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Phenolic glycosides from *Foeniculum vulgare* fruit and evaluation of antioxidative activity

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Abstract

Two diglucoside stilbene trimers and a benzoisofuranone derivative were isolated from *Foeniculum vulgare* fruit together with nine known compounds. Their structures were elucidated by spectral methods including 1D, 2D NMR and MS and chemical methods. Antioxidant activity was tested using three methods: DPPH$^{\bullet}$, total antioxidant capacity and assay of lipid peroxidation.
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Keywords: *Foeniculum vulgare*; Apiaceae; Foeniculosides; Stilbene trimer diglucosides; Benzoisofuranone derivative; 2D NMR; Antioxidant activity

1. Introduction

Fennel (*Foeniculum vulgare* Miller, Apiaceae) is a well-known Mediterranean aromatic plant, widespread in middle and southern Italy, which is used in traditional medicine and as spice. Herbal drug preparations, from numerous wild types, are active for dyspeptic complaints, bloating and flatulence (Forster et al., 1980). Diuretic, analgesic and antipyretic activity has also been found in the fennel fruit (Tanira et al., 1996) as well as antioxidant activity (Oktay et al., 2003). The leaves and fruit are mainly used to flavour fish and meat, giving them a strong aroma and taste, and as an ingredient in cosmetics. The most frequently investigated was the essential oil which showed antioxidant, antimicrobial and hepatoprotective activity (Ruberto et al., 2000; Ozbek et al., 2003). The chemical composition of the volatile oil fraction has been well described in the literature (Piccaglia and Marotti, 2001; Badoc et al., 1994; Damianova et al., 2004). Earlier investigation of *F. vulgare* fruit led to the isolation of phenolic components with antihypertensive activity (Ono et al., 1996; Nyemba et al., 1995).

The aim of this study was to determine the phytochemical composition of the non-volatile fraction of fennel fruit and evaluate antioxidant activity. In this report, we describe the isolation and structure elucidation of two new stilbene trimer diglucosides: foeniculosides X (**1**) and XI (**2**) and a new benzoisofuranone derivative (**5**) together with nine known compounds: *cis*-miyabenol C (**3**) (Ono et al., 1995), *trans*-miyabenol C (**4**) (Ono et al., 1995), *trans*-resveratrol 3-*O*-β-D-glucopyranoside (**6**) (Nyemba et al., 1995), sinapyl glucoside (**7**) (Della Greca et al., 1998), syringin 4-*O*-β-glucoside (**8**) (Park, 1996), oleanolic acid (**9**) (Seebacher et al., 2003), 7α-hydroxycampesterol (**10**) (Louter, 2004), (3β,5α,8α,22*E*) 5,8-epidioxy-ergosta-6,22-dien-3-ol (**11**) (Guyot and Durgeat, 1981), and 2,3-dihydropropylheptadec-5-enoate (**12**) (Coleman et al., 2004). The structural elucidation has been performed by spectral analysis, including various two-dimensional (2D)

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nuclear magnetic resonance (NMR) techniques and by chemical means. We also describe the antioxidant activity of crude extracts (CCl_4, $CHCl_3$, *n*-BuOH and the aqueous residue) and pure compounds **1**, **3**, **4**, **7** and **8**.

2. Results and discussion

The methanol extract of powdered fresh fruits of *F. vulgare* was subjected to Kupchan's partitioning methodology (Kupchan et al., 1973) to give four extracts: *n*-hexane, CCl_4, $CHCl_3$, *n*-BuOH and the aqueous residue. Crude extracts obtained were tested for their radical scavenging activity (Table 2).

The *n*-BuOH extract, after purification, yielded two new phenolic diglucosides **1** and **2**, the unusual benzoisofuranone derivative (**5**) and the known compounds: *cis*-miyabenol C (**3**), *trans*-miyabenol C (**4**) and *trans*-resveratrol 3-*O*-β-D-glucopyranoside (**6**).

The H_2O extract mainly contained sinapyl glucoside (**7**) and syringin 4-*O*-β-glucoside (**8**). Although it did not display antioxidant activity, the CCl_4 extract afforded oleanolic acid (**9**), 7α-hydroxycampesterol (**10**), (3β,5α,8α,22*E*) 5,8-epidioxy-ergosta-6,22-dien-3-ol (**11**), and a monoacylglycerol: 2,3-dihydropropylheptadec-5-enoate (**12**) by HPLC purification.

