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CHEMICAL COMPOSITION OF THE AERIAL PARTS OF *PIPER SARMENTOSUM* ROXB., PIPERACEAE

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Summary

Chemical Composition of the Aerial Parts of *Piper sarmentosum* Roxb., Piperaceae

From dichloromethane and ethyl acetate extracts of *Piper sarmentosum* Roxb., six compounds were isolated (**1-6**). Their chemical structures were identified as α-asarone (**1**), 5-phenyl-3-pentenamide (**2**), piperlotine B (**3**), quercetin (**4**), rutin (**5**), and β-sitosterol (**6**), based on spectral data and literature references. Compound **2** was isolated from this plant for the first time.

Keywords: *Piper sarmentosum*, *Piperlotine B, 5-phenyl-3-pentenamide.*

1. Introduction

Piper sarmentosum is a popularly cultivated plant in Vietnam, with traditional medicinal uses for rheumatism, digestive issues, lumbago, digestive troubles, vomiting, diarrhea, and others [1]. Scientific studies have shown its potential for antiplatelet aggregation, antioxidant, and antituberculosis activities [2],[3],[4], as well as a possible treatment for hypertension and diabetes mellitus [5]. The phytochemical studies have identified essential oils, alkaloids, flavonoids, lignans, and sterols present in the plant [2],[6],[8]. In this study, we isolated and identified six compounds from the aerial parts of *P. sarmentosum* Roxb. (Fig.1), α-asarone (**1**), 5-phenyl-3-pentenamide (**2**), piperlotine B (**3**), quercetin (**4**), rutin (**5**), and β-sitosterol (**6**). This is the first report of these compounds being isolated from *P. sarmentosum* cultivated in Vietnam.

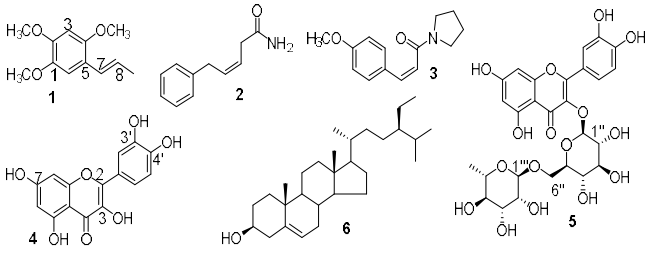

Fig. 1. Chemical structures of compounds (**1-6**)

2. Materials and methods

2.1. Plant material

The samples were collected at Hanoi Research Centre for Cultivation and Processing of Medicinal Plants in March 2022. The plant was identified as *Piper sarmentosum* Roxb. by MSc Nguyen Van Hieu, and a voucher specimen (DVLL270322) was deposited at the National Institute of Medicinal Materials.

2.2. General experiment procedures

The NMR measurements were conducted using CD_3OD, $CDCl_3$, and DMSO-d_6 as solvents on a Bruker NMR spectrometer with a frequency of 600 MHz. Tetramethylsilane was used as an internal standard, and chemical shifts were reported in δ (ppm). Electrospray Ionization Mass Spectrometry (ESI-MS) was performed using an Agilent 1100 series LC-MSD ion trap spectrometer. Column chromatography was performed using *silica gel* (70-230 or 230-400 mesh, Merck) and RP-18 as a stationary phase. Thin Layer Chromatography (TLC) was carried out on *silica gel* 60 F_{254} plates (Merck). Spots were detected by UV radiation (245, 365 nm) or by spraying with 10% H_2SO_4 followed by heating.

2.3. Extraction and isolation

The dried and pulverized aerial parts of *P. sarmentosum* (5.0 kg) were extracted with 80% ethanol (v/v) for 3 hours at 50°C, and concentrated under reduced pressure to yield total extract (1033 g, 20.7%). The total extract was suspended in water and successively extracted with *n*-hexane (nH), dichloromethane (DCM), and ethyl acetate (EtOAc). The solvent was evaporated *in vacuo* to obtain corresponding *n*-hexane (HLL, 570.2 g), dichloromethane (DLL, 90.9 g), ethyl acetate (EtLL, 9.7 g), and water (WLL, 362.2 g) extracts, respectively.

