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Distribution of a New Rosmarinic Acid Derivative in *Eryngium alpinum* L. and Other Apiaceae

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The roots of *Eryngium alpinum* L. (Apiaceae) demonstrated radical scavenging properties toward the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical in a TLC autographic assay. Isolation of the bioactive compounds allowed the identification of *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid, a new rosmarinic acid derivative. The quantitative determination of the antioxidant capacity of the compound by chemoluminescence demonstrated half the activity of *R*-(+)-rosmarinic acid. To find another source richer in this compound, a chemotaxonomic study was conducted. The higher content was found in the aerial parts of Sanicula *europeae* L., also belonging to the Saniculoideae subfamily. Although present in most of the *Eryngium* species, this compound was not detected in *Imperatoria ostruthium* L., *Pimpinella peregrina* L., and *Levisticum officinalis* L. species from the Apioideae subfamily and *Hydrocotyle asiatica* L. from the Hydrocotyloideae subfamily. The results indicate that the new derivative *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid is a potential chemotaxonomic marker of the Saniculoideae subfamily.

KEYWORDS: ***Eryngium alpinum* L.; *Sanicula europeae* L.; Saniculoideae; Apiaceae; antioxidant; chemotaxonomy; *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid**

INTRODUCTION

Free radicals produced in humans participate in many metabolic reactions. These highly reactive compounds induce chain reactions that can generally be controlled by antioxidant defense mechanisms, for example, enzymatic systems such as SOD and glutathione. However, environmental factors such as UV radiation increase the production of free radicals which then react by destroying DNA, lipids, or cell proteins, leading to different diseases and early aging (*1*). Alpine plants that are strongly exposed to UV rays are believed to protect themselves with antioxidative substances. In the search of new antioxidative compounds, alpine plants extracts were screened for their capacity to reduce radical DPPH. An active plant, *Eryngium alpinum* L., was identified, whereas the roots showed an antioxidant activity higher than in the aerial parts.

E. alpinum, a species that belongs to the Apiaceae family and to the Saniculoideae subfamily, is a protected plant with blue amethyst colored flowers. The thistle-like plant is cultivated in Switzerland (Graubünden and Valais) mainly for decorative purposes. To our knowledge, there is no known traditional use of this species. However, the fruits of *E. foetidum* L., another species from the same genus, were taken as food in Nigeria (*2*), and *Sanicula graveolens*, a related species belonging to the Saniculoideae subfamily, known as "wild coriander", is used as a spice in some rural areas in Central Chile (*3*). Moreover, *E. foetidum* L., *E. creticum* L., and *E. maritimum* L., are known for their antiinflammatory, analgesic (*4*, *5*), diuretic, antidiabetic (*6*), and antiscurvy (*7*) activities. Phytochemical investigations of the genus *Eryngium* have yielded flavonoids (*8*), coumarin derivatives (*9*), terpene aldehyde esters (*10*), as well as saponins (*2*, *11*). Chlorogenic acid and rosmarinic acid, two phenolic compounds known for their antioxidant activity, have been described for many *Eryngium* species, as well as for *Sanicula europeae* L. (*12*, *13*). The leaves of *E. alpinum*, the main species investigated in this study, are known to contain two flavonoids, quercetin and kaempferol (*14*).

This paper first describes the identification of bioactive substances in the roots of *E. alpinum*. In a second part, a chemotaxonomic study was undertaken to determine other sources enriched in these compounds.

MATERIALS AND METHODS

Materials and Reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); chlorogenic acid and rosmarinic acid were from Extrasynthese

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(Genay, France), and all solvents used were purchased from Fluka. Salviaflaside (*R*-(+)-3-*O*-*β*-D-glucopyranosyl rosmarinic acid) was a gift from Ulrike Arnold, University of Innsbruck, Austria.

HPLC (Analytical). Waters 2695 system (Waters, Rupperswil, Switzerland) equipped with auto-sampler, DAD, column thermostat; stationary phase: 150 × 3.0 mm i.d., 3.5 μm, RP18 XTerra column, guard column: 20 × 3.0 mm i.d., 3.5 μm, RP18 XTerra; temperature: 30 °C; mobile phases: water with 0.1% acetic acid and 0.9% formic acid (A), acetonitrile with 0.1% acetic acid and 0.2% formic acid (B); flow rate: 0.6 mL/min, injected sample volume 10 μL; detection at 320 and 280 nm; gradient: start, B 10%; 10 min, B 45%; 13.3 min, B 80%; 20 min, stop.

