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UNUSUAL FLAVONOIDS PRODUCED BY *CALLUS* OF *HYPERICUM PERFORATUM*

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Key Word Index—*Hypericum perforatum*; Guttiferae; tissue culture; flavonoids; 6-C-prenyl luteolin.

Abstract—6-C-prenyl luteolin, a new naturally occurring compound, together with luteolin 5,3′-dimethyl ether, luteolin 5-glucoside and luteolin 3′-glucoside were isolated from *callus* of *Hypericum perforatum* var. *angustifolium*. These flavonoids were completely different from those previously observed in *in vivo* plants of the same species. The total flavonoid content of *callus*, around 0.5–0.7 mg g^{-1} dry wt, was much lower than that found in wild growing *Hypericum perforatum* plants (14–70 mg g^{-1} dry wt).

INTRODUCTION

Hypericum perforatum L. (St John's wort) is a herbaceous perennial species widely distributed in Europe. This species has been used as a medicinal plant since the middle ages due to its reputation as an anti-inflammatory and healing agent. Nowadays plants of this species are used for their antidepressive and antiviral properties. Chemical studies on this species began in 1830 with the pioneering isolation of hypericin by Buchner, who named this compound hypericum red [1]. One of the main class of compounds present in *H. perforatum* plants is that of the flavonoids comprising the aglycone quercetin and several glycosides, namely hyperoside, rutin, isoquercitrin and quercitrin [2]. The flavonoid aglycones luteolin and kaempferol [3] as well as biflavonoids with apigenin skeleton were also found [4–5].

We initiated *callus* cultures from *in vitro* shoots of *H. perforatum* in order to study the production of secondary metabolites, namely flavonoids. During this study we observed that flavonoids were chemically different and accumulated in minor amounts when compared with those usually described for *in vivo* plants. The isolation, structural elucidation and the contents of flavonoids in *callus* cultures of *H. perforatum* are reported here.

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RESULTS AND DISCUSSION

Stem segments from *in vitro* shoot cultures of *H. perforatum* var. *angustifolium* were used to establish *callus* cultures. After the third subculture, *callus* appeared completely de-differentiated with a pale cream to green colour, friable and stabilized. Direct HPLC analysis of a freshly prepared MeOH extract from dry biomass of *callus* revealed the presence of some flavonoids, when checked with a diode-array detector. Kartenig *et al.* reported already the production of flavonoids by cell cultures of *H. perforatum* [6, 7]. According to these authors the flavonoids found in *H. perforatum* cell cultures are of the same type of those usually found in the whole plant, namely quercetin, its glycosides rutin, hyperoside, isoquercitrin and quercitrin, and the biflavonoids amentoflavone and I3,II8-biapigenin [7]. However, the flavonoids observed in the methanolic extract of our *callus* cultures have different R_f and UV spectra when compared to those observed in a similar extract from *in vivo* plant biomass (results not shown) and different from those flavonoids cited above, as confirmed with commercial standards. The results clearly indicate that *callus* produced flavonoids distinct from those of the parent plants.

In order to identify the flavonoids, a defatted crude MeOH extract from *callus* was separated by CC on Sephadex LH-20 (eluted with MeOH) and three major fractions containing the flavonoids were further investigated. These fractions were submitted to sem-

ipreparative HPLC and five pure flavonoids (**1–5**) were obtained.

Compound **1** showed a UV spectrum in methanol, and after the addition of the classical reagents [8], characteristic of a flavone with free hydroxyls at 7 and 3′ and 4′, and with a blocked hydroxyl at the 5-position. In addition these spectra were identical to those observed for an authentic sample of luteolin 5-glucoside (Roth). The blue fluorescence of this compound, when visualized under UV light (360 nm), supported the lack of a free hydroxyl in position 5. After acid hydrolysis of **1**, luteolin and glucose were observed by chromatographic techniques, and the naturally occurring compound co-chromatographed with an authentic marker of luteolin 5-glucoside, confirming its structure.

The UV study of **2** showed that this was a flavone with free hydroxyls at 5, 7 and 4′, and with an additional hydroxyl at 3′-position, which was blocked. This compound exhibited a purple fluorescence under UV light. After acid hydrolysis, glucose and luteolin were identified. In addition, this compound was not acylated, since no modification was observed on saponification. Its HPLC retention time was in the range of other luteolin monoglucosides (21.2 min under the conditions shown in Experimental), although **1** eluted with a much shorter retention time (12.8 min). This is not unexpected, since flavonoids in which the hydroxyl at 5 is blocked elute with shorter retention times in reversed-phase columns. Hence, **2** was identified as luteolin 3′-glucoside.

The remaining three compounds (**3–5**), were aglycones, as revealed by their higher retention times on HPLC, and their solubility in lipophilic solvents like Et_2O. Compound **3** was identified as luteolin, by its UV spectrum and chromatographic comparisons with an authentic marker.