The DLL extract (90 g) was chromatographed on a *silica gel* column, eluting with gradient solvents of nH/EtOAc/methanol (50/1/1-5/1/1-100%MeOH, v/v/v) to give 5 fractions (DL1-5). The DL1 fraction (1.7 g) was continuously separated on a *silica gel* column, eluting with solvents of *n*-hexane/acetone (10/1, v/v) to afford fraction DL1.2 (250 mg). This fraction was washed five times with *n*-hexane to obtain compound **1** (20 mg). Compound **6** (15 mg) was yielded from the DL2 fraction (432 mg) using a *silica gel* column with an eluent of DCM/MeOH (15/1, v/v).

The EtLL extract (9.5 g) was chromatographed on a *silica gel* column, eluting with gradient solvents of DCM/MeOH (30/1-1/1, v/v) and 100%MeOH to give 6 fractions (EL 1-6). The EL1 (350 mg) fraction was separated on a *silica gel* column, eluting with gradient solvents of *n*-hexane/acetone (15/1 - 1/1, v/v) to yield compounds **2** (9 mg) and **3** (7 mg). Compound **4** (11 mg) was isolated from the EL2 fraction (680 mg) using a *silica gel* column, eluting with solvents of nH/acetone (2/1, v/v). The EL4 (795 mg) fraction was continuously chromatographed on a *silica gel* column, eluting with solvents of DCM/MeOH (4/1, v/v) to obtain 3 fractions (EL4.1-EL4.3). Compound **5** (10 mg) was yielded from the EL4.2 fraction (58 mg) by RP-18 column with an eluent of MeOH/water (2/1, v/v).

Compound **1**: colorless crystals; ESI-MS *m/z*: 207.1 $[M-H]^-$, $C_{12}H_{16}O_3$; ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 6.48 (1H, s, H-3), 6.94 (1H, s, H-6), 6.65 (1H, dd, J = 1.2 & 15.6 Hz, H-7), 6.09 (1H, dq, J = 6.6 &15.6 Hz, H-8), 1.88 (3H, dd, J = 1.8 & 6.6 Hz, H-9), 3.84 (3H, s, 1-OCH_3), 3.86 (3H, s, 2-OCH_3), 3.79 (3H, s, 4-OCH_3); ^{13}C-NMR (150 MHz, $CDCl_3$) δ (ppm): 143.4 (C-1), 148.8 (C-2), 98.1 (C-3), 150.7 (C-4), 110.0 (C-5), 119.1 (C-6), 125.1 (C-7), 124.2 (C-8)), 18.7 (C-9), 56.5 (1-OCH_3), 56.1 (2-OCH_3), 56.6 (4-OCH_3).

Compound **2**: yellow syrup; ESI-MS *m/z*: 174.1 $[M-H]^-$, $C_{11}H_{13}NO$; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm): 3.14 - 3.16 (2H, m, H-2), 6.28 (2H, t, J = 1.8 Hz, H-3), 7.25 (1H, brs, H-4), 3.09 - 3.11 (2H, m, H-5), 7.22 - 7.32 (5H, m, phenyl); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 169.7 (C-1), 36.4 (C-2), 113.2 (C-3), 128.4 (C-4), 30.4 (C-5), 140.3 (C-1′) 119.0 - 128.7 (C-2′ - C6′).

Compound **3**: colorless syrup; ESI-MS *m/z*: 232.3 $[M+H]^+$; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm): 3.03-3.21 (4H, m, H-2 & H-5), 1.23-1.30 (4H, m, H-3 & H-4), 7.14 (2H, dd, J = 2.4 & 31,2 Hz, H-2′ & H-6′), 6.82-6.86 (2H, dm, J = 2.4 Hz, H-3′ & H-5′), 6.28 (1H, t, J = 2.4 Hz, H-a), 7.26 (1H, s, H-b), 3.79 (3H, s, 4′-OCH_3); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 169.8 (C-1), 36.7 (C-2 & C-5), 29.7 (C-3 & C-4), 132.3 (C-1′), 129.3 (C-2′), 114.0 (C-3′), 158.3 (C-4′), 114.0 (C-5′), 129.3 (C-6′), 119.0 (C-a), 113.1 (C-b), 55.3 (4′-OCH_3).

