Lupane Triterpenes from the Leaves of *Acanthopanax gracilistylus*

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Abstract: The phytochemical study on the leaves of *Acanthopanax gracilistylus* (Araliaceae) resulted in the discovery of a new lupane-triterpene compound, acangraciligenin S (**1**), and a new lupane-triterpene glycoside, acangraciliside S (**2**), as well as two known ones, 3α,11α- dihydroxy-lup-20(29)-en-23,28-dioic acid (**3**) and acankoreoside C (**4**). Their chemical structures were elucidated by mass, 1D- and 2D-nuclear magnetic resonance (NMR) spectroscopy. The chemical structures of the new compounds **1** and **2** were determined to be 1β,3α-dihydroxy-lup-20(29)-en-23, 28-dioic acid and 1β,3α-dihydroxy-lup-20(29)-en-23,28-dioic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester, respectively. The anti-neuroinflammatory activity of the selective compounds, **1** and **3**, were evaluated with lipopolysaccharide (LPS)-induced BV2 microglia. The tested compounds showed moderate inhibitory effect of nitric oxide (NO) production.

Keywords: *Acanthopanax gracilistylus*; Araliaceae; lupane-triterpene; acangraciligenin S; acangraciliside S

1. Introduction

Acanthopanax gracilistylus W. W. Smith belongs to Araliaceae, which is widely distributed in China, and its dried roots and stem barks are listed officially in the Chinese Pharmacopoeia (2015 edition) as *Acanthopanax Cortex* (named as Wujiapi), which has been used as medicine for the treatment of paralysis, arthritis, rheumatism, lameness, and liver disease [1–3]. Previous phytochemical investigations of this plant have led to the identification of triterpenoids [4–7], diterpenoids [8,9], monoterpenoids [10–12], lignans [12], steroids, cerebrosides [13], and volatile components [10,14–19], which showed diverse biological activities, such as anti-tumor [20,21], anti-inflammatory [22–24], and liver protective effects [3]. As part of our ongoing research to identify bioactive substances from the genus *Acanthopanax*, we investigated a MeOH extract of the leaves of *A. gracilistylus* and identified two new lupane triterpenoids (**1** and **2**), and two known compounds (**3** and **4**) by using high-speed countercurrent chromatography (HSCCC) in conjunction with a high-performance liquid

chromatography (HPLC) isolation system (Figure 1). In this paper, the isolation and structural elucidation of the new isolates, as well as an evaluation of their bio-activities, are reported.
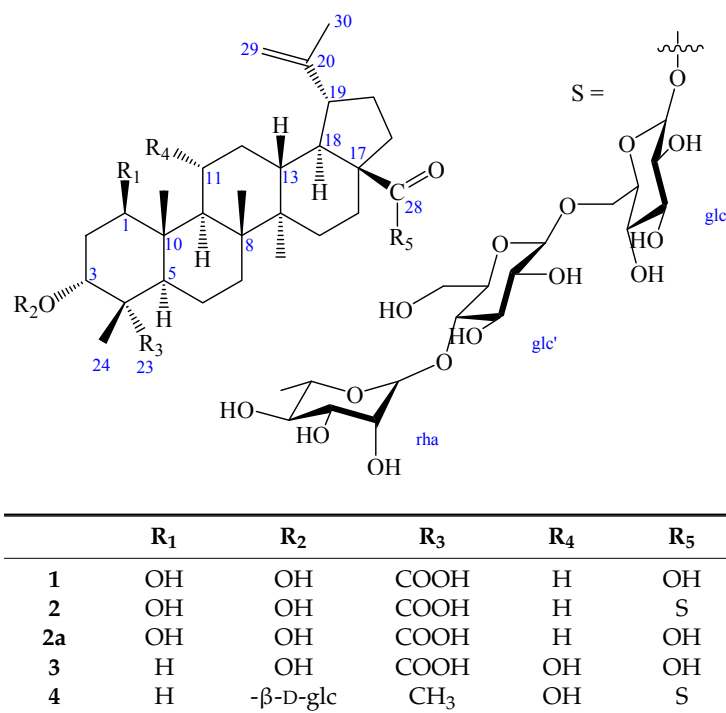

	R_1	R_2	R_3	R_4	R_5
1	OH	OH	COOH	H	OH
2	OH	OH	COOH	H	S
2a	OH	OH	COOH	H	OH
3	H	OH	COOH	OH	OH
4	H	-β-D-glc	CH$_3$	OH	S

Figure 1. Chemical structures of compounds **1**–**4**.