HPLC (Semi-prep). Waters Prep LC system, stationary phase: 100 × 19 mm i.d., 5 μm, RP 18 XTerra Prep, guard column: 19 × 10 mm i.d., 5 μm, RP 18 XTerra Prep; mobile phases: water with 0.1% acetic acid and 0.9% formic acid (A), acetonitrile with 0.1% acetic acid and 0.2% formic acid (B); flow rate: 12 mL/min; gradient: start, B 10%; 13 min, B 45%; 18 min, B 80%; 26 min, stop.

Melting points were measured on a Kofler hot-stage and were uncorrected. Optical rotation was determined with a Perkin-Elmer 341 polarimeter. FT-IR-spectra: ZnSe disk (2 mm thickness) Bruker IFS 25 FTIR-spectrometer, transmission mode within 4000−600 cm^{-1}; values in cm^{-1}. NMR: 2-D and 1-D measured at a Bruker AM-300 at 300 MHz (^1H) and 75 MHz (^{13}C); chemical shifts δ in ppm, coupling constants J in Hz; $SiMe_4$ as internal standard. MS-parameters: ESI-MS (negative mode), Esquire 3000^{plus} (Bruker Daltonics, Bremen, Germany); sample introduction by syringe pump (flow 20 μL/min); capillary temperature: 300 °C; the ion source voltage was 4 kV at a capillary offset voltage of −116.9 V; CID (collision induced dissociation) 0.3−2.0 V; nebulizer 50 psi. FAB-HR-MS (negative mode): Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA); Cs-Gun: 20 kV, 4 μA, matrix: *m*-Nitrobenzyl alcohol; $R = 6000$.

Plant Materials. The seeds of 12 species belonging to the genus *Eryngium* L. were purchased from Jelitto Staudensamen (Schwarmstedt, Germany). These species were cultivated in a greenhouse (Agroscope, Conthey, Switzerland), and their 1-year-old roots were harvested in July 2004.

Eryngium alpinum L. is commercially cultivated in Ardon and Bergün (Switzerland). Roots of *E. alpinum* L. were provided by the Swiss alpine gardens Rocher de Naye La Rambertia, Schynige Platte, alpine garden of Pont de Nant, La Thomasia, Maran/Arosa, by Agroscope (Bruson), by the botanical gardens of Freiburg and Porrentruy.

Roots of *E. amethystinum*, *E. campestre*, *E. falcatum*, *E. ilicifolium*, *E. maritimum* (all from Morocco), *E. campestre* (Germany), *E. foetidum* (Vietnam), and *E. maritimum* (Germany) were provided by Friedrich Nature Discovery FND (Germany). The botanical garden of the University of Bern provided the roots of *E. campestre*, *E. giganteum*, *E. yuccifolium*, and *E. agavifolium.* The botanical garden of the University of Zürich provided the roots of *E. amethystinum*, *E. campestre*, and *E. giganteum*. The botanical garden of the University of Freiburg (Switzerland) provided the roots of *E. bourgatii*, *E. campestre*, and *E. maritimum.* The botanical garden of the University of Basel (Spalentor) provided the roots of *E. giganteum*. *E. campestre* (Val d'Aoste (Italy), Rouffach (Alsace), Reinacher Heide (Basel)) and *E. bourgatii* (Quirbajou, Pyrenees) were collected by us with official permissions or with authorized botanists.

The aerial parts of *Sanicula europeae* L. were collected by Charles Rey in September 2004 in Savièse (Switzerland) (roots not available). The roots of the *Levisticum officinalis* L., *Pimpinella peregrina* L., and *Imperatoria ostruthium* L. were provided by Valplantes (Switzerland). Each of these species was authenticated by the botanist Charles Rey (Agroscope). The aerial parts of *Hydrocotyle asiatica* L. were purchased from the drugstore Chrüterhüsli (Basel) (roots not available). Where possible, voucher samples are kept at Alpaflor.

Extract Preparation and Isolation of *R*-(+)-Rosmarinic Acid (2) and *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid (3). Fresh roots of *E. alpinum* were washed, frozen at −80 °C, and then freeze-dried because preliminary study revealed that many compounds are decomposed when the fresh roots are first crushed. 180 g of dry roots, finely crushed (Mixer Powerblend, MX 2050), was defatted with 2 L of dichloromethane under stirring (24 h, room temperature) and extracted by maceration twice with 2 L of ethanol 50% (v/v) (16 h, 6 h, room temperature). After evaporation under reduced pressure and lyophilization, 48.7 g of crude extract was obtained. Ten extract fractionations of 1 g each were performed with a MPLC system (Büchi, Flawil, Switzerland) on a 230 × 36 mm bed of 110 g of Lichroprep RP-18 material (40−63 μm) (Merck, Darmstadt, Germany). Mobile phases: A: water with 5% methanol (0.1% acetic acid + 0.9% formic acid); B: methanol with 5% water (0.1% acetic acid + 0.2% formic acid). Gradient: start, B 5%; 32 min, B 35%; 64 min, B 40%; 96 min, B 60%; 130 min, stop; flow-rate, 20 mL/min; fractions of 20 mL were collected. Compound **3** was present in fractions 75−85 and compound **2** in fractions 92−96. After evaporation to dryness under reduced pressure, fractions of 185 mg containing (**3**) and 62 mg containing (**2**) were obtained. The stereochemistry of 2 could be determined directly without further purification. Compound **3** was purified with the preparative LC system; 43 mg of compound **3** was obtained. Compound **1** was identified by LC/MS as chlorogenic acid. This confirms the data from the literature (*12*, *13*).

