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Antiviral activities of compounds from aerial parts of *Salvia plebeia* R. Br

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ABSTRACT

Ethnopharmacological relevance: *Salvia plebeia* R. Br. is an edible plant widely spread in many countries. It has been used as a traditional medicine to treat common cold, flu, cough, hepatitis, hemorrhoids, etc. The purpose of the study is to explicate antiviral compounds responsible for its traditional use for the common cold or flu.

Materials and methods: The methanolic extract of the aerial parts of *S. plebeia* was extracted with $CHCl_3$, EtOAc, and *n*-BuOH, successively. The EtOAc and $CHCl_3$ fractions were subjected to a successive of chromatographic method, which led to the isolation of fourteen compounds. Inhibition activities of the isolated compounds were evaluated against influenza A (H1N1) neuraminidase.

Results: Chemical investigation of the methanolic extracts of *S. plebeia* resulted in the isolation of two novel benzoylated monoterpene glycosides, named as plebeiosides A (**1**) and B (**2**), together with twelve known compounds including four flavonoids (**4**–**5**, **7**, **10**), two sesquiterpenoids (**8**, **12**), four phenolics (**9**–**10**, **13**–**14**), a steroid (**6**), and a triterpenoid (**3**). Their chemical structures were elucidated based on spectroscopic data and absolute stereochemistries of **1** and **2** were determined by comparison of optical rotations of their hydrolysates with literature values. Compounds **5**, **7**, **9**, and **11** exhibited potent enzymatic inhibition against H1N1 neuraminidase (IC_{50} values ranging from 11.18 ± 1.73 to 19.83 ± 2.28 μM). Furthermore, two flavonoids (**5** and **7**) and one rosmarinic acid methyl ester (**9**) reduced cytopathic effects of the H1N1 virus during replication.

Conclusions: The antiviral activities of the flavonoids and phenolics isolated from the extracts of *S. plebeia* supported the traditional application of this medicine on common cold or flu. In this study, benzoylated monoterpene glycosides were first found to exist in this species. Moreover, the present study suggested potential of three compounds (**5**, **7**, and **9**) to be new lead structures for the development of new neuraminidase inhibitors in the future.

1. Introduction

The genus *Salvia* includes more than 900 species in the family Lamiaceae. Among this genus, *Salvia plebeia* R. Br. has been an edible plant widely spread throughout Australia, India, China, Japan, and Korea. It has been used as a traditional medicine to treat inflammatory diseases like hepatitis, common cold, flu, cough, hemorrhoid, etc (Lu and Foo, 2002). Previous phytochemical investigation resulted in the identification of some flavonoids (Gu and Weng, 2001; Gupta et al., 1975; Weng and Wang, 2000), diterpenoids (Garcia-Alvarez et al., 1986), lignans (Powell and Plattner, 1976; Plattner and Powell, 1978), caffeic acid derivatives (Lu and Foo, 2002), and sesquiterpenoids (Cao et al., 2013), which showed diverse biological activities such as antioxidant (Gu and Weng, 2001; Gupta et al., 1975), hepatoprotective (Jin et al., 2011), cytotoxic, and antimicrobial activities (Shin et al., 2001). However, to our knowledge, there has been no report on antiviral activity of *S. plebeia*.

Influenza virus (*Orthomyxoviridae*) causes annul or occasional epidemics all over the world, sometimes causing death of a number of people (Hien et al., 2004). Main strategy to cope with influenza pandemics is based on the use of antiviral agents, where neuraminidase (NA) inhibitors are key factor (Grienke et al., 2010). NA (also called sialidase) of the influenza A virus, a glycoside hydrolase enzyme, plays an important part in the hydrolysis of silaic

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acid residue from the virions of the infected host and their motility within the respiratory tract (Air and Laver, 1989; Bucher and Palese, 1975). Up to date, four NA inhibitors, which are oseltamivir, peramivir, zanamivir, and laninamivir have been approved for therapeutic and prophylactic usage in the world. Nevertheless, there have been many reports on viral resistance to these agents, their low bioavailability, or their side effects (Bantia et al., 2006; Ryan et al., 1995). For these reasons, more investigation is needed to develop novel antiviral agents derived from natural products (De Clercq, 2006). *S. plebeia* has been used to treat the common cold or flu in Korea for a long time, which might be potentially caused by its antiviral activity. It prompted us to do phytochemical investigation to figure out molecules responsible for the antiviral activity. In this study, we report on isolation of compounds from the aerial parts of *S. plebeia*, structural elucidation, and their inhibitory activities against influenza A virus (H1N1).

2. Materials and methods

2.1. General experimental procedures

Optical rotation was measured using JASCO P-2000 polarimeter, FT-IR spectra were measured on Agilent Cary 630 FTIR, and UV spectra were recorded using OPTIZEN UV–Vis spectrophotometer. NMR spectra were taken in DMSO-d_6, CD_3OD, and pyridine-d_5, and chemical shifts were referenced relative to the corresponding signals (δ_H 2.5/ δ_C 39.51 for DMSO-d_6; δ_H 3.31/ δ_C 49.15 for CD_3OD; δ_H 8.74/ δ_C 150.35 for pyridine-d_5) and measured on a Varian VNS 600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz). The high-resolution electrospray ionization mass spectra (HRESIMS) were obtained using a Bruker UHR ESI Q-TOF mass spectrometer. All HPLC were performed using Agilent series 1260 HPLC system. Open column chromatography was carried out over a silica gel 60 (70–230 mesh, Merck, Germany), a LiChroprep RP-18 gel (40–63 μm, Merck, Germany), and a Sephadex LH-20 gel (GE Healthecare, Sweden).

2.2. Plant material

The dried aerial parts of *S. plebeia* were purchased from the Yangyeongsi, Daegu, Korea, in April 2014. The aerial parts of this plant were obtained by removing root parts from the whole plants after drying them at room temperature for a week and identified by the author (S. H. Shim). A voucher specimen (NPC-15-03) was deposited at the College of Pharmacy, Duksung Women's University.

