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Bioguided Isolation and Identification of the Nonvolatile Antioxidant Compounds from Fennel (*Foeniculum vulgare* Mill.) Waste

IRENE PAREJO,[†] FRANCESC VILADOMAT,[†] JAUME BASTIDA,[†] GUILLERMO SCHMEDA-HIRSCHMANN,[‡] JESÚS BURILLO,[§] AND CARLES CODINA*,[†]

Departament de Productes Naturals, Biologia Vegetal i Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII, s/n, 08028 Barcelona, Catalunya, Spain, Instituto de Química de Recursos Naturales, Universidad de Talca, Casilla 747, Talca, Chile, and Servicio de Investigaciones Agroalimentarias, Avda. Montañana 176, 50016 Zaragoza, Spain

A bioguided isolation of an aqueous extract of fennel waste led to the isolation of 12 major phenolic compounds. Liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (LC/UV/APCI-MS) combined with spectroscopic methods (NMR) was used for compound identification. Radical scavenging activity was tested using three methods: DPPH•, superoxide nitroblue tetrazolium hypoxanthine/xanthine oxidase, and •OH/luminol chemiluminescence. In addition to products described in the literature, eight antioxidant compounds were isolated and identified for the first time in fennel: 3-caffeoylquinic acid, 4-caffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, rosmarinic acid, eriodictyol-7-*O*-rutinoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside. The structures of eriodictyol-7-*O*-rutinoside and quercetin-3-*O*-glucuronide were completely elucidated by two-dimensional NMR experiments. The isolated compounds exhibited a strong antiradical scavenging activity, which may contribute to the interpretation of the pharmacological effects of fennel.

KEYWORDS: ***Foeniculum vulgare*; bitter fennel; Apiaceae; radical scavenging activity; bioguided isolation; phenolic compounds; LC/APCI-MS**

INTRODUCTION

Antioxidants have been used as food additives to avoid degradation. Moreover, it has been widely reported that they have an important role in the prevention of aging and diseases such as cancer, atherosclerosis, and Alzheimer's, which are closely related with the production of reactive oxygen and nitrogen species (*1*). Currently, there is considerable interest in new natural antioxidants to replace synthetic ones such as BHA and butylated hydroxytoluene, because of their possible activity as promoters of carcinogenesis (*2*). Consequently, in recent years, there has been much interest in the antioxidant activity of naturally occurring substances (*3*, *4*).

Bitter fennel (*Foeniculum vulgare* Mill.) is a well-known Mediterranean aromatic plant, which has long been considered as a medicinal and spice herb. Fennel and its herbal drug preparations are used for dyspeptic problems such as mild, spasmodic gastrointestinal complaints, bloating, and flatulence (*5*). The fennel fruit has also been found to be active as a diuretic, analgesic, and antipyretic (*6*), as well as to possess antioxidant activity (*7*). The chemical composition of the essential oil of fennel and of the volatile fraction of different parts of the plant has already been described in the literature (*8*). However, the occurrence of other nonvolatile compounds, such as phenolic acids and flavonoids, has not received much attention. Thus, only some aglycones (*9*) and flavonoid glycosides (*10*−*12*) have been hitherto reported in bitter fennel, whereas chlorogenic acid (*5*), hydroxybenzoic acid derivatives (*13*), flavonoid glycosides (*5*, *12*), and some coumarins (*14*), have been identified in sweet fennel.

Although essential oils of fennel have been investigated for their antioxidant, antimicrobial, and hepatoprotective activity (*15*, *16*), very little is known about the possible presence of antioxidants in polar extracts from this plant. In a previous screening of herbs as a potential source of natural antioxidants, we found fennel to show good radical scavenging activity, especially in the waste obtained after distillation for essential oils (*17*).

The aim of this study was to determine the phenolic composition of the residue of flowering aerial parts of bitter fennel resulting from its distillation for essential oils and to evaluate the use of this waste as a potential source of natural antioxidants or antioxidant plant extracts for industrial application. To our knowledge, no information on the chemical

* To whom correspondence should be addressed. Tel: +34-93-4024493. Fax: +34-93-4029043. E-mail: ccodina@farmacia.far.ub.es.
[†] Universitat de Barcelona.
[‡] Universidad de Talca.
[§] Servicio de Investigaciones Agroalimentarias.

