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CELL WALL-CONJUGATED PHENOLICS FROM CONIFERAE LEAVES

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Key Word Index—*Abies*, *Picea*, *Pinus*, Pinaceae, insoluble phenolics, hydroxycinnamic acids, flavonol glycosides, localization, cell wall, chemotaxonomy.

Abstract—The occurrence of insoluble conjugated (ester-bound) hydroxycinnamic acids (*p*-coumaric and ferulic acids) and flavonol glycosides (kaempferol 3-*O*-β-glucopyranoside and 3-*O*-α-rhamnopyranoside, and quercetin 3-*O*-β-glucopyranoside and 3-*O*-α-arabinofuranoside) in cell wall preparations from leaves of 54 species from various Conifer taxa has been investigated the bound *p*-coumaric and ferulic acids seem to be widespread among members of the Coniferae–with *p*-coumaric acid as the major compounds in most species investigated-, while the presence of bound flavonol glycosides appears to be of chemotaxonomic relevance for members of the Pinaceae The Pinaceae species generally gave kaempferol 3-*O*-glucoside, although *Abies* gave in addition kaempferol 3-*O*-rhamnoside and *Pinus* quercetin 3-*O*-glucoside and 3-*O*-arabinoside

INTRODUCTION

In a recent communication [1] we described the isolation from Norway spruce (*Picea abies* [L.] Karst.) needles of insoluble conjugated (ester-bound) phenolics, the hydroxycinnamic acids (HCAs), *p*-coumaric and ferulic acids and the flavonoid kaempferol 3-*O*-glucoside. We presented evidence for their cell wall location, probably occurring mainly in lignin-carbohydrate complexes.

The cell wall location of HCAs is a well-known and widespread phenomenon (for example in monocotyledonous families [2]) and has been discussed to be important for the cell wall architecture (cross-linking of polymers) [3] or to play a role in the interactions with light (*E*/*Z* isomerism) resulting in changes in the structure and properties of the cell wall [4]. On the other hand, the possible cell wall location of hydrophilic flavonol glycosides, usually located in vacuoles [5, 6], is rather new [1], although the occurrence of a flavone-polysaccharide from the liverwort *Monoclea forsteri* has been discussed to be cell wall associated [7] and there is some evidence for the cell wall location of lipophilic polymethylated flavonol glucosides in *Chrysosplenium americanum* [8].

To evaluate whether the location of *p*-coumaric and ferulic acids and, more interestingly, that of kaempferol 3-*O*-glucoside in cell wall preparations from Norway spruce needles [1] might be a special feature of this plant or possibly a common phenomenon for the Coniferae, 54 species from various coniferous taxa have been investigated.

RESULTS AND DISCUSSION

In the present survey of cell wall preparations from coniferous leaves for insoluble conjugated (ester-bound) HCAs and flavonol glycosides we found, besides the known structures from *Picea abies* [1] (i.e *p*-coumaric and ferulic acids and kaempferol 3-*O*-glucoside, **1**, see scheme), three additional flavonol 3-*O*-glycosides which were isolated and identified as follows. Leaf homogenates from *Abies homolepis* and *Pinus strobus* were thoroughly extracted with various solvents, as well as sodium chloride dodecyl sulphate solutions, to obtain crude cell wall preparations which were finally treated with aqueous sodium hydroxide The latter released the phenolics from which three could readily be identified by co-TLC with the compounds isolated from cell wall preparations from leaves of *Picea abies* [1] as (*E*)-*p*-coumaric and (*E*)-ferulic acids (trace amounts of the *Z*-isomers) and **1** Three additional unknown flavonoids were purified and their structures determined by UV-Vis spectroscopy, TLC of the hydrolysis products, NMR spectroscopy and FAB mass spectrometry.