2.3. Extraction, extract fractionation and isolation of compounds

The aerial parts of *S. plebeia* (2.45 kg) were extracted with MeOH under reflux to give an extract (470 g). The methanolic extracts were fractionated into $CHCl_3$-soluble (73.0 g), EtOAc-soluble (38.0 g), and *n*-BuOH-soluble (182.0 g) parts, respectively depending on the polarity. The $CHCl_3$-soluble layer was separated by vacuum liquid column chromatography (VLC) over silica gel (SiO_2) eluted with *n*-Hexane and EtOAc gradient solvents to get seven fractions (Fr. 1-Fr. 7) as shown in Fig. 1. Fr. 5 was subjected to open column chromatography over SiO_2 eluted with *n*-Hexane and Acetone gradient solvents to afford seven sub-fractions (Fr. 5-1–Fr. 5-7). Compound **3** (40.0 mg) was crystallized from Fr. 5-1 under MeOH. The EtOAc-soluble parts were separated by VLC over SiO_2 using *n*-Hexane and EtOAc gradient solvents to provide seven fractions (Fr. E-1–Fr. E-7). Fr. E-3 (12.0 g) was further subjected to silica gel CC with the elution of $CHCl_3$and Acetone gradient solvents, to give fifteen sub-fractions (Fr. E-3-1–Fr. E-3-15). Compounds **5** (44.7 mg) and **6** (20.0 mg) were recrystallized under mixtures of $CHCl_3$ and MeOH from Fr. E-3-4 and Fr. E-3-10, respectively. Fr. E-3-3 (254 mg) was subjected to column chromatography over C18 eluted with Acetone: MeOH:H_2O gradient solvents, yielding compounds **10** (11.3 mg) and **12** (8.0 mg). Fr. E-3-8 (130 mg) was applied to reversed-phase semi-preparative HPLC to

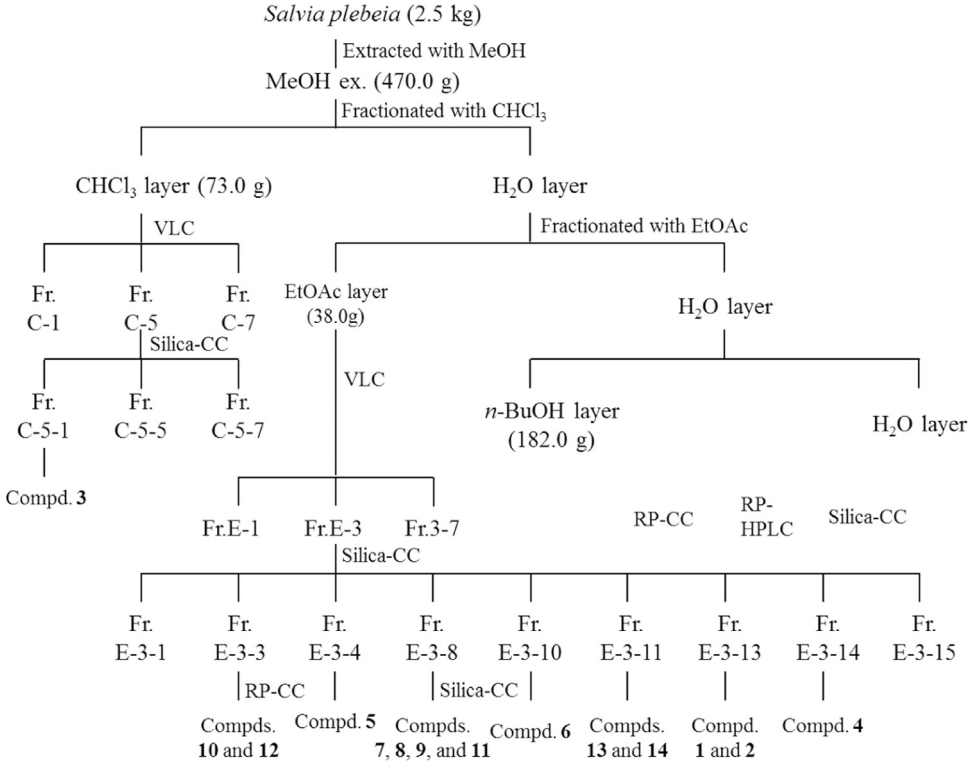

Fig. 1. Isolation scheme of the compounds from *S. plebeia*.

Table 1
NMR Data for plebeiosides A and B (**1**[a] and **2**[b]).

No.	Compound **1**		Compound **2**	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	4.26 (1H, d, 7.8)	104.8	4.23 (1H, d, 7.8)	105.1
2	3.25 (1H, t, 7.9, 8.9)	75.3	3.22 (1H, m)	75.4
3	3.37 (1H, m)	72.5	3.38 (1H, m)	78.3
4	3.41 (1H, m)	77.9	3.38 (1H, m)	72.2
5	3.60 (1H, m)	75.5	3.55 (1H, m)	75.6
6a	4.47 (1H, dd, 7.2, 12)	65.0	4.61 (1H, dd, 2.2, 12)	65.0
6b	4.59 (1H, dd, 2.5, 12)		4.40 (1H, dd, 6.6, 12)	
1′a	3.34 (1H, d, 12)	72.8		51.4
1′b	4.04 (1H, d, 9.9)			
2′		82.2	3.89 (1H, d, 9.6)	77.9
3′a	2.11 (1H, d, 3.0)	48.4	2.26 (1H, m)	39.0
3′b			0.91 (1H, dd, 3.3, 13)	
4′	1.31 (1H, m)	24.6	1.90 (1H, t, 4.4)	43.6
5′a	1.31 (1H, m)	24.2	1.22 (1H, m)	29.2
5′b			1.68 (1H, m)	
6′a	1.65 (1H, d, 2.2)	51.2	1.95 (1H, m)	27.6
6′b			1.20 (1H, m)	
7′		44.9		53.7
8′a	1.05 (1H, d, 9.8)	35.4	1.01 (3H, s)	15.8
8′b	2.05 (1H, d, 9.8)			
9′a	0.97 (3H, s)	25.9	3.89 (1H, d, 9.6)	73.7
9′b			3.25 (1H, d, 9.6)	
10′	0.75 (3H, s)	21.9	0.84 (3H, s)	14.5
1″		168.0		168.2
2″		122.3		122.4
3″	7.90 (1H, d, 8.8)	133.0	7.89 (1H, d, 8.8)	133.0
4″	6.82 (1H, d, 8.8)	116.3	6.83 (1H, d, 8.8)	116.4
5″		163.8		163.7
6″	6.82 (1H, d, 8.8)	116.3	6.83 (1H, d, 8.8)	116.4
7″	7.90 (1H, d, 8.8)	133.0	7.89 (1H, d, 8.8)	133.0

