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Montamine, a unique dimeric indole alkaloid, from the seeds of *Centaurea montana* (Asteraceae), and its in vitro cytotoxic activity against the CaCo2 colon cancer cells

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Abstract—Reversed-phase HPLC analysis of the methanol extract of the seeds of *Centaurea montana* afforded a flavanone, montanoside (**4**), six epoxylignans, berchemol (**7**), berchemol 4′-*O*-β-D-glucoside (**5**), pinoresinol (**10**), pinoresinol 4-*O*-β-D-glucoside (**8**), pinoresinol 4,4′-di-*O*-β-D-glucoside (**6**), pinoresinol 4-*O*-apiose-(1→2)-β-D-glucoside (**9**), two quinic acid derivatives, *trans*-3-*O*-*p*-coumaroylquinic acid (**1**), *cis*-3-*O*-*p*-coumaroylquinic acid (**2**), and eight indole alkaloids, tryptamine (**3**), *N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**11**), *cis*-*N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**12**), centcyamine (**16**), *cis*-centcyamine (**17**), moschamine (**13**), *cis*-moschamine (**14**) and a dimeric indole alkaloid, montamine (**15**). While the structures of two new compounds, montanoside (**4**) and montamine (**15**), were established unequivocally by UV, IR, MS and a series of 1D and 2D NMR analyses, all known compounds were identified by comparison of their spectroscopic data with literature data. The antioxidant properties of these compounds were assessed by the DPPH assay, and their toxicity towards brine shrimps and cytotoxicity against CaCo-2 colon cancer cells were evaluated by the brine shrimp lethality and the MTT cytotoxicity assays, respectively. The novel dimer, montamine (**15**), showed significant in vitro anticolon cancer activity (IC_{50}=43.9 μM) while that of the monomer, moschamine (**13**), was of a moderate level (IC_{50}=81.0 μM).

1. Introduction

Centaurea montana (family: Asteraceae alt. Compositae), an erect plant with large, reddish, blue centre flower heads, is native to Australia, Belgium and Italy, and also cultivated in many countries of the world.[1] While a number of flavonoids,[2–5] acetylenes and a lignan, arctigenin,[5] have previously been reported from the aerial parts of *C. montana*,[2–5] to our knowledge, no report on the isolation of any plant secondary metabolites from the seeds or any pharmacological properties of this plant is available to date. Many species of the genus *Centaurea* have long been used in traditional medicine to cure various ailments, e.g., diabetes, diarrhoea, rheumatism, malaria, hypertension, etc., and a variety of secondary metabolites have been reported from different species of this genus.[6] As a part of our ongoing phytochemical investigation on the species of the genus *Centaurea*,[6–11] we now report on the isolation, structure elucidation and bioactivity of a series of compounds, including a new flavanone named, montanoside (**4**), six epoxylignans, berchemol (**7**), berchemol 4′-*O*-β-D-glucoside (**5**), pinoresinol (**10**), pinoresinol 4-*O*-β-D-glucoside (**8**), pinoresinol 4,4′-di-*O*-β-D-glucoside (**6**), pinoresinol 4-*O*-apiose-(1→2)-β-D-glucoside (**9**), two quinic acid derivatives, *trans*-3-*O*-*p*-coumaroylquinic acid (**1**), *cis*-3-*O*-*p*-coumaroylquinic acid (**2**), and eight indole alkaloids, tryptamine (**3**), *N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**11**), *cis*-*N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**12**), centcyamine (**16**), *cis*-centcyamine (**17**), moschamine (**13**), *cis*-moschamine (**14**) and a novel dimer montamine (**15**) from the seeds of *C. montana*.

2. Results and discussion

Reversed-phase preparative HPLC analysis of the methanol extract of the seeds of *C. montana* afforded a new flavanone,