The UV-Vis spectroscopic analyses using the common diagnostic shift reagents [9] gave evidence for the presence of flavonols with substitution at 3-OH. Chromatography of the hydrolysis products with reference material (S1) for aglycones and S2 for sugars) showed identical R_fs for kaempferol and rhamnose from the *A homolepis* compound and quercetin and glucose or quercetin and arabinose from the two *Pinus strobus* compounds. This was finally substantiated by the NMR and MS data which identified the flavonoids as kaempferol 3-*O*-α-rhamnopyranoside **(2)**, quercetin 3-*O*-β-glucopyranoside **(3)**, and quercetin 3-*O*-α-arabinofuranoside **(4)**. Compound **3** was readily identified from inspection of the ^{1}H NMR data on comparison with a reference compound. The ^{1}H and ^{13}C NMR data of **2**, compared with literature ^{13}C data [10]. indicated the presence of the kaempferol and rhamnose moieties The ^{13}C shift of C-2 is characteristic of substitution at the C-3 position, while the couplings and chemical shifts within the rhamnose

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moiety are indicative of an α-configuration. The 1D and 2D ^1H data for **4**, on comparison with **3**, indicated a 3-substituted quercetin moiety and a sugar residue, which was a pentose from the mass spectrum. The magnitude of the coupling constants of the pentose was only compatible with either an α-arabinofuranoside or β-xylofuranoside The presence of arabinose was determined chromatographically (see above)

The survey of cell wall preparations from Coniferae (Coniferopsida) leaves for the insoluble ester-bound HCAs and flavonol glycosides (Table 1) was performed with samples from the same location, harvested at the same time of season Thus, possible climate- or habitat-dependent effects had not to be taken into account The results indicate the regular presence of *p*-coumaric and ferulic acids in the Pinaceae and the species of Cupressaceae and Taxaceae investigated. They were also found in *Araucaria araucana* with ferulic acid as the major compound *p*-Coumaric acid was regularly present as the major HCA constituent in the Pinaceae The cell wall-located accumulation of the flavonol glycosides in the Coniferae leaves seems to be a characteristic phenomenon of members of the Pinaceae (family specificity) Furthermore within this family there seems to be a genus-specific cell wall pattern All the Pinaceae investigated showed **1**, with the exception of *Pseudolarix Abies* leaves gave **1** and **2** and *Pinus* leaves **1**, **3** and **4** Thus **2** appears to be a characteristic cell wall constituent in *Abies* species, whereas **3** and **4** are regularly found in the cell walls of *Pinus* species. Figure 1 shows diagrams from three typical HPLC analyses, representative of *Pinus*, *Picea* and *Abies* Such qualitative and quantitative surveys for possible cell-wall bound phenolics, easily and quickly performed by HPLC, may be a useful contribution to studies on evolutionary relationships between the various Coniferous taxa This requires much more knowlege on the metabolism of phenolics in the Coniferae considering the present striking lack of knowledge in this field, as has already been pointed out elsewhere [11]

The data shown in Table 1 are those from leaves which are within the second year of development, with the exception of *Larix*, *Metasequoia*, *Pseudolarix* and *Taxodium* (one year-leaf development) We observed that generally the cell wall-bound phenolics reached the concentrations of their typical final quantitative pattern in the autumnal months of the first year of development (data from leaves which are in the first year of development are not shown) However, there are three remarkable exceptions with respect to the HCA accumulation. Leaves of *Cedrus brewifolia* and *C libani* gave 0 22 and

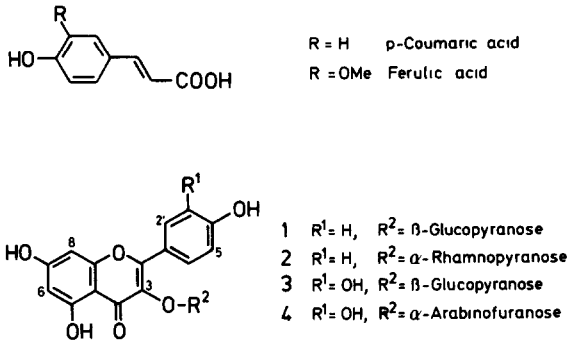
Table 1 Survey of cell wall preparations from Coniferae (Coniferopsida) leaves for insoluble ester-bound hydroxycinnamic acids (HCAs) and flavonol glycoside