[a] Data were recorded in CD_3OD at 500 MHz (^1H, COSY) and 125 MHz (^{13}C).
[b] Data were recorded in CD_3OD at 600 MHz (^1H, COSY) and 150 MHz (^{13}C).

yield compounds **7** (3.4 mg), **8** (11.8 mg), **9** (12.0 mg), and **11** (6.3 mg). Compounds **13** (6.2 mg) and **14** (2.6 mg) were isolated from Fr. E-3-11 (240 mg) by reversed-phase column chromatography. Fr. E-3-13 (60 mg) was subjected to reversed-phase HPLC with H_2O: MeOH gradient to yield compounds **1** (3.8 mg) and **2** (1.7 mg). Fr. E-3-14 (980 mg) was chromatographed over silica gel with $CHCl_3$:MeOH gradient to yield compound **4** (12 mg).

2.4. Physical and spectroscopic data of compounds

Plebeioside A (**1**): yellow solid; $[\alpha]_D^{25}$ +42.5 (*c* 1.00×10^{-3} g/mL, MeOH); IR λ_{max} 3336, 2945, 2832, 1448, 1113, 1018 cm^{-1}; UV (MeOH) λ_{max} (log ε) 257 (3.80) nm; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD), see Table 1; HMBC correlations (CD_3OD, H-# → C-#) H-1→C-4 and C-1′; H-2→C-1; H-4→C-3 and C-5; H_2-6→C-5 and C-1″; H_2-1′→C-1, C-2′ and C-4′; H_2-4′ and H_2-5′→C-2′, C-8′ and C-10′; H_2-8′→C-1′, C-2′, C-3′ and C-7′; H_3-9′→C-10, C-2′, C-6′ and C-7′; H_3-10′→C-2′, C-6′, C-7′ and C-9′; H-6″→C-2″, C-4″ and C-5″; H-7″→C-1″, C-3″ and C-5″; NOESY correlations (CD_3OD, H-# ↔ H-#) H-5 ↔ H_2-6a; H_2-1′a ↔ H-10′; H_2-1′b ↔ H_2-1′a; H-3 ↔ H_2-8′b; H_2-8′b ↔ H_2-8′a and H-9′; H-10′ ↔ H-5, H_2-8′a and H-9′; HRESIMS obsd *m/z* 475.1938 $[M+Na]^+$, calcd for $C_{23}H_{32}NaO_9$, 475.1939.

Plebeioside B (**2**): yellow solid; $[\alpha]_D^{25}$ +35.6 (*c* 1.00×10^{-3} g/mL, MeOH); IR λ_{max} 3336, 2942, 2831, 1447, 1113, 1020 cm^{-1}; UV (MeOH) λ_{max} (log ε) 258 (4.03) nm; ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1; HMBC correlations (CD_3OD, H-#→C-#) H-1→C-9′; H-2→C-3; H-4→C-3; H_2-6 C-1″; H-2′→C-8′; H_2-3′→C-2′ and C-7′; H-4′→C-1′, C-2′ and C-6′; H_2-5′ → C-3′ and C-7′; H_2-6′→C-2′; H_3-8′→C-1′, C-4′, C-7′ and C-9′; H_2-9′→C-1, C-4′, C-7′ and C-8′; H_3-10′→C-1′, C-2′, C-6′ and C-7′; H-3″→C-1″ and C-5″; H-6″→C-2″; NOESY correlations (CD_3OD, H-# ↔ H-#) H-1 ↔ H-3, H-5 and H_2-9′b; H_2-6a ↔ H-5 and H_2-6b; H_2-3′a ↔ H-2′; H_2-5′a ↔ H_2-5′b and H_2-6′a; H_2-6′a ↔ H_2-6′b; H-8′ ↔ H_2-5′b, H_2-6′b and H-10′; H_2-9′a ↔ H-3′a and H_2-9′b; H-10′ ↔ H_2-9′b; HRESIMS obsd *m/z* 475.1937 $[M+Na]^+$, calcd for $C_{23}H_{32}NaO_9$, 475.1939.

2.5. Acid hydrolysis of the compounds

To elucidate stereochemistry through optical rotation values of the glycoside moieties, compound **1** was hydrolyzed by acid to separate the glycoside moieties from the monoterpene moiety. One milligram of the compound was hydrolyzed by heating in 1 N *aq*HCl at 80 °C for 2 h, which was followed by neutralization with barium carbonate and filtration. The filtrates were extracted with EtOAc (4 mL × 3 times) to remove aglycone moiety. The aqueous layers including glucose moieties were concentrated and dissolved in 3.5 mL of MeOH to measure their optical rotations.

Acid hydrolysate of **1**. $[\alpha]_D^{25}$ +5.6 (*c* 0.3×10^{-3} g/mL, MeOH).

2.6. Viruses and cells

The influenza strain H1N1 A/PR/8/34 virus was obtained from Choong Ang Vaccine Laboratory, Korea, and stored at −80 °C. The Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 atmosphere condition. The DMEM with 0.15 μg/mL trypsin and 5 μg/mL bovine serum albumin (BSA) was used as the infection medium for the MDCK cells.

2.7. Influenza A (H1N1) neuraminidase inhibition assay

The enzyme inhibition assay was performed as previously reported methods with a slight modification (Hung et al., 2009; Ha et al., 2016). In summary, dilute virus supernatant with activated influenza NA (neuraminidase) and isolated compounds were placed in 96-well plates for the inhibition assay. After incubation, dilute 4-MU-NANA was added to each well for initiation of the enzyme reaction. Addition of stop solution (25% ethanol, 0.1 M glycine, pH 10.7) terminated the reaction after 30 min. Fluorescence of the product (4-methylumbelliferone; 4-MU) was measured with excitation and emission at 360 and 440 nm, respectively, using a spectrophotometer (Spectrmax M2[e], Molecular Devices Corporation, Sunnyvale, CA, USA). And then IC_{50} was determined. The data were analyzed using Sigmaplot 11.0 (SPCC Inc., Chicago, IL, USA).

$$\text{NA inhibition activity}(\%) = \frac{100}{1 + (\frac{IC50}{[I]})}$$

[I]=concentration of inhibitor; IC_{50}=half-maximal inhibitory concentration (μM).