2. Results and Discussion

Compound **1** was obtained as a colorless needle, m.p. 282.6 °C, and gave a positive reaction in the Liebermann–Burchard test. Its molecular formula of $C_{30}H_{46}O_6$ was determined on the basis of ESI-MS at m/z = 501.3 [M − H]$^-$ (negative) and HR-ESI-MS at m/z = 501.3221 [M − H]$^-$ (calcd. for $C_{30}H_{45}O_6$: 501.3222), indicating eight degrees of unsaturation. The ^{1}H-NMR spectrum of **1** (in acetone-d_6) showed the following signals: five tertiary methyl groups at δ 0.97, 0.99, 1.07, 1.17 and 1.71 (each 3H, s); two olefinic protons at δ 4.58 (1H, m) and 4.72 (1H, d, J = 1.68 Hz); two hydroxy-substituted methines at δ 3.82 (1H, t, J = 2.40 Hz) and 3.84 (1H, dd, J = 8.16, 3.96 Hz). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra revealed 30 carbon signals. The skeleton of **1** was recognized to be lupane triterpenoid by ^{1}H- and ^{13}C-NMR analysis (see Table 1), with the typical olefinic carbons at δ 151.89 (C-20) and 110.26 (C-29), five quaternary methyl carbons at δ 13.24, 15.40, 17.31, 17.92, and 19.72, two oxymethine carbons at δ 73.99 and 75.46, and two carboxyl signals at δ 177.84 and 178.16, respectively. The assignment of the α-hydroxyl group at C-3 was performed by comparing its spectral data with literature values [25–28]. The chemical shifts of C-3 (δ 73.99) and C-4 (δ 52.27) further confirmed the axial configuration of the 3-hydroxyl group by comparing with the corresponding data of the 3β-hydroxy-lup-20(29)-en-23,28-dioic acid [δ values for 84.4 (C-3) and 43.0 (C-4)] [29]. Additionally, the carbon chemical shifts of C-23 (δ 178.16) and C-28 (δ 177.84) were also similar to those of 3β-hydroxy-lup-20(29)-en-23,28-dioic acid [δ values for 178.7 (C-23) and 177.3 (C-28)] suggesting that the two carboxyl groups were at C-23 and C-28 [29]. In heteronuclear multiple bond connectivity (HMBC) spectrum, the H-1 proton signal at δ 3.84 (1H, dd, J = 8.16, 3.96 Hz) correlated with carbons C-2 (δ 37.36), C-9 (δ 52.98), C-10 (δ 44.33), and C-25 (δ 13.24); protons H-2 at δ 1.82 (1H, m) and H-25 at δ 0.97 (3H, s) correlated with carbon C-1 (δ 75.46); the H-3 proton signal at δ 3.82 (1H, t, J = 2.40 Hz) correlated with carbons C-5 (δ 45.25) and C-23 (δ 178.16); protons H-2 at δ 1.82 (1H, m) and H-24 at δ 1.17 (3H, s) correlated with carbon C-3 (δ 73.99); protons H-5 at δ 1.95 (1H, m) and H-24 at δ 1.17 (3H, s) correlated with carbon C-23 (δ 178.16); protons H-16 at δ 1.48 (1H, m), H-18