Compound **4**: yellow amorphous powder; ESI-MS *m*/z: 302,9 $[M+H]^+$, $C_{15}H_{10}O_7$; ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H (ppm): 6.18 (1H, d, J = 2.4 Hz, H-6), 6.40 (1H, d, J = 1.8 Hz, H-8), 7.67 (1H, d, J = 2.4 Hz, H-2'), 6.88 (1H, d, J = 8.4 Hz, H-5'), 7.54 (1H, dd, J = 1.8 & 8.4 Hz, H-6'); ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C (ppm): 146.8 (C-2), 135.7 (C-3), 175.8 (C-4), 160.7 (C-5), 98.1 (C-6), 163.8 (C-7), 93.3 (C-8), 156.1 (C-9), 103.0 (C-10), 121.9 (C-1'), 115.0 (C-2'), 145.0 (C-3'), 147.7 (C-4'), 115.7 (C-5'), 119.9 (C-6').

Compound **5**: yellow amorphous powder; ESI-MS *m/z*: 633.7 $[M+Na]^+$, $C_{27}H_{30}O_{16}$; ^{1}H-NMR (600 MHz, CD_3OD) δ_H (ppm): 6.21 (1H, d, J = 1.8 Hz, H-6), 6.39 (1H, d, J = 1.8 Hz, H-8), 7.67

(1H, d, J = 2.4 Hz, H-2′), 6.87 (1H, d, J = 8.4 Hz, H-5′), 7.63 (1H, dd, J = 2.4 & 8.4 Hz, H-6′); 5.11 (1H, d, J = 7.8 Hz, H-1″), 4.52 (1H, d, J = 1.8 Hz, H-1‴), 3.81 (1H, dd, J = 1.8 & 11.4 Hz, H-6″a), 3.40 (1H, dd, J = 6.0 & 16.8 Hz, H-6″b), 1.12 (3H, d, J = 6.0 Hz, H-6‴); ^{13}C-NMR (150 MHz, CD_3OD) δ_C (ppm): 159.3 (C-2), 135.6 (C-3), 179.4 (C-4), 163.0 (C-5), 100.0 (C-6), 166.0 (C-7), 94.9 (C-8), 158.5 (C-9), 105.6 (C-10), 123.1 (C-1′), 117.7 (C-2′), 145.8 (C-3′), 149.8 (C-4′), 116.1 (C-5′), 123.6 (C-6′), 104.7 (C-1″), 75.7 (C-2″), 78.2 (C-3″), 71.4 (C-4″), 77.2 (C-5″), 68.6 (C-6″), 102.4 (C-1‴), 72.1 (C-2‴), 72.3 (C-3‴), 73.9 (C-4‴), 69.7 (C-5‴), 17.9 (C-6‴).

Compound **6**: white needle crystals; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm): 3.52 (1H, m, H-3), 5.35 (1H, m, H-6), 1.01 (3H, s, H-18), 0.86 (3H, s, H-19), 0.92 (3H, d, J = 6.6 Hz, H-21), 0.83 (3H, d, J = 6.6 Hz, H-26), 0.81 (3H, d, J = 6.6 Hz, H-27), 0.84 (3H, t, J = 7.2 Hz, H-29).

3. Results and discussion

Compound **1**, isolated as colorless crystals, has a molecular formula of $C_{12}H_{16}O_3$ based on the ESI-MS spectrum with a peak at *m/z* 207.1 $[M-H]^-$. The ^{1}H-NMR spectrum of **1** showed the presence of 2 proton signals at δ_H 6.48 (1H, s, H-3) and 6.93 (1H, s, H-6) belong to *para* - position of the aromatic ring; conjugate *trans* double bond proton signals at δ_H 6.65 (1H, dd, J = 1.2 & 15.6 Hz, H-7) and 6.09 (1H, dq, J = 6.6 &15.6 Hz, H-8), belong to olefinic methine. Additionally, the ^{1}H-NMR spectrum displayed 3 methoxy groups at δ_H 3.84 (3H, s, 1-OCH_3), 3.86 (3H, s, 2-OCH_3), 3.79 (3H, s, 4-OCH_3) and 1 methyl group at δ_H 1.88 (3H, dd, J = 1.8 & 6.6 Hz, H-9). The ^{13}C-NMR spectrum exhibited the signals of 12 carbons and the location of the carbons was identified basic on H→C interactions of the HSQC spectrum. The HMBC correlations of H-3 (δ_H 6.48) with C-1 (δ_C 143.4), C-2 (δ_C 148.8), C-4 (δ_C 150.7), and C-5 (δ_C 110.0); H-6 (δ_C 6.93) with C-1, C-2, C-4, C-5, and C-7 (δ_C 125.1) confirmed 2 protons at H-3 and H-6 of the aromatic ring; and H-9 (δ_H 1.88) with C-8 (δ_C 124.2) confirmed the position of methyl at C-8. The position of 3 methoxy groups at C-1, C-2, and C-4 was confirmed by the HMBC correlations of 1-OCH_3 (δ_H 3.84) with C-1; 2-OCH_2 (δ_H 3.86) with C-2; and 4-OCH_3 (δ_H 3.84) with C-4. Based on these spectroscopic data and by comparison to published literature [9], compound **1** was identified as α-asarone.