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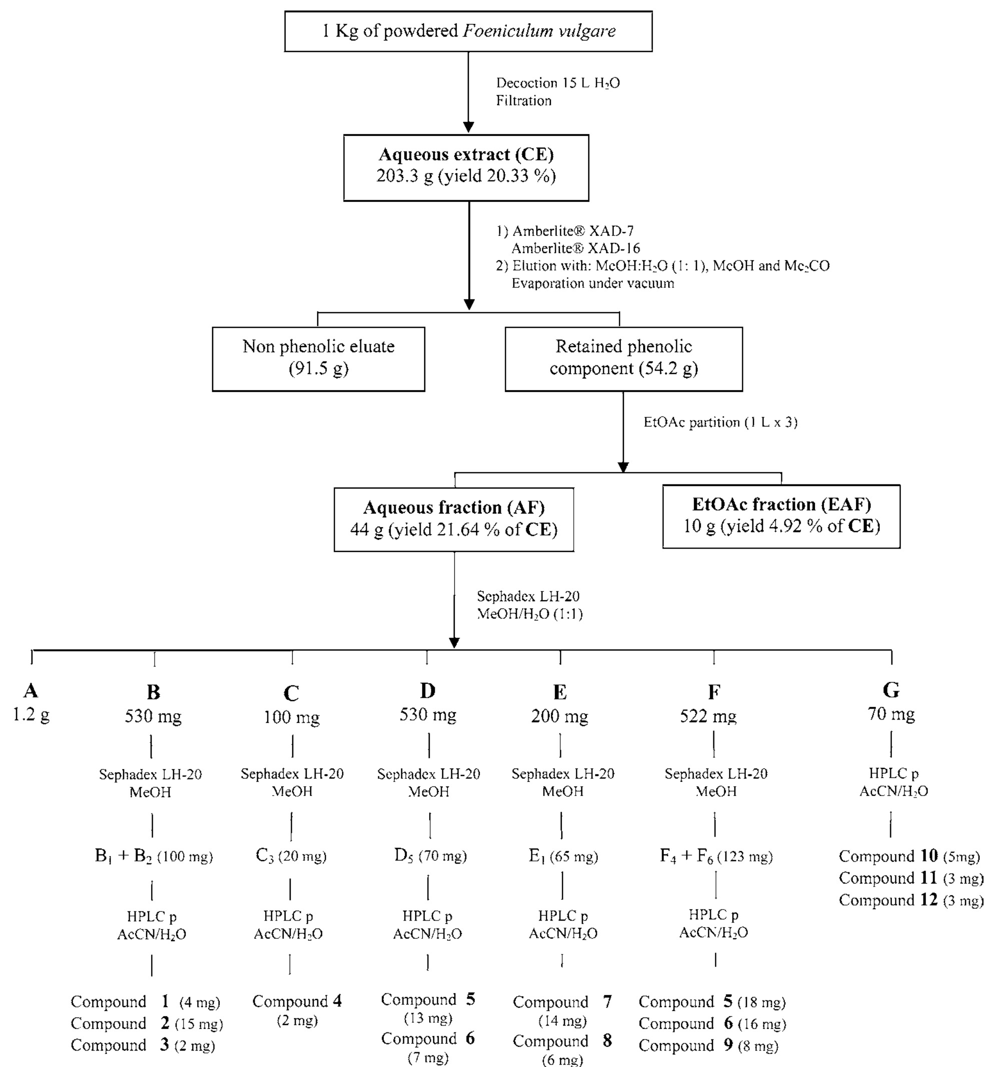

Figure 1. Scheme of the extraction and bioguided fractionation of fennel waste.

composition of this waste has been hitherto reported. The raw material was subjected to a bioguided fractionation process to isolate and identify the main antioxidant nonvolatile components of this waste. Three assays were used to evaluate the radical scavenging activity of the fractions and subfractions, as well as of the isolated compounds: the DPPH• free radical, superoxide nitro-blue tetrazolium (NBT) hypoxanthine/xanthine oxidase, and •OH/luminol CL scavenging. The TPH of the fractions was also determined by the Folin−Ciocalteu method. The identification of the phenolic compounds was performed by LC/APCI-MS and ^{1}H NMR and ^{13}C NMR spectroscopic methods, through interpretation and comparison of their spectra with those reported in the literature.

MATERIALS AND METHODS

Plant Material. Plants of *F. vulgare* Mill. var. *vulgare* (Apiaceae) were collected during the flowering period from cultures established in an experimental plot (Cetina, Zaragoza, Spain) under agronomically controlled conditions. The fresh plant material consisted of leaves, stems, and floral structures and was first extracted for essential oils by steam distillation at an industrial level following a Spanish protocol of the Ministerio de Agricultura, Pesca y Alimentación (200 L extractor, 0.5 kg/cm^{-2}, 15−20 min), registered with no. 50-41.482. After distillation, the residual material was air-dried and then powdered with a mill.

Chemicals. All chemicals and reagents used for TPH and radical scavenging assays were purchased from Sigma Aldrich (St. Louis, MO), with the exception of Folin−Ciocalteu's reagent, which was purchased from Panreac (Barcelona, Spain). All of the chemicals and reagents were of analytical grade. High-performance liquid chromatography (HPLC) grade acetonitrile (SDS, Peypin, France), analytical grade formic acid (Probus, Badalona, Spain), and ultrapure water (Milli-Q, Waters, Milford, U.S.A.) were used for mobile phase preparation in the HPLC analysis.

