Three New Iridoid Glycosides from the Aerial Parts of *Asperula involucrata*

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Three new iridoid glycosides, named involucratosides A – C (**1** – **3**), were isolated from the H$_2$O subextract of crude MeOH extract prepared from the aerial parts of *Asperula involucrata* along with a known iridoid glycoside (adoxoside), three flavone glycosides (apigenin 7-*O*-β-glucopyranoside, luteolin 7-*O*-β-glucopyranoside, apigenin 7-*O*-rutinoside) as well as two phenolic acid derivatives (chlorogenic acid and ferulic acid 4-*O*-β-glucopyranoside). Their chemical structures were established by UV, IR, 1D- (¹H, ¹³C and JMOD) and 2D- (COSY, HSQC, HMBC and NOESY) NMR experiments and HR-ESI-MS. In addition, the crude extract, subextracts and isolates were evaluated for their xanthine oxidase inhibitory and antioxidant activities in *in vitro* tests. This is the first report on the chemical composition and bioactivities of *A. involucrata*.

Keywords: *Asperula involucrata*, Rubiaceae, Iridoid glycosides, Involucratosides A – C, Xanthine oxidase inhibitory activities, Antioxidant activities.

Introduction

The genus *Asperula* L. belongs to Rubiaceae family and contains around 200 species distributed worldwide. It is represented by 41 species in the flora of Turkey with an endemism rate of 49%.[1] *Asperula involucrata* Wahlenb. is a perennial herb mainly distributed in the northern parts of Turkey.[2] Some *Asperula* species are utilized as diuretic, tonic, antidiarrheal agents as well as to reduce blood pressure and inflammation in several traditional medicines.[3][4] Previous phytochemical studies on the genus *Asperula* revealed the presence of iridoids, flavonoids, anthraquinones and phenolic acids.[5 – 7] In our previous study, we reported the isolation of new flavonoid and iridoid glycosides from *A. lilaciflora*.[8] In the continuation of our researches on the isolation of new bioactive compounds from *Asperula* species growing in Turkey, we have investigated the secondary metabolites of *A. involucrata* which has not been previously investigated in terms of its phytochemical profile and biological activities. Herein we report the isolation and structure elucidation of nine compounds including three new iridoid glycosides (**1** – **3**). Further, xanthine oxidase inhibitory and DPPH free radical scavenging activities of the crude extract, subextracts and isolates were evaluated in *in vitro* tests as some flavonoids and iridoid glucosides were previously reported to be the potential inhibitors of xanthine oxidase enzyme.[9][10]

Results and Discussion

Structure Elucidation

The aerial parts of *A. involucrata* were extracted with MeOH. The crude MeOH extract was dispersed in H$_2$O and submitted to liquid-liquid extraction with CHCl$_3$ to yield H$_2$O and CHCl$_3$ subextracts. The H$_2$O subextract was subjected to various column chromatography protocols to afford three new compounds (**1** – **3**) along with six known ones (**4** – **9**) (*Fig. 1*).