Compound **2** was isolated as yellow syrup. Its molecular formula was determined to be $C_{11}H_{13}NO$ based on the ESI-MS spectrum, which showed a peak at *m/z* 174.1 $[M-H]^-$. The ^{1}H-NMR spectrum of **2** exhibited 5 proton signals of one phenyl group at δ_H 7.22 - 7.32, conjugate *cis* double bond proton signals at δ_H 6.28 (2H, t, J = 1.8 Hz, H-3 & H-4), and 4 proton signals of two methylene groups at δ_H 3.14 - 3.16 (2H, dm, J = 7.2 Hz, H-2) and 3.09 - 3.11 (2H, dm, J = 6.6 Hz, H-5). The ^{13}C-NMR spectrum displayed 14 carbons, including one phenyl group at δ_C 140.3 (C-1′) and 119.0 - 128.7 (C-2′ - C6′); one olefinic group at δ_C 113.2 (C-3) and 128.4 (C-4); two methylene groups at δ_C 36.4 (C-2) and 30.4 (C-5); and one amid linked ketone at δ_C 169.7 (C-1). The position of the olefinic group was confirmed to be at C-3 and C-4 based on the HMBC correlations of H-2 (δ_H 3.14 - 3.16) with C-1 (δ_C 169.7). The HMBC correlations of H-5 (δ_H 3.09 - 3.11) with C-1′ (δ_C 140.3) confirmed the position of linkage of the amide group and phenyl group at C-1′. Compound **2** was identified as 5-phenyl-3-pentenamide based on the above evidence and by comparison to published literature [10]. This compound is the first report from *P. sarmentosum*.

Compound **3** was isolated as colorless syrup. The ESI-MS spectrum with a peak at *m/z* 232.3 $[M+H]^+$ suggested the molecular formula of **3** as $C_{14}H_{17}NO_2$ (M=231.1). The ^{1}H-NMR spectrum of **3** showed the presence of 4 proton signals at δ_H 7.14 (2H, dd, J = 2.4 & 31,2 Hz, H-2′ & H-6′) and 6.82-6.86 (2H, dm, J = 2.4 Hz, H-3′ & H-5′), conjugated *cis* double bond proton signals at δ_H 7.26 (1H, s, H-b) and 6.28 (1H, t, J = 2.4 Hz, H-a), and methoxy group at δ_H 3.79 (3H, s) were consistent with a p-methoxy-*Z*-cinnamoyl moiety [7]. Additionally, **3** also displayed 4 methylene groups belonging to the pyrrolidine moiety at δ_H 3.03-3.21 (4H, m, H-2 & H-5), 1.23-1.30 (4H, m, H-3 & H-4) belong to pyrrolidine moiety. The ^{13}C-NMR spectrum displayed 14 carbons. The location of the carbons was identified as basic on H→C interactions of the HSQC spectrum. The HMBC correlations of methoxy (δ_H 3.79) with C-4 (δ_C 158.3) confirmed the position of a methoxy group at C-4, and H-b (δ_H 7.26) with C-1′ (δ_C 132.9) suggested the attachment of N-pyrrolidine at C-1′. With the basic fragments of **3** established, the connectivities between them have solved the use of HMBC correlations. On the basis of the above evidence and by comparison to published literature [2], compound **3** was identified as (4-methoxy-*Z*-cinnamoyl)pyrrolidine or piperlotine B.