	Compound*					
	HCAs		Flavonol glycosides			
Taxon	Coum	Fer	1	2	3	4
PINALES						
PINACEAE						
Abietoideae						
Abies concolor (Gord) Hoopes$	0 72	0 05	0 35	0 36	--	
A Grandis (Don ex Lamb) Lindl	0 44	0 04	0 06	0 57	—	-
A holophylla Maxim	1 21	0 04	0 38	0 50	--	-
A homolepis Sieb and Zucc $	1 24	0 04	0 22	0 69		
A numidica De Lannoy	1 01	tr	0 43	0 24		-
A pinsapo glauca Boiss	1 01	0 08	0 51	0 22	--	—
A veitchii Carr	1 38	0 07	0 40	0 61		--
Picea abies (L) Karst	1 89	0 15	1 45		-	—
Pc breweriana S Wats	1 02	0 12	0 31	---	—	--
Pc engelmannii Engelm $	1 43	0 08	0 92			-
Pc glauca (Moench) Voss	1 45	0 31	0 80		-	—
Pc glehnii (Schmidt) Mast	1 17	0 25	0 86	—	- -	-
Pc jezoense (Sieb and Zucc) Carr	1 40	0 33	0 99			-
Pc omorica Purkyne$	0 79	0 65	0 37	-	—	
Pc orientalis (L) Link	1.07	0 28	1 27			--
Pc polita Carr $	1 08	0 17	0 66	-	--	
Pc pungens-glauca Engelm	0 74	0 27	0 72		-	
Pc purpurea Mast	0 86	0 26	0 64	-		- -
Pc sitchensis (Bong) Carr	1 45	0 34	1 19	—	—	
Tsuga canadensis Carr $	0 76	0 08	0 62	—	—	—
T sieboldii Carr $	1 32	0 12	0 92	- -	—	-
Laricoideae						
Cedrus brewifolia Dode$	0 55	0 12	0 60	-	—	—
C libani stenocoma Loud $	0 38	0 09	0 36	—	—	-
Larix decidua Mill	0 77	0 04	0 55	-		-
L gmelini (Rup) Litvin	0 93	0 06	0 52	-	-	-
Pseudolarix amabilis (Neb) Rehd	0 02	0 06	-	-	-	—

Table 1 (*Continued*)

