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Caffeoyl-D-Glucaric Acid Derivatives in the Genus *Gnaphalium* (Asteraceae: Gnaphalieae)

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Abstract: A chemosystematic survey was carried out to specify whether leontopodic acid and leontopodic acid B, two unique caffeoyl-D-glucaric acid derivatives, recently identified in the emblematic alpine edelweiss (*Leontopodium alpinum* Cass.) are also found in members of the genus *Gnaphalium* from the Alps. *Gnaphalium* is closely related to *Leontopodium* and both genera are assigned to the Gnaphaliinae subtribe (Asteraceae, Gnaphalieae). In all investigated *Gnaphalium* species, *G. hoppeanum* W.D.J.Koch, *G. norvergicum* Gunnerus, *G. supinum* L., *G. sylvaticum* L., and *G. uliginosum* L., both leontopodic acid and leontopodic acid B were detected. Moreover, a number of related compounds were detected by HPLC/MS and their assumed structures are discussed. The chemosystematic data reported here are of interest to explore new sources for the biologically active compounds leontopodic acid and leontopodic acid B and they also hint to the occurrence of novel caffeoyl-D-glucaric acid derivatives in *Gnaphalium* not detected in *Leontopodium*, yet.

Keywords: Asteraceae; Gnaphalieae; Gnaphaliinae; *Gnaphalium*; *Leontopodium*; phenolic acids; chemosystematics.

1. Plant Sources

In the current study, the occurrence of caffeoyl-D-glucaric acid derivatives in members of the genus *Gnaphalium* occurring in the Alps was investigated. Thereby, we intended to assess the applicability of these compounds as chemosystematic markers either for the genus *Leontopodium* or the Gnaphaliinae as a whole. Moreover, we were interested in finding new sources for these potent antioxidants, which were until now only known from the protected and difficult to cultivate alpine species *Leontopodium alpinum*.

The used nomenclature is congruent with Fischer et al. [1] and Wilhalm et al. [2]. Synonyms used in Flora Europaea [3] are also indicated. Voucher specimens of all investigated populations are preserved in the Herbarium of the Institut für Botanik, Innsbruck (IB) and the private herbarium of CZ. Scans of the voucher specimens are available as on-line only material (Figures S1-S5). The

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collection data of the plant material are given in the following format: Current scientific name; scientific name according to Flora Europaea [3]; collection site; voucher number in the herbarium of the University of Innsbruck (IB), voucher number in the herbarium of CZ.

Gnaphalium hoppeanum Koch; *Omalotheca hoppeana* (Koch) Sch.Bip. & F.W.Schultz; Juppenspitze near Lech/Bludenz/Vorarlberg/Austria; N 47°14'14.3"; E 10°06'30.2"; 2270 m; 07.08.2009; IB-33271; CZ-20090807D-1. *Gnaphalium norvegicum* Gunnerus; *Omalotheca norvegica* (Gunn.) Sch.Bip. & F.W.Schultz; Leitner Tal near Leiten/Lienz/Tirol/Austria; N 46°41'09.7", E 12°32'36.1"; 1970 m; 29.07.2009; IB-33272; CZ-20090729A-1. *Gnaphalium supinum* L.; *Omalotheca supina* (L.) DC.; Kühtai, Wiesberg/Imst/Tirol/Austria; N 47°12'51.8", E 11°02'22.1"; 2150 m; 02.08.2009; IB-33273; CZ-20090802B-2. *Gnaphalium sylvaticum* L.; *Omalotheca sylvatica* (L.) Sch.Bip. & F.W.Schultz; Patscherkofel near Patsch/Innsbruck-Land/Tirol/ Austria; N 47°12'47.0", E 11°25'20.3"; 1110 m; 12.08.2009; IB-33274; CZ-20090812A-1. *Gnaphalium uliginosum* L.; *Filaginella uliginosa* (L.) Opiz; near St. Agatha/Grießkirchen/Oberösterreich/Austria; N 48°23'18.0"; E 13°55'41.8"; 480 m; 08.08.2009; IB-33275; CZ-20090808A-1. For *L. alpinum*, refer to reference [4].