at δ 1.64 (1H, m), and H-22 at δ 1.92 (1H, m) correlated with carbon C-28 (δ 177.84); the correlations from H-29 at δ 4.58 (1H, m, Ha) and δ 4.72 (1H, d, *J* = 1.68 Hz, Hb) to C-19 (δ 48.24), C-20 (δ 151.89), and C-30 (δ 19.72); the correlations from H-30 at δ 1.71 (3H, s) to C-19 (δ 48.24), C-20 (δ 151.89), and C-29 (δ 110.26). These pieces of evidence confirmed that the double bond was at C-20/C-29 and the other hydroxyl group was at C-1 (Figure 2 and Table S1). The nuclear Overhauser enhancement spectroscopy (NOESY) spectrum of **1** showed the correlations between the proton of H-1 and H-2$_{eq}$, H-1 and H-5, H-1, and H-9, H-1 and H-11$_{eq}$, suggesting that H-1 was axial in orientation, which, in turn, suggested that the hydroxyl group at C-1 was β-positioned. Similarly, cross-peaks between H-24 and H-25, as well as H-3, H-3 and H-2$_{eq}$ as well as H-2$_{ax}$, which indicated that the methyl group (H-24) was axial and H-3 was equatorial, which, in turn, suggested that the hydroxyl group at C-3 and carboxyl group at C-4 were α-positioned. Furthermore, cross-peaks between H-19 and H-13 confirmed that the isopropenyl group at C-19 was α-positioned (Figure 3). Based on the above spectral data, the structure of **1** was determined as 1β,3α-dihydroxy-lup-20(29)-en-23,28-dioic acid, a new compound named acangraciligenin S.
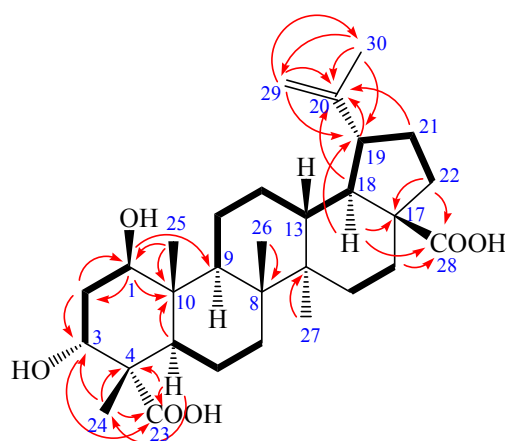

Figure 2. ^{1}H-^{1}H COSY (bold) and selected HMBC (arrow) correlations of **1**.
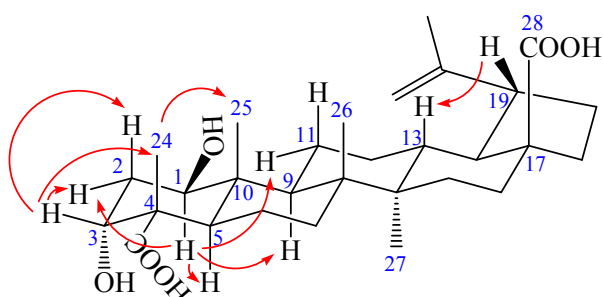

Figure 3. The key NOESY (arrow) correlations of **1**.

Compound **2** was obtained as a white amorphous powder, m.p. 230.5 °C, and gave positive responses in Liebermann–Burchard and Molish tests. Its molecular formula of $C_{48}H_{76}O_{20}$ was determined on the basis of ESI-MS at *m/z* = 972.5 [M]$^+$ and HR-ESI-MS at *m/z* = 971.4865 [M − H]$^-$ (negative) (calcd. for $C_{48}H_{75}O_{20}$: 971.4857), indicating eleven degrees of unsaturation. The ^{1}H-NMR spectrum of **2** (in methanol-d_4) showed the following signals: five tertiary methyl groups at δ 0.95, 0.98, 1.03, 1.09 and 1.70 (each 3H, s); one secondary methyl group at δ 1.25 (3H, d, *J* = 4.96 Hz), assigned to H-6″ of the rhamnose; and two olefinic protons at δ 4.58 (1H, brs) and 4.72 (1H, brs); two hydroxy-substituted methines at δ 3.67 (1H, m) and 3.80 (1H, m). In addition, three anomeric protons were at δ 4.37 (1H, d, *J* = 6.28 Hz), 4.84 (1H, overlapped), and 5.45 (1H, d, *J* = 6.56 Hz), suggesting the appearance of three sugar units. The ^{13}C-NMR and DEPT spectra revealed 48 carbon