Taxon	Compound*					
	HCAs		Flavonol glycosides			
	Coum	Fer	1	2	3	4
Pinoideae						
Pinus cembra L	1 65	0 48	1.48	—	0 17	tr
Pn. contorta latifolia Dougl	1 18	0 14	0.74	—	tr	0 11
Pn. koraiensis Sieb and Zucc	1 68	0 32	1 62	—	0 05	0 05
Pn. leucodermis Ant	2 54	0 14	0 82	—	tr	0 35
Pn. monticola Dougl	2 06	0 34	1 31	—	0 18	0 35
Pn. mugus Turra	1 75	0 17	1 02	—	0 04	0 08
Pn nigra Arn $	1 71	0 18	0 90	—	0 18	0 28
Pn pinaster Ait.	0 77	0.21	0 54	—	0 04	0 18
Pn ponderosa Dougl	0 82	0.13	0 43	—	0 69	0 13
Pn strobus L.$	1 65	0 26	1 54	—	0 27	0 06
TAXODIACEAE						
Cryptomeria japonica (L f) D Don.$	0 05	0 01	0 03	—	—	—
Cunninghamia lanceolata (Lamb.) Hook$	—	—	—	—	—	—
Matasequoia glyptostroboides Hu and Cheng	—	0 09	—	—	—	—
Sciadopitys verticellata (Thunb) Sieb. and Zucc $	0.01	0 10	—	—	—	—
Sequoia giganteum (Lindl.) Buchh $	—	—	—	—	—	—
S. sempervirens (Lamb) Endl $	—	0 04	—	—	—	—
Taxodium ascendens brongn	0 01	—	—	—	—	—
T. distichum (L.) Rich.	0 01	—	—	—	—	—
CUPRESSACEAE						
Chamaecyparis obtusa (Sieb. and Zucc.) Endl $	0 02	0 01	—	—	—	—
Ch. pisifera (Sieb. & Zucc.) bndl.$	0.13	0.03	—	—	—	—
Juniperus communis L.$	0.02	0 01	—	—	—	—
J virginiana L.$	0.02	0.03	—	—	—	—
Thuja occidentalis L $	0.32	0 09	—	—	—	—
Th. plicata D. Don $	0 12	0.12	—	—	—	—
ARAUCARIACEAR						
Araucaria araucana (Mol) K Koch$	0 03	0 85	—	—	—	—
TAXALES						
TAXACEAE						
Taxus bacata L $	0 05	0 01	—	—	—	—
T cusbidata L $	0 07	0 02	—	—	—	—
Torreya nucifera (L) Sieb & Zucc $	0 01	0.01	—	—	—	—

*μmol/g leaf fresh weight (data from one of two experiments which gave similar results), $ first and second year of leaf development investigated (only the data from the second year of leaf development are shown), otherwise only the leaves from the second year of development were investigated with the exception of Larix, Metasequioa, Pseudolarix, and Taxodium (one year-leaf development),- = not detected, tr = trace (< 0 01 μmol), Coum = (E)-p-coumaric acid, fer = (E)-ferulic acid, 1 = kaempferol 3-*O*-β-glucopyranoside, 2 = kaempferol 3-*O*-α-rhamnopyranoside, 3 = quercetin 3-*O*-β-glucopyranoside, 4 = quercetin 3-*O*-α-arabinofuranoside.



Scheme 1.

0.15 in the first year and only 0.12 and 0.09 μmol ferulic acid/g fresh weight in the second year of development, those of *Taxus baccata* 0.12 and 0 05 μmol *p*-coumaric acid in the first and second year, respectively. This indicates a further metabolism of the cell wall HCAs, possibly through conversion of non-hydrolysable linkages.

So far nothing is known about the site of attachment of these phenolics and their cell or tissue distribution within the Pinaceae leaves. There is only some evidence from our earlier investigation on *Picea abies* [1] that they might be predominantly located in 'lignin-carbohydrate complexes' [12] A feruloylated trisaccharide has been isolated [13] from bagasse lignin-carbohydrate complex. Whereas the esterification of HCAs with sugar moieties of