2.1. Foeniculoside X (*1*)

The molecular formula of foeniculoside X (**1**), was determined as $C_{54}H_{52}O_{19}$ by HRFAB-MS $[M+H]^+$ (*m*/*z* 1005.3221, calcd. 1005.3181) which was consistent with ^{13}C NMR and HSQC data. The FAB-MS (negative ion mode) gave a $[M-H]^-$ ion peak at *m*/*z* 1003 and a fragment ion peak at *m*/*z* 679 $[(M-H) - (\text{hexose} \times 2)]^-$. The NMR spectra (Table 1) suggested the presence of an oligostilbene as the basic skeleton such as *cis*-miyabenol C (**3**) and a cluster of signals typical of two monosaccharide units. On acid hydrolysis with 1 N HCl, **1** afforded glucose. The D-configuration of glucose was assigned as follows. After hydrolysis of **1**, the hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic samples (D-glucose and L-glucose) prepared in the same manner (Lee et al., 2000). The ^1H NMR spectrum showed two anomeric proton signals at δ_H 4.70 (*d*, $J = 7.6$ Hz) and 4.39 (*d*, $J = 7.7$ Hz) with a large vicinal coupling constants ($^3J_{H1,H2} = 7.2$–8.1 Hz) indicating the *trans*-diaxial orientation with respect to their coupling partners (β-configuration). Starting from the anomeric proton signals, the proton resonances of each sugar can be assigned by the aid of ^1H–^1H COSY, HSQC and by HMBC data. The low-field chemical shift of H_2-6 (δ_H 3.84 *dd* and 4.06 *dd*) in the ^1H NMR spectrum, suggests that the hydroxyl group at C-6 of glucose bears the second glucose (Glc I) unit. The interglycosidic linkage was determined by the HMBC correlation between δ_H 4.39 (H-1′ Glc I) and δ_C 68.6 (C-6 Glc). Comparison of ^1H and ^{13}C NMR signals of **1** with those of **3** showed downfield shifts of 10a-H, 12a-H, 12a-C, 14a-H and 14a-C of the aglycone moiety suggesting the glycosylation at position C-11a. Direct support of the attachment point of the disaccharide came from the results of an HMBC experiment which showed a correlation between the anomeric proton of glucose (δ_H 4.70) and C-11a of the aglycone (δ_C 160.2 ppm). In confirmation of the proposed structure, an enzymatic hydrolysis of **1** was also performed with the glycosidase mixture of *Charonia lampas*, giving compound **1a** which showed NMR data quite similar to those reported for the *cis*-miyabenol C (**3**). The *Z* double bond geometry was determined by ^1H NMR analysis of the coupling constants between H-7c (δ_H 5.66 *d*, $J = 12.2$ Hz) and H-8c (δ_H 5.81 *d*, $J = 12.2$ Hz). In the *E* stereoisomer the corresponding coupling constants have been reported as 16.5 Hz (Ono et al., 1995). The relative configuration of **1** was established by a ROESY experiment. The *trans*-orientation of H-7a and H-8a was deduced from an intense ROE between H-7a/H-14a (δ_H 5.21/ 5.96) and H-8a/ H-2(6)a (δ_H 4.11/7.08) indicating a *trans*-2,3-diaryl-2,3-dihydrobenzofuran system. A similar relationship was revealed by ROE observed between H-7b/H-14b (δ_H 5.23/ 6.10) and H-8b/ H-2(6)b (δ_H 3.76/ 6.33) that established the *trans*-orientation of the two aryl groups in position 7b and 8b. An additional ROE between H-2(6)c/H-14b (δ_H 6.61/6.10) and between H-8a/ H-8c (δ_H 4.11/5.81) indicated the spatial proximity of these protons. The spatial relationship among aromatic rings and the disaccharide moiety was also determined by cross-peak correlations observed between H-1 of Glucose (δ_H 4.70) and H-3 (δ_H 3.45), H-5 (δ_H 3.46), H-10a (δ_H 5.90), H-12a (δ_H 6.38). The second glucose unit (Glc I) showed ROEs between H-1′(δ_H 4.39) and H-3′, H-5′ and H-6 (Glc, δ_H 3.84). These results indicated that the relative configuration of **1** is *rel*-(7a*R*, 8a*R*, 7b*S*, 8b*S*) The structure of foeniculoside X (**1**) was concluded to be *cis*-miyabenol C 11a-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside Fig. 1.