Compound **1** was obtained as colorless amorphous powder. The molecular formula, C$_{27}$H$_{34}$O$_{13}$, was assigned by HR-ESI-MS (*m/z* 589.1905 ([*M* + Na]$^+$), calc. C$_{27}$H$_{34}$NaO$_{13}$ 589.1897) and from the ¹³C-NMR data. The UV spectrum of **1** contained a maximum at 221 nm typical for C(4) substituted iridoids and its IR spectrum displayed absorption bands due to hydroxy groups at 3402, ester carbonyl group at 1701,
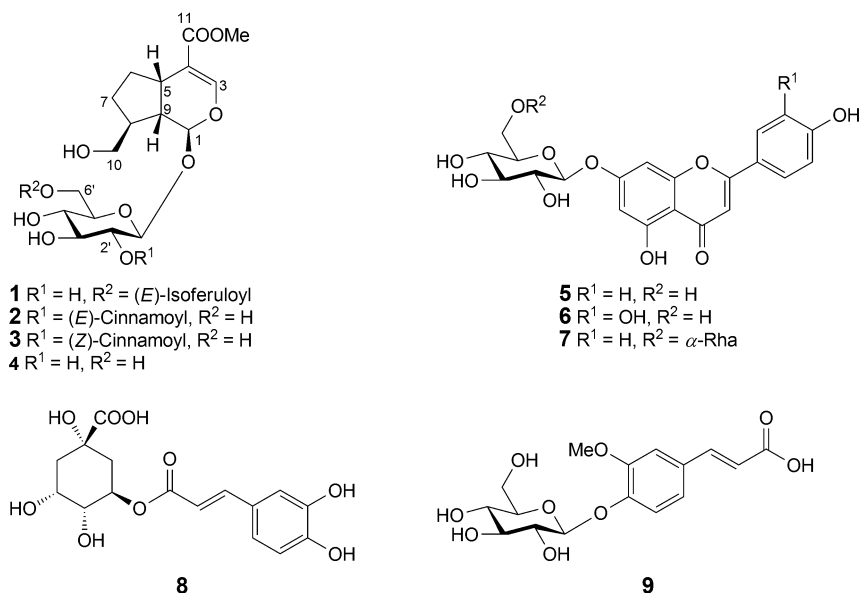
**Figure 1.** Structures of compounds (**1** − **9**) from *Asperula involucrata*.

conjugated C=C at 1632, and an aromatic ring at 1512 and 1440 cm^{-1}. The ^{1}H-NMR spectrum (*Table 1*) of **1** contained an olefinic signal at δ(H) 7.44 (*s*), along with one hemiacetal signal at δ(H) 5.02 (*d*, *J* = 7.3 Hz), one oxymethylene at δ(H) 3.52 (*dd*, *J* = 10.7, 6.1 Hz) and 3.48 (*dd*, *J* = 10.7, 6.6 Hz), three methine signals at δ(H) 2.74 − 2.78 (*m*), 2.10 − 2.15 (*m*) and 1.90 − 1.94 (*m*), two non-equivalent methylene signals at δ(H) 2.06 − 2.10 (*m*), 1.24 − 1.30 (*m*) and δ(H) 1.75 − 1.81 (*m*), 1.32 − 1.39 (*m*) which were observed in the same spin system in COSY spectrum (*Fig. 2*). Moreover, the ^{1}H-NMR spectrum also displayed an anomeric signal at δ(H) 4.69 (*d*, *J* = 7.9) arising from a β-glucopyranose and a carboxymethyl *singlet* at δ(H) 3.66 (*s*). These findings, taken together with the corresponding ^{13}C-NMR data (*Table 2*) revealed that **1** is an adoxoside type iridoid glycoside.[11] However, ^{1}H-NMR spectrum of **1** exhibited additional signals; a pair of *trans*-coupled olefinic signals at δ(H) 7.57 and 6.32 (each *d*, *J* = 15.8) as well as three aromatic signals at δ(H) 7.05 (*d*, *J* = 1.9 Hz), 7.03 (*dd*, *J* = 8.4, 1.9 Hz) and 6.93 (*d*, *J* = 8.4 Hz) as an *ABX* system indicating the presence of a (*E*)-caffeoyl derivative in **1**. ^{13}C-NMR spectrum of **1** contained 27 resonances, eleven of which were assigned to iridoid aglycone while six were attributed to β-glucopyranose, the remaining ten signals (δ(C) 168.8, 151.6, 148.0, 146.8, 128.8, 122.8, 115.8, 114.7, 112.5, 56.4) were characteristic for a *trans*-isoferuloyl moiety.[12] The chemical shift values of CH$_2$(6') signals (δ(H) 4.47 and 4.42) of β-glucopyranose unit were shifted downfield around 0.5 and 0.7 ppm in the ^{1}H-NMR spectrum of **1** indicating the