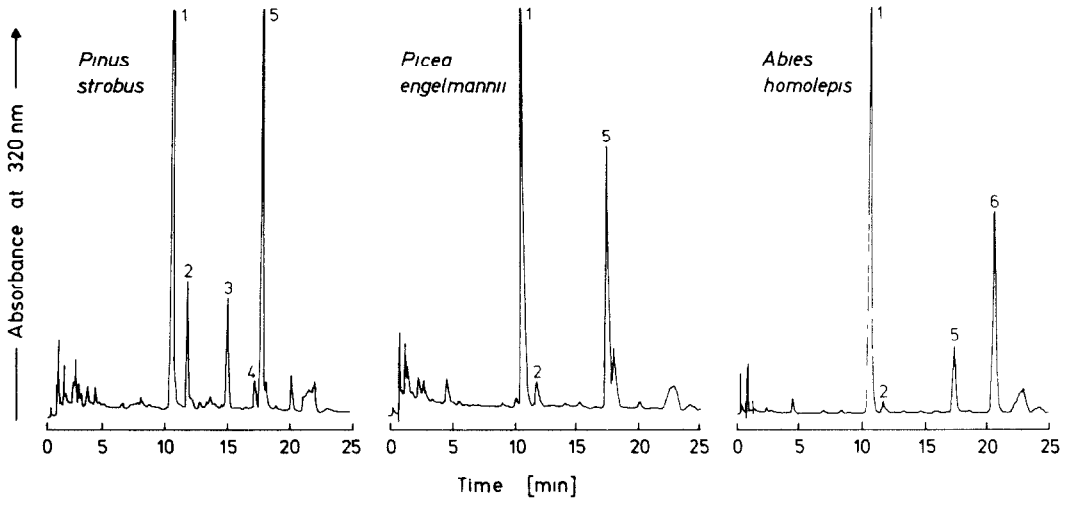


Fig 1 Diagrams from representative HPLC analyses of cell wall-bound phenolics which were isolated by saponification of cell wall preparations from Coniferae leaves Peak identification 1 = (*E*)-*p*-coumaric acid (R_t = 10 6 min), 2 = (*E*)-ferulic acid (R_t = 11 7 min), 3 = quercetion 3-*O*-glucoside (R_t = 16 7 min), 4 = quercetin 3-*O*-arabinoside (R_t = 19 0 min), 5 = kaempferol 3-*O*-glucoside (R_t = 17 5 min), 6 = kaempferol 3-*O*-rhamnoside (20 6 min) The chromatographic column used was prepacked with Nucleosil C_{18} (5 μm) (Macherey-Nagel, Duren), 250 × 4 mm i d Chromatographic conditions with a delay time of 0 6 min linear gradient elution within 20 min from 25 to 60% solvent B (MeOH–MeCN–H_2O, 1 1 1) in solvent A (1 5% H_3PO_4 in H_2O), flow rate at 1 5 ml/min, detection at 320 nm

pectines or (hemi) celluloses appears to be plausible, that of the flavonol glycosides is rather obscure It would be interesting to search for a molecule linking together the glycosidic flavonol moiety and a sugar from a cell wall-carbohydrate fraction It is quite possible that a dicarboxylic acid, frequently found as an acyl group in this class of flavonoid [14–16], may function in this way Studies on the linking structure and possible acyltransferase reactions involved in the formation of cell-wall bound phenolics are in progress. In addition, the relation between metabolically active soluble flavonol glycosides [17] and those serving as end-products in the Coniferae leaf cell walls will be investigated

EXPERIMENTAL

Plant material Leaves from various taxa of the Coniferae, identified by A Moller, were collected in June and July 1987 from the Forstbotanischer Garten (forest garden), Koln-Rodenkirchen, F R G , and were immediately frozen with liq N_2 on the spot Once in the laboratory, they were stored at −30°

Crude cell wall preparation Analytical Coniferae leaves (1 g fr wt) were treated with an Ultra-Turrax homogenizer (5–10 min, depending on the leaf material) until consistent homogeneity in 10 ml MCF (MeOH–$CHCl_3$–7 M HCO_2H, 12 5 3 [18]), allowed to stand for 1 hr with continuous stirring, and centrifuged at 2000 *g* to remove MCF-soluble components The pellet was re-extracted twice with 10 ml MCF and then consecutively treated with the following solvents (20 ml) each for 30 min with stirring followed by centrifugation) (i) × 2 MeOH, (ii) × 4 H_2O, (iii) × 6 MeOH; (iv) × 2 Me_2CO, (v) × 2 Et_2O The remaining insoluble whitish material (crude cell wall fraction) was dried at 60° (*ca* 1 hr) Preparative Leaves (100 g fr wt) from *A homolepis* and *Pn strobus* were homogenized in the presence of liq N_2 in a centrifugal mill (Retsch, type ZM 1) The resulting fine powder was suspended in 700 ml 80% aq MeOH and allowed to stand for 4 hr with continuous stirring After filtration the insoluble residue was re-extracted × 4 with the same solvent and then consecutively treated × 2 with 500 ml each of H_2O, 0 5 m aq NaCl [19], 0 5% aq SDS [20], H_2O, MeOH, Me_2O, and Et_2O The remaining material was dried overnight at 60