2.2. Foeniculoside XI (*2*)

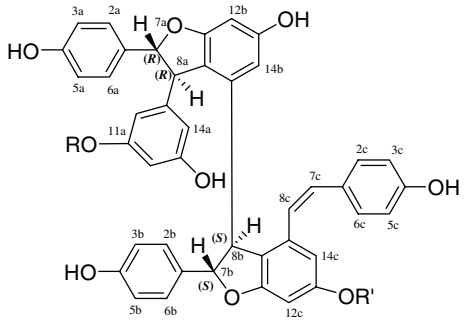
The molecular formula of foeniculoside XI (**2**), was derived as $C_{54}H_{52}O_{19}$ by HRFAB-MS. The FAB-MS (negative ion mode) gave a $[M-H]^-$ ion peak at *m*/*z* 1003 and two fragment ion peaks at *m*/*z* 841 $[M-H-\text{hexose}]^-$ and *m*/*z* 679 $[(M-H)-(\text{hexose} \times 2)]^-$ ^1H and ^{13}C spectra showed aromatic signals quite similar to compound **1** and characteristic proton and carbon resonances for two pyranose units, both identified as D-glucose after acid hydrolysis of **2** and GLC analysis on a chiral column. From these data, **2** was considered as an isomer of **1** with an oligostilbene skeleton (Table 1). An accurate analysis of ^1H–^1H COSY and HSQC data revealed significant differences in the chemical shifts of positions 7b, 8b and 12c, 14c with respect to *cis*-miyabenol C (**3**). In addition, the resonances of protons 10a and 12a were slightly shifted with respect to foeniculoside X (**1**). Among the correlation peaks observed

Table 1
^{1}H and ^{13}C NMR (CD_3OD, 500 and 125 MHz) data of compounds **1**, **2** and **3**

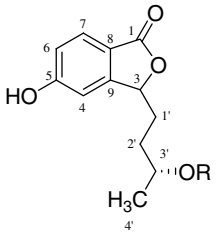
	Foeniculoside X (**1**)		Foeniculoside XI (**2**)		*cis*-Miyabenol C (**3**)	
Position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1a	–	135.1	–	135.3	–	135.6
2(6)a	7.08 *d* (8.5)	127.1	7.07 *d* (8.5)	127.2	7.05 *d* (8.5)	127.6
3(5)a	6.82 *d* (8.5)	116.0	6.80 *d* (8.5)	116.1	6.77 *d* (8.5)	116.3
4a	–	158.2	–	158.7	–	158.5
7a	5.21 *d* (3.0)	93.8	5.25 *d* (3.0)	93.9	5.23 *d* (2.6)	94.2
8a	4.11 *d* (3.0)	57.2	4.10 *d* (3.0)	57.0	4.15 *d* (2.6)	57.6
9a	–	148.6	–	148.5	–	144.3
10a	5.90 *s*	106.8	5.91 *s*	107.1	5.84 *d* (2.0)	107.2
11a	–	160.2	–	160.5	–	160.6
12a	6.38 *t* (2.0)	103.4	6.33 *t* (2.0)	103.3	6.08 *d* (2.0)	102.3
13a	–	159.3	–	159.1	–	160.6
14a	5.96 *s*	109.2	5.92 *s*	108.8	5.84 *d* (2.0)	106.9
1b	–	133.9	–	134.1	–	134.1
2(6)b	6.33 *d* (8.4)	127.2	6.27 *d* (8.4)	127.2	6.34 *d* (8.5)	127.3
3(5)b	6.50 *d* (8.4)	116.0	6.50 *d* (8.4)	116.0	6.47 *d* (8.5)	116.0
4b	–	156.8	–	157.5	–	157.8
7b	5.23 *d* (2.3)	92.8	5.17 *d* (2.3)	92.7	5.23 *d* (2.6)	93.0
8b	3.76 *d* (2.3)	53.0	3.77 *d* (2.3)	53.0	3.81 *d* (2.3)	52.8
9b	–	143.7	–	144.4	–	144.4
10b	–	119.8	–	120.3	–	120.2
11b	–	162.1	–	162.4	–	162.6
12b	6.23 *d* (2.0)	95.0	6.25 *s*	96.3	6.21 *d* (1.8)	96.3
13b	–	159.6	–	160.3	–	159.7
14b	6.10 *d* (2.0)	107.4	6.09 *s*	108.6	6.08 *d* (1.8)	107.7
1c	–	125.6	–	127.8	–	128.4
2(6)c	6.61 *d* (8.6)	131.0	6.59 *d* (8.6)	131.0	6.63 *d* (8.5)	131.2
3(5)c	6.44 *d* (8.6)	115.8	6.44 *d* (8.6)	115.8	6.43 *d* (8.5)	115.9
4c	–	157.4	–	158.2	–	158.0
7c	5.66 *d* (12.2)	131.0	5.68 *d* (12.2)	131.4	5.72 *d* (12.0)	131.8
8c	5.81 *d* (12.2)	126.0	5.80 *d* (12.2)	125.6	5.81 *d* (12.0)	126.0
9c	–	137.6	–	137.0	–	138.0
10c	–	121.8	–	122.0	–	119.8
11c	–	160.3	–	161.9	–	162.8
12c	6.24 *d* (1.8)	95.5	6.58 *s*	97.3	6.25 *d* (1.8)	96.8
13c	–	159.6	–	161.6	–	159.9
14c	6.07 *d* (1.8)	108.0	6.30 *s*	108.5	6.05 *d* (1.8)	107.5
	Glc		**Glc**			
1	4.70 *d* (7.6)	101.4	4.71 *d* (7.8)	101.1		
2	3.46	74.7	3.43	74.6		
3	3.45	77.4	3.49	77.6		
4	3.55 *t* (8.9)	70.6	3.47	70.8		
5	3.46	76.5	3.31	77.7		
6	3.84 *dd* (4.3, 11.4) 4.06 *dd* (11.4)	68.6	3.90, 3.77	61.9		
	Glc I		Glc II			
1′	4.39 *d* (7.7)	104.5	4.73 *d* (7.8)	102.4		
2′	3.25	75.0	3.42	74.6		
3′	3.31	77.5	3.44	77.7		
4′	3.35	71.2	3.48	70.8		
5′	3.25	77.7	3.36	77.4		
6′	3.68 *dd* (5.9-11.9) 3.87 *dd* (2.3-11.9)	62.4	3.82, 3.75	61.9		