esterification site of (*E*)-isoferuloyl unit to be C(6')–OH of β-glucopyranose. HMBC spectrum (*Fig. 2*) confirmed this assumption by the long-range coupling of carbonyl carbon (δ(C) 168.8) of (*E*)-isoferuloyl unit with the CH$_2$(6') of β-glucopyranose unit. Further key cross-peaks were evident between C(4'') (δ(C) 151.6) and methoxy signal at δ(H) 3.89 and between C(11) (δ(C) 169.5) and methoxy signal at δ(H) 3.66 establishing the locations of methoxy groups. The relative configuration of the molecule was elucidated as depicted by the NOE interactions of H–C(1) (δ(H) 5.02)/H–C(8) (δ(H) 2.13) and H–C(5) (δ(H) 2.76)/H–C(9) (δ(H) 1.92) in the NOESY spectrum. Furthermore, the ^{13}C-NMR data of the cyclopentan ring were compared to those of C(8) epimeric iridoids, 8-α-dihydrogeniposide and 8-β-dihydrogeniposide (adoxoside).[11] The ^{13}C data of the aforementioned signals particularly C(7) (δ(C) 28.3), C(8) (δ(C) 44.2) and C(10) (δ(C) 66.3) were found to be superimposable with those of adoxoside and showed significant differences from those of 8-α-dihydrogeniposide. These data suggested that **1** is the 6'-*O*-(*E*)-isoferuloyl ester of adoxoside and named as involucratoside A.

Compound **2** was obtained as colorless amorphous powder. The molecular formula was determined to be C$_{26}$H$_{32}$O$_{11}$ by its HR-ESI-MS (*m/z* 543.1857 ([*M* + Na]$^{+}$), calc. C$_{26}$H$_{32}$NaO$_{11}$, 543.1842) and ^{13}C-NMR data. The UV and IR spectra were typical for an ester iridoid. Its ^{1}H- and ^{13}C-NMR spectra (*Tables 1* and *2*) of **2** showed characteristic signals for adoxoside skeleton and were in good agreement with those of **1**. The only difference was due to the

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Table 1. ^{1}H-NMR (CD$_3$OD, 500 MHz) data of compounds **1** – **3**. δ in ppm, J in Hz.

Position	**1**[a]	**2**[a]	**3**[a]
Aglycone			
1	5.02 (*d*, *J* = 7.3)	5.41 (*d*, *J* = 2.5)	5.31 (*d*, *J* = 4.4)
3	7.44 (*s*)	7.30 (*s*)	7.37 (*s*)
5	2.74 − 2.78 (*m*)	2.79 − 2.83 (*m*)	2.79 − 2.83 (*m*)
6	2.06 − 2.10 (*m*)	1.88 − 1.93 (*m*)	1.99 − 2.02 (*m*)
	1.24 − 1.30 (*m*)	1.58 − 1.63 (*m*)	1.52 − 1.59 (*m*)
7	1.75 − 1.81 (*m*)	1.65 − 1.69 (*m*)	1.70 − 1.77 (*m*)
	1.32 − 1.39 (*m*)	1.33 − 1.38 (*m*)	1.34 − 1.41 (*m*)
8	2.10 − 2.15 (*m*)	1.96 − 2.01 (*m*)[b]	2.01 − 2.06 (*m*)[b]
9	1.90 − 1.94 (*m*)	1.96 − 2.01 (*m*)[b]	1.93 − 1.97 (*m*)
10	3.52 (*dd*, *J* = 10.7, 6.1)	3.53[b]	3.52 (*dd*, *J* = 10.7, 5.0)
	3.48 (*dd*, *J* = 10.7, 6.6)	3.50[b]	3.49 (*dd*, *J* = 10.7, 6.3)
COO*Me*	3.66 (*s*)	3.58 (*s*)	3.58 (*s*)
Glc			
1′	4.69 (*d*, *J* = 7.9)	4.88 (*d*, *J* = 8.0)	4.81 (*d*, *J* = 8.0)
2′	3.24 (*t*, *J* = 8.6)	4.82[b]	4.78[b]
3′	3.41 (*t*, *J* = 8.9)	3.63 (*t*, *J* = 8.9)	3.55[b]
4′	3.35 (*t*, *J* = 8.9)	3.39[b]	3.36 (*t*, *J* = 9.3)
5′	3.54 − 3.58 (*m*)	3.39[b]	3.31 − 3.34 (*m*)
6′	4.47 (*dd*, *J* = 11.8, 2.5)	3.92 (br. *d*, *J* = 11.7)	3.89 (*dd*, *J* = 11.4, 1.9)
	4.42 (*dd*, *J* = 11.8, 6.5)	3.70 (*dd*, *J* = 11.7, 4.0)	3.68 (*dd*, *J* = 11.4, 5.2)
Acyl			
2″	7.05 (*d*, *J* = 1.9)	7.60 − 7.62 (*m*)	7.60 − 7.63 (*m*)
3″	–	7.41[b]	7.32[b]
4″	–	7.41[b]	7.32[b]
5″	6.93 (*d*, *J* = 8.4)	7.41[b]	7.32[b]
6″	7.03 (*dd*, *J* = 8.4, 1.9)	7.60 − 7.62 (*m*)	7.60 − 7.63 (*m*)
α	6.32 (*d*, *J* = 15.8)	6.46 (*d*, *J* = 15.9)	5.88 (*d*, *J* = 12.7)
β	7.57 (*d*, *J* = 15.8)	7.64 (*d*, *J* = 15.9)	6.98 (*d*, *J* = 12.7)
4″-MeO	3.89 (*s*)	–	