Keywords: *Centaurea montana*; Asteraceae; DPPH assay; Cytotoxicity; MTT assay; Colon cancer; Brine shrimp lethality assay; Lignan; Flavanone; Indole alkaloids; Dimer.
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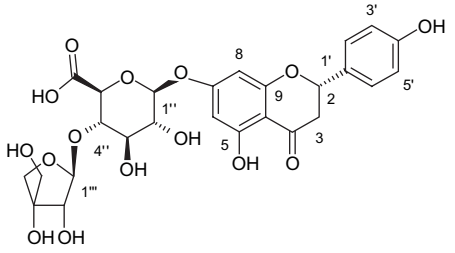
montanoside (**4**), six epoxylignans, berchemol (**7**), berchemol 4′-*O*-β-D-glucoside (**5**), pinoresinol (**10**), pinoresinol 4-*O*-β-D-glucoside (**8**), pinoresinol 4,4′-di-*O*-β-D-glucoside (**6**), pinoresinol 4-*O*-apiose-(1→2)-β-D-glucoside (**9**), two quinic acid derivatives, *trans*-3-*O*-*p*-coumaroylquinic acid (**1**), *cis*-3-*O*-*p*-coumaroylquinic acid (**2**), and eight indole alkaloids, tryptamine (**3**), *N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**11**), *cis*-*N*-(4-hydroxycinnamoyl)-5-hydroxytryptamine (**12**), centcyamine (**16**), *cis*-centcyamine (**17**), moschamine (**13**), *cis*-moschamine (**14**), and a novel dimer, montamine (**15**). The spectroscopic data of the known lignans (**5**–**10**), quinic acid derivatives (**1** and **2**) and alkaloids (**3**, **11**–**14**, **16** and **17**) were in good agreement with respective literature data.[12–22] The structures of the novel compounds, montanoside (**4**) and montamine (**15**), were established unequivocally by UV, MS and a series of 1D and 2D NMR analyses.

The ESIMS spectrum of **4** showed the *pseudo*molecular ion peak at *m*/*z* 603 $[M+Na]^+$ suggesting Mr=580. The HRCIMS gave the *pseudo*molecular ion at *m*/*z* 598.1769 $[M+NH_4]^+$ (calculated 598.1771 for $C_{26}H_{32}NO_{15}$). The UV absorptions at 213, 252 and 343 nm and the IR absorption band (1679 cm^{-1}) for a conjugated carbonyl were indicative of a flavanone skeleton.[23,24] The ^1H and ^{13}C NMR spectra (Table 1) of **4** showed the presence of one methylene [δ_H 3.12 (dd, J=13.0, 17.0 Hz) and 2.73 (dd, J=2.0, 17.0 Hz); δ_C 42.4], one oxymethine [δ_H 5.36 (dd, J=2.0, 13.0 Hz); δ_C 79.0] and two methines [δ_H 6.15 (d, J=2.0 Hz); δ_C 95.0 and 6.11 (d, J=2.0 Hz); δ_C 96.5] of the ring A, and four methines in a group of two chemically equivalent protons [δ_H 7.28 (d, J=8.4 Hz) and 6.77 (d, J=8.4 Hz)] of the ring B. These data also supported **1** being a flavanone.[25] The presence of a 1,4-disubstituted benzene ring system was evident from these signals, which was further confirmed by cross peaks between H-2′/6′ and H-3′/5′ in the ^1H–^1H COSY spectrum. The ^1H–^{13}C HMBC correlations between H-6 (δ_H 6.11) and C-8 (δ_C 95.0) and C-10 (δ_C 104.0), and H-8 (δ_H 6.15) and C-6 (δ_C 96.5), C-7 (δ_C 164.0) and C-10 (δ_C 104.0) further confirmed the flavanone skeleton. The ^1H and ^{13}C NMR spectra (Table 1) also revealed signals representing two sugar moieties. A doublet at δ_H 5.05 (d, J=7.2 Hz) and additional signals (δ_H 3.47–3.72) in the ^1H NMR implied that one of the sugars was a β-glucose derivative and the carbon signal at δ 173.0 in the ^{13}C NMR confirmed that it was a β-D-glucuronic acid moiety.[26] Five more ^{13}C NMR signals at δ 109.0, 79.9, 77.5, 74.0 and 65.0 could be assigned to the carbons of another sugar, apiose.[27] A ^3J ^1H–^{13}C long-range coupling between the anomeric proton of apiose δ_H 5.38 (H-1″) and δ_C 75.0 (C-4″) in the HMBC spectrum confirmed that the apiose was connected to glucuronic acid at C-4″. The presence of apiose/glucuronic acid was also confirmed by the loss of 309 mass units from the molecular mass of the compound in the ESIMS spectrum. The ^3J ^1H–^{13}C long-range HMBC correlation between δ_H 5.05 (H-1″) and δ_C 164.0 (C-7) confirmed that the glucuronic acid moiety was connected to C-7 of the flavanone skeleton. The specific optical rotation for **4** was found to be −48°. Literature review showed that all natural (−)-flavanones possess a *S* configuration at C-2.[28,29] Comparing with other flavanones of established absolute configuration measured by using circular dichroism spectroscopy, **4** was considered having *S* configuration at C-2 due to its levorotatory nature.[28,29] Thus, **4** was determined as a flavanone derivative, and named montanoside. To the best of our knowledge this is a new natural product.