Compound **4** showed a dark yellow fluorescence under UV light, and a UV spectrum (in MeOH and MeOH plus the classical reagents) like a luteolin derivative in which hydroxyls at 5 and 3′ were blocked, and those at 7 and 4′ were free. The EI-MS spectrum confirmed that **4** was a dihydroxy-dimethoxyflavone, and the retro-Diels-Alder fragmentation, showed that both the A and B rings had monohydroxy-monomethoxy substitution. Thus, this was identified as luteolin 5,3′-dimethyl ether.

Compound **5** showed lipophilic behaviour. Its UV spectrum in methanol was similar to that of luteolin, but in this case a bathochromic shift of 5 nm was observed in band IIa (267 → 273 nm), indicating that an additional substituent should be present either at the 6- or 8-position. The UV study indicated that free hydroxyls at 5, 7, 3′, and 4′ were present. The EI-MS spectrum showed that this was a flavone with two hydroxyls on ring B, and two hydroxyls and one isoprenyl residue on ring A. The ^1H NMR spectrum showed a typical 3′,4′-oxygenated ring B, two singlets for the protons at C-3 and either at C-6 or C-8 position, and the characteristic signals of the isoprenyl residue. This was confirmed by ^1H–^1H, COSY experiments. The ^{13}C NMR spectrum confirmed the substitution pattern of the flavone, the presence of the isoprenyl, and located the isoprenyl residue at the C-6 position. Therefore, compound **5** was identified as 6-C-prenyl luteolin, a new naturally occurring compound. To our knowledge, there is no report on the occurrence of prenylated flavonoids in *H. perforatum*. However, an isopentenylated flavonol from *H. japonicum* (9) and several prenylated xanthones have been described in *in vitro* cultures of *Hypericum* species [10–12].

The total flavonoid content (Table 1) of *callus* is very small when compared to the concentrations of 14–70 mg g^{-1} dry wt biomass reported for *in vivo* plants [13–15]. The flavonoid content is strongly dependent on the stage of maturation of *H. perforatum* plants [15] and geographical variability would be a factor to take into account [13–14]. In the present case, however, these factors do not seem to explain adequately such accentuated flavonoid concentration differences. There are some differences between secondary metabolites produced by cultured cells and those produced by intact plants. Cell culture media can favour synthesis of certain secondary metabolites being repressive to others [16]. In nature, the flavonoids play important roles as defense compounds against bacterial and fungal infections [17]. On the other hand strong evidence supports the action of flavonoids as "sunscreens" against UV radiation for vulnerable plant tissues [18]. It is possible that the protected environment that characterizes *callus* cultures may be responsible for the low flavonoid accumulation due to the lack of the stress, normally found in nature. In *in vivo* plants, phenolic synthesis occurs mainly during cell differentiation and after leaf maturation [16]. The lack of differentiation in *callus* cultures may certainly be unfavourable to flavonoid synthesis.

EXPERIMENTAL

In vitro culture

In vitro shoot cultures were induced from stem segments of *Hypericum perforatum*. var. *angustifolium* plants, collected in May 1994 in the National Park of Peneda-Gerês at North of Portugal. *Callus* cultures were established from stem segments of *in vitro* shoot cultures of *H. perforatum*. *Callus* cultures were initiated and maintained on solid MS medium supplemented with 4.5 μM of NAA and 2.3 μM of KIN. Cultures were grown at $25 \pm 1°$, 16 h light day^{-1}. The *callus*, at the end of exponential growth phase, was routinely subcultured with 4 week intervals.

Extraction and isolation

25 g Dry wt of ground lyophilized *callus* biomass with 4 week age old, after the third subculture, were

Table 1. Flavonoids in *H. perforatum callus*

Flavonoid	R_t (min) HPLC	Content (μg g^{-1} dry wt)
1 (luteolin 5-glucoside)	12.8	110 ± 39 (min. 80; max. 150)
2 (luteolin 3′-glucoside)	21.2	110 ± 37 (min. 80; max. 140)
3 (luteolin)	25.8	97 ± 38 (min. 60; max. 130)
4 (luteolin 5,3′-dimethyl ether)	28.4	95 ± 39 (min. 60; max. 130)
5 (6-C-prenyl luteolin)	39.6	140 ± 46 (min. 90; max. 180)
Total amount (μg g^{-1} dry wt)		550 ± 60 (min. 400; max. 720)

Flavonoid contents represent the means of 5 independent samples ± SD.

first defatted with *n*-hexane in a Sohxlet apparatus, during 24 h, then extracted with hot methanol (4 × 200 ml). The MeOH extracts were combined and evapd (40°, under red. pre.). The residue was dissolved in 10 ml of MeOH and fractionated over Sephadex LH-20 (column: 40 × 3 cm) using MeOH as eluent. The fractions containing flavonoids were identified by analytical HPLC-DAD and submitted to semipreparative HPLC in order to isolate them. Elution was carried out with aqueous MeOH (15 to 80%) in a reversed-phase semipreparative column Spherisorb ODS-2 (250 × 7 mm, 5μm particle size) at a flow rate of 2 ml min^{-1}, and the detection wavelength set to 350 nm.

