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Phytochemical constituents from the aerial parts of *Salvia plebeia*

Leo Adrianne Paje[1] · Hak-Dong Lee[1] · Jungwon Choi[1] · Juree Kim[1] · Ki Hyun Kim[2] · A Ram Yu[1,3] · Min-Jung Bae[3] · Sanghyun Lee[1,4,5]

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Abstract Four compounds were isolated from *Salvia plebeia* aerial parts. Silica gel open column chromatography with a gradient elution system was used to isolate and purify these compounds. Nuclear magnetic resonance spectroscopy and mass spectroscopy were used for structural elucidation and identification, while electronic circular dichroism was used to confirm the absolute configuration. The structures were determined to be *β*-sitosterol (**1**), (–)-1*S*,5*S*,8*S*,10*R*-1-acetoxy-8-hydroxy-2-oxoeudesman-3,7(11)-dien-8,12-olide (**2**), ursolic acid (**3**), and *N*-methylhydroxylamine (**4**). Compounds **2** and **4** were isolated for the first time from this plant. Compound **2** was quantitatively analyzed via HPLC/UV. The results showed that the methanol extract of *S. plebeia* had a higher content of compound **2** (1.20 mg/g) than the ethanol extract (0.55 mg/g). This study could be used as a preliminary step in conducting HPLC/UV analysis of sesquiterpenoids in *S. plebeia* extract to assess their bioavailability and potency.

Keywords Eudesmane-type sesquiterpenoid · *N*-methylhydroxylamine · *Salvia plebeia*

Sanghyun Lee (✉)
E-mail: slee@cau.ac.kr

[1]Department of Plant Science and Technology, Chung-Ang University Anseong 17546, Republic of Korea

[2]School of Pharmacy, Sungkyunkwan University, Suwon 16419, Republic of Korea

[3]Technical Assistance Department, The Food Industry Promotional Agency of Korea, Iksan 54576, Republic of Korea

[4]BET Research Institute, Chung-Ang University, Anseong 17546, Republic of Korea

[5]Natural Product Institute of Science and Technology, Anseong 17546, Republic of Korea

Introduction

Salvia plebeia R. Br. (SP), an annual or biennial plant, belongs to Lamiaceae. It is widely distributed in Asia, including China, Japan, Korea and other regions of Oceania [1]. As a highly valued plant, SP has been used for 490 years in traditional Chinese medicine (TCM) and is believed to detoxify toxins, relieve swellings, cool the blood, remove body moisture and act as a diuretic agent [2,3]. In South Korea, it is highly cultivated since it is prized for its effectiveness against asthma. Its leaves are used to treat inflammation, hepatitis, hemorrhage, and stress [4]. In traditional Asian medicine, it is used to treat several illnesses due to its anti-inflammatory, anti-microbial, anti-blastic, anti-pyretic, and anti-dynous activities [5,6].

Many studies have investigated the phytochemistry of SP and its associated pharmacological activities. In terms of its chemical constituents, several flavonoids, terpenoids, phytosterols and phenolic acids have been reported. SP has also been found to contain diterpenoids, phenylpropanoids, and sesquiterpenoids. These compounds are said to be responsible for most of its biological activities and are therefore utilized as marker compounds to evaluate the quality of SP [7]. The literature suggests that SP has anti-inflammatory, anti-bacterial, anti-oxidative, and anti-viral effects [8].

Due to the significant pharmacological effects of SP against many diseases, its novel components must be continually examined. SP has received increased attention over the past decades owing to the therapeutic effects of its chemical constituents. Several studies have performed compound isolation and chemical profiling of SP extracts [9,10].

In this study, we isolated compounds from different fractions obtained from the ethanol (EtOH) extract of SP. Column chromatography using silica gel was used to further report the isolation techniques that could be used for the future development of its marker compounds. Structural elucidation using NMR and MS was carried out to identify the isolated compounds. Quantitative analysis using HPLC/UV was also performed on the

compound of interest to examine its bioavailability in the extracts of different *Salvia* species.

Materials and Methods

Plant material

The aerial part of SP was obtained from Gimpo Agricultural Extension Center, Gimpo, Korea. A voucher specimen was deposited in the herbarium of our department.

