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Chemical constituents from *Ainsliaea acerifolia* as potential anti-obesity agents

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ABSTRACT

Bioassay-guided isolation of an ethanolic extract of the aerial parts from *Ainsliaea acerifolia* using pancreatic lipase inhibitory assay led to the isolation of a new guaianolide-type sesquiterpene lactone (**1**), a new dicaffeoylquinic acid derivative (**4**), as well as eight known secondary metabolites. Structures of the two new compounds were elucidated on the basis of spectroscopic methods. All isolated compounds were evaluated for their inhibitory effects against pancreatic lipase, and compound **2** exhibited significant inhibitory activity with an IC_{50} value of 15.3 ± 0.7 μM. Furthermore, compound **2** also exhibited potent inhibitory effects against 3T3-L1 adipocyte cells.

1. Introduction

Obesity results from disequilibrium between energy intake and expenditure and is widely considered as a major health problem. Obesity is closely associated with lifestyle-related disorders such as hypertension, hyperlipidemia, arteriosclerosis, osteoarthritis, type II diabetes, and coronary heart disease (Cooke and Bloom, 2006). Recent therapeutic approaches to obesity can be classified on the basis of their distinct mechanisms, which include inhibition of lipases, suppression of energy intake, stimulation of energy expenditure, inhibition of adipocyte differentiation, and control of lipid metabolism (Yun, 2010). Advanced strategies for the treatment of obesity involve abrogation of dietary triglyceride absorption via inhibition of pancreatic lipase, which is commonly recognized as a key enzyme in triglyceride absorption (Birari and Bhutani, 2007). This enzyme is secreted from the pancreas and hydrolyzes triglycerides into glycerol and fatty acids (Lowe, 1994). Thus, pancreatic lipase inhibitors are considered to be valuable therapeutic targets for the treatment of diet-induced obesity. As an example, orlistat is clinically effective for the treatment of obesity by inhibiting activity of gastrointestinal lipase and reducing absorption of dietary fat (Fernstrom and Fernstrom, 2002; Krempf et al., 2003). Obesity is also characterized at the cellular level by increases in the number and size of adipocytes differentiated from adipose tissues (Furuyashiki et al., 2004). Therefore, regulation and accumulation of lipid absorption mediated by disturbance of pancreatic lipase and adipocyte differentiation are suggested to be important factors in the development of an anti-obesity agent.

Ainsliaea acerifolia is a perennial herb belonging to the Compositae family, which is distributed in the mountains of South Korea. This bitter mountainous vegetable has long been used for the treatment of rheumatic arthritis and enteritis in traditional folk medicine (Choi et al., 2006). The major secondary metabolites from aerial parts of *A. acerifolia*, known as quinic acid derivatives, have been reported to possess several biological efficacies, including antioxidative, anti-diabetic, anti-viral, anti-thrombotic, hepatoprotective, and neuroprotective activities (Park, 2010). In addition, previous limited phytochemical investigation into *A. acerifolia* revealed the presence of sesquiterpene lactones and lignans (Choi et al., 2006; Miyase and Fukushima, 1984). As part of an ongoing effort to discover naturally occurring anti-obesity agents from medicinal plants, a porcine pancreatic lipase assay was carried out using an initial screening procedure. An EtOAc-soluble portion of *A. acerifolia* extract exhibited significant inhibitory