[a] Assignments are based on COSY, HSQC and HMBC experiments. [b] Overlapped signals.
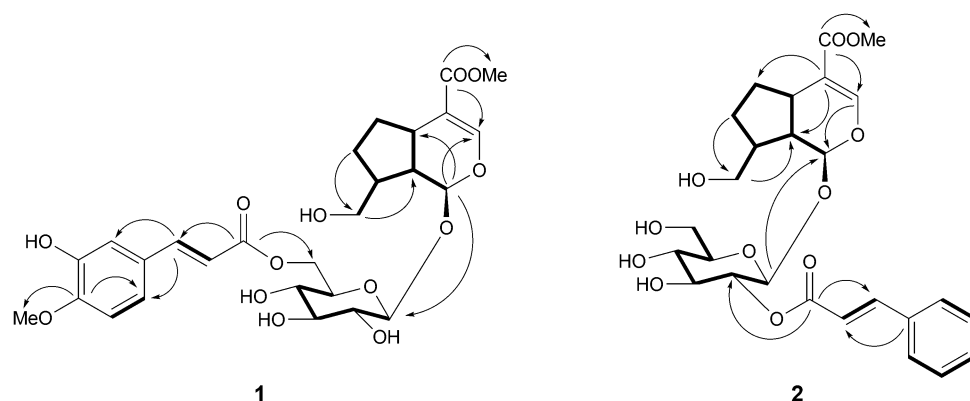
Figure 2. ^{1}H,^{1}H-COSY (bold lines) and key HMBCs (C→H, arrows) for compounds **1** and **2**.

aromatic acyl group signals. The ^{1}H-NMR spectrum of **2** exhibited characteristic signals for a *(E)*-cinnamoyl unit at δ(H) 7.64 and 6.46 (each *d*, *J* = 15.9) as an *AX* type and five aromatic signals at δ(H) 7.60 − 7.62 (*m*, 2 H) and 7.41 (3 H). These findings revealed that **2** contains *(E)*-cinnamoyl unit in its

structure instead of *(E)*-isoferuloyl unit. The linkage of the *(E)*-cinnamoyl group was determined to be C (2′)−OH of β-glucopyranose on the basis of the downfield shift for H−C(2′) (δ(H) 4.82) of β-glucopyr-anose as well as the HMBC correlation between car-bonyl carbon (δ(C) 167.4) of *(E)*-cinnamoyl unit with

Table 2. ^{13}C-NMR (CD$_3$OD, 125 MHz) data of compounds **1** − **3**. δ in ppm.