in the HMBC spectrum, the most important for the location of the glucose units were detected between H-1 of Glc (δ_H 4.71) and C-11a (160.5 ppm) and between H-1 of Glc II (δ_H 4.73) and C-13c (161.6 ppm). The C-13c glycosidic linkage was also revealed by the downfield shifts of H-12c, H-14c and C-13c. Further information have been obtained by a ROESY experiment which showed dipolar correlations between H-1 (Glc) and H-10a and between H-1′ (Glc II) with H-12c. The relative configuration of **2** was suggested by ROE data, as reported above for Foeniculoside X (**1**).

Therefore, the structure of foeniculoside XI (**2**) was proposed as *cis*-miyabenol C 11a,13c-di-*O*-β-D-glucopyranoside.



		1→6	
Foeniculoside X (**1**)	R=Glc I	→ Glc	R'=H
1a	R=H		R'=H
Foeniculoside XI (**2**)	R=Glc		R'=Glc II
cis-Miyabenol C (**3**)	R=H		R'=H



Compound **5**	R = H
Compound **5a**	R = (*R*)-MTPA
Compound **5b**	R = (*S*)-MTPA

Fig. 1. New compounds isolated from the seeds of *Foeniculum vulgare*.

2.3. (3′R)-5-Hydroxy-3-(3′-hydroxybutyl)-isobenzofuran-1(3H)-one (5)

The molecular formula of compound **5** was deduced from its positive ion FAB-MS as $C_{12}H_{14}O_4$ which showed a $[M + Na]^+$ ion at *m/z* 245 and by HRFAB-MS data. The IR spectrum displayed absorptions at 1671 cm^{-1} due to a lactone ring.