Sample Preparation for NMR. Compound **3** was isolated as sodium salt. The interfering cations were removed by filtering the sample, dissolved in 1.0 mL of MeOH and 100 μL of distilled water, through a Chromabond C18 SCX column preconditioned with 0.01% aqueous TFA/MeOH (3:4, v/v), followed by a 4.0 mL MeOH rinsing step. The dried sample was dissolved in an appropriate amount of CD_3OD.

Spectroscopic and Physical Data. Chlorogenic Acid 1. LC-online UV: $\lambda_{max} = 327$ nm, 235 nm. ESI-MS: *m*/*z* 353 $[M - H]^-$, fragment *m*/*z* 191 $[(M - H) - (C_9H_6O_3)]^-$ (-caffeoyl group). ***R*-(+)-Rosmarinic Acid 2.** Isolated as an amorphous yellow powder (62 mg). LC-online UV: $\lambda_{max} = 330$ nm, 235 nm. ESI-MS: *m*/*z* 359 $[M - H]^-$, fragment *m*/*z* 197 $[(M - H) - (C_9H_6O_3)]^-$ (-caffeoyl group); optical rotation: $[\alpha]^{20}_D = +73.74$ (MeOH; $c = 0.339$), $[\alpha]^{20}_D = +78$ (MeOH; $c = 0.40$) (*15*).

***R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid 3.** Isolated as an amorphous yellow powder (43 mg); $C_{24}H_{26}O_{13}$, HR-FAB-MS (negative mode): found *m*/*z* 521.1305 $[C_{24}H_{26}O_{13} - H]$, calculated: *m*/*z* 521.1295 $[C_{24}H_{26}O_{13} - H]$; LC-online UV: $\lambda_{max} = 329$ nm, 221 nm; FT-IR: ν_{max} cm^{-1} 3383 (−OH), 1714, 1604, 1518, 1446, 1358, 1284, 1153, 1116, 979, 851, 814; mp: 154−155 °C (decomp.); optical rotation: $[\alpha]^{20}_D = +11.24$ (MeOH; $c = 0.1$); ESI-MS: *m*/*z* 521 $[M - H]^-$, fragments: *m*/*z* 359 $[(M - H) - C_6H_{10}O_5]^-$ (-glucose), *m*/*z* 197 $[(M - H) - C_9H_6O_3]^-$ (-caffeoyl group). ^1H and ^{13}C NMR data: see **Table 1**.

Hydrolysis of *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid 3. A small amount of the isolated compound **3** (5.50 mg) was dissolved in 5.0 mL of 2 M aqueous trifluor-acetic acid and heated for 1 h to 130 °C. After being cooled, the hydrolysate was evaporated. To remove all traces of trifluoracetic acid, evaporation was repeated twice after addition of 10.0 mL of MeOH. The obtained residue was dissolved in 1.0 mL of water and used as TLC solution to prove simultaneously the identity of the sugar moiety and the reaction progress. TLC: silica gel 60 F_{254} TLC plates, EtOAc/ethanol (96%)/acetic acid/water/saturated aqueous solution of boric acid (50/20/6/4/10); detection: UV 254 nm; spraying reagent: mixture of 10 parts ethanolic resorcin solution (0.2 g in 100 mL of ethanol (96%)) and 1 part ortho-phosphoric acid; followed by 5 min heating to 120 °C; reference compounds: *β*-D-glucose (R_f: 0.40), *β*-D-galactose (R_f: 0.35), compound **2** (R_f: 0.93) and **3** (R_f: 0.61). The aqueous solution of the reaction residue was mixed three times with 1.0 mL of EtOAc to separate the free rosmarinic acid. After phase-separation, the organic layers were removed, combined, and evaporated, yielding 3.62 mg of rosmarinic acid with an optical rotation of $[\alpha]_D^{20} = +68.32$ (MeOH; $c = 0.221$).