Table 1. ^1H NMR (chemical shift, multiplicity, coupling constant J in hertz), ^{13}C NMR data and long-range HMBC correlations for montanoside (**4**)

Carbon number	Chemical shift δ in ppm		HMBC ($^1H \rightarrow {}^{13}C$)	
	^1H[a]	^{13}C[a]	^2J	^3J
2	5.36, dd, 2.0, 13.0	79.0	—	—
3	3.12, dd, 13.0, 17.0 2.73, dd, 2.0, 17.0	42.5	C-4	C-1′
4	—	196.0	—	—
5		162.0	—	—
6	6.11, d, 2.0	96.5	—	C-8, C-10
7		164.0	—	—
8	6.15, d, 2.0	95.0	C-7	C-6, C-10
9	—	162.0	—	—
10	—	104.0	—	—
1′	—	130.0	—	—
2′	7.28, d, 8.4	129.0	C-3′	C-2, C-4′, C-6′
3′	6.77, d, 8.4	115.1	C-4′	C-1′, C-5′
4′	—	158.0	—	—
5′	6.77, d, 8.4	115.1	C-4′	C-1′, C-3′
6′	7.28, d, 8.4	129.0	C-5′	C-2, C-2′, C-4′
1″	5.05, d, 7.2	99.0	—	C-7
2″	3.47, m	73.0	C-3″	—
3″	3.61, m	77.8	—	—
4″	3.72, t, 9.2	75.0	—	—
5″	3.65, m	78.0	—	C-1″
6″		173.0	—	—
1‴	5.38, d, 1.0	109.0	C-2‴	C-4″, C-3‴
2‴	3.89, m	77.5		C-4‴, C-5‴
3‴	—	79.9	—	—
4‴	3.91, m	74.0	—	C-5‴
5‴	3.47, m	65.0	C-3‴	C-4‴

[a] ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) in CD_3OD.



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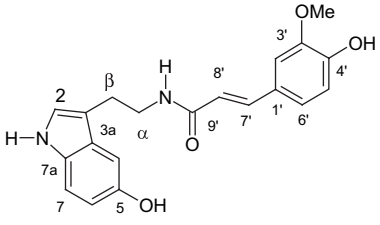
The UV and IR spectral data of **15** indicated the presence of a serotonin moiety conjugated with a cinnamic acid derivative substructure, exactly similar to that of moschamine (**13**).[6] The ^{13}C NMR spectrum of **15** (Table 2) displayed 20 carbons. The DEPT-135 indicated the presence of two methylenes (δ_C 41.0, 25.0), nine methines (δ_C 141.0, 121.0, 126.0, 117.5, 115.0, 111.5, 111.2, 111.1, 110.0), seven quarternary (δ_C 149.0, 148.0, 146.0, 133.0, 129.5, 129.0, 111.0), one carbonyl carbon (δ_C 172.0) and a methoxy group (δ 55.5). These carbon signals and the ^1H NMR data (Table 2) also supported the fact that **15** was composed of a serotonin derived substructure and a feruloyl moiety like the known alkaloid moschamine (**13**), and was further confirmed by the ^1H–^1H COSY and ^1H–^{13}C HMBC experiments. The ^1H–^1H COSY spectrum revealed four different spin systems: H-7↔H-6↔H-4, $H_2\alpha \leftrightarrow H_2\beta$, H-7′↔H-8′ and H–H-6′↔H-5′ and the ^1H–^{13}C HMBC spectrum showed key correlations between H-2 to C-3, C-3a and

Table 2. ^{1}H NMR (chemical shift, multiplicity, coupling constant J in hertz), ^{13}C NMR data and long-range HMBC correlations for montamine (**15**)