signals, of which, 30 signals were assigned to a triterpenoid sapogenol moiety and 18 signals belong to three monosaccharide moieties. The aglycone of **2** was recognized to be lupane triterpene type by ^{1}H- and ^{13}C-NMR analysis (see Table 1), with the typical olefinic carbons at δ 151.77 (C-20) and 110.41 (C-29), five quaternary methyl carbons at δ 13.17, 15.10, 17.14, 18.08, and 19.49, two oxymethine carbons at δ 74.14 and 76.33, and two carboxyl signals at δ 176.40 and 182.60, respectively. Compared with the ^{1}H- and ^{13}C-NMR data of the aglycone of **2** and compound **1**, the high similarity indicated that the aglycone of **2** was the same as that of compound **1**. Assignments of the β-hydroxyl group at C-1 (δ 76.33), α-hydroxyl group at C-3 (δ 74.14), and two carboxyl groups at C-23 (δ 182.60) and C-28 (δ 176.40) were performed by comparing its spectral data with compound **1** [δ values for 75.46 (C-1), 73.99 (C-3), 178.16 (C-23) and 177.84 (C-28)] and literature values [28]. In HMBC spectrum, the H-1 proton signal at δ 3.80 (1H, m) correlated with carbons C-2 (δ 36.79), C-3 (δ 74.14), C-9 (δ 53.06), C-10 (δ 44.47), and C-25 (δ 13.17); protons H-2 at δ 1.72 (1H, m), H-3 at δ 3.67 (1H, m), H-5 at δ 1.87 (1H, m), and H-25 at δ 0.95 (3H, s) correlated with carbon C-1 (δ 76.33); the H-3 proton signal at δ 3.67 (1H, m) correlated with carbons C-1 (δ 76.33) and C-5 (δ 46.11); protons H-2 at δ 1.80 (1H, m) and H-24 at δ 1.09 (3H, s) correlated with carbon C-3 (δ 74.14); protons H-5 at δ 1.87 (1H, m) and H-24 at δ 1.09 (3H, s) correlated with carbon C-23 (δ 182.60); protons H-16 at δ 1.44 (1H, m), H-18 at δ 1.65 (1H, m), and H-22 at δ 1.94 (1H, m) correlated with carbon C-28 (δ 176.40); the correlations from H-29 at δ 4.58 (1H, brs, Ha) and δ 4.72 (1H, brs, Hb) to C-19 (δ 48.36), C-20 (δ 151.77), and C-30 (δ 19.49); the correlations from H-30 at δ 1.70 (3H, s) to C-19 (δ 48.36), C-20 (δ 151.77), and C-29 (δ 110.41). These pieces of evidences further confirmed that the double bond was at C-20/C-29 and the two hydroxyl groups were at C-1 and C-3, respectively (Figure 4 and Table S2).
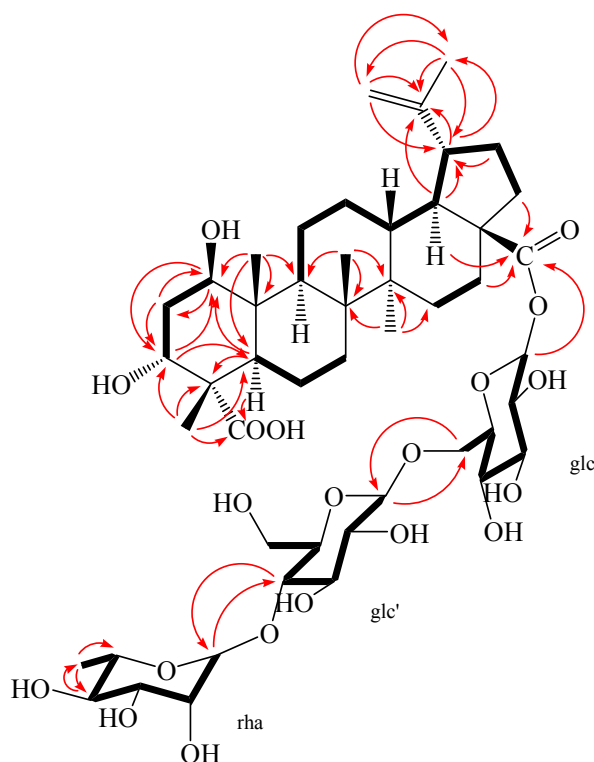

Figure 4. ^{1}H-^{1}H COSY (bold) and selected HMBC (arrow) correlations of **2**.

The NOESY spectrum of **2** showed the correlations between the proton of H-1 and H-2$_{eq}$, H-1 and H-5, H-1, and H-9, suggesting that H-1 had an *axial* orientation, which, in turn, suggested that the hydroxyl group at C-1 was β-positioned. Similarly, cross-peaks between H-24 and H-25, as well as H-3, H-3 and H-2$_{eq}$, indicated that the methyl group (H-24) was axial and H-3 was equatorial, which, in turn, suggested that the hydroxyl group at C-3 and carboxyl group at C-4 were α-positioned (Figure 5).