Determination of the Antioxidant Capacity. *TLC Decolorizing Assay/Reduction of DPPH Radical.* Methanolic solutions of the samples (1%) were chromatographed on precoated silica gel 60 F_{254} TLC plates (Merck) using EtOAc/MeOH/H_2O (60/25/15). After drying, the TLC plates were sprayed with a freshly prepared 0.1% DPPH (Sigma-Aldrich) solution in MeOH, a modified method of Cuendet et al. (*16*). Compounds showing a yellow-on-purple area were antioxidants. Chlorogenic acid had a R_f value of 0.42, *R*-(+)-rosmarinic acid a R_f

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for *R*-(+)-Rosmarinic Acid, *R*-(+)-3-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid, and *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid (CD_3OD) (*J* in Hz)

	R-(+)-rosmarinic acid[a]		*R*-(+)-3-*O*-*β*-D-glucopyranosyl rosmarinic acid[b]		*R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid	
position	^{1}H NMR (500 MHz)	^{13}C NMR (125 MHz)	^{1}H NMR (300 MHz)	^{13}C NMR (75 MHz)	^{1}H NMR (300 MHz)	^{13}C NMR (75 MHz)
			Rosmarinic Acid Moiety			
1		128.12		127.0		127.7
2	7.03 d (2.0)	115.27	7.48 br s	116.5	7.06 d (2.2)	115.6
3		146.85		146.1		146.8
4		149.5		150.1		149.7
5	6.77 dd (8.2)	116.6	6.82 d (8.0)	116.7	6.80 d (8.5)	116.5
6	6.91 dd (8.2)	123.04	7.14 dd (8.0, 2.0)	125.2	6.96 dd (8.5, 2.2)	123.4
7	7.51 d (15.5)	146.79	7.54 d (16.0)	145	7.56 d (16.0)	147.8
8	6.27 d (15.5)	115.77	6.33 d (16.0)	115.3	6.27 d (16.0)	114.4
9		169.24		167.6		168.4
1′		131.29		129.2		133.5
2′	6.77 d (2.0)	117.63	6.73 br s	117.2	6.84 d (2.2)	118.3
3′		146.08		145.7		145.9
4′		144.93		144.1		148.3
5′	6.68 d (8.0)	116.34	6.66 d (8.0)	114.9	7.14 d (8.1)	118.8
6′	6.63 dd (8.2)	121.89	6.58 br d (8.0)	120.8	6.75 dd (8.1, 2.2)	121.9
7′	3.10 dd (14.5, 3.5)	38.93	3.03 br d (14.0)	37.2	3.17 dd (4.4, 14.0)	37.9
	2.94 dd (14.5, 10)				3.07 dd (8.2, 14.0)	
8′	5.09 dd (10, 3.5)	77.79	4.92 m	76.3	5.24 dd (8.2, 4.4)	74.3
9′		177.64		176.5		173.3
			Glucose Moiety			
1″			4.80 d (8.0)	103.3	4.75 d (7.5)	104.4
2″			3.41 m	73.9	3.46 m	74.9
3″			3.48 m	76.6	3.46 m	77.6
4″			3.51 m	70.5	3.39 m	71.3
5″			3.40 m	77.5	3.40 m	78.3
6″			3.93 dd (12.0, 2.0)	61.6	3.89 br d (12.0)	62.4
			3.67 dd (12.0, 5.0)		3.71 dd (12.0, 4.9)	

[a] Reference *15*. [b] Reference *21*.

value of 0.50, and *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid a R_f value of 0.40.

Chemoluminescence Antioxidant Assay. The antioxidant capacity of substances was determined by means of the Photochem system (Analytik Jena AG., Jena, Germany) and the ACW (Antioxidative Capacity of Water soluble compounds) measuring kit (*17*). Superoxide anion radicals were produced by optical stimulation, whereupon they were scavenged proportionally to the antioxidant capacity of the substances present in the sample. The luminescence of the still active radicals was measured to determine the quantity of antioxidants in the sample. By comparison with a standard (establishment of a standard curve with ascorbic acid), quantification could be expressed in units equivalent to the ascorbic acid standard [mg/mg of sample].

Differentiation of the Isomers *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid (3) and *R*-(+)-3-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid (Salviaflaside) by TLC Analysis. Methanolic solutions of compound 2, 3, and salviaflaside were chromatographed on precoated silica gel 60 F_{254} TLC plates (Merck) using EtOAc/MeOH/H_2O/AcOH (60/15/15/1). The TLC plates were sprayed with a vanillin-sulfuric acid reagent and heated for 10 min at 100 °C. The compounds were evaluated in visible. *R*-(+)-Rosmarinic acid had a R_f value of 0.54, *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid a R_f value of 0.24, and salviaflaside a R_f value of 0.26. Attempts to separate the isomers by HPLC failed.