Compound **4** was obtained as a yellow amorphous powder. The ESI-MS spectrum with a peak at *m/z* 302,9 $[M+H]^+$ suggested the molecular formula of **4** as $C_{15}H_{10}O_7$. The ^{1}H-NMR spectrum of **4** showed the presence of 5 proton signals of aromatic rings, of which two

signals at δ_H 6.18 (1H, d, J = 2.4 Hz, H-6) and 6.40 (1H, d, J = 1.8 Hz, H-8) belonging to 2 protons at *meta* - position of A ring, three other proton signals at δ_H 7.67 (1H, d, J = 2.4 Hz, H-2'), 7.54 (1H, dd, J = 1.8 & 8.4 Hz, H-6) and 6.88 (1H, d, J = 8,4 Hz, H-5') belonging to a B ring. The ^{13}C-NMR spectrum of **4** displayed 15 carbon signals including one carbonyl group at δ_C 175.8 (C-4), nine non-hydrogenated carbons at δ_C 146.8 (C-2), 135.7 (C-3), 160.7 (C-5), 163.8 (C-7), 156.1 (C-9), 103.0 (C-10), 121.9 (C-1'), 145.0 (C-3'), and 147.7 (C-4'); five methine carbons at δ_C 98.1 (C-6), 93.3 (C-8), 115.0 (C-2'), 115.7 (C-5'), and 119.9 (C-6'), characteristic for a flavone system. Based on these spectroscopic data and by comparison to published literature [11], compound **4** was identified as quercetin.

Compound **5** was isolated as a yellow amorphous powder. The ESI-MS spectrum with a peak at *m/z* 633.7 $[M+Na]^+$ suggested the molecular formula of **5** as $C_{27}H_{30}O_{16}$. The ^{1}H-NMR spectrum of **5** was similar to that of **4**, quercetin, except for additional proton signals belonging to two sugar units, with 2 anomeric protons at δ_H 5.11 (1H, d, J = 7.8 Hz, H-1″) and 4.52 (1H, d, J = 1.8 Hz, H-1‴), a hydroxy methylene at δ_H 3.81 (1H, dd, J = 1.8 & 11.4 Hz, H-6″a) và 3.40 (1H, dd, J = 6.0 & 16.8 Hz, H-6″b), and one methyl at δ_H 1.12 (1H, d, J = 6.0 Hz, H-6‴), as well as other proton signals of hydroxymethines of typical sugars at δ_H 3.26-3.55. The ^{13}C-NMR spectrum of **5** exhibited 27 carbons, including fifteen carbons of a flavone ring system similar to **4** and 12 carbons of 2 sugars, with six carbon signals of β-D-glucopyranosyl moiety at δ_C 104.7 (C-1″), 75.7 (C-2″), 78.2 (C-3″), 71.4 (C-4″), 77.2 (C-5″), and 68.6 (C-6″), and six carbon signals of α-L-rhamnopyranosyl at δ_C 102.4 (C-1‴), 72.1 (C-2‴), 72.3 (C-3‴), 73.9 (C-4‴), 69.7 (C-5‴), and 17.9 (C-6‴). The HMBC correlations of the anomeric proton H-1″ (δ_H 5.11) with C-3 (δ_C 135.6) confirmed the attachment of the saccharide chain at C-3 of flavon, and the anomeric proton H-1‴ (δ_H 4.52) with C-6″ (δ_C 68.6) verified the positions of sugar moieties. Thus, compound **5** was identified as quercetin-3-O-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside or rutin [12].

Compound **6** was obtained as a white needle crystal. The ^{1}H-NMR spectrum showed the presence of 6 methyl groups at δ_H 1.01 (3H, s, H-18), 0.92 (3H, d, J = 6.6 Hz, H-21), 0.86 (3H, s, H-19), 0.84 (3H, d, J = 7.2 Hz, H-29), 0.83 (3H, d, J = 6.0 Hz, H-26), and 0.81 (3H, d, J = 6.6 Hz, H-27), 1 olefin proton at δ_H 5.35 (1H, m, H-6), 1 hydroxymethine proton at δ_H 3.52 (1H, m, H-3). The TLC analysis using a solvent mixture of *n*-hexane/acetone (2/1, v/v) showed a similar color and R_f to a reference standard sample of *β*-sitosterol, which further confirmed its identity. Therefore, **6** was identified as *β*-sitosterol.

