* on LPS-induced *i*NOS-dependent NO production in RAW 264.7 cells.

	Dose (μ M)	Cell viability (% of control)	NO level	NO inhibition (% of control)	IC ₅₀ (μ M)
Control	(-)	100.0 \pm 4.9	-0.5 \pm 0.1	(-)	(-)
LPS	(+)	98.7 \pm 8.0	45.4 \pm 2.7 ^{###}	(-)	(-)
1	12.5	88.0 \pm 1.5	36.6 \pm 1.9	19.3 \pm 4.2	(-)
	25	81.2 \pm 1.8	33.5 \pm 0.9	26.2 \pm 2.0	
	50	73.0 \pm 3.6 *	(-)	(-)	
	100	66.9 \pm 5.5 **	(-)	(-)	
2	12.5	98.0 \pm 2.2	34.3 \pm 4.7	24.5 \pm 10.2	50.0 \pm 0.3
	25	91.0 \pm 3.6	35.1 \pm 1.2	22.7 \pm 2.6	
	50	82.9 \pm 1.2	22.7 \pm 0.5 **	50.0 \pm 1.0	
	100	65.7 \pm 8.6 **	(-)	(-)	
3	12.5	93.5 \pm 1.8	38.5 \pm 2.3	15.2 \pm 5.0	99.8 \pm 0.4
	25	92.6 \pm 3.1	39.5 \pm 0.6	13.1 \pm 1.2	
	50	88.7 \pm 1.8	35.6 \pm 0.9 *	21.6 \pm 2.0	
	100	81.4 \pm 2.3	22.6 \pm 0.4 **	50.1 \pm 0.9	
4	12.5	82.8 \pm 2.7	38.4 \pm 0.7	15.5 \pm 1.5	(-)
	25	80.7 \pm 3.8	38.6 \pm 3.3	15.1 \pm 7.3	
	50	65.3 \pm 2.3 **	(-)	(-)	
	100	55.8 \pm 2.7 **	(-)	(-)	
5	12.5	98.9 \pm 5.2	38.3 \pm 1.3	15.5 \pm 2.8	48.6 \pm 1.2
	25	96.5 \pm 9.2	33.1 \pm 1.0 *	27.1 \pm 2.3	
	50	95.9 \pm 8.6	22.1 \pm 1.5 **	51.4 \pm 3.2	
	100	80.2 \pm 5.7	14.8 \pm 0.7 ***	67.3 \pm 1.5	
6	12.5	94.8 \pm 3.1	37.0 \pm 1.8	18.5 \pm 4.0	51.5 \pm 0.5
	25	83.7 \pm 3.5	33.1 \pm 2.4 *	27.1 \pm 5.3	
	50	83.5 \pm 3.0	22.9 \pm 1.1 **	49.5 \pm 2.4	
	100	81.3 \pm 2.8	15.1 \pm 0.3 ***	66.7 \pm 0.6	
7	12.5	98.6 \pm 3.1	42.6 \pm 0.7	6.2 \pm 1.5	55.5 \pm 0.3
	25	93.6 \pm 4.0	40.6 \pm 2.8	10.6 \pm 6.1	
	50	92.5 \pm 2.8	23.7 \pm 0.2 **	47.7 \pm 0.3	
	100	83.7 \pm 1.6	14.3 \pm 0.3 ***	68.5 \pm 0.7	
8	12.5	98.4 \pm 3.2	39.8 \pm 1.2	12.3 \pm 2.6	54.5 \pm 0.8
	25	94.0 \pm 2.6	35.4 \pm 0.8 *	21.9 \pm 1.8	
	50	88.6 \pm 2.6	23.5 \pm 0.9 **	48.2 \pm 2.0	
	100	82.4 \pm 1.2	14.4 \pm 0.3 ***	68.3 \pm 0.7	
9	12.5	99.9 \pm 4.8	42.0 \pm 1.2	7.5 \pm 2.7	(-)
	25	90.0 \pm 1.6	40.4 \pm 0.7	11.0 \pm 1.6	
	50	82.6 \pm 2.7	25.9 \pm 2.3	42.9 \pm 5.0	
	100	76.2 \pm 1.1 *	(-)	(-)	

The data were presented as mean \pm S.D. ^{###} Compared with sample of control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were compared with LPS-alone group. (-): Not determined.