Extraction and Bioguided Fractionation. One kilogram (dry weight) of fennel waste was boiled in water (15 L × 2) for 15 min. The combined aqueous extracts (18 L) were allowed to cool and then filtered through a filter paper. An aliquot of 200 mL was freeze-dried for extraction yield (% w/w) calculation. The decoction was chromatographed by consecutive passages of the aqueous extract through two 70 cm × 5 cm Amberlite columns packed with XAD-7 and XAD-16. An aliquot of the volume nonretained in the columns (16.5 L) was freeze-dried to estimate the yield of the extraction and to test it for the antiradical activity. The phenolic compounds were retained in the columns and then eluted with methanol:water 1:1 (2 L), methanol (4 L), and finally acetone (3 L) until the eluate was colorless. All of the eluates were combined and concentrated under vacuum to dryness affording 54 g of a crude extract (CE). This CE was then redissolved in water (1 L) and partitioned with ethyl acetate (1 L × 3). The ethyl acetate fraction was dried under vacuum (EAF, 10 g), and the water fraction was freeze-dried (AF, 44 g). The yields of this extraction process (expressed as % w/w of CE) are shown in **Figure 1**. An aliquot (10 g) of the freeze-dried water fraction (AF) was dissolved in MeOH:H_2O (1:1) and fractionated by gel filtration on a 50 cm × 5 cm column of Sephadex LH-20 (Pharmacia), eluted at 1 mL/min with MeOH:H_2O (1:1) to afford 96 fractions (10 mL each). The fractions were monitored

by thin-layer chromatography (TLC) on 10 cm × 10 cm Alugram silica gel plates (Macherey-Nagel) developed with EtOAc:AcOH:H_2O (10:2:3). After the plates were developed and dried, the plates were sprayed with 1% diphenylboric acid in methanol for UV enhancement of phenolic compounds and visualized under UV light at 254 and 365 nm. In addition, other TLC plates were sprayed with a DPPH$^•$ methanolic solution (20 g/L) and examined 20 min after spraying. Active compounds appeared as yellow spots against a purple background. Fractions were combined to obtain seven active fractions (from A to G). The same TLC systems were used for the monitoring of the subfractions obtained in further fractionations. The fractions A−G obtained were submitted to LC/UV/APCI-MS and subsequent bioguided isolation of the antioxidant compounds. Then, fractions from B to F were further purified on a 50 cm × 2 cm Sephadex LH-20 column eluted with MeOH at 0.5 mL/min, and fractions of 2 mL were collected. Eluents were combined on the basis of the TLC behavior as described above, and the most active subfractions obtained were then subjected to purification by semipreparative HPLC to yield the major antioxidant constituents. Fraction G was directly purified by semipreparative HPLC (**Figure 1**).

Qualitative Analyses by LC/UV/APCI-MS. Experiments were performed with a C_{18} 250 mm × 4 mm i.d., 5 μm, Nucleosil 120 column (Tecknokroma) on a Waters 2690 chromatograph (Alliance), equipped with an autosampler and photodiode array detector and coupled with a VG Platform quadropole spectrometer (Fisions Instruments, U.K.) equipped with an APCI source, with full scan acquisition. Data acquisition, processing, and instrument control were performed using MassLynx software. The gradient elution was performed with water−0.1% formic acid (solvent A) and acetonitrile−0.1% formic acid (solvent B), at a constant flow rate of 1 mL/min, using an increasing linear gradient of solvent B [*t*(min), % B]: (0, 5), (10, 15), (45, 30), (50, 100), and (55, 100). The probe and ion source parameters were as follows: source temperature, 150 °C; probe temperature, 450 °C; cone voltage, −30 V; and corona discharge, −3 KV. Negative ion spectra were adquired from *m/z* 100 to 800 with a scan time of 0.5 s.

Semipreparative HPLC Analysis. Analyses were performed on a Waters 600 liquid chromatograph system consisting of a quaternary pump, a Waters 700 autosampler, and a UV/vis detector (Jasco UV-1570). A 250 mm × 10 mm, 10 μm, C_{18} Nucleosil 120 column (Tecknokroma, Spain) was used. The gradient elution was performed with water−formic acid 0.1% (solvent A) and acetonitrile (solvent B), at a flow rate of 3 mL/min, using an increasing linear gradient of solvent B for fractions D−G [*t*(min), % B]: (0, 15), (27, 24), (30, 100), and (33, 100)] and for fractions B and C [*t*(min), % B]: (0, 10), (20, 18), (24, 22), (27, 25), (30, 100), and (33, 100)]. The compounds were monitored at 280 nm.

NMR Spectroscopy. The ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-300 NMR spectrometer. Two-dimensional (2D) NMR experiments (HMBC, HMQC, COSY, and NOESY) were recorded on an Inova 500 spectrometer. Samples were dissolved in deuterated methanol (CD_3OD).

TPH and Radical Scavenging Assays. The TPH, as well as the DPPH$^•$ free radical, superoxide NBT hypoxanthine/xanthine oxidase, and $^•$OH/luminol CL radical scavenging activity, were determined following previously used methodology (*17*). Absorbances were measured in a Hitachi U-2000 Spectrophotometer. The intensity of CL was measured as relative light units (RLU) in a Turner Designs' TD-20/20 luminometer.

Determination of Total Phenolics. The amount of total soluble phenolics (TPH) was determined according to the Folin−Ciocalteu method. The TPH was determined as GAE/mg of extract.

Free Radical Scavenging Activity. The samples were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH$^•$. The percent of DPPH decolorization of the sample was calculated according to the equation: % decolorization = $[1 - (Abs_{sample}/Abs_{control})] \times 100$. The decoloration was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50}, which is the amount of sample necessary to decrease by 50% the absorbance of DPPH.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical was produced in the Co(II)/EDTA/$^•$OH/H_2O_2−luminol system. The intensity of CL was measured as RLU. The highest CL intensity of the reaction (control light) was decreased by hydroxyl radical scavenging substances. The percent of inhibition of the CL was calculated for each concentration according to the equation: % inhibition = $[1 - (RLU_{sample}/RLU_{control})] \times 100$. The RLU was plotted against the sample extract concentration, and a linear regression was established in order to calculate the IC_{50}, which is the amount of sample necessary to decrease by 50% the CL intensity.