Position	1[a]	2[a]	3[a]
Aglycone			
1	98.7	97.0	97.7
3	153.4	152.2	152.6
4	112.0	112.7	112.7
5	36.6	34.6	35.2
6	33.8	31.3	32.1
7	28.3	27.7	28.1
8	44.2	43.4	43.8
9	43.8	45.7	45.2
10	66.3	66.4	66.4
11	169.5	168.9	169.2
COO*Me*	51.6	51.3	51.6
Glc			
1′	100.6	97.9	98.2
2′	74.6	74.9	74.6
3′	77.8	75.8	75.8
4′	71.8	71.6	71.6
5′	75.7	78.5	78.5
6′	64.4	62.6	62.6
Acyl			
1″	128.8	135.8	136.2
2″	114.7	130.8	131.2
3″	148.0	129.4	128.9
4″	151.6	131.5	130.1
5″	112.5	129.4	128.9
6″	122.8	130.0	131.2
α	115.8	118.6	120.0
β	146.8	146.3	145.0
CO	168.8	167.4	166.5
4″-MeO	56.4	−	−

[a] Assignments are based on COSY, HSQC and HMBC experiments.

the H−C(2′) of β-glucopyranose group. The NOE correlations for the iridoid aglycone were consistent with those of **1**. Thus, the structure of **2** was deduced as 2′-*O*-(*E*)-cinnamoyl ester of adoxoside and named as involucratoside B.

Compound **3** was obtained as colorless amorphous powder. It had the same molecular formula C$_{26}$H$_{32}$O$_{11}$ as **2**, determined by its HR-ESI-MS (*m/z* 543.1848 ([*M* + Na]$^+$, calc. C$_{26}$H$_{32}$NaO$_{11}$, 543.1842) and ^{13}C-NMR data. Its NMR data were very similar to those of **2**, except for the chemical shift values for olefinic protons (δ(H) 6.98 and 5.88) of the cinnamoyl unit which were shifted upfield. This finding along with the relatively small coupling constant (*J*) values (each 12.7 Hz) of these olefinic signals implied that the double bond geometry of cinnamoyl unit was (*Z*). The other NMR findings including HMBC and NOESY spectra for **3** were identical with those of **2**. Accordingly, **3** was established as

2′-*O*-(*Z*)-cinnamoyl ester of adoxoside and named as involucratoside C.

The known compounds were characterized as adoxoside (**4**),[11] apigenin 7-*O*-β-glucopyranoside (**5**), luteolin 7-*O*-β-glucopyranoside (**6**), apigenin 7-*O*-rutinoside (**7**),[13] chlorogenic acid (**8**),[14] and ferulic acid 4-*O*-β-glucopyranoside (**9**),[15] by comparison of their NMR data with those of published values.

The new iridoid glycosides obtained in this study are the esterified derivatives of adoxoside. Iridoid glycosides are regarded as significant chemotaxonomic markers particularly in dicotyledonous families and utilized for the chemotaxonomic evaluation of several genera and species.[16] Adoxoside-type iridoid glycosides were previously reported from the genera *Viburnum* (Adoxaceae),[17] *Castilleja* and *Euphrasia* (Orobanchaceae).[11] They are also being reported for the first time in the genus *Asperula*. The occurrence of such iridoid glycosides in *Asperula* may contribute to the chemotaxonomy of the genus *Asperula* which is considered to be polyphyletic[18] and may imply a relationship between *Asperula* and the genera *Viburnum*, *Castilleja* and *Euphrasia*.

