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Polar phenolic compounds in *Dryopteris filix mas* and *Dryopteris dilatata*[*])

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This study investigates for the first time in detail the pattern of flavonoids and caffeic acid derivatives in the therapeutically used ferns *Dryopteris filix mas* und *Dryopteris dilatata*. Extensive analyses using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) revealed robust differences between the pattern of these phenolics in the two species: Whereas kaempferolrutinoside and rutin occur only in *Dryopteris filix mas*, caffeoyltartronate and kaempferol-3-O-β-D-glucopyranosido-7-O-α-L-rhamnopyranoside were detected solely in *D. dilatata*. Caffeoyltartronate is a very rare natural compound that until now has been described only once in mung bean sprouts.

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Keywords: *Dryopteris filix mas, Dryopteris dilatata,* flavonoids, caffeic acid derivatives.

Introduction

Ferns from the genuses *Dryopteris*, *Athyrium*, *Polystichum* and *Thelypteris* are important remedies against rheumatic disorders, painful joints and inflammation in the traditional medicine of the alpine region of Middle Europe. Information about the use of ferns are collected in the VOLKSMED database (Saukel et al. 2006). Dried fronds are used for the preparation of bath additives or tinctures for topical use, might be directly applied to the skin or used as padding.

The determination and discrimination of species can be quite delicate for some ferns. Therefore, several studies were performed to elucidate the possibilities of discrimination by micromorphological features of leaves (Schnattinger 2008, Kainrath 2008 and Pokorny 2010) by means of the Det Morph program (Saukel et al. 2005).

Dryopteris filix- mas (L.) Schott and *Dryopteris dilatata* (L.) Hoffman are widespread ferns in Austria and the differentiation between this species is not demanding.

Phytochemical studies of *D. filix mas* and *D. dilatata* have mainly focussed on the major compounds, the phloroglucinols, which have gained a lot of interest due to their broad

[*]) Dedicated to Doz. Dr. Franz Speta with best wishes on the occasion of his 70th birthday.

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spectrum of pharmacological activities e.g. anthelmintic or antirheumatic effects (Hintersteininger 2011, Widen et al. 2001).

Polar phenolic compounds, such as flavonoids, have hardly been studied in *D. filix-mas*, and besides dryopterin (Karl et al. 1981) no structures have been published until now (Mewari & Kumar 2011). No investigations on these analytes have been performed for *D. dilatata*. Nevertheless, these compounds could provide a convenient possibility for a fast distinction of the two species e.g. in pulverized material, as flavonoid references are much more readily available than phloroglucinol standards.

Therefore, in this study the pattern of flavonoids and caffeic acid derivatives in *D. filix-mas* and *D. dilatata* was studied. Its stability in different parts of the fronds, different years and at different origins was investigated to determine its suitability to distinguish between the two species.

Material and Methods

General experimental procedures:

TLC-systems: a) silica 60 F_{254} plates (Merck, Darmstadt) using cyclohexane-dichloromethane-formic acid-ethylformiate (25+30+8+30), ethylacetate-formic acid-acetic acid-water (100+11+11+26) or chloroform-ethylacetate (60+40) as mobile phases; b) polyamide 11 F_{254} plates (Merck, Darmstadt) using toluene-petroleum ether-butanone-methanol (18+4.5+9+8) as mobile phase; c) cellulose plates (Merck, Darmstadt) using 30% acetic acid as mobile phase. Detection was performed with a 1% methanolic solution of Naturstoffreagens A and additionally with 5% ethanolic solution of PEG 400; the plates were controlled under UV_{366}.

The total flavonoid content was determined according to the flavonoid quantification in the monograph Betulae folium (Pharm. Eur.). Spectra were recorded on a Beckman DU-640 Spectrophotometer.