2.8. Cytopathic effect (CPE) inhibition assay

The CPE inhibition assay was performed as a previously reported method (Del Barrio et al., 2011). In summary, MDCK cells confluent monolayers were infected with Influenza virus in 96 well for 1 h, followed by being replenished with DMEM including trypsin and several compounds at different concentrations. After incubation, the cells were replaced with DMEM and MTT to each well and incubated in the dark. After removing the supernatant, formazan crystals were dissolved in dimethylsulfoxide (DMSO) for each well and then the absorbance was measured at 550 nm using a microplate reader (VersaMax™, Randor, PA, USA).

2.9. Cytotoxicity assay

The cell viability was evaluated using an MTT method. Briefly, MDCK cells were seeded on 96-well plates at 1×10^5 cells per well and incubated for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and replaced by new DMEM containing several compounds at different concentrations. After 48 h of incubation, 20 μL of the 2 mg/mL MTT solution was added to each well and re-incubated for 4 h in the dark. The supernatant were then removed and formazan crystals were dissolved in 100 μL DMSO. Absorbance was measured at wavelength 550 nm. All experiments were performed in triplicate. The regression analysis was applied to calculate the 50% cytotoxic concentration (CC_{50}) using Sigma Plot Statistical Analysis software.

2.10. Statistical analysis

The results are expressed as the means ± SD of three independent experiments. Statistical analysis of IC_{50}, EC_{50}, and CC_{50} was performed using Sigma Plot Statistical Analysis software. Statistical calculations of other data were examined by analysis of variance (ANOVA), followed by Tukey's range test, conducting in SPSS Statistics 20. Statistical significance was accepted at $p < 0.05$.

3. Results

Fourteen compounds including two new (**1** and **2**) and twelve known compounds (**3**–**14**) were isolated from the MeOH extracts of *S. plebeia* through successive chromatographic methods over various resins such as silica gel, Sephadex LH-20, and RP-18. Two new compounds were named plebeiosides A (**1**) and B (**2**), and twelve known compounds were identified to be ursolic acid (**3**) (Seebacher, 2003), 6-methoxynaringenin-7-*O*-β-D-glucoside (**4**) (Lee et al., 2010b), hispidulin (**5**) (Lee et al., 2010b), daucosterol (**6**) (Jung et al., 2008), nepetin (**7**) (Lee et al., 2010b), plebeiolide B (**8**) (Dai et al., 2014), rosmarinic acid methyl ester (**9**) (Jin et al., 2009), methyl *p*-hydroxyphenyllactate (**10**) (Uchida et al., 1996), luteolin (**11**) (Lee et al., 2010a), 1α-acetoxy-8α-hydroxyl-2-oxo-eudesman-3,7(11)-dien-8,12-olide (**12**) (Dai et al., 2014), protocatechuic acid (**13**) (Lee et al., 2010a), and citrusin C (**14**) (Teng et al., 2005), respectively (Fig. 2), by comparison of their spectral data with the reported values.

Molecular formula of compound **1** was deduced to be $C_{23}H_{32}O_9$ (eight unsaturations) on the basis of ^1H, ^{13}C, and HMQC data, which was verified by HRESIMS (observed at *m/z* 475.1938 $[M+Na]^+$, calculated for $C_{23}H_{32}NaO_9$, 475.1939). Twenty-seven out of the entire protons were bound to carbon based on HMQC results, suggesting that it has five exchangeable protons. Detailed analysis of ^1H, ^{13}C NMR (Table 1), and HMQC data exhibited the presence of two tertiary methyl groups at δ_H 0.75 (δ_C 21.86) and 0.97 (δ_C 25.91), three non-oxygenated methylene groups at δ_H 1.05 and 2.05 (δ_C 35.39), 1.31 (δ_C 24.18), and 1.31 (δ_C 24.60), two oxygenated methylene groups at δ_H 3.34 and 4.04 (δ_C 72.78) and 4.47 and 4.59 (δ_C 64.96), seven sp^3 methine groups at δ_H 1.65 (δ_C 51.22), 2.11 (δ_C 48.38), 3.25 (δ_C 75.30), 3.37 (δ_C 72.46), 3.41 (δ_C 77.89), 3.60 (δ_C 75.53) and 4.26 (δ_C 104.80), four aromatic methine groups at δ_H 6.82 (δ_C 116.29) and 7.90 (δ_C 133.00), one sp^3 quaternary carbon at δ_C 44.94, one oxygenated sp^3 quaternary carbon at δ_C 82.21, two sp^2 quaternary carbons at δ_C 122.34 and 163.75, and one carbonyl carbon at δ_C 168.01. Accordingly, this compound was presumed to have a bicyclic structure to fulfill the unsaturation requirement since it contained one benzoyl group and one hexose. Not only ^1H–^1H COSY correlations but also coupling constants implied the existence of a 1,4-disubstituted benzene moiety at δ_H 6.82 (2 H, d, J=8.8 Hz) and 7.90 (2 H, d, J=8.8 Hz). A glucose moiety was confirmed to be present by the chemical shift for an anomeric group (δ_C 104.80), four oxygenated sp^3 methine groups (δ_C 75.30, 72.46,