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activity, with an IC_{50} value of 75.1 ± 5.2 μg/mL. Activity-guided chromatographic separation of this EtOAc extract using pancreatic lipase assay led to the isolation and identification of a new guaianolide-type sesquiterpene (**1**) and dicaffeoyl quinic acid derivative (**4**), together with eight known compounds. The structures of two new compounds, ainsliaside C (**1**) and methyl 3,5-di-*O*-caffeoyl-*epi*-quinate (**4**), were established by spectroscopic data interpretation. The known compounds were identified as ainsliaside A (**2**) (Miyase and Fukushima, 1984), zaluzanin C (**3**) (Ando et al., 1989), 3,5-di-*O*-caffeoyl-*epi*-quinic acid (**5**) (Kim and Lee, 2005), 4,5-di-*O*-caffeoyl quinic acid (**6**) (Tatefuji et al., 1996), methyl 4,5-di-*O*-caffeoyl quinate (**7**) (Basnet et al., 1996), 3,4-di-*O*-caffeoyl quinic acid (**8**) (Timmermann and Hoffmann, 1983), chlorogenic acid (**9**) (Merfort, 1992), and caffeic acid (**10**) (Cui et al., 1990) (Fig. 1) based on spectroscopic analysis and comparison of the data with literature values. In addition, the inhibitory effects of these isolated compounds on adipocyte differentiation were evaluated using 3T3-L1 cells.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. HRESIMS analysis of compound **1** gave a sodiated molecular ion peak at *m/z* 577.2093 $[M+Na]^+$ (calcd for $C_{30}H_{34}O_{10}Na$), consistent with a molecular formula of $C_{30}H_{34}O_{10}$. The ^1H NMR spectrum of **1** (Table 1) exhibited characteristic resonances for two methine protons of a γ-lactone moiety at δ_H 4.26 (1H, t, *J* = 9.3 Hz, H-6) and 2.89 (1H, m, H-7), an oxygenated methine proton at δ_H 4.63 (1H, m, H-3), six exomethylene protons at δ_H 6.09 (1H, d, *J* = 3.0 Hz, H-13a), 5.56 (1H, d, *J* = 3.0 Hz, H-13b), 5.43 (1H, br s, H-15a), 5.35 (1H, br s, H-15b), 5.01 (1H, s, H-14a), and 4.91 (1H, s, H-14b), and two aliphatic methines at δ_H 2.99 (1H, dd, *J* = 16.8, 8.4 Hz, H-1) and 2.78 (1H, dd, *J* = 9.6, 9.6 Hz, H-5), indicating the presence of a guaiane-type sesquiterpene lactone moiety (Zedro et al., 1991) in **1**. The presence of guainolide skeleton was evidenced by ^{13}C NMR resonances corresponding to those of zaluzanin C (Ando et al., 1989) and further supported by ^1H–^1H COSY correlations of H-2/H-3, –1, H-5/H-6, –1, H-7/H-8, –6, and H-8/H-9, –7. In addition to diagnostic aliphatic signals, the ^1H NMR spectrum of **1** also showed resonances corresponding to A_2B_2 aromatic protons at δ_H 7.37 (2H, d, *J* = 7.8 Hz, H-3″, 5″) and 7.56 (2H, d, *J* = 7.8 Hz, H-2″, 6″), two *trans*-olefinic protons at δ_H 7.58 (1H, d, *J* = 15.6 Hz, H-7″) and 6.48 (1H, d, *J* = 15.6 Hz, H-8″), and characteristic anomeric protons at δ_H 4.45 (1H, d, *J* = 7.2 Hz, H-1′), indicating the presence of a β-glucose and a *trans*-*p*-coumaroyl moieties. Consistent with these ^1H NMR observations, the ^{13}C NMR and HSQC spectra of **1** closely resembled those of compound **2**, ainsliaside A (Miyase and Fukushima, 1984), except for the presence of an aromatic ring system with different substitution patterns in **1**. The linkage point of the aliphatic zaluzanin C and *trans*-*p*-coumaroyl residues on the sugar moiety in **1** was determined unambiguously from the key HMBC spectrum, which showed H-1′/C-3 and H-2′/C-9″ correlations (Fig. 2). The large coupling constants between the three methine protons ($J_{5,6}$ = 9.6 Hz, $J_{6,7}$ = 9.3 Hz) as well as the ROESY correlations between H-7/H-5, 8α suggest anti-diaxial relationships among the respective protons (Li et al., 2011; Michalska et al., 2013). The ROESY correlations between H-3 and H-5, –2α, –1 also indicated an α-orientation for the oxymethine proton at the C-3 position

Table 1
^1H and ^{13}C NMR shifts of compound **1**.[a]