Quantification. The preparative extraction method of *Eryngium alpinum* L. roots was modified and applied for the quantification of compounds from the roots of the other investigated *Eryngium* L. species, for *Levisticum officinalis* L., *Pimpinella peregrina* L., and *Imperatoria ostruthium* L. roots, as well as for the aerial parts of *Sanicula europeae* L. and *Hydrocotyle asiatica* L. (**Table 3**). Five grams of each dry root and aerial part sample, finely crushed, was defatted with 50 mL of dichloromethane under stirring (24 h, room temperature) and extracted by maceration twice with 50 mL of ethanol 50% (v/v) (16 h, 6 h, room temperature). A third extraction with this solvent was performed, and compound **3** was no more detectable at the detection limit of 0.1 mg/g. Ethanolic extracts were combined, and the solvent was evaporated. Each extract was then analyzed by HPLC ($n = 3$) taking the extraction yield into account. The quantitative analysis was carried out by the establishment of a standard curve. The purified compounds **2** ($y = 228\,206x + 118\,673$; $R^2 = 0.97$) and **3** ($y = 100\,829x + 81\,304$; $R^2 = 0.99$) were used as external standards. The results are expressed in mg/g dry weight of plant material. Statistical analysis could not be realized rigorously because the plant material available was limited; one sample for each species was analyzed.

Table 2. Determination of the Antioxidant Capacity (Photochem) of the *E. alpinum* L. Extract and Compounds **1**, **2**, and **3**[a]

tested samples [compounds/extract]	ascorbic acid equivalent [mg/mg sample]
Eryngium alpinum root extract	0.16 ± 0.00
chlorogenic acid (**1**)	4.21 ± 0.04
R-(+)-rosmarinic acid (**2**)	3.86 ± 0.03
R-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid (**3**)	1.98 ± 0.00

[a] Values are means ± s.d, $n = 2$.

RESULTS AND DISCUSSION

Identification of Active Substances. For a further refinement of the screening results and a preliminary identification of the antioxidative substances in the root extract of *Eryngium alpinum*, a DPPH/TLC decolorizing assay was performed. This identified three spots that showed antioxidant activities. HPLC analysis of the same *Eryngium* extract showed the presence of three major compounds. Two of these could be identified by LC/MS

Table 3. Quantitative Study of *R*-(+)-Rosmarinic Acid (**2**) and *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid (**3**) in Different Species of the Saniculoideae, Apioideae, and Hydrocotyloideae Subfamilies[a]

	2 [mg/g]	**3** [mg/g]
Saniculoideae		
E. agavifolium L.	124 ± 2.8	n.d.[b]
E. alpinum L.	27 ± 0.0	11 ± 0.0
E. bourgatii L.	15 ± 0.1	0.7 ± 0.0
E. campestre L.	0.6 ± 0.0	0.4 ± 0.0
E. eburneum L.	272 ± 2.7	0.9 ± 0.0
E. giganteum L.	12 ± 0.2	n.d.
E. pandanifolium L.	103 ± 2.6	0.3 ± 0.1
E. planum L.	0.4 ± 0.0	16 ± 0.4
E. planum var. BC	0.4 ± 0.0	14 ± 0.0
E. spinalba L.	8.8 ± 0.1	0.2 ± 0.0
E. tripartitum L.	2 ± 0.0	10 ± 0.2
E. varifolium L.	39 ± 0.6	0.1 ± 0.0
E. yuccifolium L.	72 ± 1.1	11 ± 0.2
Sanicula europeae L.[c]	297 ± 0.2	96 ± 0.0
Apioideae		
Imperatoria ostruthium L.	14 ± 0.0	n.d.
Levisticum officinalis L.	0.5 ± 0.0	n.d.
Pimpinella peregrina L.	0.1 ± 0.0	n.d.
Hydrocotyloideae		
Hydrocotyloideae asiatica L.[c]	n.d.	n.d.