2. Previous Studies

Leontopodium alpinum Cass. was originally described as *Gnaphalium leontopodium* L. and thus, also as a member of the genus *Gnaphalium*. Today, the generic status of *Leontopodium* is undisputed but the close relationship of *Gnaphalium* and *Leontopodium* is reflected in the fact that both genera are currently assigned to the subtribe Gnaphaliinae within the Gnaphalieae tribe of the Asteraceae family. According to the latest molecular data, the Gnaphaliinae comprise approximately 180 to 190 genera, including the European genera *Antennaria*, *Filago*, *Gnaphalium*, and *Helichrysum* [5]. The cosmopolitan genus *Gnaphalium* comprises around 50 species [6], seven of which occur naturally in Europe [3]. Five of these European species are also occurring in the Alps [1].

Recently, two unique caffeoyl-D-glucaric acid derivatives, leontopodic acid and leontopodic acid B, were identified in *Leontopodium alpinum* Cass. [4,7]. Subsequent studies demonstrated a chemoprotective bio-activity of leontopodic acid against toxins aflatoxin B1 and deoxynivalenol [8]. Other secondary metabolites of the genus *Leontopodium* known so far include flavonoids [4], lignans [9], polyacetylenes [9], and sesquiterpenes [10]. From *Gnaphalium* mainly diterpenes [11-13] and flavonoids [14-15] were reported. To the best of our knowledge, no reports on phenolic acids from *Gnaphalium* have been published yet.

3. Present Study

500.0 mg of dry ground plant material were sonicated with 25 mL of a mixture of CH_3OH and H_2O (1/1, v/v) for 30 minutes. After centrifugation the solution was transferred to a 100 mL volumetric flask. The procedure was repeated twice and the combined extracts were diluted to 100.0 mL with a mixture of CH_3OH and H_2O (1/1, v/v). Finally 20.0 mL of this solution were brought to dryness in vacuo, re-dissolved in 2.00 mL of a mixture of CH_3OH and H_2O (1/1, v/v), filtered and used for HPLC analysis.

Phenolics were analyzed as reported before [4]. HPLC: HP 1100 system (Agilent, Waldbronn, Germany) equipped with an auto sampler, DAD and column thermostat; stationary phase: Phenomenex Synergy Polar RP 80A (150 x 4.6 mm); guard column: LiChroCART 4-4, with Merck LiChrospher 100 RP 18 (5 µm); mobile phase: solvent A: H_2O with 0.9 % formic acid, 0.1 % acetic acid, 1.5 % 1-butanol (all v/v); solvent B: acetonitrile with 30 % methanol and 0.9 % formic acid, 0.1 % acetic acid (all v/v); solvent C: methanol; DAD: 350 nm, temp.: 45°C; injection volume: 5 µL; flow: 1.00 mm/min; solvent gradient: start: 90 % A, 10 % B, 0% C; 15 min: 87 % A, 13 % B, 0 % C; 40 min: 84 % A, 16 % B, 0 % C; 41 min: 74 % A, 16 % B, 10 % C; 50 min: 70 % A, 16 % B, 14 % C; 60 min: 55 % A, 25 % B, 20 % C; 65 min: 2 % A, 78 % B, 20 % C; stop time: 70 min; post time: 10 min. MS-parameters: LC-MS: Esquire 3000^{plus} (Bruker Daltonics, Bremen, Germany); split: 1:5; ESI,

neg. mode; spray voltage: –4.5 kV, 325°C; dry gas: 8.00 l/min; nebulizer 30 psi; full scan mode: *m/z* 100-1500.