Position	1		Position	1	
	δ_H (J in Hz)[b]	δ_C, mult.		δ_H (J in Hz)[b]	δ_C, mult.
1	2.99 (dd, 16.8, 8.4)	46.2	15a	5.43 (br s)	113.6
2α	2.33 (m)	38.6	15b	5.35 (br s)	
2β	1.96 (m)		1′	4.45 (d, 7.2)	103.1
3	4.63 (m)	81.3	2′	3.87 (m)	78.2
4	–	150.8	3′	3.37 (t, 8.4)	75.3
5	2.78 (dd, 9.6, 9.6)	51.8	4′	3.28 (m)	71.8
6	4.26 (dd, 9.6, 9.3)	85.2	5′	3.26 (m)	77.9
7	2.89 (m)	46.4	6′a	3.86 (dd, 12.0, 2.4)	62.8
8α	2.26 (m)	31.6	6′b	3.68 (dd, 12.0, 5.4)	
8β	1.45 (m)		1″	–	130.9
9α	2.18 (m)	34.4	2″	7.56 (d, 7.8)	129.0
9β	2.50 (m)		3″	7.37 (d, 7.8)	130.0
10	–	150.0	4″	–	150.1
11	–	142.1	5″	7.37 (d, 7.8)	130.0
12	–	172.3	6″	7.56 (d, 7.8)	129.0
13a	6.09 (d, 3.0)	120.4	7″	7.58 (d, 15.6)	144.6
13b	5.56 (d, 3.0)		8″	6.48 (d, 15.6)	121.7
14a	5.01 (s)	114.7	9″	–	172.3
14b	4.91 (s)				

[a] ^1H NMR measured at 600 MHz, ^{13}C NMR measured at 150 MHz; obtained in CD_3OD with TMS as internal standard. Assignments based on HMQC and HMBC NMR spectra.
[b] *J* values (Hz) are given in parentheses.

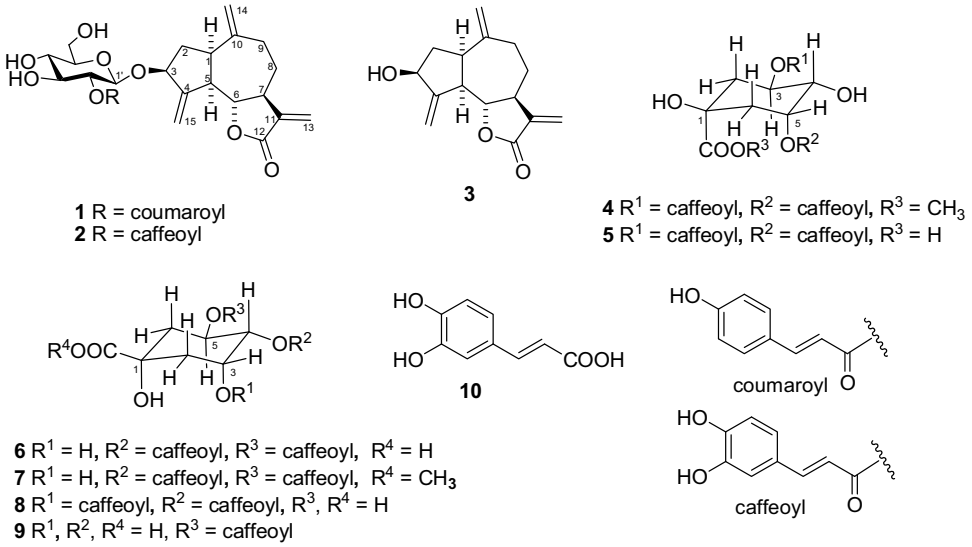

Fig. 1. Structures of isolated compounds **1**–**10**.