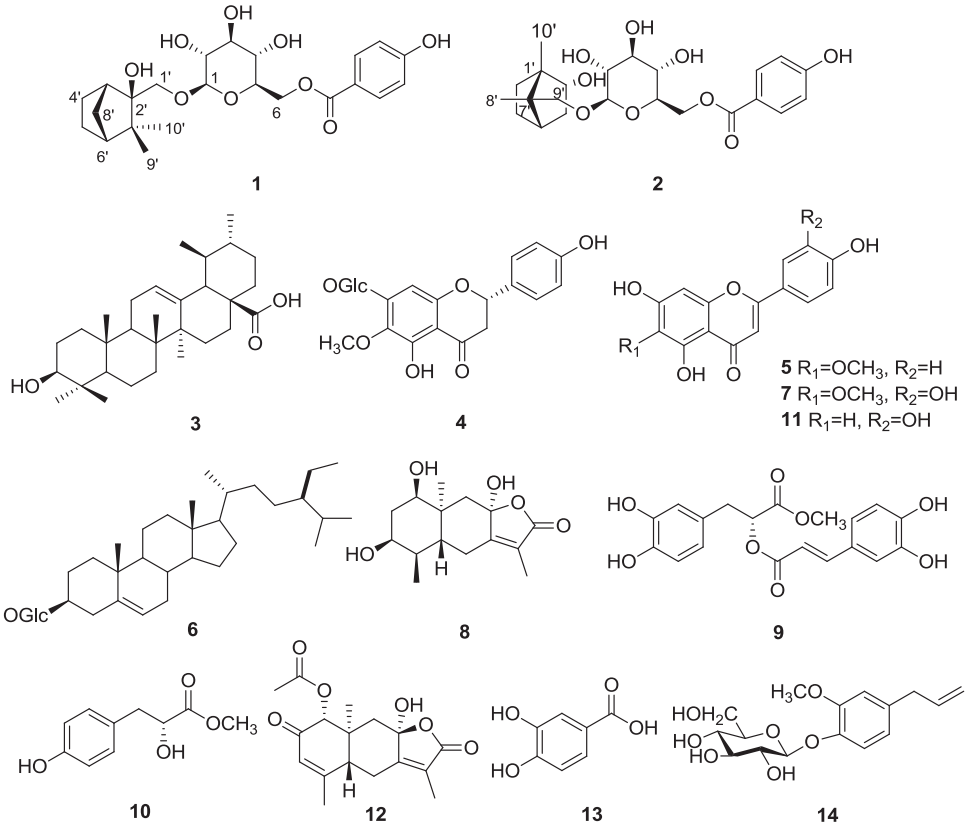

Fig. 2. Structures of the isolated compounds from *S. plebeia*.

77.89, 75.53), and an oxygenated methylene group (δ_C 64.96). The downfield-shifted proton resonances at δ_H 4.47 and 4.59 suggested acylation of C-6 in the glucose moiety. Besides the 1,4-disubstituted benzoyl group and the glucose moiety, ten carbons remained unexplained, suggesting that it possibly had a monoterpene skeleton. An isolated CH-CH_2-CH_2-CH-CH_2 spin-system corresponding to the C3′-C4′-C5′-C6′-C8′ unit was identified by the ^{1}H–^{1}H COSY spectrum of **1**, indicating the presence of a cyclopentane ring. The connection of the spin-system and attachment of functional groups were verified by HMBC correlation (Fig. 3(A)). The HMBC correlations of two tertiary methyl protons at δ_H 0.97 and 0.75 with a quaternary carbon at δ_C 44.94, an oxygen-bearing quaternary carbon at δ_C 82.21, and an sp^3 methine carbon at δ_C 51.22 indicated that the quaternary carbon (C-7′) bearing two tertiary methyl groups (C-9′ and C-10′) was positioned between the hydroxylated quaternary carbon (C-2′) and the sp^3 methine carbon (C-6′) of the cyclopentane ring. The HMBC correlation of H_2-4′ (δ_H 1.31) with C-2′ (δ_C 82.21) and C-8′ (δ_C 35.39) suggested that the cyclopentane ring corresponding to C3′-C4′-C5′-C6′-C8′ was fused with another cyclopentane ring corresponding to C2′-C7′-C6′-C8′-C3′, bearing C6′-C8′-C3′ linkage in common. In addition, attachment of the fused bicyclic monoterpene moiety to C-1 of glucose was evident from the HMBC correlations of H_2-1′ at δ_H 3.34, 4.04 with C-2′ at δ_C 82.21 and C-1 at δ_C 104.80. The hydroxyl group was predicted to be attached to C-5′ of the disubstituted benzene from fulfillment of the molecular formula, along with its downfield shifted ^{13}C resonance (δ_C 163.75). Finally, the HMBC correlation of H_2-6 at δ_H 4.47, 4.59 with C-1′ at δ_C 168.01 allowed the linkage of the *p*-hydroxybenzoyl group to the C-6 of the glucose. Thus, the planar structure of **1** was established.

The relative stereochemistry of **1** was elucidated based on coupling constants and NOESY data (Fig. 3(A)). The NOESY correlations between H_3-10′ and H-6′ indicated that the methyl group H_3-10′ was in the opposite face of the methylene bridge (C3′-C8′-C6′). In addition, the NOESY correlations between H_3-10′ and H-1′b indicated that the methylene bridge was in the same face as the hydroxyl group at C-2′. Regarding the glucose moiety, a large coupling constant (7.8 Hz) of the anomeric proton suggested that the glucose moiety was in β configuration. Therefore, the structure of compound **1** was explicated as shown in Fig. 3(A). This compound was named as plebeioside A.