Instruments, reagents, and chemicals

Silica gel (60-200 μm, Merck Co., Darmstadt, Germany) open column chromatography and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) was used to isolate compounds. TLC analysis was performed on a glass pre-coated with silica gel 60 F_{254} (Merck, Sigma-Aldrich). The NMR spectra were recorded on an AVANCE NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 500 MHz. A JEOL-MS spectrometer (Jeol, Tokyo, Japan) was used to record the MS spectra. Electronic circular dichroism (ECD) spectrum in methanol (MeOH) was acquired in a quartz cuvette with an optical path length of 1 mm on a JASCO J-1500 spectropolarimeter (Tokyo, Japan). An HPLC system (PerkinElmer Flexar QUATERNARY Pump, Shelton, CT, USA) equipped with an auto-sampler, pump, and UV detector (PerkinElmer PDA LC Detector) was used for chromatographic analysis.

Extraction and fractionation

The SP aerial parts (1.6 kg) were dried and ground to obtain a powder. Then it was extracted using reflux method in 95% EtOH at 80 °C for 3 h repeated three times. The solution was filtered and concentrated using a rotary evaporator to obtain the SP crude EtOH extract (SPE). The same procedure was used to obtain the SP crude MeOH extract (SPM). The SPE (340 g) was suspended in distilled water (H_2O) and successively partitioned with *n*-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to obtain fractions of *n*-hexane (21 g), $CHCl_3$ (5 g), EtOAc (17 g), and *n*-BuOH (22 g).

Isolation of compounds

Open column chromatography packed with silica gel was used to isolate the compounds. The fractions were initially eluted with a varying solvent mixture in a stepwise gradient manner (100:0 to 0:1 v/v). The *n*-hexane fraction (5.0 g) was primarily eluted with *n*-hexane/EtOAc followed by EtOAc/MeOH to obtain 148 small fractions. The 148 fractions were analyzed by TLC (*n*-hexane/EtOAc) and grouped into four sub-fractions with the same Rf value (SPH1-3). Compound **1** was isolated from fraction 3 (SPH3) by MeOH recrystallization. The $CHCl_3$ fraction (4.5 g) was chromatographed in an open column eluted with a gradient solution of *n*-hexane/EtOAc to obtain 100 fractions. The 100 fractions were then analyzed by TLC (*n*-hexane/EtOAc) and regrouped into four sub-fractions (SPC1-4) having the same Rf value. Vials 3-5 of SPC1 were re-chromatograped in silica gel eluted with *n*-hexane/EtOAc (6:4) to afford compound **2**. Compound **3** was obtained from fraction 2 (SPC2) vials 6-1 by MeOH recyrstallization. The EtOAc fraction (10.0 g) was chromatographed in a silica gel column eluted with a stepwise gradient solution of increasing MeOH in $CHCl_3$ and collected to obtain fractions. Sub-fraction 1 (SPEA-1) was purified using a Sephadex LH-20 column eluted with a gradient solution of H_2O/MeOH (5:1, 3:1, 1:1, 1:3, and 1:5) as the mobile phase to obtain compound **4**.

Compound **1**: $C_{29}H_{50}O$; EI-MS: *m/z* 414 $[M]^+$ (100.0), 396 (28.0), 381 (19.5), 369 (19.0), 351 (20.0), 329 (22.0), 303 (31.0), 271 (32.0), 255 (55.0), 213 (29.0), 159 (31.0), 145 (32.0), 133 (30.5), 107 (48.0), 81 (48.5), 55 (49.0); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.53 (1H, m, H-3), 5.35 (1H, d, *J*=5.5 Hz, H-5), 0.68 (3H, s, H-18), 1.01 (3H, s, H-19), 0.94 (3H, d, *J*=14.5 Hz, H-21), 0.86 (3H, d, *J*=14.5 Hz, H-21), 0.86 (3H, s, H-26), 0.84 (3H, d, *J*=2.0 Hz, H-27), 0.82 (3H, d, *J*=4.5 Hz, H-28), 1.01 (3H, s, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 37.5 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4), 141.0 (C-5), 121.9 (C-6), 32.1 (C-7), 29.4 (C-8), 50.3 (C-9), 36.7 (C-10), 20.5 (C-11), 40.0 (C-12), 42.5 (C-13), 57.0 (C-14), 23.3 (C-15), 28.5 (C-16), 56.3 (C-17), 12.1 (C-18), 19.6 (C-19), 36.4 (C-20), 19.0 (C-21), 34.2 (C-22), 24.5 (C-23), 46.0 (C-24), 26.3 (C-25), 20.0 (C-26), 19.3 (C-27), 21.3 (C-28), 12.2 (C-29).