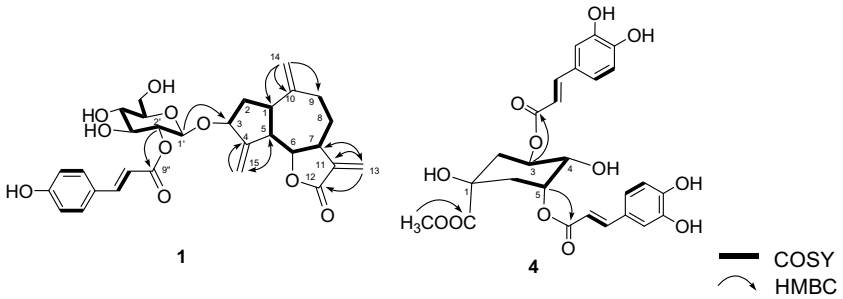

Fig. 2. ^{1}H–^{1}H COSY and key HMBC correlations of **1** and **4**.

(Wang et al., 2009) (Fig. 2). Therefore, the relative structure of compound **1** was assigned as ainsliaside C, which was previously unknown in the literature.

Compound **4** was obtained as a yellow amorphous powder, $[\alpha]^{20}_{D}$ −120.0° (MeOH). HRFABMS analysis showed a pseudomolecular ion peak at *m/z* 531.1506 $[M+H]^{+}$, corresponding to the molecular formula $C_{26}H_{27}O_{12}$. The UV spectrum of **4** exhibited maximum absorptions at 330 and 296 nm, which are characteristic of chlorogenic acid derivatives (Wu et al., 2012). The presence of a caffeoylquinic acid nucleus was further demonstrated by the ^{1}H NMR spectrum of **4** for three oxygenated methine protons at δ_H 5.43 (1H, m, H-3), 5.39 (1H, m, H-5), and 3.96 (1H, dd, *J* = 9.0, 3.0 Hz, H-4), two methylenes at δ_H 2.31 (1H, dd, *J* = 14.4, 3.6 Hz, H-6_{eq}), 2.23 (1H, dd, *J* = 13.6, 3.0 Hz, H-2_{eq}), 2.11 (1H, dd, *J* = 13.6, 9.0 Hz, H-2_{ax}), and 2.06 (1H, m, H-6_{ax}), which are coupled to each other in the ^{1}H–^{1}H COSY spectrum, and a methoxyl group at δ_H 3.69 (3H, s, OCH_3-7). In addition to diagnostic aliphatic signals, the spectrum also included characteristic *trans*-caffeoyl group signals attributable to two pairs of ABX-type aromatic protons at δ_H 7.07 (1H, d, *J* = 2.0 Hz, H-2′), 7.06 (1H, d, *J* = 2.0 Hz, H-2″), 6.97 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 6.96 (1H, dd, *J* = 8.4, 2.0 Hz, H-6″), 6.78 (1H, d, *J* = 8.4 Hz, H-5′), and 6.77 (1H, d, *J* = 8.4 Hz, H-5″) and four *trans*-oriented olefinic protons at δ_H 7.61, 7.54 (each 1H, d, *J* = 15.6 Hz, H-7′, −7″) and 6.34, 6.21 (each 1H, d, *J* = 15.6 Hz, H-8′, −8″). The linkage points of two caffeoyl residues and a methoxyl group on the quinic acid moiety in **4** were determined unambiguously from the key HMBC correlations between H-3/C-9′, H-5/C-9″, and OCH_3/C-7 (Fig. 2). Most of the NMR signals in **4** were nearly identical to those of the 3,5-di-*O*-caffeoyl-quinic acid methyl ester (Lee et al., 2010), except for slightly different chemical shifts for H-2_{eq} and H-4_{eq} in the quinic acid moiety.

The ROESY correlations among OCH_3-7/H-2_{eq}, −6_{eq}, H-3/H-2_{eq}, and H-5/H-4, −6_{ax} clearly indicated an α-configuration for the C-7 carbonyl group on the cyclohexane ring (Fig. 3). The absolute configuration of the quinic acid moiety in **4** was determined as (−)-epiquinic acid based on negative specific rotation values as well as a negative first Cotton effect at 289 nm and positive second Cotton effect at 341 nm in the circular dichroism (CD) spectral comparison with authentic analogues (Kim and Lee, 2005). Thus, the new structure of methyl-3,5-di-*O*-caffeoyl-*epi*-quinate (**4**) was assigned.