Alkaline hydrolysis of **2** with 5% KOH in MeOH gave a sapogenol (**2a**), a colorless needle, mp 282.5 °C, together with a mixture of sugars. The sugar mixture was identified to be composed of D-glucose and L-rhamnose by thin-layer chromatography (TLC) with their authentic sample. The ^{1}H and ^{13}C NMR data of **2a** (see Experimental Section) were the same as those of **1**, which further confirmed that this aglycone (**2a**) was 1β,3α-dihydroxy-lup-20(29)-en-23,28-dioic acid. Moreover, the HMBC correlations between the inner glc H-1 (δ 5.45) and C-28 of the aglycone (δ 176.40), between outer glc H-1′ (δ 4.37) and inner glc C-6 (δ 69.55), between rha H-1″ (δ 4.84) and glc C-4′ (δ 79.51) were observed (Figure 4). In addition, the NOESY correlations between the inner glc H-1 and glc H-5, between outer glc H-1′ and glc H-3′ as well as glc H-5′, between rha H-1″ and rha H-2″, rha H-5″ and rha H-3″, as well as rha H-6″, rha H-4″ and rha H-6″, which further confirmed the inner glucose, outer glucose, and rhamnose were *β*-D, *β*-D, and *α*-L positioned, respectively (Figure 5). These results suggest the sequence of sugar linkages of **2**. The carbon signals of the sugar moiety were superimposable on those of characteristic triterpene glycosides isolated from *Acanthopanax* species [4–7,25–28]. Consequently, the structure of **2** was determined as 1β,3α-dihydroxy-lup-20(29)-en-23,28-dioic acid 28-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester, a new compound named acangraciliside S.
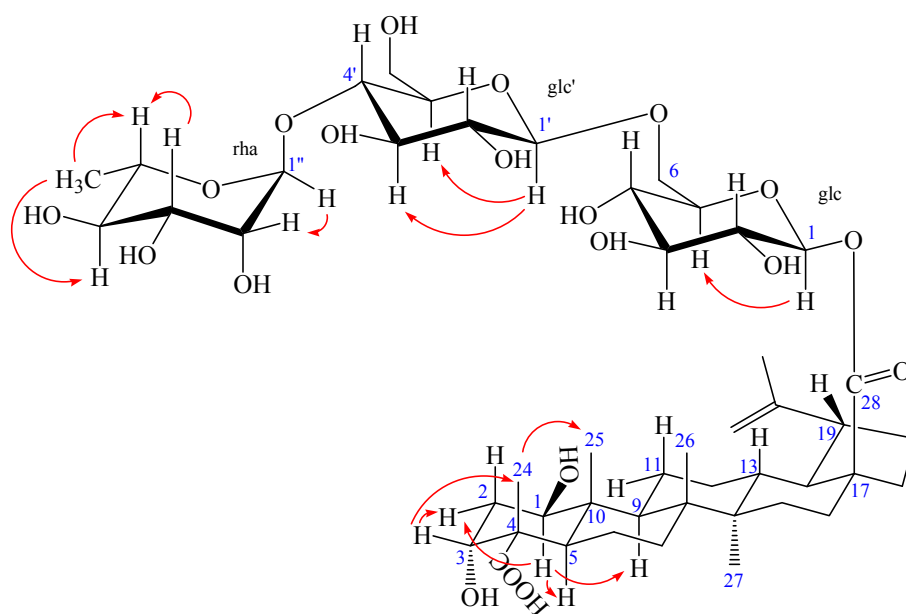

Figure 5. The key NOESY (arrow) correlations of **2**.

Table 1. NMR spectral data of compounds **1**–**2**.

Position	1 δ$_C$ [a,c]	δ$_H$ [a,d] [mult. (*J* in Hz)]	2 δ$_C$ [b,c]	δ$_H$ [b,d] [mult. (*J* in Hz)]
Aglycone				
1	75.46	3.84 (1H, dd, 8.16, 3.96)	76.33	3.80 (1H, m)
2	37.36	1.82 (1H, m); 1.87 (1H, m)	36.79	1.72 (1H, m); 1.80 (1H, m)
3	73.99	3.82 (1H, t, 2.40)	74.14	3.67 (1H, m)
4	52.27	-	52.24	-
5	45.25	1.95 (1H, m)	46.11	1.87 (1H, m)
6	22.09	1.35 (1H, m); 1.57 (1H, m)	22.17	1.26 (1H, m); 1.58 (1H, m)
7	35.19	1.31 (1H, m); 1.56 (1H, m)	35.09	1.30 (1H, m); 1.55 (1H, m)
8	42.76	-	42.96	-
9	52.98	1.72 (1H, dd, 10.08, 2.68)	53.06	1.72 (1H, m)
10	44.33	-	44.47	-
11	24.68	1.32 (1H, m); 2.43 (1H, brd, 9.80)	24.82	1.36 (1H, m); 2.28 (1H, m)