Compound **2**: $C_{17}H_{20}O_6$; EI-MS: *m/z* 320 $[M]^+$ (14.0), 302 (14.5), 278 (74.0), 260 (100.0), 242 (63.0), 207 (49.0), 179 (28.0), 163 (53.0), 135 (34.0), 107 (25.0), 91 (22.0), 72 (20.0); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 323 (−0.9), 257 (−0.6), 249 (+0.6), 205 (−1.1) nm; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 5.16 (1H, s, H-1), 5.95 (1H, br, s, H-3), 2.94 (1H, br, d, *J*=13.0 Hz, H-5), 3.03 (1H, dd, *J*=13.0, 3.5 Hz, H-6α), 2.38 (3H, br, t, *J*=13.3 Hz, H-6β), 1.63 (3H, d, *J*=13.0 Hz, H-9α), 2.27 (1H, d, *J*=13.5 Hz, H-9β), 1.80 (3H, s, H-13), 1.06 (3H, s, H-14), 2.05 (1H, br, s, H-15), 7.30 (1H, s, OH-8), 2.13 (3H, s, OAc); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 82.2 (C-1), 192.0 (C-2), 124.7 (C-3), 162.6 (C-4), 47.9 (C-5), 22.6 (C-6α), 159.0 (C-7), 102.7 (C-8), 45.4 (C-9α), 42.4 (C-10), 122.0 (C-11), 171.4 (C-12), 8.1 (C-13), 12.3 (C-14), 21.7 (C-15), 20.4, 169.6 (OAc).

Compound **3**: $C_{30}H_{48}O_3$; EI-MS: *m/z* 456 $[M]^+$ (2.0), 300 (1.5), 248 (100.0), 203 (33.0), 164 (10.0), 133 (19.0), 91 (5.0), 69 (4.5); ^{1}H-NMR (500 MHz, DMSO-d_6): δ 5.51 (1H, t, *J*=3.25 Hz, H-12), 3.48 (1H, dd, *J*=10.0, 6.0 Hz, H-3α), 2.66 (1H, d, *J*=11.0 Hz, H-18), 1.08 (3H, s, H_3-27), 1.03 (3H, m, H_3-30), 1.04 (3H, s, H-23), 1.03 (3H, s, H-25), 0.97 (3H, d, *J*=6.0 Hz, H_3-29), 0.98 (3H, s, H_3-26), 0.87 (3H, s, H_3-24); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 180.5 (C-28), 139.7 (C-13), 126.1 (C-12), 78.7 (C-3), 56.3 (C-5),

54.1 (C-18), 48.5 (C-9), 48.5 (C-17, overlap), 43.0 (C-14), 43.0 (C-8, overlap), 40.0 (C-19), 39.9 (C-1), 38.4 (C-4), 39.5 (C-20), 37.9 (C-10), 37.7 (C-22), 34.0 (C-7), 31.5 (C-21), 29.3 (C-23), 29.1 (C-15), 28.6 (C-2), 25.4 (C-16), 24.4 (C-27), 24.1 (C-11), 21.9 (C-30), 19.2 (C-6), 18.0 (C-29), 17.9 (C-26), 17.0 (C-24).

Compound **4**: CH_5NO; EI-MS: *m/z* 318 $[M]^+$ (11.0), 284 (12.0), 256 (18.0), 241 (5.0), 213 (18.0), 182 (11.0), 167 (19.0), 129 (18.0), 84 (100.0), 73 (23.0), 66 (99.0), 57 (21.0); ^{1}H-NMR (500 MHz, DMSO-d_6): δ 4.10 (1H, s, H-1), 3.17 (1H, s, H-2); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 48.6 (C-1).

Sample preparation and chromatographic condition

The methanol extracts of *Salvia japonica* (SJM), *Salvia officinalis* (SOM), and *Salvia splendens* (SSM) were acquired from KRIBB, Daejeon, Korea. SPE, SPM, SJM, SOM, and SSM were dissolved in MeOH (20 mg/mL). One milligram of compound **2** was also dissolved in MeOH (1 mL). Quantitative analyses of compound **2** in the extracts were performed in an HPLC system eluted with a gradient system using a reverse-phase YMC-Pack Pro C18 column (4.6×250 mm, 5 μm). The mobile phase consisted of 0.5% acetic acid in water (A) and acetonitrile (B). The gradient elution system are as follows; 95% of solvent A at 0 min, 75% at 20 min, 50% at 45 min, 10% at 55 min. It was then increased to 65% at 65 min and maintained until 70 min. The column was kept at a constant temperature of 25 °C. The injection volume was 10 μL, and the flow rate was set to 1 mL/min. An UV wavelength of 238 nm was used for detection.