The molecular formula of compound **2** was deduced as $C_{23}H_{32}O_9$ (eight unsaturations) on the basis of NMR data and verified by HRESIMS. The 27 protons out of the entire protons were bound to carbon based on HMQC results, suggesting that it has five exchangeable protons. The ^{1}H and ^{13}C NMR spectra for **2** were resembled to those of **1** (Table 1). Through detailed analysis of NMR data, **2** was found to have a *p*-hydroxy benzoyl group [δ_H 7.89 (δ_C 133.01) and 6.83 (δ_C 116.36)], a glucose moiety [an anomeric group at δ_H 4.23 (δ_C 105.11), and four oxygenated methines at δ_H 3.22 (δ_C 75.36), 3.38 (δ_C 78.30), 3.38 (δ_C 72.24), and 3.55 (δ_C 75.59), and an oxygenated methylene group at δ_H 4.61 and 4.40 (δ_C 65.04)], and a monoterpene moiety [two tertiary methyl groups at δ_H 1.01 (δ_C 15.79) and 0.84 (δ_C 14.48), four methylene groups at δ_H 2.26 and 0.91 (δ_C 38.98), 1.68 and 1.22 (δ_C 29.21), 1.95 and 1.20 (δ_C 27.56), and 3.89 and 3.25 (δ_C 73.72), an oxygenated methine group at δ_H 3.89 (δ_C 77.86), an non-oxygenated methine group at δ_H 1.90 (δ_C 43.60), and two sp^3 quaternary carbons at δ_C 51.40 and 53.74] like in compound **1**. The interpretation of ^{1}H–^{1}H COSY spectrum for **2** suggested the presence of an isolated CH_2-CH_2-CH-CH_2-CH spin-system corresponding to the C6′-C5′-C4′-C3′-C2′ unit. The HMBC correlations of a methyl group (H-10′) at δ_H 0.84 with a methylene carbon C-6′ (δ_C 27.56), a quaternary carbon C-1′ (δ_C 51.40), and an oxygenated methine carbon C-2′ (δ_C 77.86) indicated that C-6′ of the spin system was linked to C-2′ through the methyl group-bearing quaternary carbon C-1′. HMBC correlations of H_3-8′ (δ_H 1.01) with the sp^3 methine carbon C-4′ (δ_C 43.60) and two quaternary carbons C-7′ and C-1′ (δ_C 53.74 and 51.40) indicated that the methyl group-bearing quaternary carbon C-7′ was positioned between C-4′ and C-1′, indicating that this C4′-C7′-C1′ linkage was dividing the cyclohexane ring corresponding to C1′-C2′-C3′-C4′-C5′-C6′, forming bicyclic ring system. In addition, HMBC correlations of the oxygenated methylene protons (H_2-9′) with the quaternary carbon C-7′ and the methyl group C-8′ suggested that the oxygenated methylene group was attached to C-7′ together with the methyl group C-8′. Attachment of the hydroxyl group to C-2′ of the cyclopentane ring was predictable to fulfill the molecular formula and verified by its downfield shifted ^{13}C NMR resonance (δ_C 77.86). Finally, attachment of the fused bicyclic moiety to C-1 of glucose was evident from the HMBC correlations of the anomeric proton of the glucose with the carbon (C-9′) at δ_C 73.72. As in compound **1**, the *p*-hydroxybenzoyl group was found to be attached to C-6 of the glucose moiety by HMBC data (Fig. 3(B)). Thus, the planar structure of **2** could be obtained as shown.

The NOESY study (Fig. 3(B)) was tried to elucidate the relative stereochemistry of compound **2**. The NOESY correlations between H-1 and H-9′b/H_3-10′ implied that the bridge C1′-C8′-C4′ was positioned on the upper face of the molecule. Even though the proton signals for H-2′ and H-9′b appeared exactly at the same resonances at δ_H 3.89, no NOESY correlations between H-2′ and H-5′a/H-5′b as well as H-2′ and H-4′ suggested that the hydroxyl group at C-2′ was positioned in α orientation as shown in Fig. 3(B). This compound was named as plebeioside B.

The absolute stereochemistries of compounds **1** and **2** were elucidated by measuring optical rotations after acid hydrolysis. Compound **1** was hydrolyzed with acid to get *p*-hydroxybenzoylated glucose moiety together with monoterpene moiety. Positive optical rotation value of the hydrolysate obtained from compound **1** indicated that it had a D-glucose. Compound **2** was presumed to also have D-glucose. With reference of the glucose carbons, the absolute stereochemistries of the chiral carbons in compounds **1** and **2** could be elucidated based on the relative stereochemistry as shown in the Figs. 2 and 3.

Compounds **1** and **2** have a partial structure in common. They consist of a monoterpene containing a fused bicyclic ring (monoterpene), O-linked glucose moiety, and *p*-hydroxybenzoyl group. The difference between **1** and **2** is the chemical structures of the monoterpene moieties. Both of them have fused bicyclic ring systems, however, **1** has the camphene skeleton of monoterpene while **2** has the camphane (bornane) skeleton of monoterpene, differing in the position of the isopropyl group. To date, a structurally close compound to **1** and **2** is salviaplebeiaside which was isolated from *S. plebeia*. It contains O-linked glucose and *p*-hydroxybenzoyl group without a fused bicyclic group (Jin et al., 2009). Both camphene and camphane monoterpenes could be naturally found in essential oils such as terpentine, cypress oil, camphor oil, citronella oil, neroli, ginger oil, and valerian. It was reported to be synthesized from camphor and have been widely reported as intermediates for the synthesis of organic derivatives (Stoyanova et al., 2013). To our knowledge, the existence of the camphene monoterpene in **1** was only once reported as an intermediate in organic synthesis (Yang et al., 2007). The camphane monoterpene in **2** was vicodiol, which was isolated from *Vicoa indica* DC. (Compositae)(Vasanth et al., 1990). The structurally closest compound to **2** was (1 *R*,2*S*,4*R*,7*S*)-vicodiol 9-*O*-β-D-glucopyranoside, which was isolated from the seed of *Amomum xanthioides* (Zingiberaceae)(Kitajima and Ishikawa, 2003). The difference between them is that **2** has *p*-hydroxybenzoyl group attached to C-6 of the glucose moiety, while (1*R*,2*S*,4*R*,7*S*)-vicodiol 9-*O*-β-D-glucopyranoside does not have a *p*-hydroxybenzoyl group.