Isolation of insoluble phenolics Analytical Crude cell wall preparations were suspended in 8 ml hot (80°) 1 M aq NaOH and allowed to stand for *ca* 17 hr at room temp The hydrolysates were centrifuged and aliquots (2 ml) of the supernatants were acidified (*ca* pH 2) with 200 μl H_3PO_4 200 μl aliquots were centrifuged and 20 μl of the clear supernatants analysed by HPLC for the presence of phenolics Preparative The materials from the preparative cell wall preparations from *A homolepis* and *Pn strobus* were treated for *ca* 17 hr with 400 ml 0 5 m aq NaOH at 70° The filtrates were acidified (pH 1–2) with concd HCl, the liberated phenolics extracted with Et_2O, and kaempferol 3-*O*-rhamnoside (**2**), quercetin 3-*O*-glucoside (**3**) and quercetin 3-*O*-arabinoside (**4**) isolated as follows The Et_2O fractions were dried *in vacuo* and the residues redissolved in 4 ml 50% aq MeOH These were fractionated on a polyamide column using H_2O, 40% aq MeOH, 80% aq MeOH, 100% MeOH, and 0 02% NH_3 in MeOH The flavonol glycosides to be identified (**2** from *A homolepis* and **3** and **4** from *Pinus strobus*) were obtained with the 80% aq MeOH fraction and separated on TLC in S1 **2** (R_f 0 33, UV deep purple, UV/NH_3 yellow green fluorescence), **3** (R_f 0 15, UV deep purple, UV/NH_3 yellow fluorescence), and **4** (R_f 0 18, UV deep purple, UV NH_3 orange fluorescence) The TLC-flavonol glycoside bands were scraped off and eluted from the cellulose with 50% aq MeOH Final purification was achieved on a Sephadex LH-20 column eluting with 50% aq MeOH

Acid hydrolysis and product identification Compounds **2**–**4** were dissolved in 20% aq HCO_2H and kept for 60 min at 100 The aglyca were separated from the liberated sugars by repeated extraction with Et_2O TLC of hydrolysis products gave kaempferol (R_f 0 44 in S1) and rhamnose (R_f 0 82 in S2) from **2**,

quercetin (R_f 0 21 in S1) from **3** and **4**, glucose (R_f 0 63 in S2) from **3** and arabinose (R_f 0.64 in S2) from **4**.

TLC On microcrystalline cellulose ('Avicel', Macherey-Nagel, Duren) with S1 ($CHCl_3$–HOAc, 3 2, H_2O satd) and on silica gel (impregnated with 0 5 M NaH_2PO_4 in H_2O–MeOH, 3 1) with S2 (*iso*-PrOH–Me_2CO–0 1 M lactic acid, 2 2 1) according to Hansen [21] Flavonols were detected under UV light (350 nm) without and with NH_3 vapour (UV/NH_3) and sugars in day light after spraying with aniline–diphenylamine–Me_2O–H_3PO_4 (2 ml 2 g 100 ml 15 ml) and subsequent heating at 110° until colour development (arabinose = violet, glucose = blue-green, rhamnose = olive-green) Kaempferol and quercetin (Roth, Karlsruhe) and glucose, rhamnose and arabinose (Serva, Heidelberg) were used as reference material