Calibration curve

The standard stock solution of compound **2** was prepared by dissolving it in MeOH (1 mg/mL). It was then serially diluted to prepare the different concentrations used to construct the calibration curve. The calibration curve was calculated by plotting the peak area (Y) against the concentration (X, mg/mL). The calibration equation was used to calculate the concentrations of compound **2** in the samples. All values are reported as the mean (n =3) ± standard deviation.

Results and Discussion

Repeated open column chromatography using silica gel as the adsorbent material was used to obtain compounds **1-4** from SP (Fig. 1). Previous studies have mostly focused on the isolation of several flavonoids from SP, as they are considered to be marker compounds with several biological activities. However, in addition to flavonoids, SP was also found to be rich in terpenoids of many types as well as phytosterols. These classes of compounds are equally significant for evaluating the medicinal value of SP.

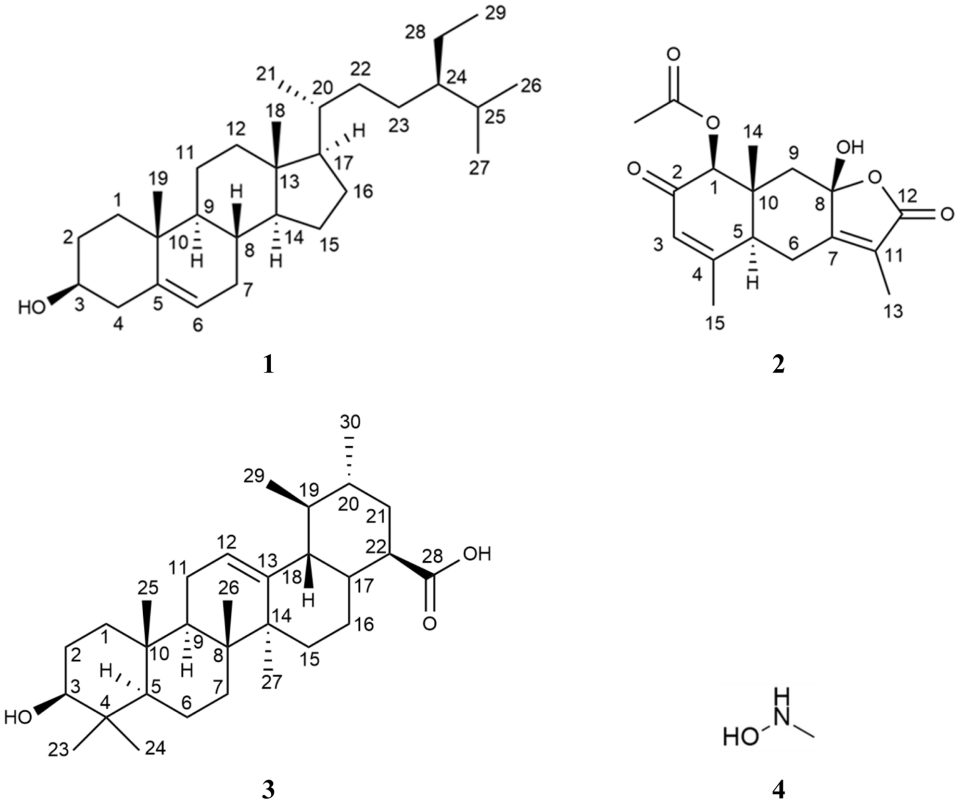

Fig. 1 Chemical structures of compounds **1-4** from *S. plebeia*

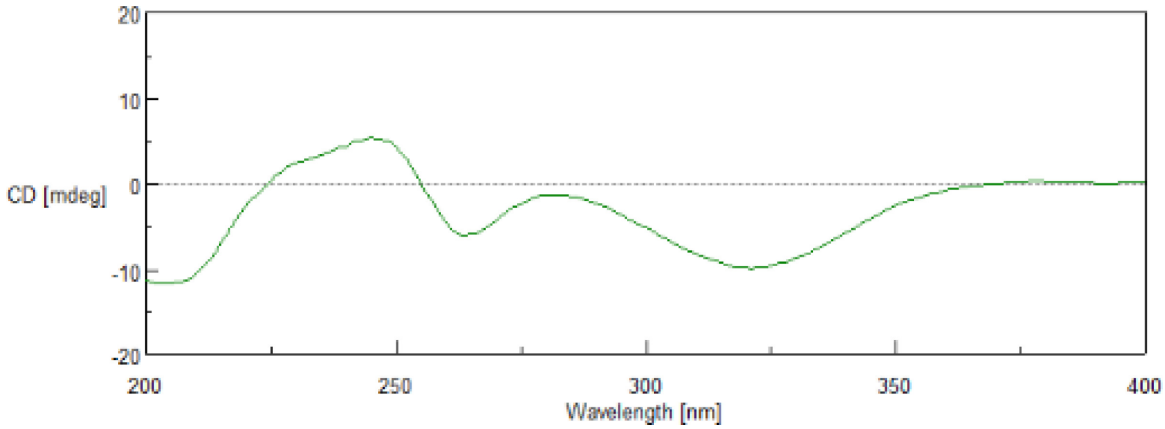

Fig. 2 The electronic circular dichroism (ECD) spectrum of compound **2**

Phytosterols have been analyzed in several *Salvia* species, and their content and composition have also been determined [11,12]. Several sesquiterpenoids from SP have been investigated in terms of their structure and configuration [13].

The EI-MS data of compound **1** showed a molecular ion peak at *m/z* 414 which corresponds to $C_{29}H_{50}O$. The ^{1}H-NMR spectroscopic data of compound **1** exhibited six methyl signals that appeared as two methyl singlets at δ_H 0.68 and 1.01 which corresponds to the angular methyl singlets. Three methyl doublets appeared at δ_H 0.86, 0.86, and 0.94. The substance also showed one olefinic proton at δ_H 5.35 and a methyl triplet at δ_H 0.86 corresponding to the methyl protons. Moreover, a multiplet appeared at δ_H 3.53 ppm corresponding to the hydroxyl group. The ^{13}C-NMR showed recognizable signals at δ_C 141.1 ppm and 121.9 ppm which are typical of alkene double bonds. The values at δ_C 19.0 and 12.1 ppm correspond to angular methyl carbon atoms. The signal at δ_C 72.0 ppm can be attributed to the β-hydroxyl group attached to the carbon at position 3 of compound **1**. The ^{13}C-NMR spectrum also showed twenty-nine carbon signals consisting of six methyl, eleven methylenes, nine methane, and three quaternary carbon signals. Based on these results and by comparison with existing literature, the peaks are typical of *β*-sitosterol [8]. *β*-Sitosterol is present in a variety of dietary and non-dietary plants. It is a phytosterol with a wide distribution among plant species [9]. Isolation of the *β*-sitosterol complex from *Salvia* species has already been reported [10]. A recent study investigated the *β*-sitosterol content of seeds from different *Salvia* species influenced by various environmental parameters [11]. Several studies have shown that *β*-sitosterol modulates anti-oxidant enzymes and human estrogen receptor [14]. It also exhibits anti-inflammatory activity in rats and human aortic cells. Other therapeutic potentials of this compound include anti-cancer, antipyretic, anti-diabetic, and anti-microbial effects [15].

Compound **2** was identified as $C_{17}H_{20}O_2$ based on its EI-MS peak at *m/z* 320. The ^{1}H-NMR data showed the presence of an acetate group verified by the signal of a three-proton singlet at δ_H 2.13 (3H, s, OAc). The spectrum also signaled for two methyl groups on double bonds at δ_H 1.80 and 2.05. One-proton singlet at δ_H 5.16 (1H, s, H-1) was assigned to the geminal proton of an acetate group, and a broad singlet at δ_H 5.95 (1H, br s, H-3) corresponded to the *α* proton of an *α*,*β*-unsaturated ketone. A vinyl proton indicated that the second double bond was tetra-substituted. These data are in accordance with those of previous studies on eudesmane-type sesquiterpenoids [16,17]. Thus, compound **2** was deduced to have a planar structure of 1-acetoxy-8-hydroxy-2-oxoeudesman-3,7(11)-dien-8,12-olide. The absolute configuration of compound **2** was confirmed by comparing its experimental ECD spectrum with the reported ECD data [18]. Compound **2** exhibited negative Cotton effects at 323, 257, and 205 nm and a positive Cotton effect at 249 nm, which was consistent with the ECD curve of (–)-1*S*,5*S*,8*S*,10*R*-1-acetoxy-8-hydroxy-2-oxoeudesman-3,7(11)-dien-8,12-olide (Fig. 2), rather than its enantiomer [18]. The chemical structure of compound **2**, including its absolute configuration, was elucidated as (–)-1*S*,5*S*,8*S*,10*R*-1-acetoxy-8-hydroxy-2-oxoeudesman-3,7(11)-dien-8,12-olide. Previous reports regarding sesquiterpenoid isolation in the genus *Salvia* have already been reported [19]. The bioactivity of the sesquiterpenoids was evaluated. Studies have shown that it possesses anti-oxidant and anti-proliferative activities [13]. Jang *et al.* investigated sesquiterpenoids and revealed that they inhibited IL-6-induced STAT3 activation [20]. Further studies are needed to isolate more sesquiterpenoids from SP as most of the reports have focused on flavonoids, diterpenoids, and triterpenoids [17].