HPLC was performed on a system including a SIL-20AC HT autosampler, a DGU-20A5 degasser, a LC-20AD pump, a CTO-20AC column oven, a SPD-M20A diode array dector and a CBM-20A interface (all Shimadzu). Stationary phase: Agilent Hypersil BDS C-18, 5µm, 4 x 250 mm; mobile phase: A) aqueous formic acid pH 2.7, B) acetonitrile with HCOOH conc. (0.23 mL/L). The analyses were performed at 30°C with a flow rate of 1.0 ml/min. under gradient elution (0 - 17 min from 12 to 20.5 % B, 17 - 30 min from 25.5 to 43.9% B).

ESI-MS were recorded on a PE Sciex API 150 EX single quadrupole instrument (Applied Biosystems, Foster City, CA, USA) configurated for negative ionization. Full scan spectra were acquired over the range 180 – 800 amu/2 sec. Analyst 1.4.1 software was used for data analysis.

NMR spectra were recorded on a Avance DRX 600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) using a 5 mm switchable quadruple probe (QNP, ^{1}H, ^{13}C, ^{19}F, ^{31}P) with z-axis gradients and automatic tuning and matching accessory. The resonance frequency for ^{1}H NMR was 600.13 MHz, for ^{13}C NMR 150.92 MHz. All measurements were performed for a solution in D_2O or fully deuterated methanol at 300 K. Standard 1D and gradient enhanced (ge) 2D experiments like double quantum filtered (DQF) COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC, were used as supplied by the manufacturer. The chemical shifts are referenced internal to the residual, non-deuterated solvent

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signal for ^{1}H (D_2O: δ = 4.75 ppm; CD_3OD: δ = 3.31 ppm), or to the carbon signal of the solvent for ^{13}C (CD_3OD: δ = 49.00 ppm). The analysis of the ^{1}H-^{1}H coupling constants (given in Hertz) was supported by the spin-simulation program DAISY within the software TOPSPIN (Bruker BioSpin).

Plant material:

Plant material of *Dryopteris filix mas* and of *Dryopteris dilata* was collected in Austria from three different origins in two years (see Table 1). Vouchers are kept in the herbarium of the Department of Pharmacognosy, University of Vienna.

Extraction for analytical purposes:

200 mg pulverized dried fronds were extracted exhaustively with 200 ml aqueous methanol (40%) under reflux at 80°C for 1 hour. After filtration and evaporation 30 mg of the extracts were dissolved in 0.3 ml 20% methanol and applied to Varian Mega BE-C18 cartridges (50 mg; 1 ml; conditioned with 2 volumes (RV) methanol and 3 RV water). After withdrawal of the solvent by suction (fraction I), the cartridges were eluted with 2 RV water (fraction II), 6 RV 20% methanol (fraction III) and 4 RV 80% methanol (fraction IV). All fractions were evaporated to dryness.

Extraction for fractionation:

Fronds of *Dryopteris dilata* were collected at Ebenwaldhöhe, Lower Austria, in 2007. The authenticated dried plant material was milled.

For the isolation of compound **1** the drug (20 g) was extracted with 200 ml aqueous methanol (40%) under reflux at 80°C for 1 hour. After filtration the extracts were evaporated resulting in 2.15 g dry extract.

Tab. 1: Plant material. – Tab. 1: Pflanzenmaterial.

Species	Origin	Year	Voucher No.
Dryopteris dilatata	Ebenwaldhöhe	2006	EWH-A
Dryopteris dilatata	Ebenwaldhöhe	2006	EWH-C
Dryopteris dilatata	Ebenwaldhöhe	2006	EWH-D
Dryopteris dilatata	Ebenwaldhöhe	2006	EWH-E2
Dryopteris dilatata	Ebenwaldhöhe	2006	EWH-F
Dryopteris dilatata	Schneeberg	2007	S07-1
Dryopteris dilatata	Schneeberg	2007	S07-4
Dryopteris dilatata	Schneeberg	2007	S07-7
Dryopteris dilatata	Schneeberg	2007	S07-10
Dryopteris dilatata	Schneeberg	2007	S07-14
Dryopteris dilatata	Lauschützsee	2007	L07-1Dd
Dryopteris filix mas	Ebenwaldhöhe	2006	Dfm-0
Dryopteris filix mas	Ebenwaldhöhe	2007	Dfm-1
Dryopteris filix mas	Ebenwaldhöhe	2007	Dfm-2
Dryopteris filix mas	Ebenwaldhöhe	2007	Dfm-3
Dryopteris filix mas	Ebenwaldhöhe	2007	Dfm-4
Dryopteris filix mas	Ebenwaldhöhe	2007	Dfm-5
Dryopteris filix mas	Turrachbach	2007	T07-1
Dryopteris filix mas	Lanschützsee	2007	L07-1fm