Superoxide Anion Scavenging Activity. The superoxide radicals were generated in vitro by the hypoxanthine/xanthine oxidase system. The scavenging activity of the extract was determined by the NBT reduction method. In this method, $O_2^{•-}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the purple NBT formation. The results are expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without sample (buffer only) and were calculated by the equation: % inhibition = $\{[(C_{abs} - CB_{abs}) - (S_{abs} - SB_{abs})]/(C_{abs} - CB_{abs})\} \times 100$, where S_{abs}, SB_{abs}, C_{abs}, and CB_{abs} were the absorbances of the sample, the blank sample, the control, and the blank control, respectively.

Table 1. TPH and Radical Scavenging Activity of the Different Extracts and Fractions of Fennel Waste

fraction	weight (g)	TPH[a]	DPPH[b]	CL[b]	superoxide[c]
CE	203.25	138.70 ± 3.15	16.42 ± 1.04	20.30 ± 1.24	70.21 ± 3.22
EAF	10	38.05 ± 1.86	46.06 ± 2.36	120.30 ± 5.36	23.40 ± 0.96
AF	44	297.10 ± 18.9	9.94 ± 0.26	15.23 ± 0.25	58.63 ± 1.26
A	1.9	125.60 ± 2.36	75.90 ± 3.0	86.35 ± 4.56	54.70 ± 1.23
B	0.85	385.00 ± 10.1	16.87 ± 0.66	7.14 ± 0.74	65.30 ± 159
C	0.16	364.25 ± 10.9	21.60 ± 0.36	10.18 ± 0.44	81.00 ± 2.36
D	0.53	443.50 ± 17.9	20.00 ± 0.08	3.92 ± 0.30	61.70 ± 1.01
E	0.2	308.40 ± 2.67	41.31 ± 1.95	18.59 ± 1.28	29.20 ± 0.36
F	0.52	368.95 ± 10.7	9.40 ± 0.25	4.58 ± 0.41	93.60 ± 3.02
G	0.071	361.91 ± 29.1	6.45 ± 0.02	25.33 ± 1.36	71.00 ± 1.47

[a] Values expressed as μg gallic acid (GAE)/mg extract. [b] Values expressed as IC_{50} (μg/mL). [c] Values expressed as percentage of inhibition at 50 μg/mL. See **Figure 1** for the identification of extracts and fractions. The results are the mean of three determinations ± SD (standard deviation).

RESULTS AND DISCUSSION

The initial extraction and fractionation of fennel waste led to an aqueous fraction (**Figure 1**), which exhibited the highest TPH, and both free radical and hydroxyl radical scavenging activity (**Table 1**). The nonphenolic eluate did not exhibit radical scavenging activity in any of the three methods used. A further bioguided fractionation of the aqueous fraction (AF) on Sephadex LH-20 led to seven active fractions (A−G), which were first evaluated for their TPH and radical scavenging activity and then analyzed by LC/UV/APCI-MS. The characteristic UV bands and fragment ions of the main peaks are listed in **Table 2**. Fraction A was not found to be active. Subsequent bioguided chromatographic separation of fractions from B to G, and further purification by preparative HPLC, afforded 12 antioxidant phenolic compounds (**Figure 2**). Radical scavenging activities of the isolated compounds are shown in **Table 3**.

Characterization of the Phenolic Compounds. LC/UV/APCI-MS experiments revealed the presence of three compounds (**1**−**3**) with *m/z* 353 in fraction B (**Figure 3**). MS spectra of these three componds exhibited the same characteristic ion fragments at *m/z* 179 [caffeic acid − H]$^-$ and *m/z* 191 [M − H − caffeic acid]$^-$, corresponding to quinic acid, although the relative intensities of the fragment ions were quite different depending on the quinic acid substitution. Thus, it was not possible to differentiate the isomers on the basis of fragments in the mass spectrum. Compounds **1**−**3** were isolated (see **Figure 1**) and identified as 3-*O*-caffeoylquinic acid (**1**), 5-*O*-

Table 2. UV and MS Data of Fennel Compounds Obtained from LC/UV/APCI-MS Analysis

compound	no.	fraction	UV (nm)	MW	ions full scan MS (*m/z*)	
					[M – H]⁻	fragments
3-caffeoylquinic acid	1	B	328, 212, 292 sh[a]	354	353	191, 179
5-caffeoylquinic acid	2	B	328, 212, 292 sh	354	353	191, 179
4-caffeoylquinic acid	3	B	328, 212, 292 sh	354	353	191, 179
rosmarinic acid	4	C	322, 218, 292 sh	360	359	197, 179, 161
eriodictyol -7-*O*-rutinoside	5	D, F	200, 284	596	595	287, 151
quercetin-3-*O*-glucuronide	6	D, F	257, 354, 256 sh	478	477	301
quercetin-3-*O*-rutinoside	7	E	257, 354, 256 sh	610	609	301
kaempferol-3-*O*-rutinoside	8	E	266, 348, 290 sh	594	593	285
1,5-*O*-dicaffeoyl quinic acid	9	F	328, 212, 292 sh	516	515	353, 191, 179
quercetin-3-*O*-galactoside	10	G	257, 354, 256 sh	464	463	301
quercetin-3-*O*-glucoside	11	G	257, 354, 256 sh	464	463	301
kaempferol-3-*O*-glucoside	12	G	266, 348, 290 sh	448	447	285

[a] sh, shoulder.