The molecular formula of compound **3** was deduced to be $C_{30}H_{48}O_3$ according to its FAB-MS, revealing a molecular ion peak at *m/z* 456. The ^{1}H-NMR spectrum showed seven methyl resonances that can be identified into five singlets at δ_H 0.67, 0.74, 0.86, 0.89 and 1.03 ppm together with two doublets at δ_H 0.81 (3H, *J*=6.4 Hz) and 0.90 (3H, *J*=6.0 Hz). The ^{13}C-NMR revealed the presence of 30 carbon resonances distinguished into seven methyl resonances (δ_C 15.3, 16.1, 16.9, 17.0, 21.1, 23.3, and 28.3 ppm), nine methylene moieties (δ_C 18.0, 22.9, 23.9, 27.0, 27.6,

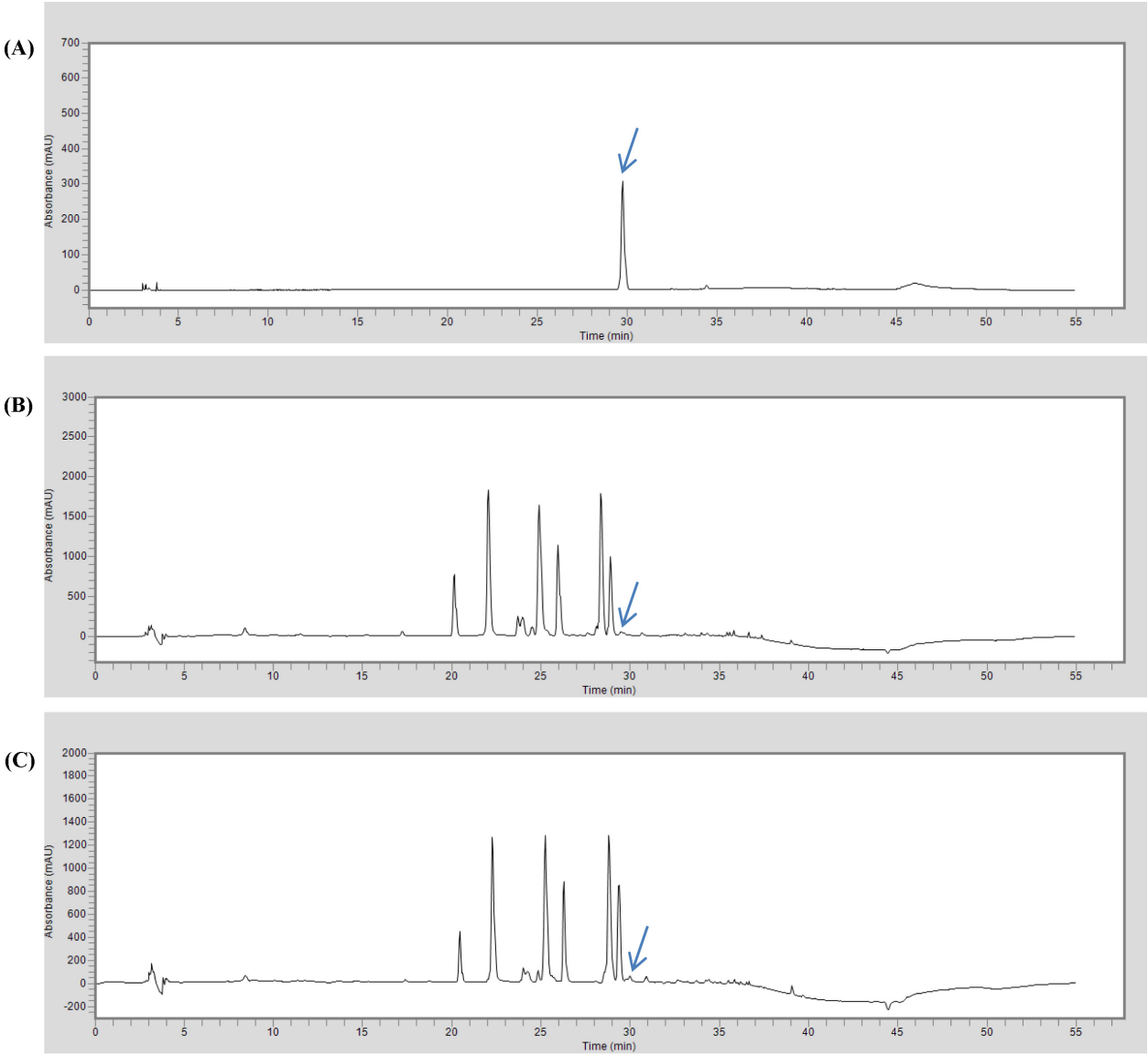

Fig. 3 HPLC/UV chromatograms of compound **2** (A), SPE (B), and SPM (C)

Table 1 Calibration curve of compound **2**

Compound	t_R	Calibration equation	Correlation factor (r^2)
2	29.74	Y = 13742X + 7093.2	0.9997

t_R = retention time
Y = peak area, X = concentration of the standard (μg/mL)

30.2, 32.7, 36.4, and 38.8 ppm), and seven methine carbons (δ_C 38.5, 38.6, 47.1, 52.4, and 54.8 ppm) in addition to two carbon resonances at δ_C 76.9 and 124.6 ppm representing one oxygenated aliphatic (C-3) and one olefinic (C-12) carbons, respectively. Seven quaternary carbons (δ_C 36.6, 38.4, 40.2, 41.7, 46.9, 138.2, and 178.3 ppm) were also observed. The signals at δ_C 124.6 and 138.2 ppm indicate the presence of a double bond. A carboxylic function was also observed corresponding to the downfield signal at δ_C 178.3. The compound was identified to be ursolic acid [21]. Ursolic acid is a terpenoid compound widespread in plants. It is mainly present in the stem bark, leaves or fruit peel. This compound is known to exhibit numerous biological activities. One of the most significant roles of ursolic acid is that it can help in treating and preventing cancer [22].

Compound **4** was identified to be an inorganic compound which is a derivative of hydroxylamine. The ^{1}H- and ^{13}C-NMR showed signals typical to that of *N*-methylhydroxylamine where one of the hydrogens of the amino groups is replaced by a methyl