The ^1H NMR spectrum showed two one-proton doublets at δ_H 7.65 ($J = 8.4$ Hz) and 6.87 ($J = 1.7$ Hz) and a one-proton double doublet at δ_H 6.93 ($J = 8.4$, 1.7 Hz), suggesting a typical 1,2,4-trisubstituted aromatic ring. The ^{13}C NMR data indicated the presence of 12 carbons (one methyl, two methylenes, five methines, three quaternary and one carbonyl carbons) by an HSQC experiment. Inspection of the COSY spectrum allowed us to detect two distinct spin systems, one of them belonging to the aromatic moiety and the second relative to an oxygenated chain. The data arising from the HMBC experiment were used to interconnect the partial structures. When the signal of the methyl protons (δ_H 1.17) was used as a starting point, a sequence with a oxygenated methylene proton (δ_H 3.75) and then with two methylene protons (H_2-2′ and H_2-3′) was identified. The last methylene protons (δ_H 2.22–1.72) showed in turn a cross-peak with a multiplet at δ_H 5.47 that, in the HSQC spectrum, correlated with the carbon signal at δ_C 82.3. The location of the oxygenated chain was determined by the aid of the HMBC experiment where H-3 (δ_H 5.47) showed correlations with C-9, C-1, C-1′ and C-2′. These data were also indicative of the iso-benzofuranone moiety and ^3J and ^2J correlations were observed for H-7 (δ_H 7.65) with C-1 and C-9, respectively (Section 3). The absolute configuration at C-3′ was determined to be *R* by a modified Mosher's method after esterification with MTPA [α-methoxy-α-(trifluoro-methyl)-phenyl acetate] (Ohtan et al., 1991). $\Delta\delta S$–$\Delta\delta R$ values in Hz are shown in Fig. 2. This finding suggested that the structure of **5** was (3′*R*)-5-hydroxy-3-(3′-hydroxybutyl)-isobenzofuran- 1(3H)-one.

2.4. Antioxidant activity

We have investigated the antioxidant activity of crude extracts and of pure compounds **1**, **3**, **4**, **7** and **8**. The total polyphenolic content of crude extracts (CCl_4, $CHCl_3$, *n*-BuOH and the aqueous residue) obtained from fresh fruit of *F. vulgare*, ranged from 10.3 and 14.4 mg/g, respectively for $CHCl_3$ and CCl_4, to 103 mg/g for *n*-BuOH extracts. The results are shown in Table 2. The *n*-BuOH extract showed a moderate activity in the lipid peroxidation assay but exclusively at the higher tested concentration: 10^{-5} M (with values eight times higher than quercetin used as standard). Compound **3** showed limited antioxidant capacity at 10^{-6} M which increased at 10^{-5} M (from six to three times that of the standard). Compound **4**, exhibited moderate activity at low concentrations, when tested toward lipid peroxidation. Among the extracts tested for radical scavenging activity, the aqueous residue also exhibited some activity.

In general, pure compounds showed higher antioxidant activity then the crude extracts, but were weaker than the reference compound, i.e. quercetin.

Therefore, our results do not reveal strong antioxidant activities of isolated *F. vulgaris* components, in particular as related to the radical scavenging activity of polar (water and ethanol) extracts. In turn, even though we fractionated and isolated compounds of novel structure from *F. vulgare*, none of the isolated compounds appear to be particularly exploitable from the antioxidant point of view.

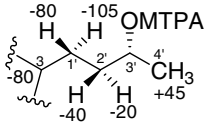


Fig. 2. Results of the modified Mosher's method for **5**. The $\Delta\delta$ values are in Hz (δS–δR, 500 MHz).

Table 2
Antioxidant activity tests of crude extracts and pure compounds **1**, **3**, **4**, **7** and **8**

Sample	Polyphenols (mg/g extract)	TBARS (nmol TBARS/mg LDL)		Antioxidant capacity (mEq. Uric acid)		DPPH (EC_{50})
		10^{-6} M	10^{-5} M	10^{-6} M	10^{-5} M	
Quercetin	n.a.	3.85 ± 0.5	0.75 ± 0.04	0.75 ± 0.06	2.17 ± 1.35	4.37×10^{-6} M
CCl_4 extract	14.4					
$CHCl_3$ extract	10.3					
n-BuOH extract	103.13	50.53	7.16	0.045	0.29	4.36×10^{-5} M
Foeniculoside X (**1**)		18.80	6.30	0.021	0.19	3.87×10^{-5} M
cis-Miyabenol (**3**)		70.11	17.52	0.12	0.65	2.56×10^{-5} M
trans-Miyabenol (**4**)		10.79	5.24	0.056	0.51	7.08×10^{-5} M
Aqueous extract	21.74	73.54	13.46	0.039	0.27	1.45×10^{-5} M
Synapil glucoside (**7**)		54.59	54.49	0.042	0.27	4.17×10^{-5} M
Syringin 4-*O*-β-glucoside (**8**)		53.95	52.35	0.01	0.05	3.78×10^{-5} M

3. Experimental

3.1. General experimental procedures

Fast atom bombardment mass spectrometry (FAB-MS), electron ionization mass spectrometry (EI-MS) high-resolution (HR) FAB-MS were recorded on a Fisons VG Prospec instrument. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. ^1H and ^{13}C NMR spectra were determined on a Varian Unity INOVA spectrometer at 500.13 and 125.77 MHz, respectively, equipped with an indirect detection probe. Chemical shifts were referenced to the solvent signals: deuterated methanol (CD_3OD) and deuterated chloroform ($CHCl_3$). Residual CHD_2OD: δ_H 3.31, δ_C 49.0; residual $CHCl_3$: δ_H 7.26, δ_C 77.0.