Biological Studies

The crude extract, subextracts and isolates were evaluated for their xanthine oxidase inhibitory activities (*Table 3*). Only H$_2$O subextract displayed weak xanthine oxidase inhibitory with IC_{50} of 137.3 µg/ml, while none of the compounds isolated thereof were found to be active. Although some flavonoids were previously reported as xanthine oxidase inhibitory agents from several medicinal plants, the flavonoids obtained in this study didn't exert notable inhibitory activity against xanthine oxidase. The absence of the activity of the tested flavonoids might be due to their glycosidic structures, as the flavonoid aglycones such as apigenin, luteolin, chrysoeriol, diosmetin, hispidulin, eupatilin, kaempferol and quercetin were found to inhibit the xanthine oxidase enzyme significantly.[10] [19][20] Hence, it can be concluded that the glycosidation or an increase in the polarity of the flavonoids may lead to a decrease or loss in their xanthine oxidase inhibitory activity. The same samples were also tested for their antioxidant activities by DPPH method (*Table 3*). Among the extracts, crude MeOH extract and H$_2$O subextract displayed activity with IC_{50} values of 43.6 and 37.7 µg/ml. Regarding the isolates, only phenolic compounds showed moderate DPPH free radical scavenging activities with IC_{50} values ranging from 3.6 to 26.8 µg/ml.

Table 3. *In vitro* xanthine oxidase (XO) inhibitory and antioxidant activities of extract, subextracts and isolates (**1** − **9**) from *Asperula involucrata*

Sample	XO-Inhibitory activity (IC_{50}, µg/ml ± SD)	Antioxidant activity (IC_{50}, µg/ml ± SD)
MeOH extract	186.9 ± 47.4	43.6 ± 0.90
H$_2$O subextract	137.3 ± 32.7	37.7 ± 0.13
CHCl$_3$ subextract	356.5 ± 37.1	NA
1 − **3**, **5**	NA	NA
4	NA	26.8 ± 2.19
6	NA	19.9 ± 0.89
7	NA	10.4 ± 0.10
8	NA	3.60 ± 0.15
9	NA	20.4 ± 0.52
Ascorbic acid		0.60 ± 0.13
Allopurinol	7.5 ± 0.1 µM	

NA, not active.

Experimental Section

General

TLC: Precoated SiO$_2$ 60F$_{254}$ plates (*Merck*, Darmstadt, Germany); visualized under UV light and by spraying with 1% vanillin/H$_2$SO$_4$ soln., followed by heating at 105 °C for 2 − 3 min. Column chromatography (CC): SiO$_2$ 60 (0.063 − 0.200 mm; *Merck*, Darmstadt), *Polyamide* (*Sigma–Aldrich*, St. Louis, MO, USA), and *Sephadex LH-20* gel (*Sigma–Aldrich*, St. Louis, MO, USA). Medium-pressure liquid chromatography (MPLC): *Sepacore®* *Flash Systems X10/X50* (*Büchi Labortechnik AG*, Flawil, Switzerland), *Redi sep* columns (*LiChroprep C₁₈*, 130 and 43 g, SiO$_2$ 12 and 4 g; *Teledyne Isco*, Lincoln, Nebraska, USA). Semiprep. HPLC: *Waters 2487* (*Waters*, Milford, Massachusetts, USA). The reversed-phase HPLC column (C$_{18}$, 5 µm, 250 × 4 mm i.d., *Merck*, Darmstadt, Germany). Optical rotations: *PerkinElmer 341* polarimeter (*PerkinElmer*, Waltham, Massachusetts, USA). UV Spectra: *HP Agilent 8453* spectrophotometer (*Agilent Techonologies*, Santa Clara, CA, USA); λ_{max} in nm. IR Spectra (KBr): *PerkinElmer 2000* FT-IR spectrometer (*PerkinElmer*, Waltham, Massachusetts, USA); v in cm^{-1}. NMR Spectra: *Bruker Avance DRX 500* instrument (Billerica, MA, USA; 500 (^{1}H) and 125 MHz (^{13}C)) in CD$_3$OD; δ in ppm rel. to Me$_4$Si as internal standard, *J* in Hz. HR-ESI-MS: *Q Exactive Orbitrap* (*Thermo Fisher Scientific*, Waltham, MA, USA) in MeOH; positive-ion mode; in *m/z*.

Plant Material

The aerial parts of *Asperula involucrata* WAHLENB. (Rubiaceae) were collected from Kayışdağı, İstanbul, in May 2015. The plant material was authenticated by Dr.

Hasan Kırmızıbekmez. A voucher specimen (YEF 15007) has been deposited with the Herbarium of Faculty of Pharmacy, Yeditepe University, İstanbul.