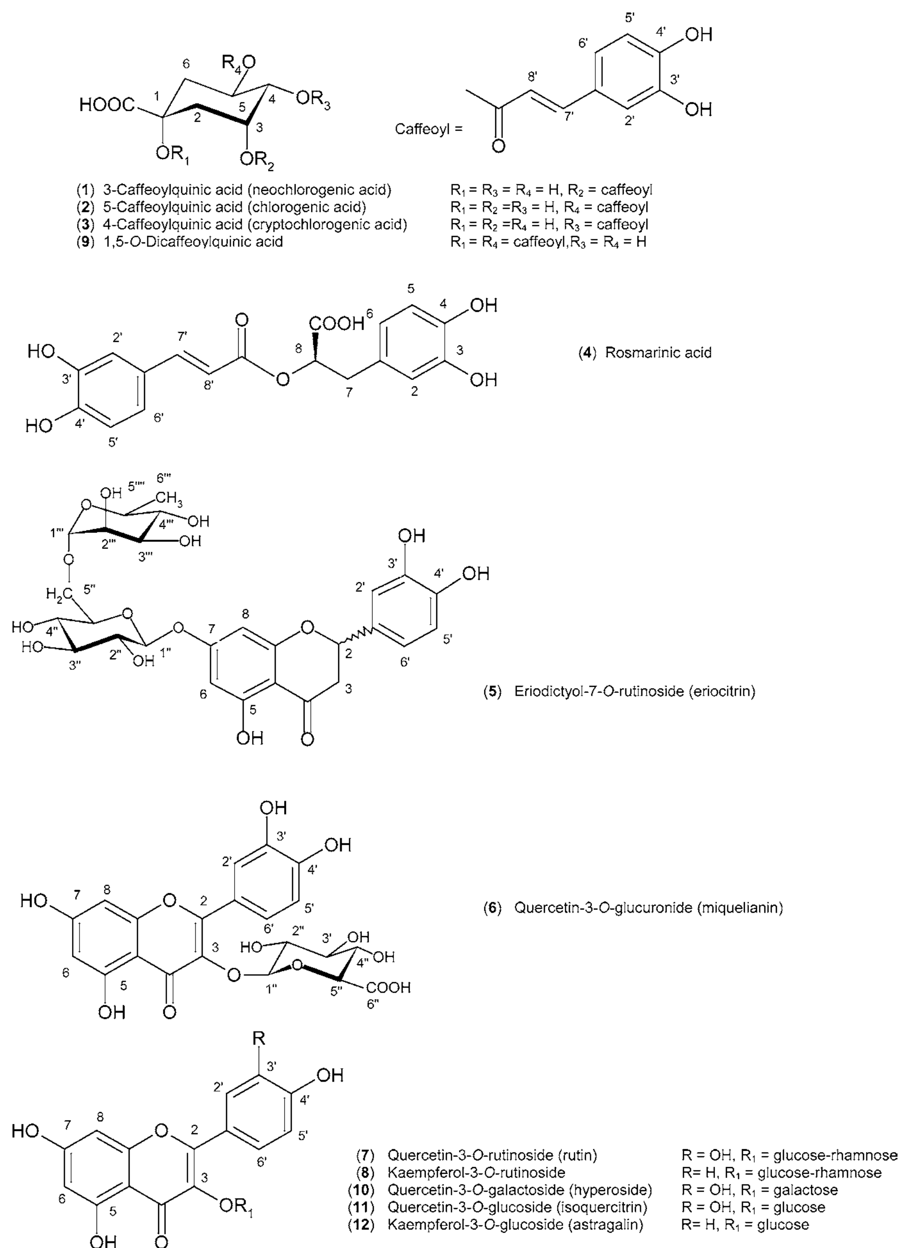

Figure 2. Chemical structures of the phenolic compounds isolated from *F. vulgare.*

caffeoylquinic acid (**2**), and 4-*O*-caffeoylquinic acid (**3**) by direct comparison of their spectroscopic data (^{1}H NMR) with those previously reported (*18*).