HPLC The liquid chromatography (two-pump system) and the data processor (Chromatopac C-R3A) for quantitative calculations used were from Pharmacia LKB (Freiburg) and Shimadzu (Kyoto, Japan), respectively Injections were performed via a Rheodyne (Cotati, CA, U.S A) rotary valve with a 20 μl loop For details of the chromatographic conditions see Fig 1 *p*-Coumaric and ferulic acids, kaempferol 3-*O*-rutinoside and quercetin 3-*O*-rutinoside (Roth) were used as ext std for quantification All the quantitative experiments were performed in duplicate The data shown in Table 1 were taken from one of the two experiments which gave similar results (10–20% variation)

UV/Vis spectroscopy UV/Vis spectra were recorded in MeOH solutions and the flavonol glycosides **1–4** gave results (± shift reagents) identical to those described by Mabry *et al* [9]

NMR spectroscopy and MS The ^{1}H and ^{13}C NMR spectra were recorded at ambient temp locked to the major deuterium resonance of the solvent Chemical shifts are reported in ppm relative to TMS and coupling constants in Hz Kaempferol 3-*O*-α-rhamnopyranoside **(2)** ^{1}H NMR (CD_3OD), δ 7 810 ["*d*", H-2', H-6', J(2'–3') + *J*(2'–5') 8 9], 6 976 ["*d*", H-3', H-5'], 6 417 [*d*, H-8, *J*(8–6) 2 1], 6 240 [*d*, H-6], 5 417 [*d*, H-1'', *J*(1''–2'') 1.7], 4 261 [*d,d*, H-2'', *J* (2''–3'') 3 4], 3 751 [*d,d*, H-3'', *J*(3''–4'') 9.3], 3 43–3 30 [*m*, H-4'', H-5''], 0 961 [*d*, H-6'', *J*(6''–5'') 5 8] ^{13}C NMR (CD_3OD) δ = 179 63 (*s*, C-4), 166 11 (*s*, C-7), 163 22 (*s*, C-5) 161 57 (*s*, C-4'), 159 28, 158 60 (*s* × 2, C-2, C-9), 136 24 (*s*, C-3), 131 88 (*d*, C-2', C-6'), 122 70 (*s*, C-1'), 116 55 (*d*, C-33', C-5'), 105 92 (*s*, C-10), 103 54 (*d*, C-1''), 99 94 (*d*, C-6), 94 83 (*d*, C-8), 73 25, 72 20, 72 03, 71 94 (*d* × 4, C-2'' to C-5''), 17 64 (*q*, C-6''). FABMS *m/z* 431 $[M-H]^-$ Quercetin 3-*O*-β-glucopyranoside **(3)** ^{1}H NMR (CD_3OD), δ 7 749 [*d*, H-2', *J*(2'–6') 2 1], 7 633 [*d, d*; H-6', *J*(6'–5') 8 5], 6 910 [*d*, H-5'], 6 437 [*d*, H-8, *J*(8–6) 2.1], 6 245 [*d*, H-6], 5 303 [*d*, H-1'', *J*(1''–2'') 7 4], 3 756 [*d,d*, H-6''A, *J*(6''A-5'') 2 1, *J*(6''A–6''B) 11 9], 3 614 [*d,d*, H-6''B, *J*(6''B–5'') 5.3], 3 55–3 25 (*m*, H-2'' to H-5''] Quercetin 3-*O*-α-arabinofuranoside **(4)** ^{1}H NMR (CD_3OD, δ 7 568 [*d*, H-2', *J*(2'–6') 2 1], 7.539 [*d,d*, H-6', *J*(6'–5') 8 3], 6 942 [*d*, H-5'], 6 434 [*d*, H-8, *J*(8–6) 2 1], 6 247 [*d*, H-6], 5 509 [*d*, H-1'', *J*(1''–2'') 0 8], 4 370 [*d,d*, H-2'', *J*(2''–3'') 2 9], 3 950 [*d,d*, H-3'', *J*(3''–4'') 5 4], 3 908 [*m*, H-4''], 3.54 [*m*, H-5''AB] FABMS *m/z* 433 $[M-H]^-$

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