PERGAMON

Phytochemistry 53 (2000) 699–704

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Unusual phenolic glycosides from *Cotoneaster orbicularis*

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Received 14 July 1999; received in revised form 5 November 1999

Abstract

The whole plant of *Cotoneaster orbicularis* contains the novel di-*C*-glycosylflavone, 4″,4‴-di-*O*-β-glucopyranosyl-vicenin II, or 6,8-di-*C*-β-Cellobiosylapigenin, as well as the hitherto unknown natural phenolic glucoside, gentisic acid 2-*O*-β-glucopyranoside, or orbicularin. Further phenolics are protocatechuic, anisic, caffeic, *p*-coumaric acids, catechin, epicatechin, 2″-*O*-α-rhamnopyranosylvitexin, vitexin, rutin, isoquercetrin, hyperin and naringenin. All structures were determined by routine methods of analysis and confirmed mostly by ^{1}H- and ^{13}C-NMR.

Keywords: *Cotoneaster orbicularis*; Rosaceae; Whole plant; *C*-Glycosylflavones; 6,8-di-*C*-Cellobiosylapigenin; Phenolic acid-*O*-glucoside; Gentisic acid; 2-*O*-glucopyranoside; Orbicularin; ESI–MS; NMR

1. Introduction

Extracts of many species in the genus *Cotoneaster* (Rosaceae) are noted for their cytotoxic, antitumor activity; antispasmodic, cardiotonic actions; antiviral and diuretic properties [Palme, Bilia, Vincenzo & Morelli, 1994; Boulos, 1983]. Among these species, *Cotoneaster orbicularis* has never been subjected to phytochemical investigation. The plant is a thorny evergreen shrub with small leaves and red fruits. It grows wild in the southern part of the Sainai peninsula. In the present paper the phytochemical investigation of *Cotoneaster orbicularis* phenolics from an aqueous alcoholic extract of the whole plant is described. This investigation resulted in the isolation and characterisation of the new unusual di-*C*-glycosylapigenin, 6,8-di-*C*-cellobiosylapigenin (**1**) as well as the unknown natural phenolic, 2,5-dihydroxybenzoic acid-2-*O*-β-$^{4}C_{1}$-glucopyranoside, or orbicularin, (**2**), previously known as a synthetic product (Boulos, 1983). The known phenolics, gentisic (**3**), protocatechuic (**4**), anisic **(5)**, caffeic **(6)**, *p*-coumaric **(7)** acids, catechin (**8**), epicatechin (**9**), 2″-*O*-α-rhamnopyranosylvitexin (**10**), vitexin (**11**), rutin (**12**), isoquercetrin (**13**), hyperin (**14**) and naringenin (**15**) were also isolated and identified. It should be noted however, that *C*-glycosyl flavones (**10** and **11**) have previously been found in the genus *Cotoneaster* (Palme, Bilia, Vincenzo & Morelli, 1994).

The analytical data of (**1**) was compared with that of the known positional isomer, 6,8-di-*C*-sophorosylapigenin, isolated once before from *Ephedra aphylla* (Hussein, Barakat, Nawwar & Willuhn, 1997). The new cellobiosylapigenin is interesting because it contains the unusual interglucosidic linkage (1 → 4). This linkage has been determined before in two flavone *O*-glucosylglucosides characterized from *Salvia uliginosa* (Veitch, Grayer, Irwin & Takeda, 1998) and in an *O*-glucosyl-*C*-glucosylflavone, namely, 4″-*O*-glucosylswertisin (zivulgarin) from *Zizyphus spinosus* (Jay, 1986) as well.

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00598-1

It should be mentioned also, that gentisic acid glycosides have recently been identified as derivatives of salisylic acid, a compound now known to be synthesised in many plants during defence responces. The acid has been encountered in nature only as its 5-*O*-glucoside (Fujii, Aoki, Komoto & Munakata, 1971; Zane & Wender, 1964), but not as the 2-*O*-glucoside.