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The extract was fractionated in 5 portions of 400 mg, each, by SPE using Varian Mega BE-C18 cartridges (5 g; 20 ml). After conditioning with 2 volumes (RV) methanol and 3 RV water, the extract dissolved in 20 % methanol (400mg/10 ml) was applied. The solvent was removed by suction (fraction I). Elution was performed with 2 RV water (fraction II), 6 RV 20% methanol (fraction III) and 4 RV 80% methanol (fraction IV). The corresponding fractions of these separations were combined resulting in 887 mg fraction I, 407 mg fraction II, 186 mg fraction III and 236 mg fraction IV.

For the isolation of compound **2** a higher amount of the drug (50 g) was extracted with 500 ml aqueous methanol (40%) in the same way resulting in 8.4 g dry extract. Fractionation by SPE (Varian Mega BE-C18 cartridges 10 g; 60 ml) was performed in 8 portions of 1g extract (dissolved in 20 ml 20% methanol), each, in the same manner as described above. The amounts of the combined fractions were 4.57 g (fraction I), 1.42 g (fraction II), 1.02 g (fraction III) and 0.74 g (fraction IV).

Isolation and identification of Compounds 1 and 2:

Caffeoyltartronate (**1**)

By gel permeation chromatography (GPC) on Sephadex LH 20 (24 x 1 cm) under elution with water 400 mg fraction II were separated and resulted in the isolation of 29 mg compound **1**.

TLC R*f*: 0.14 (system a; mobile phase: ethylacetate-formic acid-acetic acid-water 100+11+11+26). *Rt*-HPLC: 3.50 min. UV λ_{max} in RP-HPLC solvent nm: 236, 330.

Kaempferol-3-O-β-D-glucopyranosido-7-O-α-L-rhamnopyranoside (**2**)

700 mg of fraction IV were submitted to GPC on Sephadex LH 20 (45 x 2 cm) and eluted with methanol-water mixtures of decreasing polarity. The resulting fraction 8 (30 mg) contained enriched compound **2** which was finally purified by another GPC with the same stationary (26 x 1cm) and mobile phase (7 mg).

TLC R*f*: 0.50 (system a; mobile phase: ethylacetate-formic acid-acetic acid-water 100+11+11+26). *Rt*-HPLC: 10.70 min. UV λ_{max} MeOH nm: 262, 348; +$AlCl_3$+HCl: 275, 298sh, 346, 400. Negative ESI-MS ($C_{27}H_{30}O_{15}$) m/z: 593 $[M-H]^-$, 447 $[M-H-146]^-$, 431 $[M-H-162]^-$.

Results and discussion

An important pre-requisite in this first detailed investigation of polar phenolic compounds in *D. filix mas* and *D. dilatata* was the proof, that the pattern of these substances is stable in different parts of the fronds, in different plants from one origin, in plants from one origin in different years and finally at different locations. For this purpose aequeous methanolic (40%) extracts were prepared and studied by TLC whether directly or after enrichment by solid phase extraction (SPE).

First of all, one frond of each species was divided into stalk, upper part, medium part and lower part (see Figure 1). TLC of the extracts on silica under use of the mobile phase ethylacetate-formic acid-acetic acid-water (100+11+11+26) showed a conform pattern of polar phenolics in the three leaf samples. As expected, no secondary metabolites were detected in the stalks. This result confirmed that extracts of even small leaf samples can provide a definite conclusion on the composition these analytes in the plant material.