All pure isolates in the present systemic investigation (Kim et al., 2011; Eom et al., 2013; Park et al., 2013) were evaluated for their anti-obesity properties against pancreatic lipase (Kim et al., 2007) using orlistat as a positive control (Table 2). Three guaianolide-type sesquiterpenes **1–3** exhibited higher activities, showing IC_{50} values ranging from 15.3 ± 0.7 to 47.8 ± 1.4 μM, with ainsliaside A (**2**) as the most potent secondary metabolite. When pancreatic lipase inhibitory activities were compared among structurally related guaianolide-type sesquiterpenes **1**–**3**, it was found that the presence of a phenylpropanoid moiety along with more aromatic hydroxyl groups linked to the glucose residue may affect the activity of this type of compound. Caffeoylquinic acid analogues **4** and **7** containing a C-7 carboxyl group replaced by a methoxyl group were found to be more effective compared with parent compounds **5** and **6**. Futhermore, compound **4** showed a higher inhibitory activity against pancreatic lipase than its C-1 epimer **7**, implying that the C-1 conformation of the quinic acid moiety may influence this effect. The five active isolates (**1**–**4**, **7**) against pancreatic lipase were tested for their inhibitory activities against preadipocyte differentiation of 3T3-L1 cells, which was evaluated by measuring fat accumulation with Oil Red O staining

Table 2
Pancreatic lipase inhibitory effects of compounds **1**–**10**.

Compound	IC_{50} value (μM)[a]	Compound	IC_{50} value (μM)[a]
1	36.5 ± 1.5	**7**	135.7 ± 1.7
2	15.3 ± 0.7	**8**	>200
3	47.8 ± 1.4	**9**	>200
4	127.1 ± 1.5	**10**	>200
5	>200	Orlistat[b]	0.6 ± 0.2
6	>200		

[a] All compounds were examined in triplicate experiments.
[b] Used as positive control.

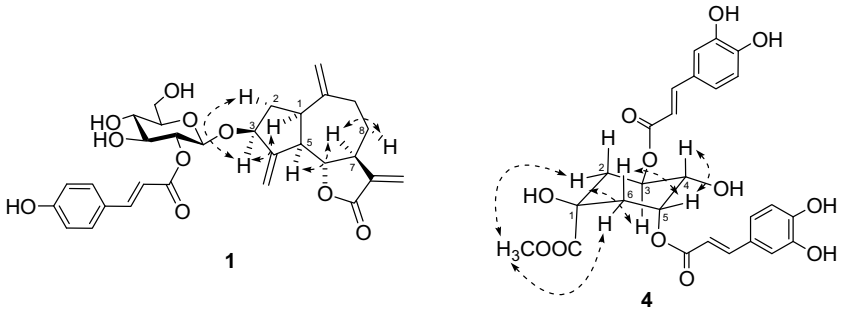

Fig. 3. Selected ROESY correlations observed for compounds **1** and **4**.

(Liu et al., 2011). Non-toxic concentrations of the tested compounds were established in 3T3-L1 preadipocytes at concentrations of 100, 50, 25, and 10 μM. After 2 days of incubation, cell viability was measured by MTT assay, and no cytotoxic effect was observed for compounds **1**–**3** up to 50 μM and for compounds **4** and **7** up to 100 μM. As shown in Fig. 4, compared to the non-treated control, the guaianolide-type sesquiterpene glucoside **2** showed the most potent inhibitory effect against preadipocyte differentiation with an IC_{50} value of 32.7 μM. However, structurally related zaluzanin C derivatives **1** and **3** were much less potent than **2** against 3T3-L1 cells. The tested quinic acid methyl esters **4** and **7** showed relatively weak inhibitory effects against 3T3-L1 preadipocytes differentiation. Previous studies have documented that dihydroxyl and trihydroxyl groups containing phenolic acids strongly inhibit pancreatic lipase and lipid accumulation, whereas other close analogues of these compounds do not (Imai et al., 2015). This finding is consistent with our observations, which indicated that the number of aromatic hydroxyl groups in the molecules is correlated with an increase in biological activity. For more accurate estimation of the structure-activity relationship, further studies should investigate the inhibitory effects of other guaianolide-type sesquiterpenes and quinic acid derivatives with more structural diversity against pancreatic lipase and preadipocyte differentiation.