Extraction and Isolation

The shade-dried and powdered aerial parts of *A. involucrata* (100 g) were extracted with MeOH for 2 h (1.1 l × 2) at 45 °C. The combined MeOH extracts were concentrated under vacuum to yield a crude extract (27 g) which was suspended in H$_2$O (40 ml) and partitioned with CHCl$_3$ (3 × 40 ml) to obtain H$_2$O (16.3 g) and CHCl$_3$ (7.1 g) subextracts. The H$_2$O subextract was fractionated over *Polyamide* column (75 g, 3.3 × 62 cm) eluting with a gradient solvent system H$_2$O/MeOH (100:0 − 0:100) to give six main fractions, *Frs. A − F. Fr. B* (282 mg) was subjected to C$_{18}$-Medium Pressure Liquid Chromatography (*LiChroprep C₁₈*-MPLC, 43 g) eluting with stepwise H$_2$O/MeOH gradient (95:5 → 20:80) to give ferulic acid 4-*O*-β-glucopyranoside (**9**, 2 mg). *Fr. D* (656 mg) was submitted to C$_{18}$-MPLC (130 g) eluting with H$_2$O/MeOH gradient (90:10 → 0:100) to yield eight main fractions, *Frs. D₁ − D₈*. Rechromatography of *Fr. D₂* (35 mg) by *Sephadex LH-20* CC (6 g, 1.3 × 26 cm) eluting with MeOH gave apigenin 7-*O*-rutinoside (**7**, 3 mg). *Fr. D₄* (70 mg) was applied to Medium Pressure Liquid Chromatography (SiO$_2$, 12 g) eluting with CHCl$_3$/MeOH gradient (95:5 → 70:30) to obtain adoxoside (**4**, 3 mg). *Fr. D₆* (50 mg) was chromatographed on SiO$_2$ (7 g, 1.3 × 20 cm) column using the gradient mixture of CHCl$_3$/MeOH (100:0 → 92:8) as mobile phase to afford involucratoside A (**1**, 8 mg). Similarly, separation of *Fr. D₈* (113 mg) by SiO$_2$ column chromatography using CH$_2$Cl$_2$/MeOH/H$_2$O (100:0:0 → 85:15:1) gradient yielded involucratoside B (**2**, 6 mg) along with a fraction (32 mg) containing impure **3**. Purification of this fraction by SiO$_2$-MPLC (4 g) using a gradient solvent system of CHCl$_3$/MeOH (100:0 → 80:20) gave involucratoside C (**3**, 3 mg). *Fr. E* (480 mg) was subjected to C$_{18}$-MPLC (43 g) eluting with stepwise H$_2$O/MeOH gradient (90:10 → 30:70) to obtain chlorogenic acid (**8**, 20 mg). *Fr. F* (699 mg) was subjected to semi-preparative RP-HPLC (MeOH/H$_2$O 35:65) to purify apigenin 7-*O*-β-glucopyranoside (**5**, 3 mg) and luteolin 7-*O*-β-glucopyranoside (**6**, 2 mg).

Involucratoside A (= **Methyl (1*S*,4a*S*,7*S*,7a*S*)-1,4a, 5,6,7,7a-Hexahydro-1-({6-*O*-[(2*E*)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoyl]-β-D-glucopyranosyl}-oxy)-7-(hydroxymethyl)cyclopenta[*c*]pyran-4-carboxylate**; **1**). Amorphous powder. $[\alpha]_D^{26} = -68$ (*c* = 0.1, MeOH). UV (MeOH): 221, 239, 297 (sh), 326. IR (KBr): 3402, 2952, 1701, 1632, 1512, 1440. ^{1}H-NMR: (*Table 1*).

^{13}C-NMR (*Table 2*). HR-ESI-MS (pos.): 589.1905 ([M + Na]$^+$, C$_{27}$H$_{34}$NaO$_{13}^+$; calc. 589.1897).