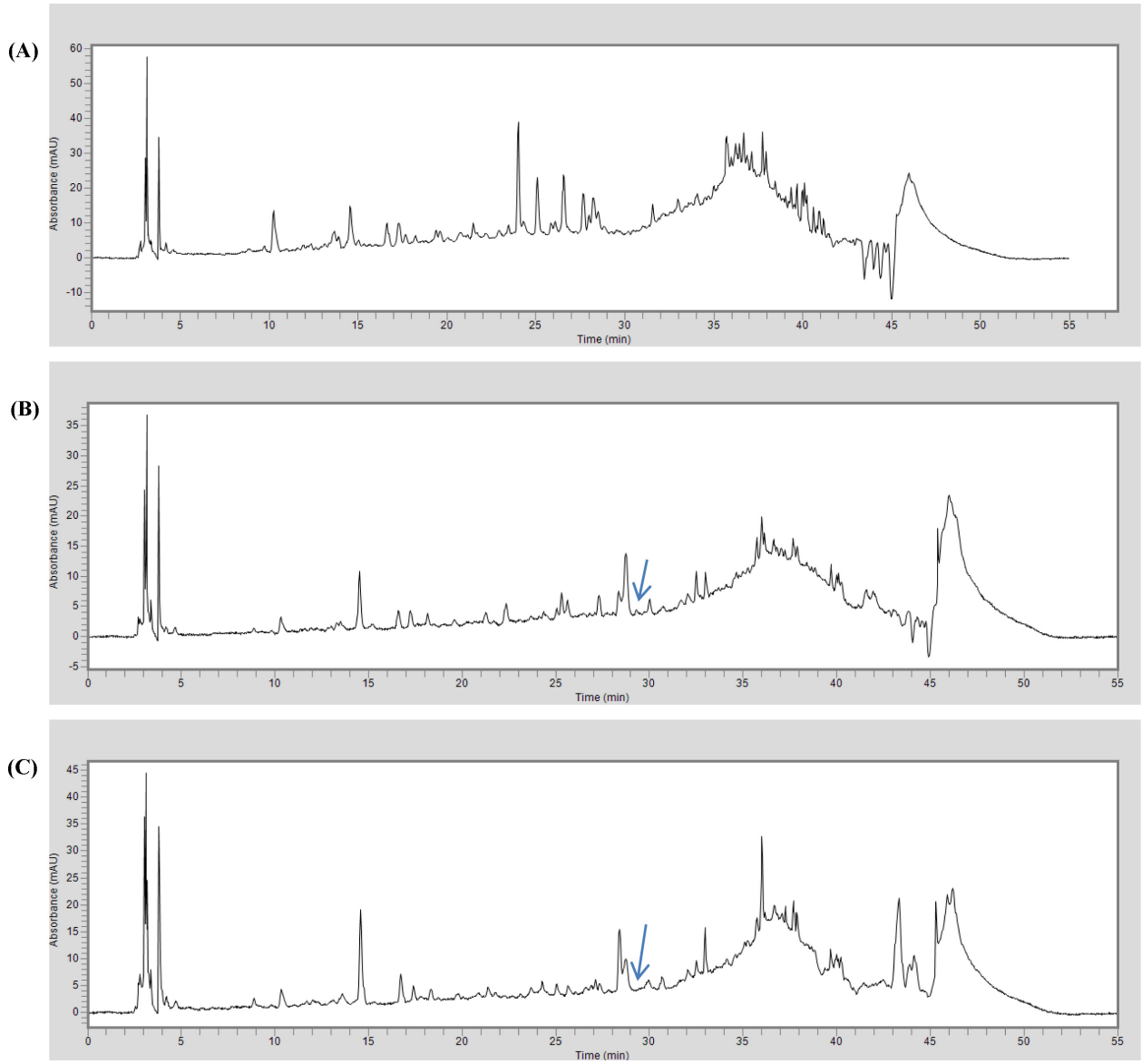

Fig. 4 HPLC/UV chromatograms of SJM (A), SOM (B), and SSM (C)

Table 2 Content of compound **2** in *Salvia* species

Compound	Content (mg/g)				
	SPE	SPM	SJM	SOM	SSM
2	0.55 ± 0.01	1.20±0.01	ND	tr	tr

ND = not detected; tr = trace

group. Studies related to *N*-hydroxylamine are limited but there are already reports regarding its potential use. Commercially, this compound is used as an inorganic catalyst used in the transamidation of primary amides with amines. To our knowledge, this is the first report of hydroxylamine derivatives in SP.

This study also quantitatively analyzed compound **2** in SPE and SPM. Our HPLC analysis showed a good separation of the peak of interest (Fig. 3). The retention time of the compound was recorded at 29.74 min. The linearity of the method was achieved with a correlation coefficient r^2 value of 0.9997 (Table 1). The optimum wavelength for the detection of compound **2** was 238 nm. Table 2 shows the content of compound **2** in SPE and SPM. As shown in Table 2, the compound had a higher content in SPM (1.20 mg/g) than in SPE (0.55 mg/g). This suggests the use of methanol as an extraction solvent to obtain a higher yield of this compound. To the best of our knowledge, there have been few to

no reports of sesquiterpenoids being quantitatively analyzed in the SP extract despite their potential bioactivities. Furthermore, compound **2** is not commonly detected in the natural environment, and it was isolated for the first time from SP. This study provides a preliminary screening of the presence of a sesquiterpenoid compound in SP using HPLC/UV as an analytical technique.