HPLC analyses

These were performed with a Beckman System Gold apparatus equipped with a Diode Array Detector and a UV/Vis detector (for quantification purposes). MeOH samples were analyzed on an end-capped LichroCart RP18 supersphere column (Merck, Darmstadt) (125 × 4 mm, 4μm particle size), with a pre-column of the same material. The mobile phase was a two solvent system consisting of solvent A (5% HCOOH) and a solvent B (MeOH) at 1.0 ml min^{-1}. The elution program was a gradient consisting: 0 min 20% B, 8 min 30% B, 15 min 35% B, 25 min 50% B, 35 min 70% B, linear for 5 min, 60 min 95% B, linear for 5 min, 70 min 20% B. Quantification of the flavonoids were performed by the external standard method, at 350 nm. The different flavonoids were quantified as luteolin equivalents.

Flavonoid identification

Compound **1**. UV λ_{max}^{MeOH}: 252, 262i, 344; +NaOMe: 260, 396; +$AlCl_3$: 246, 274, 282i, 314, 365; +$AlCl_3$/HCl: 247, 274, 281, 317, 359; +NaOAc: 262, 393; +NaOAc/H_3BO_3: 256, 365. Acid hydrolysis: luteolin + glucose.

Compound **2**. UV λ_{max}^{MeOH}: 252i, 265, 341; +NaOMe: 254, 276, 329i, 395; +$AlCl_3$: 263, 277i, 303i, 352, 388i; +$AlCl_3$/HCl: 260, 278i, 303i, 345, 388i; +NaOAc: 270, 325i, 390; +NaOAc/H_3BO_3: 269, 288i, 347. Acid hydrolysis: luteolin + glucose.

Compound **4**. UV λ_{max}^{MeOH}: 255, 268i, 348; +NaOMe: 268, 278i, 330i, 403; +$AlCl_3$: 275, 283i, 316, 363; +$AlCl_3$/HCl: 275, 282i, 315, 362; +NaOAc: 269, 394; +NaOAc/H_3BO_3: 255, 267i, 350. EI-MS probe 70 eV (*m/z*, rel. int.): 314 $[M]^+$ (100), 313 $[M-H]^+$ (30), 286 [M-28] (5), 165 A_1+H (4), 148 B_1 (35).

Compound **5**. UV λ_{max}^{MeOH}: 255, 273, 348; +NaOMe: 270, 279i, 341i, 405; +$AlCl_3$: 276, 283i, 308i, 368, 417; +$AlCl_3$/HCl: 276, 282i, 304i, 361; +NaOAc: 271, 404; +NaOAc/H_3BO_3: 265, 273i, 373, 427i. EI-MS probe 70 eV (*m/z*, rel. int.): 355 $[M+H]^+$ (34), 354 $[M]^+$ (12), 340 $[M+H-15]^+$ (12), 339 $[M-15]^+$ (6), 338 $[M+H-17]^+$ (6), 326 $[M-28]^+$ (5), 313 (27), 312 $[M+H-43]^+$ (100), 311 $[M-43]^+$ (10), 301 (21), 300 $[M+H-55]^+$ (68), 299 $[M-55]^+$ (8), 137 B_2 (6), 134 B_1 (9). ^{1}H NMR (300 MHz, DMSO-d_6, ppm/TMS): 13.2 (1H, *s*, 5-OH), 7.45 (1H, *dd*, J = 9.3; J = 2.0 Hz, H-6′); 7.38 (1H, *d*, J = 2 Hz, H-2′), 6.87 (1H, *d*, J = 9.3 Hz, H-5′), 6.65 (1H, *s*, H-3), 6.51 (1H, *s*, H-8), 5.17 (1H, *m*, H-2″), 3.20 (2H, *d*, J = 7.6 Hz, H-1″), 1.71 (3H, *s*, H-4″), 1.61 (3H, *s*, H-5″). ^{1}H,^{1}H-COSY connectivities confirmed the proton assignments. ^{13}C NMR (70.5 MHz, DMSO-d_6, ppm/TMS): 181.6 (C-4), 163.7 (C-2), 161.8 (C-7), 158.3 (C-9), 155.1 (C-5), 149.6 (C-4′), 145.7 (C-3′), 130.6 (C-3″), 122.2 (C-6′),121.4(C-2″), 119.1 (C-1′), 116.0 (C-5′), 113.5 (C-2′), 110.8 (C-6), 103.5 (C-10), 102.3 (C-3), 93.0 (C-8), 25.5 (C-5″), 22.1 (C-1″), 17.7 (C-4″).

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