Using the methodology described above and taking into account the UV-characteristics of caffeic acid derivatives displayed in reference [16], eleven major caffeic acid derivatives were detected in extracts of members of the genus *Gnaphalium* and *L. alpinum*. These compounds were characterized with regards to their on-line ESI-MS spectra in the negative mode (*m/z*) and HPLC retention times in the system established for the phenolic constituents of *L. alpinum* [4]. In orders of increasing HPLC retention times (rt) these compounds were characterized as follows: **1** rt = 5.7 min, *m/z* = 353 [M - H]$^-$; **2** rt = 27.8 min, *m/z* = 515 [M - H]$^-$; **3** rt = 30.5 min, *m/z* = 515 [M - H]$^-$; **4** rt = 36.5 min, *m/z* = 601 [M - H]$^-$; **5** rt = 39.0 min, *m/z* = 695 [M - H]$^-$; **6** rt = 43.8 min, *m/z* = 601 [M - H]$^-$; **7** rt = 44.6 min, *m/z* = 781 [M - H]$^-$; **8** rt = 48.5 min, *m/z* = 781 [M - H]$^-$; **9** rt = 49.4 min, *m/z* = 781 [M - H]$^-$; **10** rt = 57.8 min, *m/z* = 857 [M - H]$^-$; and **11** rt = 59.0 min, *m/z* = 763 [M - H]$^-$.

In comparison with the data published earlier [4, 17] and in comparison with an extract of *L. alpinum* analyzed in the same sequence as the extracts of the different *Gnaphalium* species, compounds **1-3**, **5**, and **8** were identified as **1** chlorogenic acid, **2** 3,5-dicaffeoylquinic acid, **3** 4,5-dicaffeoylquinic acid, **5** leontopodic acid B, and **8** leontopodic acid. The remaining compounds were not unambiguously identified. Based on their UV-spectra which indicate that all of them contain at least one caffeoyl moiety and their masses measured by on-line ESI-MS as indicated above, the following considerations on the structures of these compounds seem justified: Compounds **7** and **9** are probably isomers of leontopodic acid with the same D-glucaric acid backbone and the same number of substituents but with these substituents [three caffeoyl moieties and one (3*S*)-3-hydroxybutanyl moiety] attached in a different order to the D-glucaric acid moiety than in leontopodic acid. Compound **11** (*m/z* = 763, [M - H]$^-$) can easiest be envisioned as a dehydro-derivative of leontopodic acid (or one of its isomers), i.e. the (3*S*)-3-hydroxybutanoyl moiety of leontopodic acid is probably replaced by a butenoyl moiety in this compound. Compounds **4** and **6** (*m/z* = 601, [M - H]$^-$) are conceivable as derivatives of **11** (or one of its isomers) with one caffeoyl moiety less than **11**, i.e. butenoyl-dicaffeoyl derivatives of D-glucaric acid. Finally, compound **10** (*m/z* = 857, [M - H]$^-$) is based on its UV-spectrum and its mass spectrum tentatively identified as 2,3,4,5-tetracaffeoyl-D-glucaric acid.

Table 1 gives an overview about the occurrence of compounds **1-11** in the five *Gnaphalium* and the one *Leontopodium* species analyzed. Interestingly, leontopodic acid B **5**, leontopodic acid **8**, and its tentative isomer **9** were detected not only in *L. alpinum* but also in all five investigated *Gnaphalium* species. Moreover, the common caffeoyl quinic acid derivatives chlorogenic acid **1**, 3,5-dicaffeoylquinic acid **2**, and 4,5-dicaffeoylquinic acid **3** were found in all taxa investigated. Compound **4** was found in all five *Gnaphalium* species investigated but not in *L. alpinum*. Compound **6** was detected in all *Gnaphalium* species investigated except *G. hoppeanum* and was also not detected in *L. alpinum*. Compound **7**, another tentative isomer of **8**, was missing in *Leontopodium* but detected in all *Gnaphalium* species except *G. uliginosum*. Compounds **10** and **11** were both only found in *G. norvegicum*, *G. sylvaticum*, and *G. uliginosum*.

The presented findings indicate that caffeoyl-D-glucaric acid derivatives are not restricted to the genus *Leontopodium* but also occur in other taxa of the Gnaphalieae. Within the genus *Gnaphalium* compounds of this type seem to be widespread and their structural diversity appears to be higher than in *Leontopodium*. In chemosystematics, caffeoyl-D-glucaric derivatives indeed seem to have a potential to characterize certain groups within the Gnaphalieae and these compounds also might be suitable to distinguish between different species within the genus *Gnaphalium*.