Involucratoside B (= **Methyl (1S,4aS,7S,7aS)-1,4a,5,6,7,7a-Hexahydro-7-(hydroxymethyl)-1-({2-O-[(2E)-3-phenylprop-2-enoyl]-β-D-glucopyranosyl}oxy)-cyclopenta[c]pyran-4-carboxylate**; **2**). Amorphous powder. $[\alpha]_D^{26} = -99$ (*c* = 0.1, MeOH). UV (MeOH): 218, 223, 278. IR (KBr): 3524, 2921, 1705, 1637, 1450. ^{1}H-NMR: (*Table 1*). ^{13}C-NMR (*Table 2*). HR-ESI-MS (pos.): 543.1857 ([M + Na]$^+$, C$_{26}$H$_{32}$NaO$_{11}^+$; calc. 543.1842).

Involucratoside C (= **Methyl (1S,4aS,7S,7aS)-1,4a,5,6,7,7a-Hexahydro-7-(hydroxymethyl)-1-({2-O-[(2E)-3-phenylprop-2-enoyl]-β-D-glucopyranosyl}oxy)cyclo-penta[c]pyran-4-carboxylate**; **3**). Amorphous powder. $[\alpha]_D^{26} = -47$ (*c* = 0.1, MeOH). UV (MeOH): 218, 223, 275. IR (KBr): 3432, 2917, 1733, 1708, 1632, 1437. ^{1}H-NMR (500 MHz, CD$_3$OD) and ^{13}C-NMR (125 MHz, CD$_3$OD): see *Tables 1* and *2*. HR-ESI-MS: 543.1848 ([M + Na]$^+$, C$_{26}$H$_{32}$NaO$_{11}^+$; calc. 543.1842).

Xanthine Oxidase Inhibitory Assay

Xanthine oxidase isolated from bovine milk (lyophilized powder) and xanthine powder were purchased from *Sigma–Aldrich*. The production of uric acid by xanthine oxidase was measured at 290 nm for 3 min in 96-well plate, using the plate reader *FluoSTAR OPTIMA* (*BMG LABTECH*) Fluorescence, in a total volume of 300 µl as described in the literature.[21] Stock solutions were prepared as recommended: 50 mM potassium buffer, pH 7.5, 0.15 mM xanthine solution, pH 7.5 and XO enzyme 0.04 units/ml. The stock solutions of the extracts (12 g/ml) were prepared in DMSO solution. 140 µl of buffer solution and 100 µl of xanthine solution were added to the wells, to give a final concentration of 33 and 0.05 mM, resp. Extracts and compounds were added in appropriate volumes so that the final concentration of DMSO in the assay did not exceed 3.3% of the total volume. The reaction was initiated by automatic addition of 50 µl of XO solution to a final concentration of 0.006 units/ml. Each sample was tested in triplicate. Allopurinol, the positive control, was tested in different concentrations, started from 10 to 0.3125 µg/ml in microdilution, to achieve the IC_{50} value. The IC_{50} values were calculated by analyzing the inhibitory percentage values of each concentration using GraphPad Prism 5.04 software (*GraphPad Software Inc.*) with nonlinear regression.

Antioxidant Activity Assay

The antioxidant activity of the extracts and pure compounds (isolates) were evaluated by using DPPH free radical scavenging method as described previously.[22] Microdilution series were made on a 96 well microplate from the sample solutions (1 mg/ml, prepared with HPLC grade MeOH) beginning from 100 µl, in three parallel copies. To each well 100 µl of DPPH (2,2-diphenyl-1-picrylhydrazyl, *Sigma–Aldrich*, Germany) solution (100 µM, dissolved in HPLC grade MeOH) was added, to gain 200 µl final volume. After 30 min of storing at r.t. in dark conditions, the absorbance was measured at 550 nm with a *BMG Labtech FluoStar Optima* plate reader. As positive control 0.1 mg/ml ascorbic acid solution was used. The evaluation of EC_{50} values were carried out with the help of Graphpad Prism 6.05.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201600288.

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Conflict of Interest

The authors declare no conflicts of interest.

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