Guaianolide-type sesquiterpenes are a large and structurally diverse group of plant secondary metabolites (Heinrich et al., 1998) with significant biological properties, including anti-malarial, anti-inflammatory, and anti-tumor effects (Drew et al., 2009). Recent research has also reported that guaianolide-type sesquiterpenes from *Artemisia douglasiana* are major inhibitors of 3T3-L1 adipocyte differentiation (Galvis et al., 2011). In this investigation, two new compounds, ainsliaside C (**1**) and methyl 3,5-di-*O*-caffeoyl-*epi*-quinate (**4**), as well as eight previously known compounds were isolated from this biomass. Their structures were established on the basis of spectroscopic data interpretation. Among the tested compounds, compound **2** exhibited more potent anti-adipogenic activity with much lower IC_{50} values against both pancreatic lipase and 3T3-L1 adipocytes. The results of this investigation indicate that compound **2** might be a potential anti-obesity agent of *A. acerifolia* as well as a possible anti-adipogenic agent.

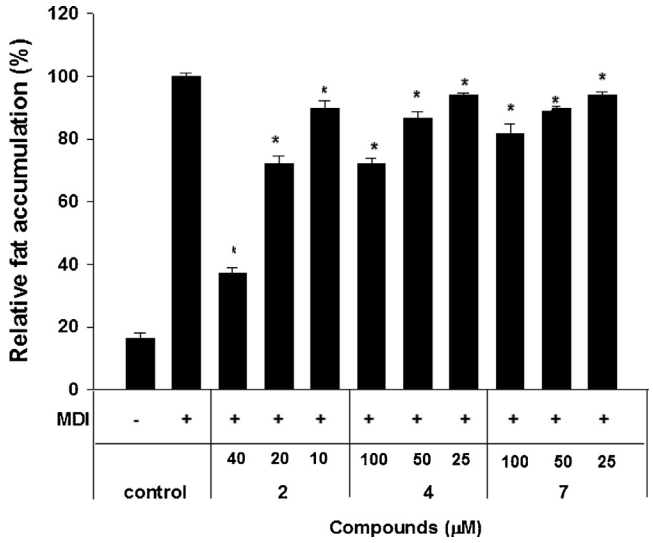

Fig. 4. Effect of compounds **2**,**4**, and **7** on fat accumulation in 3T3-L1 preadipocytes. Results are expressed as mean ± SD of three independent experiments, each performed using triplicate wells. *$p < 0.01$ compared with control and N.D. mean not detected.

3. Experimental

3.1. General experimental procedures

UV spectra were obtained using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan), and CD spectra were run on a JASCO J-720W spectrometer (JASCO, Tokyo, Japan). IR spectra were obtained with a Nicolet Nexus 670 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Varian VNS600 instrument (Varian, Palo Alto, CA, USA) operated at 600 and 150 MHz, respectively. Chemical shifts are given in δ (ppm) values relative to those of the solvent CD_3OD (δ_H 3.35; δ_C 49.0) on a tetramethylsilane (TMS) scale. The standard pulse sequences programmed into the instruments were used for each 2D measurement. The J_{CH} value was set at 8 Hz in the HMBC spectra. HRESIMS and HRFABMS were obtained on a Micro Mass Auto Spec OA-TOF spectrometer (Micromass, Manchester, UK). Column chromatography was performed using Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and YMC GEL ODS AQ 120-50S (YMC Co., Kyoto, Japan). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates (0.25 mm layer thickness, Merck, Darmstadt, Germany), and spots were detected by UV irradiation (254, 365 nm) as well as spraying with 10% H_2SO_4 reagent.

3.2. Plant materials

A whole plant sample of *A. acerifolia* was collected at Yeongyang-gun, Gyeongsangbuk-do, Korea, in May 2013. A voucher specimen representing this collection was identified by Prof. Youn-Moon Park and deposited at the Natural Product Chemistry Laboratory of Daegu University.