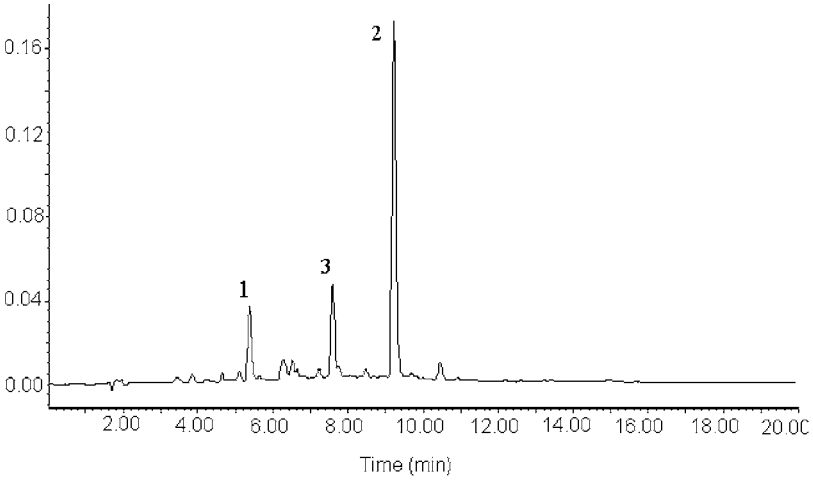
[a] Values are means ± s.d, $n = 3$. [b] Not detected at a detection limit of 0.1 mg/g. [c] Aerial parts were analyzed.

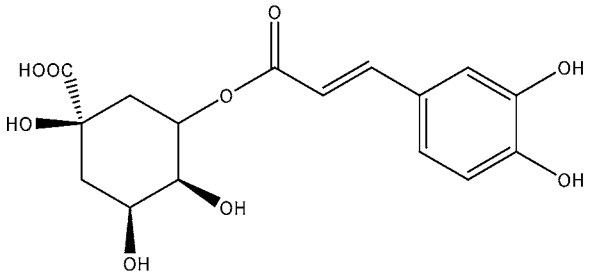
(negative mode) and HPLC spiking experiments as chlorogenic acid **1** and rosmarinic acid **2** (**Figure 1**). Repetition of the DPPH/TLC analysis with the crude extract and the corresponding pure compounds confirmed the presence of the assigned compounds in the extract as well as their antioxidant activity. For a definitive stereochemical assignment of compound **2**, the substance was isolated and the optical rotation measured. The optical rotation of +73.74 (MeOH; $c = 0.339$) established the isolated compound **2** as *R*-(+)-rosmarinic acid (*15*). The third antioxidative substance showed an R_f value and HPLC retention time between those of chlorogenic and rosmarinic acid. For further characterization, compound **3** was isolated by means of MPLC and preparative HPLC. During the isolation process of **3**, the substance turned out to be unstable in a MeOH/H_2O (1/1) solution. One-third of its quantity was degraded after 11 h at room temperature. HPLC analysis of this solution showed the presence of only one UV detectable degradation product, which was identified as rosmarinic acid.

ESI mass spectrum (negative mode) of the unchanged substance **3** exhibited a pseudo molecular ion with an *m*/*z* value of 521 $[M - H]^-$ and a fragment assignable to a rosmarinic acid moiety (*m*/*z* 359 $[(M - H) - 162]^-$), a caffeoyl group (*m*/*z* 197 $[(M - H) - C_9H_6O_3]^-$), and a sugar moiety (*m*/*z* 162). HR-FAB-MS (negative mode) showed a molecular formula of $C_{24}H_{26}O_{13}$ (*m*/*z* 521.1305 $[C_{24}H_{26}O_{13} - H]$, calculated *m*/*z* 521.1295 $[C_{24}H_{26}O_{13} - H]$). The ^1H NMR spectrum of **3** displayed two resonance clusters of aromatic protons at δ_H 7.14−6.18, for two ABX type proton systems, but with distinctly different shift values in comparison to those of rosmarinic acid **2**. The deviating signals and six additional signals in the range of δ_H 4.75−3.39, that is, the resonance for an anomeric sugar proton (δ_H 4.75 d, $J = 7.5$ Hz), indicated the presence of a *β*-D-glucose moiety. An HMBC experiment (**Figure 2**) allowed assignment of the signals for the ABX systems, the carboxylic acid moiety (δ_C 168.4 and 173.3), and the aliphatic (δ_H/δ_C 5.24/74.3, δ_H/δ_C 3.17, 3.07/37.9) and unsaturated (δ_H/δ_C 6.27/114.4, δ_H/δ_C 7.56/147.8) units of the rosmarinic acid aglycone. Additionally, a cross-peak between the anomeric proton signal of the *β*-D-glucose moiety and the carbon signal of C-3′ established the linkage between the two subunits. Hydrolysis of compound **3** confirmed simultaneously the presence of a *β*-D-glucose moiety as well as the suggested stereochemistry *R*-(+) of the rosmarinic acid unit deduced from similar optical rotation values of compound **3** ($[\alpha]_D^{20} = +11.24$; MeOH; $c = 0.1$) and salviaflaside (*R*-(+)-3-*O*-*β*-D-glucopyranosyl rosmarinic acid, $[\alpha]_D^{20} = +18.5$; MeOH; $c = 0.065$ (*18*)). Thus, compound **3** is *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid as confirmed by ^{13}C NMR data, HMQC, and DQFCOSY data.