4. Conclusion

Six compounds were obtained from the DCM and EtOAc extracts of the aerial parts of *P. sarmentosum* including *α*-asarone (**1**), 5-phenyl-3-pentenamide (**2**), piperlotine B (**3**), quercetin (**4**), rutin (**5**), and *β*-sitosterol (**6**). The structures of these compounds were established by spectral data and literature references. Among them, compound **2** was isolated from this plant for the first time.

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CHEMICAL CONSTITUENTS FROM THE TUBER ROOT OF *STEMONA TUBEROSA*

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Summary

Chemical Constituents from the Tuber Root of *Stemona tuberosa*

Phytochemical investigation of the tuber root of *Stemona tuberosa* Lour resulted in the isolation of five compounds. They are composed of two alkaloids, a phenolic acid, an anthraquinone, and another unidentified compound. The structures of these compounds were confirmed using spectroscopic methods such as ESI-MS and NMR, and by comparing them with published spectroscopic data. The isolated compounds were identified as tuberostemonine O (**1**), neotuberostemonine (**2**), *p*-hydroxybenzoic acid (**3**), hydroxymethylfurfural (**4**), and physcion (**5**). Among them, compounds **1**, **3**, and **5** were isolated for the first time from *S. tuberosa* collected in Vietnam.

Keywords: *Stemona tuberosa, Alkaloid, Tuberostemonine O.*

1. Introduction

Stemona tuberosa Lour (Stemonaceae) is known as a traditional medicine in China and certain regions of South Asia [1]. In Vietnam, this plant, named as Bach Bo, which is a hairless, perennial herb that can reach lengths of 4-10 m [2]. The tuberous root of *S. tuberosa* (Radix Stemonae) has long been used in traditional medicine for its antitussive properties, lung moisturizing, and cough-suppressing effects, as well as its ability to combat parasites [3]. Phytochemical investigations on *S. tuberosa* have revealed the presence of various components including stilbenoids, tocopherols, and alkaloids (predominantly stemonine, stemine, tuberostemonine, oxytuberostemonine, isotuberostemonine, etc.). Alkaloids and stilbenoids have been recognized for their antitussive and antibacterial activities, while dehydrotocopherol derivatives exhibit antioxidant properties [4]. Recent attention has been drawn to the extracts from *S. tuberosa* due to their diverse biological functions, particularly their potential as anti-cancer agents [3]. Despite the wide distribution and significant utilization of *S. tuberosa* in Vietnam, there have been limited studies on its chemical composition [5],[6],[7],[8],[9]. Therefore, further investigations on the chemical constituents of this important medicinal plant in Vietnam are warranted. The present study aims to investigate the composition of tuber roots of *S. tuberosa*, and this article describes the isolation and structural elucidation of five compounds from the dichloromethane and ethyl acetate extracts of *S. tuberosa*.

2. Materials and methods

2.1. Plant material

The tuber roots of *Stemona tuberosa* Lour were collected in Phu Tho province in January 2022, and were taxonomically identified by MSc. Phan Van Truong (Department of Medicinal Material Resources, NIMM).

2.2. General experimental procedures

NMR spectra were recorded on Bruker AM 500 and 600 FT-NMR spectrometers (Karlsruhe, Germany) with TMS as an internal standard, *J* in Hz at 294 K. MS data were analyzed with an LC-MS/MS (Shimadzu, Japan). *Silica gel* (Merck, 0.040 - 0.063 mm particle size) and RP-C_{18} (Merck, 30 - 50 µm particle size) were used for column chromatography (CC). Thin-layer chromatography (TLC) was carried out with *silica gel* 60F_{254} (Merck, *silica gel*, 0.25 mm) and RP-18F_{254} (Merck, 0.25 mm) plates. Spots were detected under UV 254 and 366 nm or by spraying with 10% sulfuric acid in 96% ethanol (EtOH) and heating or Dragendorff's reagent.

2.3. Extraction and isolation

The tuber roots of *S. tuberosa* (5.0 kg) were extracted with 70% ethanol by refluxing for 3 hours. After three repetitions, the combined extracts were combined and concentrated under reduced pressure at 50°C, resulting in a residue. The residue was then sequentially extracted with *n*-hexane, dichloromethane, and ethyl acetate, followed by