3.3. Extraction and isolation

Fresh *A. acerifolia* (5.0 kg) plants were ground and extracted exhaustively using 70% EtOH (25 L × 3) at room temperature, and the solvent was evaporated *in vacuo*. This concentrated extract (203.3 g) was suspended in 10% MeOH (4.0 L) and then partitioned in turn with *n*-hexane (4 L × 3), EtOAc (4 L × 3), and *n*-BuOH (4 L × 3) to yield dried *n*-hexane- (73.8 g), EtOAc- (56.0 g), *n*-BuOH- (28.0 g), and H_2O-soluble (186.0 g) residues. In the pancreatic lipase inhibition assay, EtOAc-soluble extract was found to be most active, with an IC_{50} value of 75.1 ± 5.2 μg/mL. Regarding chromatographic separation performance, fractions were monitored by reversed-phase HPLC. A portion (20.0 g) of the EtOAc extract was chromatographed over a Toyopearl HW-40 column (coarse grade; 2.8 cm i.d. × 51 cm) with H_2O containing increasing amounts of MeOH in stepwise gradient mode and fractioned into five subfractions MP01—MP05, respectively. Bioactive subfraction MP02 was subjected to column chromatography over a YMC GEL ODS AQ 120-50S column (1.1 cm i.d. × 40 cm) with aqueous MeOH to yield pure compound **3** (188.4 mg, t_R 8.4 min). In a similar fashion, the active subfraction MP03 was chromatographed over a YMC GEL ODS AQ 120-50S column (1.1 cm i.d. × 40 cm) with aqueous MeOH to yield pure compounds **1** (3.4 mg, t_R 12.9 min), **2** (8.6 mg, t_R 15.4 min), **4** (13.1 mg, t_R 6.5 min), **5** (41.5 mg, t_R 5.0 min), **6** (35.0 mg, t_R 4.3 min), **7** (27.3 mg, t_R 8.2 min), **8** (85.5 mg, t_R 5.6 min), **9** (2.3 mg, t_R 2.5 min), and **10** (24.2 mg, t_R 8.9 min). HPLC analysis was performed on a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm; YMC Co., Kyoto, Japan), and the solvent system consisted of a linear gradient that started with 20% (v/v)

MeCN in 0.1% HCOOH/H_2O (detection: UV 254 nm; flow rate: 1.0 mL/min; temperature: 40 °C), increased to MeCN over 20 min.

3.4. New compound information

Ainsliaside C (**1**): white amorphous powder. $[\alpha]^{20}_D$ +23.0° (*c* 0.1, MeOH); UV λ max MeOH nm (log ε): 207 (4.25), 246 (sh 4.05), 301 (sh, 4.11); IR ν_{max} (film) 3400, 1746, 1705, 1512, 1435, 1255, 1151 cm^{-1}; ^1H and ^{13}C NMR data shown in Table 1. HRESIMS *m/z* 577.2093 $[M+Na]^+$ (calcd for $C_{30}H_{34}O_{10}Na$, 577.2050).