Table 1. *Cont.*

Position	1 δ_C [a,c]	δ_H [a,d] [mult. (*J* in Hz)]	2 δ_C [b,c]	δ_H [b,d] [mult. (*J* in Hz)]
12	26.87	1.12 (1H, dd, 10.52, 3.76); 1.68 (1H, m)	26.94	1.12 (1H, m); 1.68 (1H, m)
13	39.14	2.34 (1H, td, 10.32, 3.0)	39.13	2.27 (1H, m)
14	43.72	-	43.82	-
15	30.75	1.20 (1H, m); 1.46 (1H, m)	30.86	1.15 (1H, m); 1.54 (1H, m)
16	33.15	1.48 (1H, m); 2.25 (1H, dt, 9.84, 2.8)	32.95	1.44 (1H, m); 2.33 (1H, m)
17	56.98	-	57.93	-
18	50.30	1.64 (1H, m)	50.60	1.65 (1H, m)
19	48.24	3.05 (1H, td, 8.76, 4.0)	48.36	3.00 (1H, m)
20	151.89	-	151.77	-
21	31.61	1.34 (1H, m); 1.90 (1H, m)	31.55	1.37 (1H, m); 1.94 (1H, m)
22	37.84	1.48 (1H, m); 1.92 (1H, m)	37.68	1.48 (1H, m); 1.94 (1H, m)
23	178.16	-	182.6	-
24	17.92	1.17 (3H, s)	18.08	1.09 (3H, s)
25	13.24	0.97 (3H, s)	13.17	0.95 (3H, s)
26	17.31	0.99 (3H, s)	17.14	0.98 (3H, s)
27	15.40	1.07 (3H, s)	15.10	1.03 (3H, s)
28	177.84	-	176.40	-
29	110.26	4.58 (1H, m); 4.72 (1H, d, 1.68)	110.41	4.58 (1H, brs); 4.72 (1H, brs)
30	19.72	1.71 (3H, s)	19.49	1.70 (3H, s)
C-28 O-glc				
1			95.26	5.45 (1H, d, 6.56)
2			74.00	3.33 (1H, m)
3			78.28	3.42 (1H, m)
4			70.95	3.43 (1H, m)
5			78.06	3.54 (1H, m)
6			69.55	3.81 (1H, m); 4.11 (1H, dd, 9.48, 1.36)
glc′(1→6)glc				
1′			104.56	4.37 (1H, d, 6.28)
2′			75.32	3.23 (1H, m)
3′			76.71	3.45 (1H, m)
4′			79.51	3.53 (1H, m)
5′			76.89	3.30 (1H, m)
6′			61.90	3.65 (1H, m); 3.80 (1H, m)
rha(1→4)glc′				
1″			102.92	4.84 (1H, overlapped)
2″			72.44	3.81 (1H, m)
3″			72.16	3.62 (1H, m)
4″			73.75	3.38 (1H, m)
5″			70.64	3.95 (1H, m)
6″			17.84	1.25 (3H, d, 4.96)

Note: Assignments were performed by HMQC, HMBC, ^{1}H-^{1}H COSY, and NOESY experiments; Glc: D-glucopyranosyl; Rha: L-rhamnopyranosyl; [a] Measured in CD$_3$COCD$_3$; [b] Measured in CD$_3$OD; [c] 100 MHz; [d] 400 MHz.

Compounds **3** and **4** were identified as 3α,11α-dihydroxy-lup-20(29)-en-23,28-dioic acid [30] and acankoreoside C [31], respectively, by comparing their NMR and mass spectral data with the literature values. (The ^{1}H- and ^{13}C-NMR data of **3** and **4** see Supplementary Materials: Table S3)

Moreover, the cytotoxicity and inhibition of production of nitric oxide (NO) of selected isolates (**1** and **3**) from *A. gracilistylus* were investigated on LPS-stimulated BV2 microglias. Compounds **2** and **4** were excluded from biological evaluation because of sample shortage. As shown in Table 2, the production of NO was down-regulated moderately when their concentration was 80 μM with the inhibition as 38.6% and 40.4%, respectively.

Table 2. Inhibitory effects of compounds **1** and **3** against LPS-Induced NO production in murine microglia BV2 cells [a].

Compounds	Inhibition	Cell Viability
1	38.6% in 80 μM	86.44%
3	40.4% in 80 μM	83.90%

[a] Data is presented as the mean of three experiments ± SD.