Carbon number	Chemical shift δ in ppm		HMBC (^{1}H→^{13}C)	
	^{1}H[a]	^{13}C[a]	^2J	^3J
2	6.93, s	126.0	C-3	C-3a, C-7a
3	—	111.0	—	—
3a	—	129.5	—	—
4	6.83, d, 2	111.2	C-5	C-7a
5	—	148.0	—	—
6	6.82, dd, 2, 8	111.1	C-5	C-7a
7	7.26, d, 8	111.5	C-7a	C-3a, C-5
7a	—	133.0	—	—
β-CH_2	2.27, m 2.19, m	25.0	C-2, C-α-CH_2	C-3
α-CH_2	2.86, m 2.77, m	41.0	C-β-CH_2	C-3, C-9′
1′		129.0	—	—
2′	6.99, d, 2	110.0	—	C-6, C-4′, C-7′
3′	—	146.0	—	—
4′	—	149.0	—	—
5′	6.70, d, 8.4	115.0	—	C-1′, C-3′
6′	6.91, dd, 2, 8.4	121.0	—	C-2′, C-4′, C-7′
7′	7.27, d, 15.6	141.0	—	C-2′, C-6′, C-9′
8′	6.31, d, 15.6	117.5	C-9′	C-1′
9′	—	172.0	—	—
OCH_3 (3′)	3.78, s	55.5	—	C-3′

[a] ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in CD_3OD.

C-7a, H-4 and C-5 and C-7a, H-6 and C-5 and C-7a, H-7 and C-3a and C-5, H-8′ and C-1′ and C-9′. All spectroscopic data suggested that **15** possessed a moschamine (**13**) type substructure. However, the α-CH_2 and β-CH_2 protons of **15** gave rise to significantly different resonances in the ^{1}H NMR spectrum compared to those of **13** (Table 3). For moschamine (**13**), the signals for α-CH_2 and β-CH_2 protons were observed at δ_H 3.53 (t, J=7.2 Hz) and 2.88 (t, J=7.2 Hz), whereas they were found at more upfield region δ_H 2.86, m and 2.27, m, respectively, for **15**. The ESIMS spectrum showed that **15** had a molecular mass of 702 instead of 352. The HRESIMS gave $[M+Na]^+$ at 725.2587 (required 725.2587), counted for the molecular formula $C_{40}H_{38}N_4O_8$. The HRESIMS and ^{1}H NMR spectra confirmed that **15** was a symmetrical dimer of moschamine (**13**), formed through an N–N linkage. The N–N linkage could be either of these three: (a) between two ring N of the indole skeleton; (b) one ring N of the indole skeleton and the other from the serotonin side chain N; or (c) two N from the serotonin side chain. As only one set of ^{1}H and ^{13}C NMR data were observed for **15**, option (b) could be ruled out because it would generate an asymmetric dimer. Options (a) and (c) could generate symmetrical dimers, but only option (a) could explain the upfield shift of the methylene resonances in the ^{1}H NMR spectrum. The upfield shift was due to the fact that in **15**, the formation of a N–N dimer brought these methylenes into close proximity of the aromatic electron clouds, and the rotation about N–N bond was somewhat restricted. A trivial name, montamine, has been proposed for this dimer. Although the N–N dimer formation between two tryptamine/serotonin derivatives is not common, there is precedence of such dimer formation in the case of the indole alkaloid, schischkiniin, which was isolated from *Centaurea schischkinii*.[7] Montamine (**15**), isolated from *C. montana*, is a new natural product.



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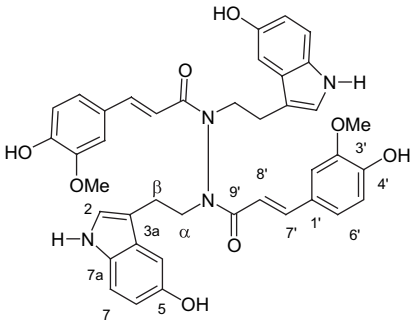

15

The DPPH assay[30] is an easy and straightforward method for determining the free radical scavenging property of a compound. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour, which is typical of the free DPPH radical, decays and the change in absorbance at 517 nm is monitored spectrophotometrically. All the compounds (**1**–**15**) showed low to moderate levels of free radical scavenging activity (IC_{50}=16.0×10^{-2}–2.02×10^{-3} mg/mL) (Table 4). Among eight alkaloids (**3** and **11**–**17**), moschamine (**13**), montamine (**15**) and centcyamine (**16**) showed the most prominent antioxidant property, which could be attributed to the presence of the highest number of phenolic hydroxyl groups (four –OH) in the molecule (Table 4).