2. Results and discussion

2 D-PC screening of the aqueous ethanolic (1:3) whole *Cotoneaster orbicularis* plant extract revealed an oligoglycosylated flavonoid [(**1**), dark purple spot on PC under UV light, turned light yellow when fumed with ammonia, with high R_f-values in aqueous solvents and low mobility in organic ones], together with a complicated mixture of phenolic, phenylpropenoid acids (characterisitic fluorescence under short and long UV light on PC) and flavonoid glycosides (dark purple spots on PC under UV light, changing to yellow or orange colour when fumed with ammonia). All compounds (**1**–**15**) were isolated and identified by polyamide CC followed by applying a combination of Sephadex LH-20 column fractionation and preparative PC. Two of the separated compounds (**1** and **2**) are new. The remaining compounds (**3**–**14**) are known and gave chromatographic, UV spectral and hydrolytic data identical with those of gentisic, protocatechuic, anisic, caffeic, *p*-coumaric acids, catechin, epicatechin, vitexin-2″-α-*O*-$^1C_4$-rhamnopyranoside, vitexin, rutin, isoquercetin, hyperin and naringenin, respectively. The structures of these known compounds were confirmed by ^{1}H- and ^{13}C-NMR spectral analysis.

Compound (**1**), was isolated as a yellowish white amorphous powder which possessed a molecular ion of $[M\text{-}H]^-$ m/z = 917 in negative ESI–MS, corresponding to a M_r of 918 amu. Chromatographic properties and UV spectral analysis (see Table 1) in methanol and in the presence of diagnostic shift reagents (Harborne & Williams, 1975; Mabry, Markham & Thomous, 1969) together with the results of the ESI–MS analysis suggested for (**1**) an oligo-*C*-hexosylapigenin structure whose molecule contains four hexose moieties. Complete acid hydrolysis (methanolic 2 M HCl, 7 h) of (**1**) gave 6,8-di-*C*-glucosyl apigenin, or vicenin II (CoPC, UV, ^{1}H- and ^{13}C-NMR analysis) and glucose (CoPC). The former also was released from (**1**) on β-glucosidase enzymic hydrolysis. Consequently (**1**) is proved to be a di-*O*-β-glucosylvicenin II, which possessed quite distinct R_f-values (Table 1) from that of its positional isomer, 6,8-di-*C*-sophorosylapigenin, isolated previously from *Ephedra aphylla* (Hussein, Barakat, Nawwar & Willuhn, 1997).

^{1}H-NMR spectral analysis of (**1**) failed to unravel the ambiguity about the site of attachment of the two *O*-glucoside moieties to vicenin II in the molecule of (**1**). The measured spectrum (room temperature, DMSO-d_6) was closely similar to that of the positional isomer 6,8-di-*C*-sophorosylapigenin. It revealed four β-anomeric glucose proton resonances, all appearing as doublets (J = 8 Hz) at δ ppm 4.95, 4.85, 4.75 and 4.72. In addition, a broad multiplet, located in the region from 3.22 to 3.85 ppm, was assigned to the remaining 24 sugar proton resonances of the four glucosyl moieties and to the hydroxyl and water protons as well. In this spectrum the typical pattern, of proton resonances which characterises 6,8-disubstituted apigenin was also recognised [δ ppm 6.6 (*s*, H-3), 6.8 (*d*, J = 8 Hz, H-3″ and H-5″), 7.9 (*d*, J = 8 Hz, H-2″ and H-6″)].

Precise determination of the site of attachment of the two *O*-glucosyl moieties to vicenin II in the molecule of (**1**) was then achieved through ^{13}C-NMR, including HMBC spectral analysis. The ^{13}C-NMR spectrum revealed two *O*-β anomeric glucosyl carbon resonances at δ ppm 103.3 and 103.9, comparable to 103.3 ppm showed by the β-anomeric *O*-glucosyl carbon in free cellobiose (Breitmayer & Voelter, 1978). The spectrum also exhibited a total of 18 distinct sugar carbon resonances in the region from δ ppm 60.0 to 82.0. The resonances at δ ppm 60, 60.1, 61.0 and 61.3 were obviously due to the four unsubstituted methylenic glucose carbons in the four sugar moieties of (**1**), while resonances at 77.0, 77.1, 69.1, 69.8, 77.8 78.1 are attributable to C3‴″, C-3‴″′, C-4‴″, C-4‴″′, C-5‴″ and C-5‴″′ in two terminal *O*-glucosyl moieties, attached to the 6-C and 8-C primary glucosyl moieties,

Table 1
Chromatographic and UV data of compounds **1** and **2**

Compound	Chromatographic properties ($R_f s$ (×100)				UV spectral data λ_{max} (nm)			
	H_2O	HOAc	BAW	MeOH	NaOAc	NaOAc–H_3BO_3	$AlCl_3$	MeONa
1	32	45	17	272, 332	283, 390	284, 391	282, 300, 345, 382	275, 360, 402
Vicenin-II	23	55	33	272, 333	282, 393	283, 390	280, 305, 345, 383	275, 361, 402
2	35	48	19	236, 315				238, 326
Gentisic acid	68	70	78	239, 334				

respectively. Besides these, two other carbon resonances have been recognised, in this spectrum at δ ppm 73.4 and 74.1 and were assigned to the C-2‴″ and C-2‴″′ in the two terminal glucosyl moieties.