The Heteronuclear Single-Quantum Coherence (HSQC) spectra were optimized for an average $^1J_{CH}$ of 140 Hz; the gradient-enhanced Heteronuclear Multiple Bond Correlation (HMBC) experiment were optimized for a $^3J_{CH}$ of 8 Hz. Nuclear Overhauser Effect (NOE) measurements were performed by 2D ROESY experiment (Kessler et al., 1987). GC analyses were performed on a L-Chirasil-Val column (0.32 mm × 25 m). Droplet counter-current chromatography (DCCC) was performed on a DCC-A apparatus (Tokyo Rikakikai Co., Tokyo-Japan) equipped with 250 glass-columns. HPLC was performed using a Waters 510 pump equipped with a Waters U6K injector and a Waters 401 differential refractometer as detector, using a 30 cm × 3.9 mm; i.d., C_{18} μ-Bondapak (Waters, Milford, MA, USA) columns; flow rate was 1 ml min^{-1} and a 150 mm × 4.60 mm i.d, 3 μ, Luna C-18 (Phenomenex, Torrance, CA, USA).

3.2. Plant material

F. vulgare Miller (Apiaceae) was harvested in September 2005 in the mountainous northern part of Campobasso (Italy). Plants were identified in the Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio (University of Molise) and a voucher specimen is deposited under No. FV-05127 in the Herbarium of University of Molise (Pesche, Isernia).

3.3. Extraction and isolation

The powdered, fresh fruit (225 g) was freeze-dried immediately and then extracted in MeOH at room temperature (1.5 l). Evaporation of MeOH extracts afforded 18 g of a glassy material, which was then subjected to Kupchan's partitioning methodology (Kupchan et al., 1973) as follows. The methanol extract was dissolved in 10% aqueous methanol and partitioned against *n*-hexane. The water content (% v/v) of the MeOH was adjusted to 20% and 40% and partitioned against CCl_4 and $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. Four extracts were obtained: *n*-hexane (3.8 g), CCl_4 (3.2 g), $CHCl_3$ (2.8 g), *n*-BuOH (2.7 g) and an aqueous residue (3.5 g) which were tested for antioxidant activity (Table 2).

The CCl_4 extract was loaded onto an MPLC silica gel column (150 g, 3.5 × 45 cm), using a solvent step gradient system *n*-hexane–EtOAc (95:5–30:70), giving three fractions. Fractions were combined and examined by TLC on silica gel plates with $CHCl_3$–MeOH–H_2O (80:18:2) as eluent. Fraction 1 (*n*-hexane–EtOAc 75:25), fraction 2 (*n*-hexane–EtOAc 7:3) and fraction 3 (*n*-hexane–EtOAc 55:45) were purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm; i.d) with MeOH–H_2O (85:15) to yield compound **9** (7.3 mg; from 1) compound **11** and **12** (0.9 mg and 1.2, respectively; from 2) and compound **10** (0.7 mg, from 3).

The *n*-BuOH extract was submitted to DCCC with *n*-BuOH–Me_2CO–H_2O (3:1:5) in the descending mode (the upper phase was the stationary phase), to give six main fractions. The obtained fractions were monitored by TLC on silica gel plates with *n*-BuOH–HOAc–H_2O (12:3:5) and $CHCl_3$–MeOH–H_2O (80:18:2). Fractions obtained were then separated by HPLC (C_{18} μ-Bondapak column; 30 cm × 3.9 mm; i.d). Fraction 2 contained compound **8** (4.5 mg) and was purified by HPLC with MeOH–H_2O

(2:8) as eluent; fraction 3 contained compound **2** (1.2 mg) and **6** (1.6 mg) and fraction 4 contained compound **5** (0.9 mg) and **1** (1.8 mg) and were both purified by HPLC with MeOH–H_2O (35:65). Fraction 5 contained compound **4** (1.4 mg) and **3** (4.6 mg) and was processed by HPLC with a Luna C_{18} column MeOH–H_2O (45:55).

The aqueous residue (3.5 g) was chromatographed on a Amberlite XAD-7, using stepwise elution, H_2O 100%, MeOH–H_2O (1:1) and MeOH 100% to give three fractions. Fractions 2 and 3 were purified by HPLC on a C_{18} μ-Bondapak column with MeOH–H_2O (2:8) to yield compound **7** (5.1 mg) and compound **8** (2.5 mg).