The brine shrimp lethality assay, which has been proven to be an effective and rapid assay method to screen compounds for potential general toxicity and cytotoxic activity,[31,32] was used to determine the general toxicity of compounds **1**–**17**. The LD_{50} values of quinic acid derivatives and epoxylignans were between 6.5×10^{-2} and 10.3×10^{-2} mg/mL (Table 4),

Table 3. Major differences in NMR data of **13** and **15**

Molecule	Group	^{1}H	^{13}C	HMBC (^{1}H→^{13}C)	HRESIMS $[M+Na]^+$
Moschamine (**13**)[6]	α-CH_2	3.53, t, 7.2	40.3	C-2, C-9′	375.1321
	β-CH_2	2.88, t, 7.2	25.2	C-2	
Montamine (**15**)	α-CH_2	2.86, m 2.77, m	41.0	C-3, C-9′, C-β-CH_2	725.25869
	β-CH_2	2.27, m 2.19, m	25.0	C-2, C-3, C-α-CH_2	

Table 4. Antioxidant (DPPH assay) and cytotoxic (MTT assay) activities, and brine shrimp toxicity (Brine Shrimp Lethality assay) of compounds **1–17**

Compounds	Antioxidant activity IC_{50} (mg/mL)	Cytotoxicity IC_{50} (μM)	Brine shrimp toxicity LD_{50} (mg/mL)
1	7.6×10^{-2}	146.4	7.8×10^{-2}
2	10.0×10^{-2}	325.0	10.3×10^{-2}
3	6.4×10^{-2}	198.0	8.3×10^{-2}
4	5.0×10^{-2}	153.4	7.2×10^{-3}
5	2.1×10^{-2}	1260.0	10.3×10^{-2}
6	$>5\times10^{-2}$	843.2	7.2×10^{-1}
7	3.2×10^{-2}	833.0	3.1×10^{-2}
8	3.6×10^{-2}	705.0	6.8×10^{-2}
9	3.0×10^{-2}	1130.0	8.3×10^{-2}
10	1.4×10^{-2}	233.0	6.5×10^{-2}
11	1.6×10^{-2}	125.0	7.5×10^{-2}
12	4.8×10^{-2}	411.0	8.2×10^{-2}
13	2.2×10^{-3}	81.0	20×10^{-3}
14	4.5×10^{-3}	213.0	19.2×10^{-3}
15	3.6×10^{-2}	43.9	3.5×10^{-3}
16	2.8×10^{-3}	82.2	15×10^{-3}
17	3.2×10^{-3}	213.0	13.8×10^{-3}
Methanol extract of *Centaurea montana*	32.7×10^{-2}	56.4	62.5×10^{-2}
Quercetin	2.88×10^{-5}	—	—
Podophyllotoxin	—	—	2.79×10^{-3}

and montanoside (**4**), moschamine (**13**) and montamine (**15**) were found to be the most toxic of all the test compounds towards brine shrimp ($LD_{50}=7.2\times10^{-3}$, 20×10^{-3}, 3.5×10^{-3} mg/mL, respectively). The toxicity of these compounds (**4**, **13** and **15**) was comparable to that of the positive control podophyllotoxin ($LD_{50}=2.79\times10^{-3}$ mg/mL), a well known cytotoxic lignan.