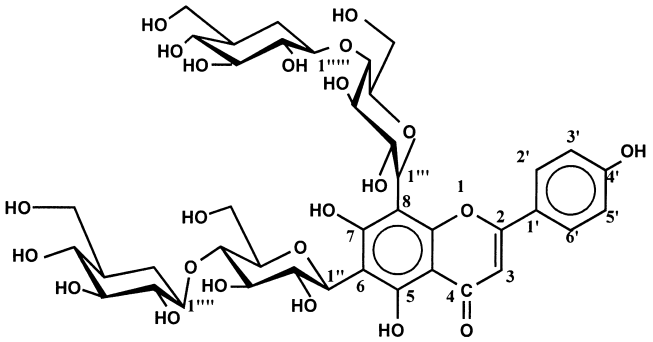
Comparison of the chemical shifts of the primary glucose carbon resonances in β-glucosyl (1 → 4) -β-glucose, (β-cellobiose) and those of free β-glucose itself led to the deduction of substituent additive rules which when applied to the chemical shifts of the carbon resonaces of the 6-C and 8-C-glucosyl moieties in vicenin II (Nawwar, El-Mousallamy, Barakat, Buddrus & linscheid, 1989), would give calculated chemical shifts which could be compared with those recorded for the two primary 6-C and 8-C-glucosyl moieties of compound (**1**). Both calculated and recorded values were in close agreement, thus proving that resonances in the recorded spectrum located at δ ppm 72.8, 73.3, 70.6,71.0, 77.5, 78.9, 81.5, 82.0, 80.9 and 81.0 are assignable to C-1″, C-1‴, C-2″, C-2‴, C-3″, C-3‴, C-4″, C-4‴, C-5″ and C-5‴, respectively (taking into account that chemical shifts with the same number, but with different superscript may be interchanged). Furthemore, the absence of any sugar carbon resonances with chemical shift lower than 82.0 ppm except those of the *O*-β-anomeric carbons would directly eliminate the possibility of the presence of a laminaribiosyl (glucosyl 1 → 3 glucose) moiety in the molecule of (**1**), (Karl, Pederesen & Mueller, 1980). The two *C*-glucosyl moieties linked to the flavonoid carbons C-6 (at 107.5 ppm) and C-8 (at 105.3 ppm) were unambiguously confirmed, in an HMBC spectrum, by a long-rang correlation (^2J) between their anomeric protons (H-1″ at 4.95 ppm and H-1‴ at 4.85 ppm) and these flavonoid carbons. This latter spectrum also allowed the interconnectivity, across three bonds (^3J), of the remaining anomeric glucose protons, H-1‴″ at 4.75 ppm and 1‴″′ at 4.72 ppm with the primary glucose carbons, C-4″ and C-4‴ at 81.5 and 82.0 ppm.

Consequently, compound (**1**) is identified to be 4″,4‴-di-*O*-β-glucopyranosylvicenin II, or 6,8-di-*C*-cellobiosylapigenin, which represents to the best of our knowledge, a new natural product.