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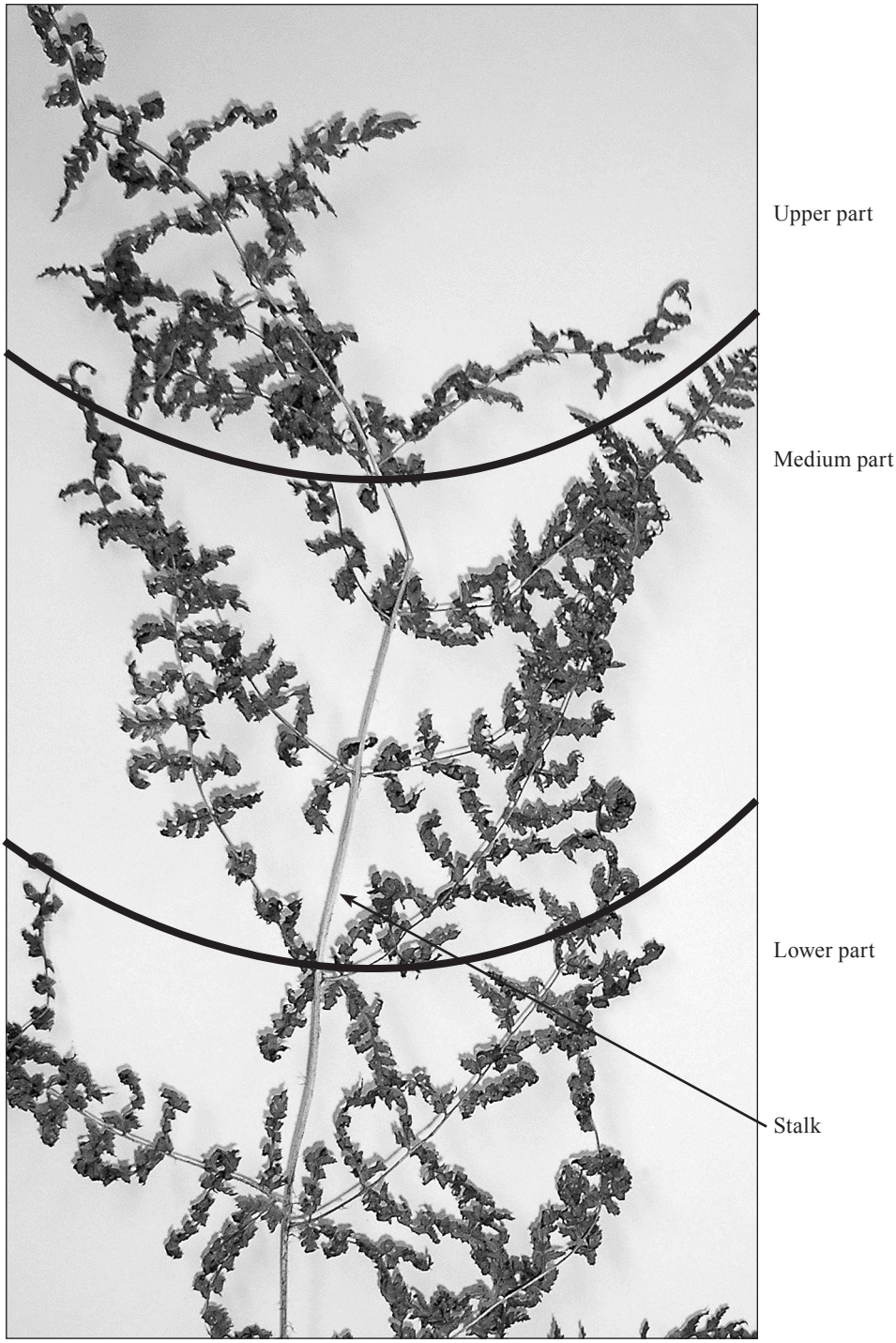

Fig. 1: Parts of fronds analyzed separately. – Abb. 1: Getrennt untersuchte Teile der Farnwedel.