The discovery of caffeoyl-D-glucaric acid derivatives in the genus *Gnaphalium* might also prove of practical importance. Leontopodic acid is an antioxidant compound patented as an anti-wrinkle cosmetic [18] and *L. alpinum* is cultivated in Switzerland [19] to obtain extracts rich in this bioactive [8] compound. The finding that taxa widespread in Central Europe and not confined to alpine regions such as *G. sylvaticum* and *G. uliginosum* also contain these compounds will make access to leontopodic acid easier as these species are more amenable to cultivation.

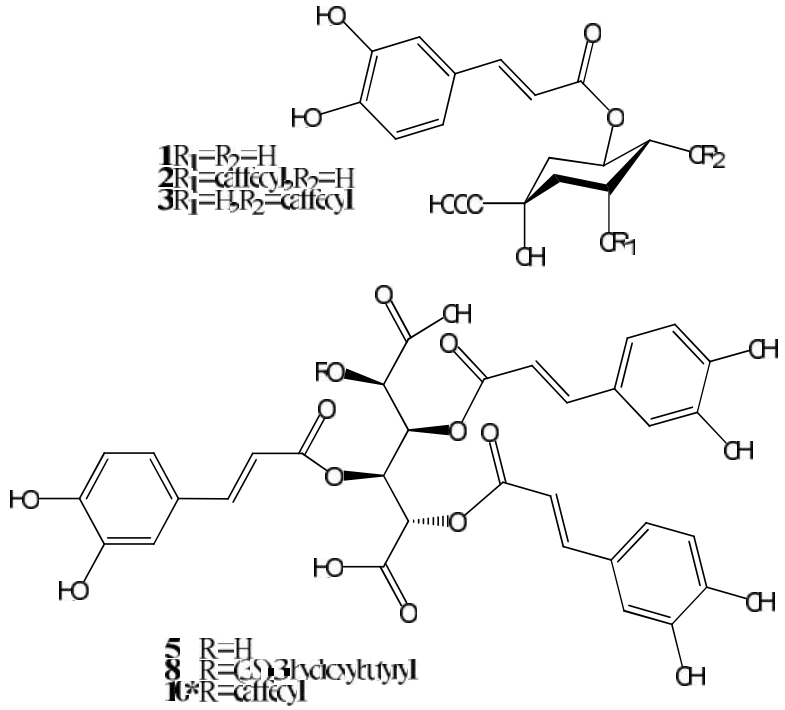

Figure 1. Caffeic acid derivatives from *Gnaphalium* and *Leontopodium* (* assigned tentatively).

Finally, up to now unidentified compounds with a higher number of *ortho*-dihydroxyphenolic moieties, such as the supposed tetracaffeoyl-D-glucaric acid derivative **10**, might even surpass the anti-oxidant activity of leontopodic acid. Further studies to unambiguously identify caffeoyl derivatives **4**, **6**, **7**, **9**, **10**, and **11** and to assess their bioactivities are therefore warranted.

Table 1. Occurrence of caffeic acid derivatives (**1-11**) in members of the genera *Gnaphalium* and *Leontopodium* from the Alps based on HPLC/MS/DAD data: (+) traces, + present.

Taxon/Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
retention time (min)	5.7	27.8	30.5	36.5	39.0	43.8	44.6	48.5	49.4	57.8	59.0
m/z [M - H]$^-$	353	515	515	601	695	601	781	781	781	857	763
G. hoppeanum	+	+	+	+	+		+	+	+		
G. norvegicum	+	+	+	+	(+)	+	+	+	(+)	(+)	+
G. supinum	+	+	+	+	+	(+)	+	+	(+)		
G. sylvaticum	+	+	+	+	(+)	+	+	+	(+)	+	+
G. uliginosum	+	+	+	+	+	(+)		+	(+)	+	+
L. alpinum	+	+	+		+			+	+		

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP

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