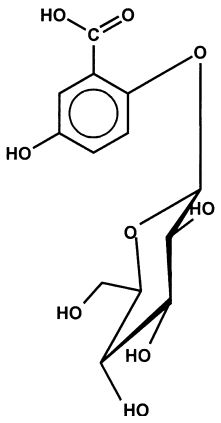
The new compound (**2**) was obtained as an amorphous off-white powder which possesses phenolic characters (dirty blue colour with $FeCl_3$) and UV spectral maxima: 236, 315 nm in MeOH and 238, 326 nm in MeOH + MeONa. Negative FAB mass spectral analysis established that (**2**) was a dihydroxybenzoic acid hexoside ([M-H]$^-$ at $m/z = 315$) of a M_r = 316 amu. This could also be deduced from the EI-M spectrum of the compound which revealed a fragment ions at m/z: 154, 136 and 107 consistent with a dihydroxybenzoic acid as the aglycone moiety. On normal acid hydrolysis (2 N aqueous HCl, 3 h, 100°C) compound (**2**) yielded 2,5-dihydroxybenzoic acid, gentisic acid [coPC, UV (Table 1), ^{1}H, ^{13}C and ^{1}H–^{13}C coupled NMR spectral analysis] together with glucose (coPC). The former was released from (**2**) on β-glucosidase enzymic hydrolysis. To find out how the gentisic acid and glucose moieties are incorporated in the molecule of (**2**) NMR spectral analysis were carried out. The ^{1}H-NMR spectrum (room temperature. DMSO-d_6) revealed, in addition to the characteristic proton resonances pattern of gentisic acid moiety [δ ppm 7,2 (*d*, J = 2 Hz, H-6′), 6.9 (*dd*, J = 2 Hz and J = 7.5 Hz, H-4′, 6.85 (*d*, J = 7.5 Hz, H-3′)] another different pattern of proton resonances belonging to a β-glucose moiety [δ ppm 4.7 (*d*, J = 8 Hz, H-1), 3.2-3.85 (*m*, six glucose protons hidden by hydroxyl and water protons]. Glucosidation at C-2′ of the gentisic acid moiety was evidenced by the lowfield shift of H-3′ proton resonance ($\Delta\delta = 0.2$ ppm) when compared with the corresponding signal in the spectrum of free gentisic acid. The value of the coupling constant of the anomeric glucose proton resonance (J = 8 Hz) indicated that the sugar moiety adapts a $^4C_1$ conformation and a β-configuration (Nawwar, Ishak, Michael & Buddrus, 1984). The weight of evidences given above proved that (**2**) is gentisic acid 2-*O*-β-$^4C_1$-glucoside. Confirmation of this structure was then achieved through ^{13}C and ^{1}H–^{13}C coupled NMR analysis. The ^{13}C spectrum contained, as expected a total of 13 distinct carbon resonances. Assignments were aided by comparison with the ^{13}C-NMR data reported for similar phenolic *O*-glucosides (e.g. flavones 5-*O*-glucosides and gentisic acid itself) (Merkham & Chari, 1982). The β-anomeric glucose carbon resonance was readily identified from its characteristic δ value at 102 ppm, while the most upfield glucose carbon resonance, in this spectrum, located at δ 60.7, ppm was assigned to the methylenic carbon. Other resonances in the sugar region exhibited δ-values which were in accordance with those reported for glucose in previously reported phenolic *O*-glucosides (Merkham & Chari, 1982; Nawwar, Buddrus & Bauer, 1982). The ^{1}H–^{13}C coupled spectrum of (**2**) showed, in the aromatic region, one doublet for the gentisic acid carbons C-1′ (J = 7 Hz), one doublet for C-6′ (J = 162 Hz), a double doublet for C-3′ (J = 158.6 Hz and J = 6.5 Hz) and another double doublet for C-4′ (J = 161 Hz, J = 6.5 Hz) at δ ppm 115.3, 117.7, 117.4 and 124.5, respectively. It also showed a doublet (J = 7.Hz) for carbons C-2′, a clear triplet (J = 6.5 Hz) for C-5′ and a doublet (J = 6 Hz) for C-7′ at δ ppm 147.9, 156.4 and 171.6, respectively. Comparison of these chemical shift values with those recorded for gentisic acid (room temperature DMSO-d_6), [112.9 (C-1), 149.6 (C-2), 114.9 (C-3), 124.1 (C-4), 154.4 (C-5), 118.1 (C-6) and 172.0 (C-7)] showed an upfield shift, $\Delta\delta = 1.73$ ppm for the C-2′ resonance accompanied by downfield shifts of $\Delta\delta = 4.4$ and 2.5 ppm for the resonances of the *ortho* carbons C-1′ and C-3′, respectively. These changes in chemical shifts are

obviously due to the introduction of a glucose moiety at position 2′ of the gentisic acid moiety to form the molecule of **(2)**, (see Formula).

Conformation of the glucose moiety as $^4C_1$ followed from the β-configuration discussed above. Also, the chemical shift values of the resonances of all glucose carbons confirmed the pyranose form of this sugar moiety. Consequently, compound (**2**) is finally identified to be gentisic acid 2-*O*-β-$^4C_1$-glucopyranoside, a compound which is known as a synthetic product (Wagner, 1958), but which has not been reported, previously as a natural product.