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For a comparison of different plants five samples from one origin of *D. filix mas* (collected at Ebenwaldhöhe) and *D. dilatata* (collected at Schneeberg) were studied and the extracts fractionated by SPE on C-18 cartridges to enrich the analytes. In fractions I and II mainly caffeic acid derivatives were eluted. Fraction III contained only very small amounts of

D.f.m. 2006 | Astragalin | *D.f.m.* 2007 | chlorogenic acid

Fig. 2: TLC-comparison of extracts from *D. filix mas* collected in 2006 and 2007 on silica plates using ethylacetate-formic acid-acetic acid-water (100+11+11+26) as mobile phase. – Abb. 2: DC-Vergleich der Extrakte von *D.filix mas* und *D. dilatata* , gesammelt 2006 und 2007, auf Kieselgel-Platten mit der mobilen Phase Ethylacetat-Ameisensäure-Essigsäure-Wasser (100+11+11+26).

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phenolics, whereas in fraction IV the flavonoids were enriched. TLC of the fractions (see Material and Methods) of each plant proved only minor quantitative differences in the pattern of polar phenolics. A similar result was observed in TLC comparison of samples from *D. filix mas* collected in 2006 and 2007 at Ebenwaldhöhe confirming the stability of the flavonoid pattern at one origin (see Figure 2).

Material of both species from three different collection sites (see Table 1) was investigated for the conformity of the analytes by TLC as well and the main flavonoids were consistent in all samples.

By HPLC-DAD- and TLC-comparison with authentic substances the polar compounds in *D. filix mas* were identified. The main compound in fraction I and II was chlorogenic acid. In fraction IV astragalin, isoquercitrin and rutin were identified with astragalin being the major flavonoid. Additionally small amounts of kaempferol-3-O-rutinoside were detected (see Figure 3).

In the *D. dilatata* extract chlorogenic acid was one main compound in fractions I and II besides a very polar substance, which showed similar fluorescence after detection with Naturstoffreagent A (see Figure 3). This substance was isolated by GPC on Sephadex® LH 20 using water as mobile phase. By UV-, MS- and NMR-data the compound was unambiguously identified as caffeoyl-tartronate (see Figure 4) with ^{13}C-NMR data available for the first time (see Table 2).