Methyl 3,5-di-*O*-caffeoyl-*epi*-quinate (**4**): yellow amorphous powder. $[\alpha]^{20}_D$ −120.0° (*c* 0.2, MeOH); UV λ_{max} MeOH nm (log ε): 296 (4.07), 330 (4.27); IR ν_{max} (film) 3410, 1685, 1605, 1520, 1160, 976, 810 cm^{-1}; CD (MeOH) λ_{max} (Δε): 289 (+9.8), 341 (−19.5); HRFABMS *m/z* 531.1506 $[M+H]^+$ (calcd for $C_{26}H_{27}O_{12}$, 531.1503); ^1H NMR (CD_3OD, 600 MHz) δ 7.61, 7.54 (each 1H, d, *J* = 15.6 Hz, H-7′, −7″), 7.07 (1H, d, *J* = 2.0 Hz, H-2′), 7.06 (1H, d, *J* = 2.0 Hz, H-2″), 6.97 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 6.96 (1H, dd, *J* = 8.4, 2.0 Hz, H-6″), 6.78 (1H, d, *J* = 8.4 Hz, H-5′), 6.77 (1H, d, *J* = 8.4 Hz, H-5″), 6.34, 6.21 (each 1H, d, *J* = 15.6 Hz, H-8′, −8″), 5.43 (1H, m, H-3), 5.39 (1H, m, H-5), 3.96 (1H, dd, *J* = 9.0, 3.0 Hz, H-4), 3.69 (3H, s, OCH_3-7), 2.31 (1H, dd, *J* = 14.4, 3.6 Hz, H-6_{ax}), 2.23 (1H, dd, *J* = 13.6, 3.0 Hz, H-2_{eq}), 2.11 (1H, dd, *J* = 13.6, 9.0 Hz, H-2_{ax}), 2.06 (1H, m, H-6_{eq}); ^{13}C NMR (CD_3OD, 150 MHz) δ 180.1 (C-7), 168.0 (C-9′, −9″), 148.1 (C-4″), 147.9 (C-4′), 145.5 (C-3″), 145.4 (C-3′), 145.3 (C-7′, −7″), 126.7 (C-1″), 126.5 (C-1′), 121.5 (C-6′, −6″), 111.5 (C-5′, −5″), 114.7 (C-8″), 114.2 (C-8′), 113.8 (C-2′, −2″), 74.9 (C-1), 73.1 (C-5), 71.8 (C-4), 71.1 (C-3), 52.9 (C-7 MeO), 39.5 (C-2), 36.3 (C-6).

3.5. Biological assays

3.5.1. Assay of pancreatic lipase activity

The ability of the compounds to inhibit porcine pancreatic lipase was evaluated using the previously reported method with minor modification (Kim et al., 2007). Briefly, an enzyme buffer was prepared by addition of 6 μL (10 units) of a solution of porcine pancreatic lipase (Sigma, St. Louis, MO) in 10 mM MOPS (morpholinepropanesulphonic acid) and 1 mM EDTA (pH 6.8) to 169 μL of Tris buffer (100 mM Tris-HC1 and 5 mM $CaCl_2$, pH 7.0). Then, 20 μL of each compound at the test concentration or orlistat (Roche, Basel, Switzerland) was mixed with 175 μL of the enzyme-buffer, followed by incubation for 15 min at 37 °C, with 5 μL of the substrate solution (10 mM of *p*-nitrophenylbutyrate in dimethyl formamide) added and the enzymatic reactions allowed to proceed for 15 min at 37 °C. Pancreatic lipase activity was determined by measuring hydrolysis of *p*-nitrophenylbutyrate to *p*-nitrophenol at 405 nm using an ELISA reader (Infinite F200; Tecan Austria GmBH, Grödig, Austria). Inhibition of lipase activity was expressed as the percentage decrease in optical density (OD) when porcine pancreatic lipase was incubated with the test compounds.

3.5.2. Assay of adipocyte differentiation in 3T3-L1Cells

3T3-L1 mouse embryo fibroblasts were cultured in DMEM supplemented with 10% FBS until confluence. Two days after confluence (day 0), cell differentiation was stimulated using differentiation medium containing DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μM insulin, and 1 μM dexamethasone for 2 days (day 2). Cells were then maintained in DMEM supplemented with 10% FBS and 2 μM insulin for another 6 days (day 8), followed by culturing with DMEM containing 10% FBS for an additional 4 days (day 8). All media contained 100 IU/mL of penicillin and 100 μg/mL of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Cultures were treated with test samples for the whole culture period (days 0–8) for the general experiment (Liu et al., 2011). Lipid droplets in cells were stained with Oil Red O. Eight days after differentiation induction, cells were washed two times with PBS and then fixed with 10% formalin at room temperature for 1 h. After fixation, cells were washed once with 60% isopropyl alcohol and stained with freshly diluted Oil Red O solution (three parts 0.6% Oil Red O in isopropyl alcohol and two parts water) for 1 h. Cells were then washed twice with water and visualized. For quantitative analysis, Oil Red O stain was dissolved with isopropyl alcohol, and optical density was measured at 500 nm by an ELISA plate reader.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.04.005.

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