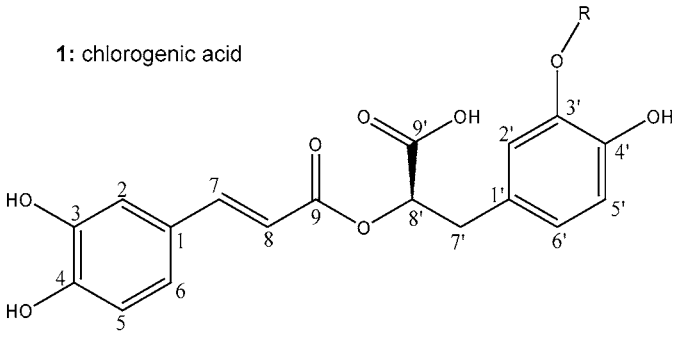
Antioxidant Activity. Isomers of **3**, the *R*-(+)-3-*O*-*β*-D-glucopyranosyl rosmarinic acid (salviaflaside), have been described in the roots of *Salvia flava* L., *Salvia deserta* Schang, *Salvia miltiorhiza* Bunge, and in *Horminium pyrenaicum* L., all belonging to the Lamiaceae family (*18*−*21*). In the fruits of *Helicteres isora* L. (Sterculiaceae), the presence of *R*-(+)-4-*O*-*β*-D-glucopyranosyl rosmarinic acid and 4,4′-di-*O*-*β*-D-glucopyranosyl rosmarinic acid has been shown (*22*). Of these isomers, only the antioxidant capacity of 4,4′-di-*O*-*β*-D-glucopyranosyl rosmarinic acid was determined. This study was done by the measurement of the scavenging activity against superoxide anion produced with xanthine and xanthine oxidase (*22*). Its activity was shown to be higher than that of its aglycone. After confirmation of the antioxidant activity of compound **3** by a DPPH/TLC decolorizing assay, the antioxidant activity of the crude extract of *Eryngium alpinum*, chlorogenic acid **1**, *R*-(+)-rosmarinic acid **2**, and *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid **3** was determined quantitatively (**Table 2**), to compare their respective activities. Related to the activity of ascorbic acid, the values show that compound **3** is half as active as *R*-(+)-rosmarinic acid **2**. Satake et al. (*22*) and our results indicate that the position and/or the number of glycosyl groups present in the molecule plays a significant part in the antioxidant activity (*23*).

Chemotaxonomic Study of *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl Rosmarinic Acid. Rosmarinic acid is a compound occurring in many plant families, especially in the Lamiaceae and Boraginaceae families. Due to its wide distribution, it cannot be used as a chemotaxonomic marker (*24*). In contrast, the new glycosylated derivative of the rosmarinic acid **3** could be of a chemotaxonomic interest. For this reason and to find a species richer in **3** than *E. alpinum*, a quantitative determination of *R*-(+)-rosmarinic acid **2** and *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid **3** was performed in several species of the Apiaceae family with the three subfamilies Saniculoideae, Apioideae, and Hydrocotyloideae. First, these two compounds were quantified in 13 species from the genus *Eryngium* as well as *Sanicula europeae* L., all species from the subfamily Saniculoideae. For this purpose, it was necessary to grow *Eryngium* plants from seeds, and samples were taken from 1-year-old plants. Because the aerial parts showed only traces of compounds **2** and **3**, the chemotaxonomic investigation of the *Eryngium* species was carried out with the roots that showed a distinctly higher content of phenolic compounds. As further representative of the same subfamily, we investigated a sample of the aerial parts of *Sanicula europeae* L., as the root material was not available. Additionally to samples of the Saniculoideae subfamily, roots of the following species of the Apioideae subfamily were analyzed: *Imperatoria ostruthium* L., *Pimpinella peregrina* L., and *Levisticum officinalis* L. As representative from the Hydrocotyloideae subfamily, we investigated a sample of the commercially available aerial parts of *Hydrocotyle asiatica* L. The results are shown in **Table 3**. Compounds **2** and **3** were detected in all analyzed *Eryngium* species, except





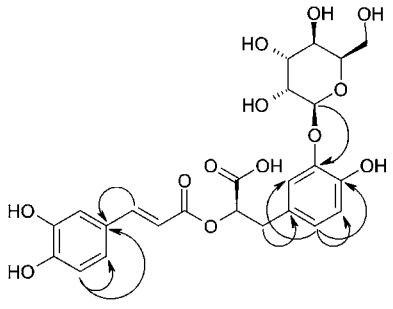
1: chlorogenic acid



2: R = H; *R*-(+)-rosmarinic acid

3: R = β-D-Glucopyranosyl; *R*-(+)-3′-*O*-β-D-glucopyranosyl rosmarinic acid

Figure 1. HPLC analysis of *Eryngium alpinum* L. extract, detected at 320 nm, and structures of compounds **1**, **2**, and **3**.