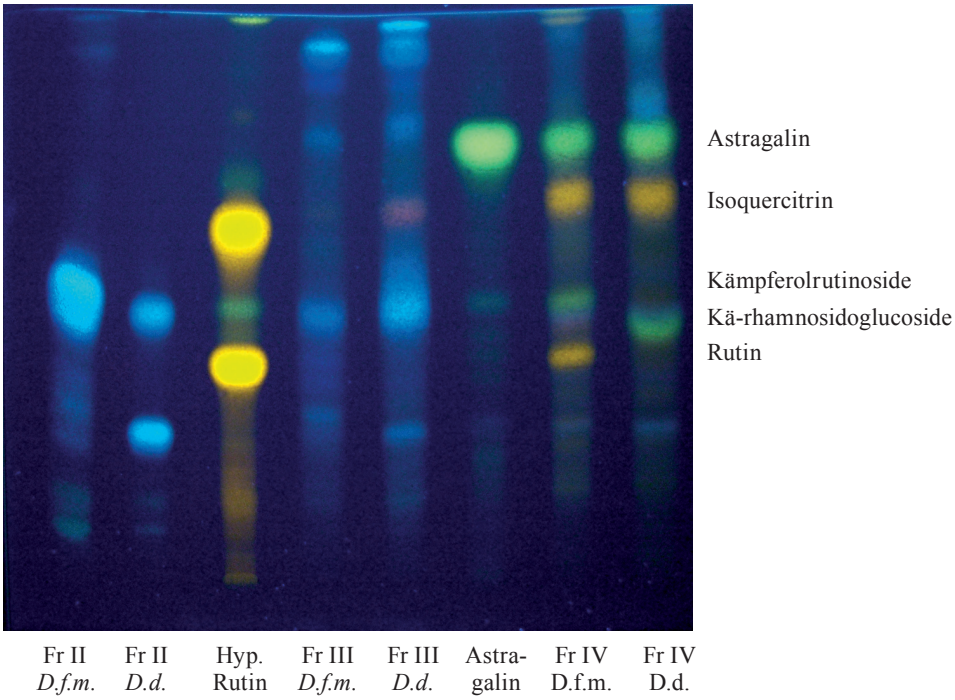

Fig. 3: TLC-comparison of SPE fractions II, III and IV from *D.filix mas* and *D. dilatata* on silica plates using ethylacetate-formic acid-acetic acid-water (100+11+11+26) as mobile phase. – Abb. 3: DC-Vergleich der SPE-Fraktionen II, III und IV von *D.filix mas* und *D. dilatata*auf Kieselgel-Platten mit der mobilen Phase Ethylacetat-Ameisensäure-Essigsäure-Wasser (100+11+11+26).

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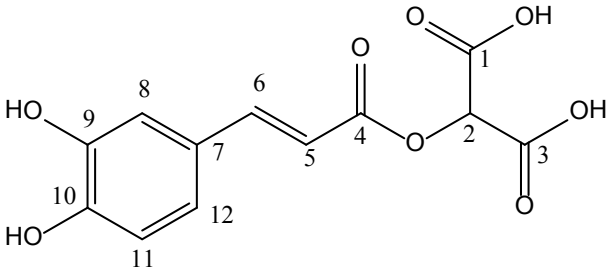
Tab. 2: ^{1}H NMR and ^{13}C NMR shifts (ppm) of caffeoyltartronate in D_2O and CD_3OD. – Tab. 2: ^{1}H-NMR- und ^{13}C-NMR-Verschiebungen (ppm) von Kaffeoyltartronat in D_2O and MeOD.

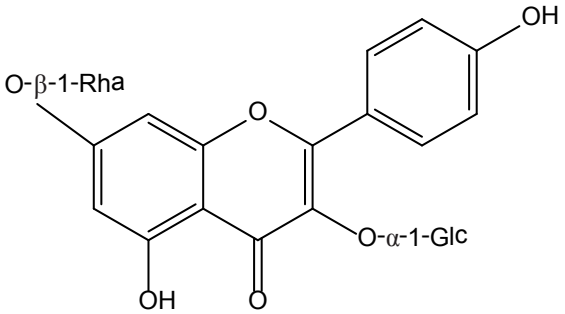
	^{1}H		^{13}C
	D_2O	CD_3OD	D_2O
1/3	---	---	173.32
2	5.159 (s)	5.365 (s)	77.83
4	---	---	168.98
5	6.458 (d, 16.0)	6.451 (d, 15.9)	114.80
6	7.674 (d, 16.0)	7.645 (d, 15.9)	146.71
7	---	---	127.43
8	7.201 (d, 2.0)	7.081 (d, 2.0)	115.49
9	---	---	144.65
10	---	---	147.51
11	6.903 (d, 8.3)	6.765 (d, 8.2)	116.60
12	7.122 (dd, 8.3 / 2.0)	6.957 (dd, 8.2 / 2.0)	123.15

To the best of our knowledge this is only the second report of this compound from a natural source. For the first time caffeoyl-tartronate had been isolated from sprouts/primary leaves from *Vigna radiata* (L.) Wilcek [STRACK et al. 1985].

The flavonoid pattern of *D. dilatata* showed some further differences to the one of *D. filix mas*. Similarly to the latter, astragalin as the main flavonoid glycoside and isoquercitrin as



caffeoyltartronate



kaempferol-3-O-β-D-glucopyranosid-7-O-α-L-rhamnopyranoside

Fig. 4: Structure of caffeoyltartronate and kaempferol-3-O-β-D-glucopyranosid-7-O-α-L-rhamnopyranoside. – Abb. 4: Stuktur von Caffeoyltartronate und Kaempferol-3-O-β-D-glucopyranosid-7-O-α-L-rhamnopyranoside.