A main peak of *m/z* 359 with characteristic ion fragments at *m/z* 197 and 161 was observed in fraction C (**Figure 3**). After purification by semipreparative HPLC, its chemical structure

Table 3. Radical Scavenging Activities of the Major Compounds Isolated from Fennel Waste and of Some Reference Substances; Results Are the Mean of Three Determinations ± SD

name	DPPH[a]	CL[b]	superoxide[c]
3-caffeoylquinic acid	3.09 ± 0.23	31.23 ± 0.25	84.93 ± 1.12
5-caffeoylquinic acid	3.82 ± 0.33	28.36 ± 0.42	84.30 ± 1.68
4-caffeoylquinic acid	3.20 ± 0.14	30.11 ± 0.22	82.40 ± 1.32
rosmarinic acid	1.17 ± 0.10	5.95 ± 0.26	46.43 ± 4.03
eriodyctiol-7-*O*-rutinoside	24.78 ± 1.24	26.21 ± 2.21	49.74 ± 2.68
quercetin-3-*O*-glucuronide	20.00 ± 5.12	32.88 ± 0.70	67.70 ± 3.69
quercetin-3-*O*-rutinoside	9.85 ± 0.23	30.04 ± 0.36	21.66 ± 1.72
kaempferol-3-*O*-rutinoside	8.21 ± 0.36	24.78 ± 0.58	19.56 ± 1.25
1,5-*O*-dicaffeoylquinic acid	19.36 ± 1.33	17.61 ± 1.80	56.00 ± 2.58
quercetin-3-*O*-galactoside	7.52 ± 0.01	27.58 ± 1.89	76.30 ± 3.19
quercetin-3-*O*-glucoside	7.40 ± 0.02	26.21 ± 1.52	75.90 ± 4.69
kaempferol-3-*O*-glucoside	9.23 ± 0.13	22.14 ± 0.86	65.98 ± 3.55
reference compounds			
caffeic acid	3.34 ± 0.07	25.39 ± 0.38	84.11 ± 1.72
quercetin	6.11 ± 0.53	5.13 ± 0.12	97.45 ± 3.25
epicatechin	4.08 ± 0.04	8.34 ± 0.38	88.44 ± 2.64
BHA	9.70 ± 0.92	2.14 ± 0.01	67.51 ± 0.29

[a] Values expressed as IC_{50} (μg/mL). [b] Values expressed as percentage of inhibition at 50 μg/mL.

was fully characterized as rosmarinic acid (**4**) by comparison of its MS, ^{1}H NMR, and ^{13}C NMR spectra with those reported in the literature (*19*).

Fraction D revealed the presence of two major peaks, **5** and **6**, *m/z* 595 and 477, respectively, and fraction F revealed that of three major active peaks corresponding to compounds **5**, **6**, and **9** (**Figure 3**), the last one with *m/z* 515. These compounds could not be totally characterized by MS, and subsequent isolation was carried out (**Figure 1**). Negative APCI-MS of compound **9** showed a pseudomolecular ion at *m/z* 515 $[M - H]^-$ and characteristic fragments at *m/z* 353 $[M - H -$ caffeic acid$]^-$, *m/z* 179 [caffeic acid $- H]^-$, and *m/z* 191 $[M - H -$ caffeic acid$]^-$ corresponding to a dicaffeoylquinic acid. After comparison of spectroscopic data (MS, ^{1}H, and ^{13}C NMR) with those reported in the literature (*20*), compound **9** was identified as 1,5-*O*-dicaffeoylquinic acid. Negative ion APCI-MS of compound **5** provided a pseudomolecular ion at *m/z* 595 $[M - H]^-$, a second fragment at *m/z* 287 $[M - H -$ rhamnoglucosyl$]^-$, corresponding to the aglycone produced by the cleavage of the glycosidic bond with the loss of 308 u, and a characteristic ion fragment at *m/z* 151 due to the retro-Diels–Alder fragmentation of the aglycone moiety. The ^{1}H NMR and ^{13}C NMR spectra exhibited signals similar to those obtained for the flavanone eriodyctiol (*21*), as well as to those corresponding to the glucosyl and rhamnosyl groups. Sugar linkage $\alpha1 \rightarrow 6$ (rutinoside) was determined by 2D NMR experiments. Naturally occurring flavanones usually have the 2*S* configuration, but racemization can occur during the extraction procedure (*22*), which explains why many signals are duplicated. Compound **5** was found to be a mixture of both diasteriomers 2*S* and 2*R*. On the basis of the NMR and MS results, compound **5** was characterized as eriocitrin (eriodictyol-7-*O*-rutinoside). Negative ion APCI-MS of compound **6** provided a pseudomolecular ion at *m/z* 477 $[M - H]^-$ and the aglycone ion fragment at *m/z* 301 $[M - H -$ glucuronosyl$]^-$ due to the cleavage of the glycosidic bond and the loss of 176 u. Both the ^{1}H and the ^{13}C NMR spectroscopic data of compound **6** exhibited a characteristic pattern of quercetin aglycone with a sugar attached at position 3 (*23*). The sugar moiety was completely elucidated, thus allowing this compound to be identified as quercetin-3-*O*-glucuronide. The ^{1}H NMR and ^{13}C NMR data of compounds **5** and **6** are shown in **Tables 4** and **5**, since there is little clearly published NMR data directly comparable to our results measured in methanol (CD_3OD).