Compound (1) : 6,8-di-C-cellobiosylapigenin



3. Experimental

^{1}H-NMR 270 MHz. ^{1}H-NMR resonances were measured in DMSO-d_6, relative to TMS and ^{13}C-NMR were converted to TMS scale by adding 39.5. Typical conditions: spectral width = 4000 for ^{1}H, 13,000 Hz for ^{13}C, 32 K data points and a flip angle of 45°. Negative ESI—M spectra were measured on SSQ finnigan MAT 4600 mass spectrometer. PC was carried out on Whatman no. 1 paper, using solvent systems: (1) H_2O; (2) 15% HOAc–H_2O; (3) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, top layer); (4) C_6H_6-*n*-BuOH–H_2O-pyridine, 1:5:3:3, top layer). Solvents 1–3 were used for prep. PC on Whatman no. 3 MM paper and solvents 3 and 4 for sugar analysis.

3.1. Plant material

Fresh shrubs of *Cotoneaster orbicularis* Schlecht. were collected from the mountains of south Sinai around Saint Catharine, Egypt, in November and authenticated by Dr. E. El-Garf, Teacher of Botany, Faculty of Science, Cairo University. A voucher specimen is deposited in the Herbarium of The NRC.

3.2. Isolation and identification

Shrubs of *C. orbicularis,* dried in the shade in an air-draft, were comminuted to powder (2 kg) and exhaustively extracted with EtOH/H_2O (3:1). The extract, dried in vacuo (204 g) was analysed by 2 D-PC. A 29.5 g portion of the extract, dissolved in 50 ml MeOH was applied to a polyamide 6S CC (Riedel-De Haen AG, Seelze Hannover, Germany) and eluted by H_2O followed by H_2O/EtOH mixtures of decreasing polarities to afford 10 major frs (I-X).

Compounds **1** (103 mg) and **2** (92 mg) were isolated from fr. I by repeated CC over Sephadex LH-20, using H_2O and *n*-BuOH saturated with H_2O for elution. Prep. PC, using BAW as solvent system, yielded pure sample of each. Compound **3** (76 mg), **4** (88 mg) and **5** (57 mg), were separated from fr. III by repeated CC on Sephadex LH-20, using 95% EtOH as an eluent. Compound **6** (102 mg) and **7** (86 mg) were isolated from fr. IV by CC fractionation on polyamide, using a mixture of MeOH/$C_6H_5CH_3$/H_2O (60:38:2) as an eluent and compounds **8** (144 mg) and **9** (78 mg) from fr. V, by CC fractionation over Sephadex LH-20, using EtOH/H_2O (1:2) for elution. Compounds **10** (122 mg), **11** (106 mg) and **12** (152 mg) were obtained from fr. VI through CC over Sephadex LH-20, using *n*-BuOH saturated with H_2O as solvent. Compounds **13** (87 mg) and **14** (52 mg) were separated from fr. VII through prep. PC, using *n*-BuOH saturated with H_2O as solvent and compound **15** (85 mg) were isolated from fr. VIII through polyamide column fractionation using EtOAc saturated with H_2O as solvent for elution.

3.3. 6,8-di-C-cellobiosylapigenin (*1*)

R_f*s*, R_f and UV spectral data: Table 1. M_r: 918, ESI–MS: negative ion: *m*/*z* (rel. inten.): 917.4(55), [M-H]$^-$, 593(100), [vicenin II-H]$^-$. **1** was hydrolysed with 2 M aqueous methanolic HCl (1:1) at 100°C for 7 h to give vicenin II and glucose. The former was precipitated from the cold aqueous hydrolysate after removal of MeOH. Vicenin II: R_f values and UV data: Table 1; ^{1}H-NMR: aglucone moiety: δ ppm 6.76 (*s*, H-3), 6.92 (*d*, *J* = 8 Hz, H-3′ and H-5′), 8.0 (*d*, *J* = 8 Hz, H-2′

Table 2
^{13}C-NMR chemical shifts (ppm) of the new *Cotoneaster orbicularis* and the related compounds