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Tab. 3: ^{1}H NMR and ^{13}C NMR shifts (ppm) of kaempferol-3-O-β-D-glucopyranosid-7-O-α-L-rhamnopyranoside in CD_3OD. – Tab. 3: ^{1}H-NMR- und ^{13}C-NMR Verschiebungen (ppm) von Kämpferol-3-O-β-D-glucopyranosid-7-O-α-L-rhamnopyranosid in CD_3OD.

	^{1}H	^{13}C
	CD_3OD	CD_3OD
Kaempferol		
2	–	159,59
3	–	135,62
4	–	179,72
5	–	162,92
6	6,474 (d, 2.2)	100.58
7	–	163,57
8	6,770 (d, 2.2)	95,53
9	–	158,09
10	–	107,46
1‘	–	122,66
2‘/6‘	8,089 (d, 9.0)	132,39
3‘/5‘	6,899 (d, 9.0)	116,14
4‘	–	161,77
α -rhamnose		
1	5,571 (d, 1.7)	99,85
2	4,020 (dd, 1.7, 3.5)	71,70
3	3,830 (dd, 3.5, 9.4)	72,07
4	3,579 (dd, 9.4, 9.5)	73,59
5	3,588 (dd, 9.5, 6.2)	71,28
6	1,251 (d, 6.2)	18,06
β-glucose		
1	5,339 (d, 7.6)	103,62
2	3,446 (dd, 7.6, 9.3)	75,76
3	3,418 (dd, 9.3, 9.2)	71,42
4	3,299 (dd, 9.2, 9.8)	71,42
5	3,212 (ddd 9.8, 2.3, 5.7)	78,52
6	3,705 (dd, 2.3, 11.9)	62,63
	3,522 (dd, 5.7, 11.9)	

well as chlorogenic acid were proven in *D. dilatata* as well. In contrary, rutin and kaempferol-3-O-rutinoside were not detected in this species. Another kaempferol-glycoside namely kaempferol-3-O-β-D-glucopyranosido-7-O-α-L-rhamnopyranoside (see Figure 4) was isolated by twofold CC on Sephadex® LH 20 with 20% methanol as mobile phase. The UV spectra in methanol as well as under addition of $AlCl_3$ solution and HCl, the fragmentation pattern in MS and the shifts in the ^{1}H- and ^{13}C-NMR (see Table 3) correlated excellently with data from literature [Iwashina et al. 1995; Pauli 2000; Suzuki et al. 2008].

The total flavonoid content in *D. filix* mas and *D. dilatata* as determined by the method of the European Pharmacopoeia for drugs containing quercetin- and kaempferol-glycosides was low, 0.066% and 0.055%, respectively.

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Conclusion

This study investigated in detail the pattern of flavonoids and caffeic acid derivatives in *D. filix mas* and *D. dilatata*. By comprehensive experiments on different parts of the fronds, single plants from one origin, material of the same origin from different years and samples from different origins, the stability of the pattern of these analytes in both species was proven. For the first time the major flavonoids and caffeic acid derivatives in *D. filix mas* and *D. dilatata* were identified by TLC and/or HPLC comparison with authentic compounds. In both species astragalin, isoquercitrin and chlorogenic acid were detected. Significant differences in the pattern of the two species were: I) the occurrence of caffoyltartronate and kaempferol-3-O-β-D-glucosido-7-O-α-L-rhamnoside exclusively in *D. dilatata* and II) of rutin and kaempferol-3-O-rutinoside exclusively in *D. filix mas*. Thus, the pattern of polar phenolics allows a distinction of these two species by TLC. For the sample preparation an enrichment of the analytes by SPE is recommended.

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