3. Experimental Section

3.1. General Experimental Procedures

Melting points (uncorrected) were measured using a Boetius micromelting point apparatus (Beijing Jingjing Science and Technology Co., Ltd., Beijing, China). ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) and 2D-NMR were recorded at room temperature in CD_3COCD_3 or CD_3OD using Bruker DRX-400 NMR spectrometer (Bruker Biospin, Zurich, Switzerland) and chemical shifts were presented in δ (ppm) values relative to tetramethylsilane (TMS) as internal standard. HR-ESI-MS was performed on an API QSTAR spectrometer (Agilent Technologies, Santa Clara, CA, USA). The high-speed countercurrent chromatography (HSCCC) instrument was performed in the present study using a model an TBE-300A HSCCC (Shanghai Tauto Biotech Co., Ltd., Shanghai, China). Semi-preparative HPLC was performed on an LC-20A liquid chromatograph (Shimadzu Technologies, Kyoto, Japan). Column chromatography was carried out on silica gel (200–300 mesh and 100–200 mesh, Qingdao Marine Chemical Co., Ltd., Qingdao, China), and Sephadex LH-20 (Merck, Darmstadt, Germany). RP-TLC was measured on a precoated RP-18F$_{254s}$ (Merck, Darmstadt, Germany) plates. TLC was conducted on self-made silica gel G (Qingdao Marine Chemical Industry, Qingdao, China) plates and spots were visualized by spraying with 10% H_2SO_4 in ethanol (*v/v*) followed by heating at 105 °C. The standard D-glucose and L-rhamnose were purchased from Beijing North Carolina Souren Biotechnology Research Institute (Beijing, China).

3.2. Collection and Identification of Biological Materials

The leaves of *A. gracilistylus* were collected from its natural habitat in Yuanling, Hunan Province, China, in July 2015 and identified by X.Q. Liu, the corresponding author of this work. A voucher specimen has been deposited in the Herbarium of Hunan University of Chinese Medicine, Hunan, China (no. 201507).

3.3. Extraction and Isolation

The dried, powdered leaves of *A. gracilistylus* (600 g) were extracted with methanol under reflux three times (3 × 3 L). The solvent was then removed under reduced pressure to yield a residue (120 g), which was suspended in distilled water and successively partitioned with petroleum ether (PE, 60–90), EtOAc, and *n*-BuOH, respectively. The EtOAc extract (28 g) was subjected to column chromatography (CC) on silica gel eluting with a gradient of $CHCl_3$-MeOH (35:1 to 10:1, *v/v*) to obtain the discolored fraction (E1). E1 was partitioned by HSCCC using two-phase solvent system composing of n-hexane-ethyl acetate-methanol-water (1:2:1.6:1, *v/v/v/v*) with the flow rate of mobile phase of 2.0 mL/min, rotation rate of 800 r.p.m, to generate five fractions (F1–F5). Compounds **1** (5.0 mg) and **3** (6.0 mg) were yielded from F2 (25 mg) by using Pre-HPLC with CH_3CN-H_2O (40:60, *v/v*) for further purification. Similarly, the *n*-BuOH extract (25 g) was partitioned by HSCCC using two-phase solvent system composing of ethyl acetate-butanol-methanol-water (3:0.3:0.8:4, *v/v/v/v*) with the flow rate of mobile phase of 2.0 mL/min, rotation rate of 900 r.p.m, to obtain five fractions (F6–F10). F6 (35 mg) was further chromatographed on Pre-HPLC with CH_3CN-H_2O (25:75, *v/v*) to produce two compounds **2** (11.3 mg) and **4** (5.3 mg).

Acangraciligenin S (**1**) was obtained as a colorless needle: mp. 282.6 °C; ^{1}H- and ^{13}C-NMR data: (Table 1); (−)-HR-ESI-MS *m/z* 501.3221 [M − H]$^-$ (calcd. for $C_{30}H_{45}O_6$: 501.3222).

Acangraciliside S (**2**) was obtained as a white amorphous powder: mp. 230.5 °C; ^{1}H- and ^{13}C-NMR data: (Table 1); (−)-HR-ESI-MS *m/z* 971.4865 [M − H]$^-$ (calcd. for $C_{48}H_{75}O_{20}$: 971.4857).

3.4. Alkaline Hydrolysis of **2**

Compound **2** (10 mg) was hydrolyzed with 8 mL of 5% KOH in MeOH for 2 h at 80 °C. The reaction mixture was neutralized with 2 M HCl in H_2O and extracted with EtOAc. The aqueous layer was filtered, concentrated and chromatographed on TLC plate in which D-glucose and L-rhamnose were detected by comparing with standard samples [4]. The EtOAc layer was evaporated *in vacuo* and the residue was performed on a preparative HPLC column by using elution with CH_3CN-H_2O (40:60, *v/v*) to obtain sapogenol fraction **2a** (2 mg).