Carbon	4″,4‴-di-*O*-glucosyl-vicenin II[a]	Vicenin II	Gentisic acid 2-*O*-β-*glucoside*	Gentisic acid
1			102.0	
2	164.2	164.1	73.3	
3	103.9	102.6	76.5	
4	182.4	182.3	69.7	
5	158.6	158.5	77.1	
6	107.5	107.5	60.7	
7	161.3	161.2		
8	105.3	105.3		
9	155.1	155.1		
10	103.9	103.8		
1′	121.5	121.5	115.3	112.9
2′	129.1	129.0	147.9	149.6
3′	115.9	115.8	117.4	114.9
4′	160.8	160.7	124.5	124.1
5′	115.9	115.8	156.4	154.4
6′	129.1	129.0	117.7	118.1
7′			171.6	172.0
1″ and 1‴	72.8 and 73.3	74.0 and 73.3		
2″ and 2‴	70.6 and 71.0	71.9 and 70.8		
3″ and 3‴	77.5 and 78.9	78.8 and 77.8		
4″ and 4‴	81.5 and 82.0	70.5 and 69.1		
5″ and 5‴	80.9 and 81.0	81.8 and 80.8		
6″ and 6‴	61.0 and 61.3	60.5 and 61.3		
1⁗ and 1⁗′	103.6 and 103.2			
2⁗ and 2⁗′	73.4 and 74.1			
3⁗ and 3⁗′	77.0 and 77.1			
4⁗ and 4⁗′	69.1 and 69.8			
5⁗ and 5⁗′	77.8 and 78.1			
6⁗ and 6⁗′	60.0 and 60.1			

[a] Chemical shifts of carbonc with the same number but with different superscript may be interchanged.

and H-6′); sugar moieties: 4.84 (broad *s*, $\Delta\nu_{1/2} = 16$ Hz, H-1′ and H-1‴), 3.08–3.88 (*m*, 12 sugar protons hidden by hydroxyl and water proton resonances). Hydrolysis of **1** with β-glucosidase (Nawwar, Hussein & Merfort, 1994), (from sweet almond meal, Merck) yielded vicenin II (coPC). ^{1}H-NMR of **1**: aglucone moiety: 6.6 (*s*, H-3), 6.8 (*d*, J = 8 Hz, H-3′ and H-5′), 7.9 (*d*, J = 8 Hz, H-2′ and H-6′); sugar moieties: δ ppm 4.95, 4.85, 4.75 and 4.72 each as *d*, J = 8 Hz, H-1″, H-1‴, H-1⁗ and H-1⁗′; 3.22–4.2 (broad *m*, 24 sugar protons hidden by hydroxyl and water protons). HMBC: Connectivities of most ^{1}H and ^{13}C sugar resonances were determined, because of signals ovelap. ^{13}C-NMR of **1**: Table 1.

3.4. 2,5-Dihydroxybenzoic acid 2-O-β-$^4C_1$-glucopyranose, gentisic acid 2-O-β-$^4C_1$-glucopyranose, orbicularin (2)

$R_f s$: Table 1. UV data: Table 1. M_r of **1**, 316, negative ESI–MS, [M-H]$^-$: m/z (rel. intent): 315 (48). Fragment ions in EI–MS, m/z (rel. inten.): 154 (76), 136 (100), 110 (87) and 107 (1). On normal acid hydrolysis (2 N aq., HCl, 3 h, 100°C) **1** yielded glucose (coPC) and gentisic acid. The latter was extracted by EtOAc. Gentisic acid: $R_f s$ and UV data: Table 1; ^{1}H-NMR in DMSO-d$_6$: δ ppm 6.75 (*d*, J = 7.5 Hz, H-3), 6.95 (*dd*, J = 7.5 and J = 2.5 Hz, H-4), 7.15 (*d*, J = 2.5 Hz, H-6); ^{13}C-NMR: Table 2. β-glucosidase enzymic hydrolysis of **2** yielded gentisic acid (coPC). ^{1}H-NMR of **2** in DMSO-d$_6$: gentisic acid moiety: δ ppm 6.85 (*d*, J = 7.5 Hz, 3′), 6.9 (*dd*, J = 7.5 and J = 2.5 Hz, H-4′), 7.2 (*d*, J = 2.5 Hz, H-6′); glucose moiety; 4.7 (*d*, J = 8 Hz, H-1), 3.2–3.85 (broad *m*, 6 sugar proton resonances hidden by sugar hydroxy proton and water proton resonances). ^{13}C-NMR of **2**: Table 2.

Acknowledgements

This work was excuted through project no. W-383-/6-II of Deutsche Forschungsgemeinschaft for which the authors feel very grateful.

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