Among the isolated compounds, **6**, **10**, **13**, and **14** were isolated

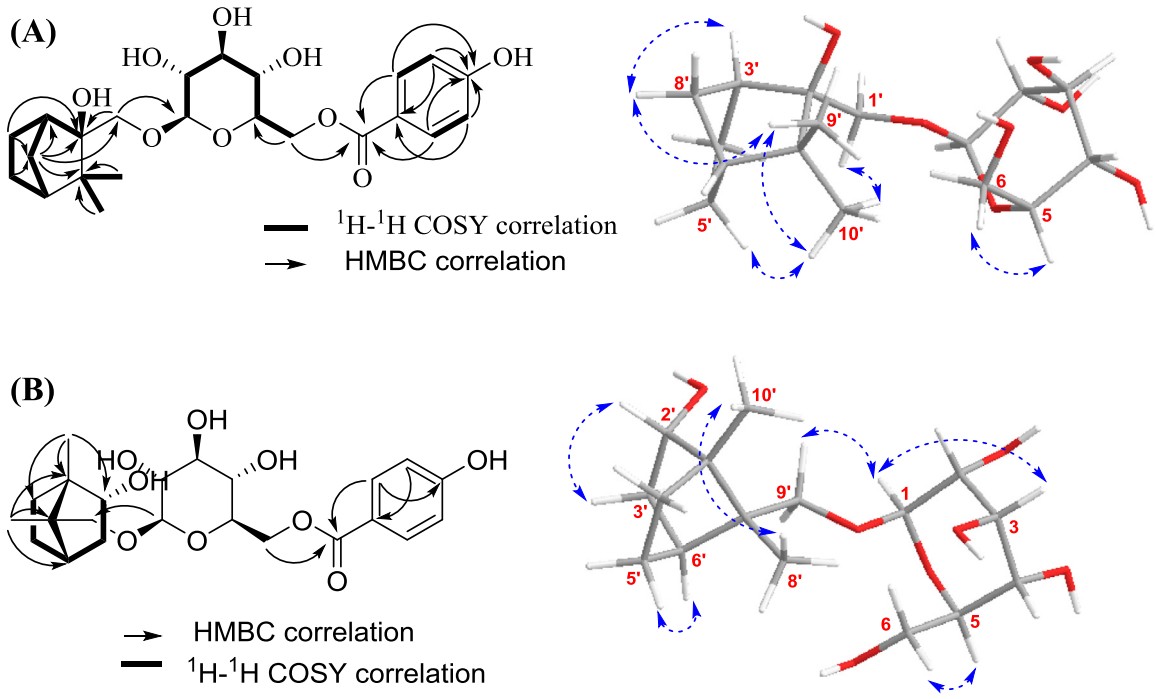

Fig. 3. Key HMBC and NOESY correlations observed for compounds **1** (**A**) and **2** (**B**).

from this plant for the first time. Especially, compound **10** was previously reported from a fungus just one time, so this is the first report on the presence of compound **10** in plants. Compounds **10** and **14** belong to phenylpropanoids, which have been isolated from the seeds of this plant. Compound **13** is one of phenolic compound found in many plants, and it has been known to have a variety of biological activities including antiviral activities (Álvarez, et al., 2012).

During our ongoing screening campaign for natural products inhibited influenza viruses, the methanol extract and fractions from aerial parts of *S. plebeia* were found to exhibit the enzymatic inhibitory activity against neuraminidase (NA) of the H1N1 influenza virus. As shown in Fig. 4(A), methanol extract and its fractions exhibited potent inhibitory effects on the neuraminidase enzyme in dose-dependent manner. These candidates were then examined in further against CPE of influenza H1H1 A/PR/8/34 in MDCK cells. The cells were incubated with test samples after inoculation with H1N1 virus for 2 h. After 3 days of incubation, the cell viability was evaluated using MTT method. The data in Fig. 5 (A) indicated that the chloroform fraction had strong cytotoxic in MDCK cells, while other fractions (ethyl acetate and butanol fractions) and total methanol extract showed promising effects against CPE of influenza H1H1 in MDCK cells at concentration of 20 μg/mL.

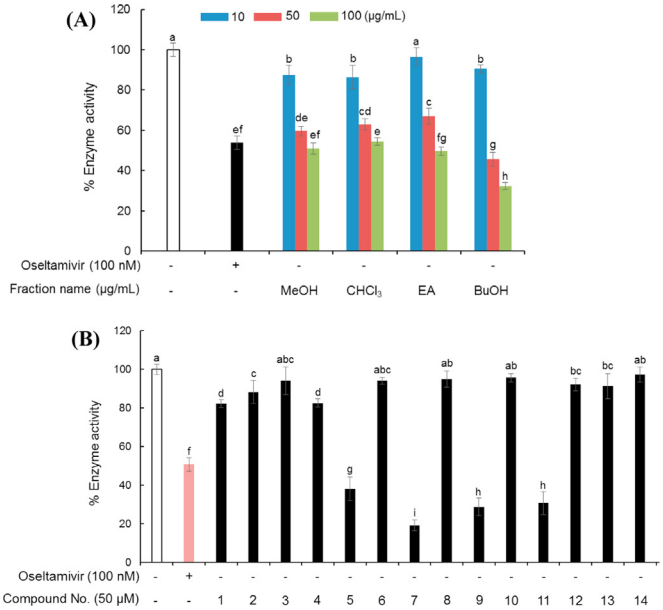

Fig. 4. Effects of the methanol extract and fractions of *S. plebeia* (**A**) and the isolated compounds (**1**–**14**) (**B**) on the H1N1 neuraminidase activity.

Based on above results, fourteen compounds isolated in this study were screened for their NA inhibitory activities. As shown in Fig. 4(B), some flavonoids or phenolic (**5**, **7**, **9**, and **11**) showed strong inhibitory activities than other compounds at concentrations of 50 μM. Two new compounds did not show strong inhibitory activities. The potential candidates were then checked detail the inhibitory effect on neuraminidase activity and H1N1-induced CPE assay. As shown in Table 2, compounds **5**, **7**, **9**, and **11** exhibited moderate the NA inhibitory activity with an IC_{50} ranging from 16.65 ± 0.91 to 19.83 ± 2.28 μM, compared with the inhibitory effects of the positive control (oseltamivir) ($IC_{50} = 0.10 \pm 0.02$ μM). Furthermore, these compounds also were confirmed the viral inhibition effects using cytopathic effect (CPE) assay.

The data in Fig. 5(B) indicated that compounds **5**, **7**, and **9** (at concentration of 20 μM) reduced the cytopathic effects on H1N1-infected MDCK cells, while compound **11** showed slightly cytotoxic effect in MDCK cells. In addition, some compounds (**5**, **7**, **9**, and **11**) were also determined the EC_{50} and CC_{50}, compared to oseltamivir as positive control. The results in Table 2 show that the selective index (SI) values of these flavonoids (**5**, **7**, and **9**) exhibited with an SI value ranging from 8.90 ± 0.76 to 11.47 ± 0.37, while compound **11** could not be determined the SI value because of the cytotoxic effect in MDCK cells.