3. Experimental

3.1. General Procedures

Melting point was determined using an Electrothermal IA-9200 melting point measuring apparatus without correction. The UV spectrum was recorded on an Agilent UV-VIS recording spectrophotometer. The IR spectra (KBr) were obtained, on a Hitachi 270-30 type spectrometer. Optical rotations were measured with a Jasco DIP-1000 KUY polarimeter. The electrospray ionization (ESI) mass spectra were determined using an Agilent 1200 LC-MSD Trap spectrometer, and the HR-ESI-MS was completed with the aid of a Bruker APEX II mass spectrometer. ^1H - and ^{13}C -NMR, COSY, NOESY, HMQC, and HMBC spectra were recorded on a Bruker Avance-500 NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, E. Merck, Darmstadt, Germany).

3.2. Plant Materials

The leaves of *Annona reticulata* L. (Annonaceae) were collected from Tiengiang, Vietnam, during October 2010 and the plant materials were identified and authenticated by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnamese Academy of Science and Technology. A voucher specimen (Viet-TSWu-20101015) was deposited at the Herbarium of the Vinh University.

3.3. Extraction and Isolation

The leaves of *Annona reticulata* L. (5.0 kg) were air-dried and powdered and soaked (24 h) with methanol (20 L \times 3) at room temperature, and the combined extracts were concentrated under reduced pressure to give deep brown syrup (316.0 g). The crude extract was suspended into water (1 L) and partitioned with *n*-hexane (1 L \times 5), ethyl acetate (1 L \times 5), and *n*-butanol (1 L \times 5), successively to afford *n*-hexane (31.0 g), ethyl acetate (82.0 g), *n*-butanol (47.0 g), and water solubles (52.0 g), respectively, after removal of the corresponding solvent.

The *n*-hexane soluble extracts were purified by silica gel column chromatography eluted with *n*-hexane and acetone gradients to afford 14 fractions. Fraction 1 was subjected to silica gel column chromatography eluted with *n*-hexane/acetone (25:1) to yield kaurenoic acid (**2**, 968 mg). Fraction 2 was isolated by silica gel column chromatography eluted with *n*-hexane/acetone (15:1) to afford taraxerol (**3**, 78 mg). Purification of fraction 3 by column chromatography with silica gel eluted by step gradients of *n*-hexane/acetone (15:1 and 9:1) afforded β -sitosterol (**4**, 302 mg). Fraction 4 was subjected to silica gel column chromatography eluted with *n*-hexane/acetone (15:1) to yield 16 α -hydro-19-*al-ent*-kauran-17-oic acid (**5**, 26 mg). Fraction 5 was purified by silica gel column chromatography eluted with *n*-hexane/acetone (9:1) to yield 6 β -hydroxystigmast-4-en-3-one (**6**, 31 mg). Isolation of fraction 6 by column chromatography with silica gel eluted by *n*-hexane/acetone (7:1) yielded 17-acetoxy-16 β -*ent*-kauran-19-oic acid (**7**, 22 mg). Silica gel column chromatography of

fraction 9 with step gradient elution of *n*-hexane/acetone (7:1 and 5:1) led to the purification of annonaretin A (**1**, 789 mg).

The ethyl acetate soluble extracts were applied to silica gel column chromatography with chloroform and methanol gradients (20:1 to 5:1) to afford eight fractions. Fraction 1 was subjected to silica gel column chromatography eluted with *n*-hexane/acetone (9:1) to yield 16 α -hydro-*ent*-kauran-17,19-dioic acid (**8**, 38 mg). Silica gel column chromatography of fraction 6 with step gradient elution of chloroform and methanol (9:1 and 5:1) led to the isolation of (2*S*)-di-*O*-methylquiritigenin (**9**, 23 mg).