3.3.1. Foeniculoside X (*1*)

Yield: 1.8 mg. $[\alpha]_D^{25}$ +40° (MeOH, *c* 0.1). FAB-MS and HRFAB-MS are given in the text. ^1H and ^{13}C NMR are given in Table 1.

3.3.2. Enzymatic hydrolysis of *1* to give *1a*

Compound **1** (1.0 mg) in a citrate buffer (1 ml; pH 4.5) was incubated with a glycosidase mixture (3.5 mg) of *Charonia lampas* (Shikagaku Kogyo, CO. LTD, Tokyo, Japan) at 37 °C. After 3 days, the TLC analysis showed that the starting material had disappeared and was replaced by one major spot. The mixture was passed through a C-18 Sep-Pak cartridge, washed with H_2O and eluted with MeOH. The MeOH was evaporated to dryness, and the residue was submitted to HPLC on the Luna C_{18} column in MeOH–H_2O (45:55) to give compound **1a**; FAB-MS (negative ion mode) *m/z* 679 $[M-H]^-$.

3.3.3. Foeniculoside XI (*2*)

Yield: 1.2 mg. $[\alpha]_D^{25}$ +42.7° (MeOH, *c* 0.1). FAB-MS are given in the text. HRFAB-MS *m/z* 1005.3226 $[M+H]^+$ (calcd. for $C_{54}H_{53}O_{19}$ 1005.3181). ^1H and ^{13}C NMR are given in Table 1.

3.3.4. Acid hydrolysis of *1* and *2*

A solution (0.5 mg each) of **1** and **2**, in 1 N HCl (0.25 ml) was stirred at 80° C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethyl-silyl)-imidazole and pyridine (0.1 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue was separated by water and CH_2Cl_2 (1 ml, v:v = 1:1). The CH_2Cl_2 layer was analyzed by GC using a L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven; the initial temperature was maintained at 100 °C for 1 min and then raised to 180 °C at the rate of 5 °C/min. The peak of the hydrolysate of **1** was detected at 14.71 min and the peak of the hydrolysate of **2** was detected at 14.70 min. Retention times for authentic samples after being treated simultaneously with 1-(trimethyl-silyl)-imidazole in pyridine gave single peaks at 14.72 min for D-glucose and 14.67 min for L-glucose. Co-injection of the hydrolysate **1** and hydrolysate **2** with the authentic silated D-glucose gave single peak at 14.73 min and 14.71 min, respectively.

3.3.5. (3′*R*)-5-Hydroxy-3-(3′-hydroxybutyl)-isobenzofuran-1(3*H*)-one (*5*)

Yield: 0.9 mg. $[\alpha]_D^{25}$ −8.89° (MeOH, *c* 0.09). $\upsilon_{max}^{CHCl_3}$ cm^{-1}: 3404, 2950, 1671, 1615. HRFAB-MS *m/z* 245.0832 $[M+Na]^+$ (calcd. for $C_{12}H_{14}O_4Na$: 245.0790). ^1H NMR (500 MHz, CD_3OD) δ_H (ppm): 7.65 (1H, *d*, *J* = 8.4 Hz, H-7), 6.93 (1H, *dd*, *J* = 1.7, 8.4 Hz, H-6), 6.87 (1H, *d*, *J* = 1.7 Hz, H-4), 5.47 (1H, *dd*, *J* = 3.9, 7.5 Hz, H-3), 3.75 (1H, *m*, H-3′), 2.22 (1H, *m*, H-1′), 1.72 (1H, *m*, H-1′), 1.57 (1H, *m*, H-2′), 1.47 (1H, *m*, H-2′), 1.17 (3H, *d*, *J* = 6.2 Hz, H_3-4′). ^{13}C NMR (125 MHz, CD_3OD) δ_H (ppm): 172.9 (C-1), 166.8 (C-5), 154.8 (C-9), 127.9 (C-7), 119.0 (C-6), 116.5 (C-8), 109.3 (C-4), 82.5 (C-3), 68.2 (C-3′), 35.0 (C-2′), 32.1 (C-1′), 23.5 (C-4′).