The in vitro cytotoxicities of all the compounds isolated and characterised in this work were determined by the MTT assay against colon cancer cell line, CaCo-2 (Table 4).[33] The dimeric indole alkaloid, montamine (**15**), exhibited significant in vitro anticancer activity with an IC_{50} value of 43.9 μM. It is interesting to note that the dimerisation of moschamine (**13**) leading to the formation of montamine (**15**) increased the cytotoxicity two-fold. The unique structural features of **15** can certainly be exploited as a template for generating compounds with enhanced anticancer activity. The new flavanone, montanoside (**4**) displayed low levels of cytotoxicity with an IC_{50} value of 153.4 μM. However, all the isolated epoxylignans demonstrated low levels of activity against colon cancer cells in vitro. Among the epoxylignans, the presence of a sugar moiety in the molecule tends to reduce significantly the anticancer activity of these compounds. It can be assumed that the presence of a sugar group may prevent the effective transport of these compounds through the cell membrane, hence their reduced biological activities. It is noteworthy that the degree of brine shrimp toxicity displayed by the test compounds in the brine shrimp lethality assay corresponded well with the cytotoxic potentials of these compounds observed in the MTT assay using colon cancer cell line.

3. Conclusion

Compounds (**1–17**) isolated and identified from the seeds of *C. montana* showed various levels of activities in the DPPH, the brine shrimps lethality and the MTT cytotoxicity assays. However, the most significant finding is the discovery of the novel dimeric indole alkaloid, montamine (**15**), which exhibited significant in vitro anticancer activity against the CaCo2 cell line with an IC_{50} value of 43.9 μM.

4. Experimental

4.1. General procedures

UV spectra were obtained in MeOH using a Hewlett–Packard 8453 UV–vis spectrometer. MS analyses were performed on a Quattro II triple quadrupole instrument. NMR spectra were recorded in CD_3OD on a Varian Unity INOVA 400 MHz NMR spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C) using the residual solvent peaks as internal standard. HPLC separation was performed using a Dionex prep-HPLC system coupled with Gynkotek GINA50 autosampler and Dionex UVD340S Photo-Diode-Array detector and/or A JASCO PU-1580 Intelligent HPLC Pump, coupled with JASCO DG-1580-53 Degasser and JASCO LG-1580-02 Ternary Gradient Unit. A Luna C_{18} preparative (10 μm, 250 mm×21.2 mm) and/or a Luna C_{18} semi-preparative HPLC column (5 μm, 250 mm×10 mm) were used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractions. HMBC spectra were optimised for a long-range J_{H-C} of 9 Hz and the NOESY experiment was carried out with a mixing time of 0.8 s.

4.2. Plant material

The seeds of *C. montana* were collected from B & T, World Seeds Sarl, Paguignan, 34210 Olonzac, France. A voucher specimen PHSH80006 has been retained in the herbarium of the Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

4.3. Extraction and isolation of compounds

Ground seeds of *C. montana* (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane and methanol (MeOH) (1 L each). The MeOH extract was fractionated by solid phase extraction method using a Sep-Pak C_{18} (10 g) cartridge eluting with a step gradient: 30, 40, 60, 80 and 100% MeOH in water (200 mL each). Preparative-HPLC (eluted with a linear gradient-water/MeCN=90:10–60:40 over 50 min followed by 40% MeCN for 10 min, 20 mL/min) of the Sep-Pak fraction, which was eluted with 30% MeOH, yielded seven fractions: **F1** (30.4 mg, t_R=6.1 min), **F2** (78.2 mg, t_R=11.2 min), **F3** (50.9 mg, t_R=12.2 min), **F4** (60.0 mg, t_R=18.2 min), **F5** (925.0 mg, t_R=19.0 min), **F6** (45.4 mg, t_R=26.5 min) and **F7** (68.2 mg, t_R=27.3 min). Following the same HPLC procedure, 40% Sep-Pak fraction of the MeOH extract yielded compound **10** (14.1 mg, t_R=31.5 min) in addition to previous seven fractions. Compounds **1–4** (7.0 mg, t_R=9.0 min; 4.5 mg, t_R=10.0 min; 3.0 mg, t_R=16.0 min and 2.0 mg; t_R=23.0 min, respectively), **5**,**6** (12.3 mg, t_R=42.0 min and 9.8 mg, t_R=70.0 min, respectively), **9** (19.9 mg, t_R=48.0 min) and **11–14** (3.5 mg, t_R=40.5 min; 4.5 mg, t_R=49.0 min; 6.0 mg, t_R=56.0 min and 22.5 mg, t_R=67.0 min, respectively) were further purified by semi-prep HPLC (isocratic elution