Compound **2a**: Colorless needles, m.p. 282.5 °C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ: 3.84 (1H, dd, *J* = 8.16, 3.96 Hz, H-1), 3.82 (1H, t, *J* = 2.40 Hz, H-3), 1.95 (1H, m, H-5), 1.72 (1H, m, H-9), 2.34 (1H, td, *J* = 10.32, 3.0 Hz, H-13), 1.64 (1H, m, H-18), 3.05 (1H, td, *J* = 8.76, 4.0 Hz, H-19), 1.17 (3H, s, H-24), 0.97 (3H, s, H-25), 0.99 (3H, s, H-26), 1.08 (3H, s, H-27), 4.59 (1H, m, H-29a), 4.73 (1H, d, *J* = 1.68 Hz, H-29b), 1.71 (3H, s, H-30); ^{13}C-NMR (100 MHz, CD_3COCD_3) δ: 75.45 (C-1), 37.35 (C-2), 73.98 (C-3), 52.26 (C-4), 45.23 (C-5), 22.08 (C-6), 35.18 (C-7), 42.75 (C-8), 52.97 (C-9), 44.32 (C-10), 24.66 (C-11), 26.85 (C-12), 39.13 (C-13), 43.71 (C-14), 30.73 (C-15), 33.14 (C-16), 56.96 (C-17), 50.29 (C-18), 48.22 (C-19), 151.88 (C-20), 31.59 (C-21), 37.82 (C-22), 178.14 (C-23), 17.90 (C-24), 13.23 (C-25), 17.29 (C-26), 15.38 (C-27), 177.82 (C-28), 110.25 (C-29), 19.70 (C-30).

3.5. MTT Assay for Cell Viability

BV2 microglias were maintained at 5×10^5 cells/mL in Dulbecco minimum essential medium (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 mg/L), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was determined by adding 100 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to 1 mL of a cell suspension (1×10^5 cells/mL in 96-well plates) and incubated for 30 min. The formazan formed was dissolved in acidic 2-propanol, and the optical density was measured at 540 nm.

3.6. Nitrite Assay

The concentration of nitric oxide (NO) in the conditioned media was determined by a method based on the Griess reaction [32]. An aliquot of each supernatant (100 mL) was mixed with the same volume of Griess reagent (0.1% (*w/v*) *N*-(1-naphathyl)-ethylenediamine and 1% (*w/v*) sulfanilamide in 5% (*v/v*) phosphoric acid) for 10 min at room temperature. The absorbance of the final product was measured spectrophotometrically at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. The nitrite concentration in the samples was determined from a standard curve of sodium nitrite prepared in phenol red-free DMEM.

3.7. Statistical Analysis

All values are expressed as the mean ± S.D. Differences between mean values of normally-distributed data were assessed with one-way analysis of variance (ANOVA) (Newman Keuls *t*-test). Statistical analysis was performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was accepted at *p* < 0.05.

4. Conclusions

In summary, in this work, a phytochemical study on the leaves of *A. gracilistylus* (Araliaceae) by using HSCCC-HPLC combinatorial chromatography method resulted in the discovery of four

lupane-triterpenoids, including two new compounds, acangraciligenin S (**1**) and acangraciliside S (**2**), as well as two known species, 3α,11α-dihydroxy-lup-20(29)-en-23,28-dioic acid (**3**) and acankoreoside C (**4**). Two selected compounds were also evaluated for their inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine microglia BV2 cells. Compounds **1** and **3** showed moderate abilities to inhibit NO production and had no influence on cell viability, as determined by the MTT method.

Supplementary Materials: The following ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, ESI-MS, and HR-ESI-MS spectra are available as supporting data. Supplementary materials are available online.

Author Contributions: Xiang-Qian Liu designed the experiments and revised the paper; Xiao-Jun Li and Qin-Peng Zou performed the experiments, analyzed the data, and wrote the paper; Xiang Wang, Mao-Fang Lu, and Sung-Kwon Ko help to analyze data of experiment; Kwan-Woo Kim and Youn-Chul Kim contributed to bioassay reagents, materials and analyzed the data; and Chang-Soo Yook revised the paper. All authors read and approved the final manuscript.

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Sample Availability: Samples of the compounds are available from the authors.