3.3.6. Preparation of (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (MTPA) esters (*5a*, *5b*) from *5*

A solution of **5** (1 mg) in 1 ml of dry CH_2Cl_2 was reacted with (+)-MTPA-Cl (5 μl) in the presence of triethylamine (10 μl) and a catalytic amount of 4-*N*,*N*′-(dimethylamino)-pyridine (DMAP), and the mixture was stirred at 25 °C for 30 min. After the solvent was removed, the mixture was purified by Si gel column chromatography performed in a Pasteur pipet filled with a slurry of Si gel using $CHCl_3$ as the eluent to afford **5a**. Compound **5b** was then prepared through a similar procedure from **5** (1 mg) using (−)-MTPA-Cl (5 μl), triethylamine (10 μl) and DMAP.

3.3.7. 3′-(*R*)-MTPA ester (*5a*)

FAB-MS (positive ion) *m/z* 461 $[M+Na]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ_H (ppm) 7.31–7.58 (5H, aromatic protons), 5.62 (1H, *dd*, *J* = 3.9, 7.5 Hz, H-3), 5.22 (1H, *m*, H-3′), 2.25 (1H, *m*, H-1′), 1.88 (1H, *m*, H-2′), 1.80 (1H, *m*, H-2′), 1.79 (1H, *m*, H-1′), 1.30 (3H, *d*, *J* = 6.2 Hz, H_3-4′).

3.3.8. 3′-(*S*)-MTPA ester (*5b*)

FAB-MS (negative ion) *m/z* 437 $[M]^-$. ^1H NMR (500 MHz, $CDCl_3$) δ_H (ppm) 7.30–7.58 (5H, aromatic protons), 5.46 (1H, *dd*, *J* = 3.9, 7.5 Hz, H-3), 5.19 (1H, *m*, H-3′), 2.04 (1H, *m*, H-1′), 1.80 (1H, *m*, H-2′), 1.76 (1H, *m*, H-2′), 1.63 (1H, *m*, H-1′), 1.39 (3H, *d*, *J* = 6.2 Hz, H_3-4′).

3.4. Antioxidant activity tests

3.4.1. Determination of polyphenolic content

The total polyphenolic content of the extracts was determined colorimetrically by the Folin–Ciocalteau method, using gallic acid as the reference compound (Visioli et al., 1995). Consequently, molarity in this paper is referred to as gallic acid equivalents.

3.4.2. DPPH scavenging test

Extract and pure compounds, in concentration ranging from 5×10^{-6} to 5×10^{-4} M, were added to a 15 μM ethanol solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). After 15 min of incubation in the dark, the absorbance was read at 517 nm (Visioli and Galli, 1998). The EC_{50} was calculated by employing Prism® 4 (GraphPad Software Inc.).

3.4.3. Total antioxidant capacity

The total antioxidant capacity of the extracts and the pure compounds (at concentrations of 10^{-5} and 10^{-6} M) was evaluated by a validated assay based upon the reduction of Cu^{2+} to Cu^{+} and its subsequent chelation by bathocuproine (BIOXYTECH® AOP-490™, OxisResearch™, Portland, OR) (Visioli et al., 2001). The results are shown as mEq uric acid, i.e. the reference compound.

3.4.4. Isolation of human low density lipoprotein (LDL)

LDL was isolated from plasma of healthy volunteers by sequential ultracentrifugation (Havel et al., 1995).

Total protein was estimated by the Bradford method, with bovine serum albumin as the standard (Bradford, 1976). For the experiments, LDL was diluted to a concentration of 200 μg protein/ml in PBS 10 mM.

3.4.5. Assay of lipid peroxidation

The thiobarbituric acid-reactive substance (TBARS) content of lipoproteins was employed as a measure of lipid peroxidation. Briefly, 0.5 ml of sample containing 100 μg lipoprotein were added with 10^{-5} M and 10^{-6} M concentrations of the extracts or the pure compounds and were incubated for 15 min at 37 °C. LDL oxidation was triggered by the addition of $CuSO_4$ (5 μM, final concentration) and samples were incubated at 37 °C for 3 h (Perugini et al., 2000). Samples (300 μl) were then analyzed by the addition of 600 μl thiobarbituric acid reagent (0.375 g thiobarbituric acid, 2.08 ml 12 N HCl, 15 ml trichloroacetic acid 100% and distilled water to a final volume of 100 ml) (Buege and Aust, 1978). After heating at 100 °C for 15 min, the samples were cooled to room temperature and centrifuged at 10,000 *g* for 10 min. The clear supernatants were analysed spectrophotometrically at 532 nm and the results are shown as nanomoles of thiobarbituric acid-reactive substances/mg of LDL protein (Balla et al., 1991).

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