R-(+)-3′-*O*-β-D-glucopyranosyl rosmarinic acid (**3**).

Figure 2. Important HMBC correlation of *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid (**3**).

E. giganteum L., which was devoid of **3**. The highest content of substance **3** in the investigated *Eryngium* species was measured in *E. planum* L. (16 mg/g), *E. planum* BC (14 mg/g), *E. yuccifolium* L. (11 mg/g), and *E. alpinum* L. (11 mg/g). A clearly higher content of the quantified rosmarinic acid and its derivative was found in aerial parts of *Sanicula europeae* L. (96 mg/g of **2** and 297 mg/g of **3**). The investigated representatives of the subfamily Apioideae (*Imperatoria ostruthium* L., *Pimpinella peregrina* L., and *Levisticum officinalis* L.) contained only *R*-(+)-rosmarinic acid; the analyzed Hydrocotyloideae representative *Hydrocotyle asiatica* L. contained neither **2** nor **3**. The second part of the study consisted of the investigation of the roots of many *Eryngium* species obtained from different sources (botanical gardens, alpine gardens, commercial sources, and collected). As the parameters age, culture and period of harvest were not controlled; only the qualitative determination of compounds **2** and **3** is given. Results are shown in **Table 4**. Quantification results of compound **3** showed distinct concentration variations including its total absence. The highest differences were observed in *E. amethystinum*, *E. maritimum*, and *E. campestre*. *R*-(+)-3′-*O*-*β*-D-Glucopyranosyl rosmarinic acid **3** was missing in all investigated *E. giganteum* specimens. This result is in good accordance with the analyses of *E. giganteum* grown from seeds (**Table 3**).

The presence of *R*-(+)-3′-*O*-*β*-D-glucopyranosyl rosmarinic acid in the *Eryngium* L. and *Sanicula* L. genus and its absence in the three Apioideae species and *Hydrocotyle asiatica* L. analyzed suggest its potential as a chemotaxonomic marker of

Table 4. Analyses of the Roots of *Eryngium* Species for the Presence of *R*-(+)-Rosmarinic Acid (**2**) and *R*-(+)-3′-*O*-β-D-Glucopyranosyl Rosmarinic Acid (**3**)

species (origin)	2	3
E. agavifolium (Bot. Garden, Bern)	+	n.d.[a]
E. alpinum (Maran/Arosa)	+	+
E. alpinum (Bot. Garden Porrentruy)	+	+
E. alpinum (La Thomasia)	+	+
E. alpinum (Bergün)	+	+
E. alpinum (Ardon)	+	+
E. alpinum (Agroscope, Bruson)	+	+
E. alpinum (Bot. Garden, Freiburg)	+	+
E. alpinum (La Rambertia)	+	+
E. alpinum (Schynige Platte)	+	+
E. amethystinum (FND, Morocco)	+	+
E. amethystinum (Bot. Garden, Zürich)	+	n.d.
E. bourgatii (Bot. Garden, Bern)	+	+
E. bourgatii (Pyrenees)	+	+
E. campestre (Val d'Aoste)	+	n.d.
E. campestre (Bot. Garden, Zürich)	+	+
E. campestre (Bot. Garden, Bern)	+	+
E. campestre (Rouffach)	+	+
E. campestre (Bot. Garden, Reinach)	n.d.	n.d.
E. campestre (FND, Morocco)	+	+
E. campestre (Bot. Garden, Freiburg)	+	+
E. campestre (Sachsen Anhalt)	n.d.	n.d.
E. falcatum (FND, Morocco)	+	n.d.
E. foetidum (FND, Vietnam)	n.d.	n.d.
E. giganteum (Bot. Garden, Bern)	+	n.d.
E. giganteum (Bot. Garden, Basel)	+	n.d.
E. giganteum (Bot. Garden, Zürich)	+	n.d.
E. ilicifolium (FND, Morocco)	n.d.	n.d.
E. maritimum (FND, Morocco)	+	n.d.
E. maritimum (FND, Germany)	+	+
E. maritimum (Bot. Garden, Freiburg)	+	+
E. yuccifolium (Bot. Garden, Bern)	n.d.	n.d.

[a] Not detected at a detection limit of 0.1 mg/g.

the Saniculoideae subfamily. To confirm these results, a more systematic study considering different parts of the plants will be done.

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