with MeCN in water, 2.0 mL/min), respectively, from fractions **F1** (10% MeCN in water), **F2** (12% MeCN in water), **F4** (17% MeCN in water) and **F6** (20% MeCN in water). From fraction **F5**, compounds **7** (25.0 mg, t_R=74.0 min) and **8** (20.0 mg, t_R=84.0 min) were obtained by prep-HPLC (isocratic elution with 15% MeCN in water, 20 mL/min). Similar prep-HPLC purification of the 60% Sep-Pak fraction produced two fractions **F8** (32.0 mg, t_R=17.5 min) and **F9** (33.0 mg, t_R=19.8 min). **F8** was further purified by semi-prep HPLC (isocratic elution with 25% MeCN in water, 2.0 mL/min) to obtain **15** (4.0 mg, t_R=92.0 min). However, **16** (5.0 mg, t_R=31.0 min) and **17** (4.0 mg, t_R=34.0 min) were obtained from fraction **F9** by prep-HPLC (isocratic elution with 30% MeCN in water, 20 mL/min).

4.3.1. Montanoside (4). Yellow amorphous (yield 0.002%); 5.0 mg; $[\alpha]_D^{23}$ −48 (*c* 0.021, MeOH); UV λ_{max} (MeOH): 213, 252, 343; IR ν_{max} (neat): 3459, 1679, 1246 cm^{-1}; ESIMS *m/z* 603 $[M+Na]^+$, 307, $[glucose+apiose+2H]^+$, 271 $[M-glucose-apiose]^+$, 104, 60; HRCIMS *m/z* 598.1769 $[M+NH_4]^+$ (calculated 598.1771 for $C_{26}H_{32}NO_{15}$); ^1H NMR (400 MHz, CD_3OD): see Table 1; ^{13}C NMR (100 MHz, CD_3OD): see Table 1.

4.3.2. Montamine (15). Gum (yield 0.004%); UV λ_{max} (MeOH): 213, 271, 274 nm; IR ν_{max} (neat): 3434, 3380, 2362, 1652, 1590, 1516, 1460, 1366, 1270, 1201, 1120, 1032 cm^{-1}; HRESIMS: 725.2587 $[M+Na]^+$ (calculated 725.2587 for $C_{40}H_{38}N_4O_8Na$); ^1H NMR: see Table 2; ^{13}C NMR: see Table 2.

4.4. Free radical scavenging activity: DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al.[30] was adopted with appropriate modifications. DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL.

4.4.1. Qualitative assay. Test compounds (**1–17**) were applied on a TLC plate and sprayed with DPPH solution using an atomiser. The plate was allowed to develop for 30 min. The colour change (purple on white) was noted.

4.4.2. Quantitative assay. Test compounds (**1–17**) were dissolved in MeOH to obtain a concentration of 0.5 mg/mL each. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, quercetin, a well known natural antioxidant.

4.5. Brine shrimp lethality assay

Shrimp eggs were purchased from The Pet Shop, Kittybrewster Shopping Complex, Aberdeen, UK. The bioassay was conducted following the procedure described by Meyer et al.[31] The eggs were hatched in a conical flask containing 300 mL artificial seawater. The flasks were well aerated with the aid of an air pump and kept in a water bath at 29–30 °C. A bright light source was left on and the nauplii hatched within 48 h. The compounds **1–17** were dissolved in 20% aq DMSO to obtain a concentration of 1 mg/mL. These were serially diluted two times and seven different concentrations were obtained. A solution of each concentration (1 mL) was transferred into clean sterile universal vials with pipette, and aerated seawater (9 mL) was added. About 10 nauplii were transferred into each vial with pipette. A check count was performed and the number alive after 24 h was noted. LD_{50} values were determined using the Probit analysis method.[32]

4.6. MTT cytotoxicity assay

CaCo2 cells were maintained in Earle's minimum essential medium (Sigma), supplemented with 10% (v/v) foetal calf serum (Labtech Int.), 2 mM L-glutamine (Sigma), 1% (v/v) non-essential amino acids (Sigma), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 72 h before the addition of drugs. Stock solution of compounds was initially in DMSO or H_2O and further diluted with fresh complete medium.

The growth-inhibitory effects of the compounds (**1–17**) were measured using standard tetrazolium MTT assay.[33] After 72 h of incubation at 37 °C, the medium was removed and 100 μL of MTT reagent (1 mg/mL) in serum free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μL) was added to each well. The metabolised MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation y=50 (50% value).

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