Finally, considering CC_{50}, EC_{50}, and SI values as analyzed in CPE assay, the morphology of MDCK cells showed the effect of compounds **5**, **7**, and **9** on H1N1-induced CPE were also observed (S-

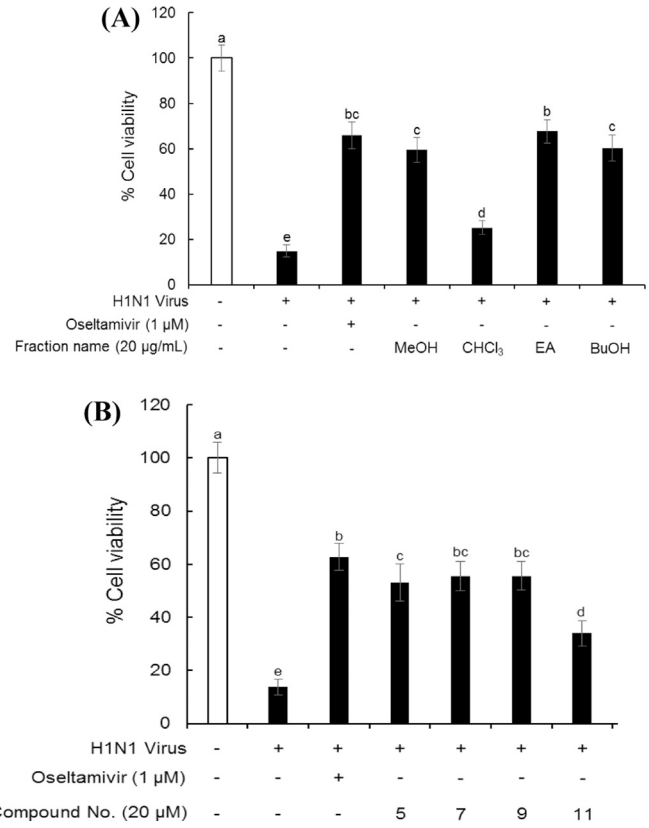

Fig. 5. Inhibitory activities of the methanol extract and fractions of *S. plebeia* (**A**) and some compounds (**B**) against CPE of influenza H1H1 A/PR/8/34 in MDCK cells.

Table 2
The inhibitory effects of bioactive compounds on neuraminidase and H1N1-induced CPE.

Compound	IC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	SI
5	19.83 ± 2.28	22.62 ± 1.79	> 200	> 8.90 ± 0.76
7	11.18 ± 1.73	17.45 ± 0.54	~200	~11.47 ± 0.37
9	16.65 ± 0.91	22.60 ± 2.76	~200	~8.98 ± 1.23
11	17.96 ± 2.38	Cytotoxic[a]	–	–
Oseltamivir	0.10 ± 0.02	0.55 ± 0.06	–	–

[a] See in supporting data (Fig. S2).

Fig. 3(A)). Without treated with our compounds, the H1N1-infected MDCK cells were completely destroyed after 3 days of incubation. However, the proportion of cell survival was significantly increase in the presence of compounds **5**, **7**, and **9** at concentration of 40 μM. Moreover, compound **5** also recovered the chromosome condensation induced by H1N1 viral infection onto MDCK cells (S-Fig. 3(B)).

4. Discussion

Phytochemical investigations of *S. plebeia* led to the isolation of fourteen compounds including two new compounds (**1**-**2**), monoterpenes with *p*-hydroxybenzoylated glucose moiety. To our knowledge, plebeioside A (**1**) is the first natural monoterpenoid with camphene skeleton. It was previously reported to be an intermediate in organic synthesis. Among the isolated compounds, compounds **6**, **10**, **13**, and **14** were isolated from this plant for the first time.

All the isolated compounds were screened for inhibitory activities against Influenza A (H1N1) neuraminidase. Among them, compounds **5**, **7**, **9**, and **11** exhibited potent inhibitory activities in dose-dependent manners. Compounds **5**, **7**, and **11** had flavone skeletons while compound **9** had an esterified phenyl propanoid skeleton. Compounds **7** and **11** with a hydroxyl group at C-4′ of flavone skeleton showed greater inhibitory activity than compound **5** without a hydroxyl group at C-4′. A previous structure-activity relationship (SAR) study on neuraminidase inhibition of flavonoids demonstrated that flavonoids should have hydroxyl groups at both C-7 and C-4′ and more importantly, an α, β-unsaturated carbonyl group at C-2, C-3, and C-4 of C ring in flavonoids for potent antiviral activities (Quy et al., 2016). Notably, these requirements agreed with the structures of compounds **5**, **7**, and **11**. Compound **9** is a methyl ester of rosmarinic acid, which is a caffeic acid ester of salvianic acid A (3,4-dihydroxyphenyllactic acid). There have been some reports on findings of caffeic acid derivatives as novel influenza neuraminidase inhibitors (Xie et al., 2013a, 2013b; Hung et al., 2009). According to molecular docking analysis, the 3,4-dihydroxyphenyl moiety of caffeic acid was reported to be important for the activity, suggesting that exploration of potent NA inhibitors from caffeic acid derivatives is promising to deal with influenza virus (Xie et al., 2013a, 2013b). As expected from the previous reports, compound **9** with two units of caffeic acid moiety definitely exhibited strong NA inhibitory activity. Inhibitory effects of compounds **5**, **7**, and **9** on viral replication were further evaluated through a cytopathic effect (CPE) reduction assay. It was observed that treatment with compounds **5**, **7**, and **9** brought about protection of the cell whereas cells without inhibitors were thoroughly destroyed during H1N1 viral replication.

The active compounds (**5**, **7**, and **9**) were not isolated from the extract of the plant in quantities, implying that there are possibly more compounds to be responsible for antiviral activities of the plant. Therefore more chemical investigation is required to find out more active compounds from the plant in quantities.

5. Conclusions

In conclusion, compounds **5**, **7**, and **9** with strong NA inhibitory activities in the extract of aerial parts of *S. plebeia* could scientifically explain the empirical treatment of this plant for the remedy of the flu or common cold in Korea. Furthermore, these three active compounds in this plant could enable exploitation for further study on the analogs synthesis for the development of novel NA inhibitors in the future. Therefore, the anti-H1N1 molecules obtained in this research could provide noteworthy candidates for further investigation the development of novel NA inhibitors in the future.

Contributions

Sunghee Bang (scbsh4331@hanmail.net)
– Isolation and structure determination of the compounds from the extracts of *Salvia plebeia*.
Thi Kim Quy Ha (htkquy@ctu.edu.vn)
– Biological work of antiviral activities.
Changyeol Lee (jaber29@naver.com)
– Running NMR for structure determinations of new compounds.
Wei Li (liwei1986@kiom.re.kr)
– Structure elucidation of new compounds.
Won-Keun Oh (wkoh1@snu.ac.kr)
– Investigator of the antiviral assays.
Sang Hee Shim (sangheeshim@duksung.ac.kr)

– Principal investigator of chemical work in this study. Most of manuscript writing was done by Prof. Shim.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.09.030.

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