Table 4. NMR Spectroscopic Data of Compound **5**, Eriodictyol-7-*O*-rutinoside (Eriocitrin)[a]

attribution	^{1}H NMR (*J*, MHz)		^{13}C NMR	
2	5.35 dd (12.5, 3.0)	5.33 dd (12.5, 3.0)	80.7 d	80.5 d
3 axial	3.12 dd (17.0, 13.0)	3.11 dd (17.0, 13.0)	44.3 t	44.0 t
3 equatorial	2.76 dd (17.0, 3.0)	2.74 dd (17.0, 3.0)	44.3 t	44.0 t
4			198.4 s	
5			164.9 s*	
6	6.17 br s+		97.0 d+	
7			166.8 s	
8	6.18 d (2.0)		97.9 d+	
9			164.4 s*	
10			104.9 s	
1′			131.5 s	131.4 s
2′	6.96 br s	6.93 br s	114.8 d	114.6 d
3′			146.4 s°	
4′			146.9 s°	
5′	6.79 d (8.0)		116.2 d	
6′	6.80 dd (8.0, 1.5)		119.3 d	
1″-glucosyl	4.94 d (7.0)	4.93 d (7.0)	101.0 d	
2″	3.40 m		74.6 d	
3″	3.42 m		77.8 d	
4″	3.34 m		71.3 d	
5″	3.57 m		77.1 d	
6a″	3.99 dd (9.0, 3.5)		67.4 t	
6b″	3.59 m		67.4 t	
1‴-rhamnosyl	4.69 d (1.5)	4.68 d (1.5)	102.1 d	
2‴	3.90 dd (3.5, 1.0)	3.87 dd (3.5, 1.0)	72.0 d	
3‴	3.69 dd (9.0, 3.5)	3.67 dd (9.0, 3.5)	72.3 d	
4‴	3.33 m		74.1 d	
5‴	3.62 m		69.7 d	
6‴	1.19 d (5.5)	1.18 d (5.5)	17.9 q	

[a] *, +, and ° values maybe exchanged.

Table 5. NMR Spectroscopic Data of Compound **6**, Quercetin-3-*O*-glucuronide (Miquelianin)

attribution	^{1}H NMR (*J*, MHz)	^{13}C NMR
2		159.0 s
3		135.4 s
4		179.2 s
5		162.9 s
6	6.19 s	99.9 d
7		165.9 s
8	6.38 s	94.7 d
9		158.4 s
10		105.6 s
1′		122.8 s
2′	7.69 s	117.3 d
3′		145.9 s
4′		145.8 s
5′	6.84 d (8.5)	116.0 d
6′	7.58 d (8.5)	123.3 d
1″-glucuronosyl	5.32 d (8.0)	104.2 d
2″	3.53 dd (9.0, 8.0)	75.4 d
3″	3.47 dd (9.5, 9.0)	77.7 d
4″	3.59 t (9.5)	72.9 d
5″	3.72 d (9.5)	77.2 d
6″		173.0 s

Compounds **7** and **8** from fraction E and compounds **10**–**12** from fraction G (**Figure 3**) were identified by comparison of their spectroscopic data (MS, ^{1}H, and ^{13}C NMR) with those reported in the literature (*23*).

Radical Scavenging Activity of the Isolated Compounds. Radical scavenging properties of compounds **1**–**12** were evaluated, and the results were compared with those of some standard compounds (**Table 3**). Thus, chlorogenic acid isomers and rosmarinic acid, which are recognized for their antioxidant