3.4. Spectral Data

Annonaretin A (**1**): colorless powder; m.p. 120–121 °C (CHCl₃); ¹H-NMR (CDCl₃) δ 0.40 (1H, d, J = 4.5 Hz, H-20), 0.62 (1H, d, J = 4.5 Hz, H-20), 0.78 (1H, m, H-6), 0.80 (3H, d, J = 9.5 Hz, CH₃-32), 0.82 (3H, s, CH₃-18), 0.91 (3H, s, CH₃-21), 0.93 (3H, d, J = 6.0 Hz, CH₃-33), 0.97 (3H, s, CH₃-22), 0.98 (3H, s, CH₃-19), 1.04 (1H, m, H-7), 1.10 – 1.20 (3H, m, H-1, -25, -16), 1.31–1.43 (7H, m, H-1, -12, -26, -5, -17, -25), 1.52–1.69 (9H, m, H-31, -11, -8, -15, -27, -6, -30, -16), 1.60 (3H, s, CH₃-30), 1.86 – 2.05 (3H, m, H-23, -26, -7), 3.01 (1H, d, J = 9.5 Hz, H-3), 3.40 (1H, br s, OH), 3.54 (1H, dd, J = 11.0, 6.0 Hz, H-24), 3.64 (1H, br dd, J = 16.0, 9.0 Hz, H-2), 3.70 (1H, br d, J = 11.0 Hz, H-24), 4.64 (1H, br s, H-29), 4.77 (1H, br s, H-29); ¹³C-NMR (CDCl₃) δ 15.1 (C-18), 18.1 (C-19), 19.0 (C-30), 19.2 (C-10), 19.3 (C-21), 20.7 (C-33), 20.9 (C-6), 21.3 (C-32), 25.1 (C-9), 25.7 (C-22), 25.7 (C-1), 26.5 (C-7), 26.8 (C-16), 27.3 (C-26), 27.7 (C-25), 29.7 (C-20), 30.2 (C-31), 31.8 (C-15), 35.3 (C-12), 39.7 (C-11), 40.3 (C-4), 43.1 (C-17), 45.0 (C-13), 46.2 (C-23), 47.1 (C-5), 47.7 (C-8), 48.7 (C-14), 55.4 (C-27), 62.8 (C-24), 71.0 (C-2), 83.2 (C-3), 111.9 (C-29), 147.4 (C-28); ESI-MS m/z (*rel. int.*) 523 ([M+Na]⁺, 100); HR-ESI-MS m/z 523.4122 [M+Na]⁺ (calcd for C₃₃H₅₆O₃Na, 523.4127).

3.5. Determination of Inhibitory Effects on NO Production

3.5.1. Cell Culture

A murine macrophage cell line RAW264.7 (BCRC No. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO₂ incubator (5% CO₂ in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca²⁺-, Mg²⁺- free phosphate-buffered saline (DPBS).

3.5.2. Cell Viability

Cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with samples in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μ L of 0.5 mg/mL MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded and 100 μ L dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3.5.3. Measurement of Nitric oxide/Nitrite

NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction [28]. The cells were incubated with a test sample in the presence of LPS (100 ng/mL) at 37 °C for 24 h. Then, cells were dispensed into 96-well plates, and 100 µL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices). By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from absorbance at 540 nm.

3.5.4. Statistical Analysis

Experimental results were presented as the mean \pm standard deviation (SD) of three parallel measurements. Statistical comparisons were made by Student's t-test. IC₅₀ values were estimated using a non-linear regression algorithm (SigmaPlot 8.0; SPSS Inc. Chicago, IL, USA). Statistical significance is expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

4. Conclusion

In summary, nine compounds were characterized from the leaves of *A. reticulata* L. and their inhibitory activity on NO production was examined. The results provide a potential explanation for the use of the leaves of *A. reticulata* as a herbal medicine in the treatment of inflammatory diseases, and they may be potentially useful in developing new anti-inflammatory agents.

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Sample Availability: Samples of the compounds **1–9** are available from the authors.

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