A quantitative analysis of compound **2** in the extracts of three different *Salvia* species namely SJM, SOM, and SSM was also performed (Fig. 4). However, the results showed that in SJM, compound **2** was barely detected and only a trace was found in SOM and SSM (Table 2). The peaks in the extracts were not sufficiently resolved to quantify compound **2**. A thorough HPLC/UV analysis is further needed to confirm the presence of compound **2** in other *Salvia* species.

SP is considered to be a medicinally valuable plant. Therefore, in-depth research is required to give additional evidence for its therapeutic benefits and to develop novel drugs to improve human health [7]. In this study, four compounds were isolated by silica gel column chromatography. The compounds were successfully determined by structural elucidation using NMR spectroscopy. The *n*-hexane fraction of the SP EtOH extract afforded a biologically active phytosterol, *β*-sitosterol. Ursolic acid was isolated from $CHCl_3$ fraction. Additionally, a previously known sesquiterpenoid was also obtained from the $CHCl_3$ fraction, which was confirmed by comparing the NMR spectra with previous literature [21,23]. Among all the four compounds, (–)-1*S*,5*S*,8*S*, 10*R*-1-acetoxy-8-hydroxy-2-oxoeudesman-3,7(11)-dien-8,12-olide (**2**) and *N*-methylhydroxylamine (**4**) were isolated for the first time from this plant.

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