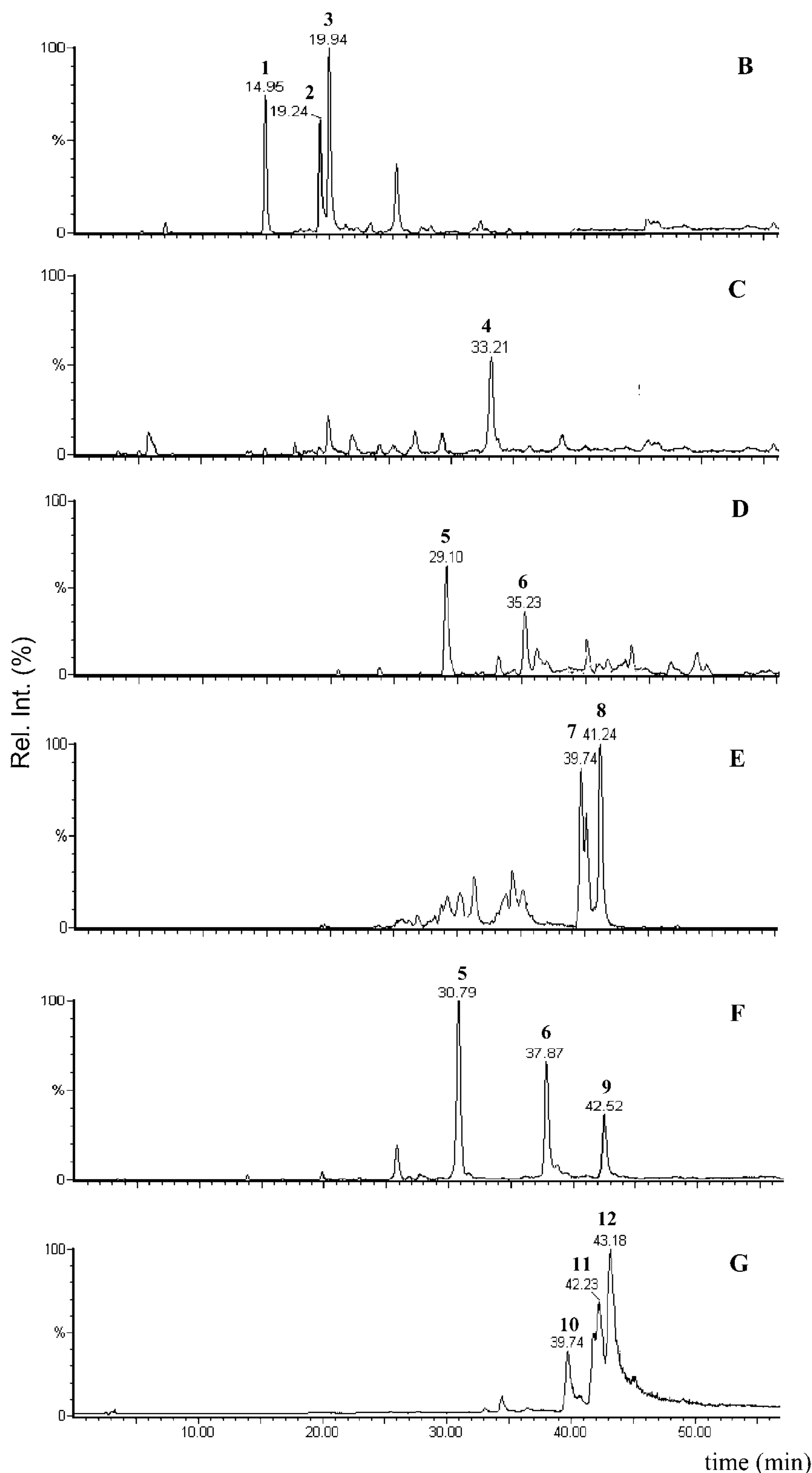

Figure 3. UV chromatograms of the Sephadex fractions (B–G) of the fennel waste.

activity (*18*, *24*) showed an activity comparable to caffeic acid and quercetin. Regarding flavonoid compounds (**5**−**12**), it is generally assumed that their radical scavenging and/or antioxidant activity is mainly due to the catechol structure in the B ring, the 2,3-double bond in conjugation with a 4-carbonyl function, and the additional presence of both 3- and 5-hydroxyl groups for a maximal radical scavenging capability. These sites can be considered as the active centers or the prerequisite factors for the scavenging of free radicals. It is generally proposed that glycosylation of flavonoids reduces their activity when compared with the corresponding aglycones (*25*). In our work, glycosylation of the hydroxyl group at position 3 of the quercetin aglycone decreased all of the radical scavenging activities measured. As shown in **Figure 2**, the isolated quercetin derivatives possess the structural characteristics described above and differ from each other in the sugar attached at position 3. Their ability to scavenge the superoxide radical showed a descending pattern in the following order: galactose, glucose, glucuronic acid, and rhamnose−glucose (**Table 3**), thus suggesting that the sugar may determine the radical scavenging activity of a compound

by changing the electron distribution and participating in the electron delocalization (*26*). Kaempferol and quercetin derivatives differ from each other in the substituent in the B ring (pyrogallol and catechol, respectively). Because the kaempferol derivatives isolated in this work (**8** and **12**) were found to show an antiradical activity similar to that of quercetin derivatives, it can be concluded that such a substitution does not have much effect on the activity of flavonoids.

On the other hand, compound **5** exhibited a moderate radical scavenging activity probably due to the different flavonoid structure, as eriodyctiol aglycone contains neither the C_3−OH group nor the 2,3-double bond, which allows the electron delocalization, one of the prerequisites for maximum effectiveness. Finally, compound **9** showed a moderate radical scavenging activity as compared to the monocaffeoyl-substituted chlorogenic acid isomers, thus revealing how the antiradical activity of quinic acid derivatives is affected by the caffeoyl substitution. Glycosylation of flavonoids, however, should not have any in vivo effects, since glycosidic bonds are probably hydrolyzed to afford the free hydroxyl groups.

In this work, LC/UV/APCI-MS experiments and subsequent bioassay-guided fractionation led to the identification of 12 major radical scavenging constituents in fennel waste. The chemical identification of eriodictyol-7-*O*-rutinoside and quercetin-3-*O*-glucuronide has been here ascertained by 2D NMR experiments. The occurrence of 3-caffeoylquinic and 4-caffeoylquinic acids, rosmarinic acid, eriodictyol-7-*O*-rutinoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, and 1,5-*O*-dicaffeoylquinic acid in bitter fennel is reported for the first time, together with four phenolic compounds already described in the literature. On the other hand, quercetin and kaempferol-3-*O*-arabinosides, which have been described as occurring in fruit fennel (*6*), were not found in this fennel waste. The identification of these compounds in *F. vulgare* reveals the connection between its radical scavenging activity and chemical composition. In addition, these results may contribute to the interpretation of the pharmacological effects of this medicinal and aromatic plant and support the possibility that fennel has protective effects on human health. Furthermore, apart from its use in the Mediterranean diet as a spice or in folk medicine, the waste after its distillation for essential oils can also constitute an easily accessible source of natural antioxidants.

ABBREVIATIONS USED

APCI-MS, atmospheric pressure chemical ionization mass spectrum; BHA, butylated hydroxyanisole; CL, chemiluminescence; DPPH•, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalents; IC_{50}, inhibitory concentration 50; TPH, total phenolic content.

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