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ANTIFUNGAL PHLOROGLUCINOL DERIVATIVES AND LIPOPHILIC FLAVONOIDS FROM *HELICHRYSUM DECUMBENS*

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Key Word Index—*Helichrysum decumbens*; Compositae; prenylated phloroglucinols; antifungal compounds; lipophilic flavonoids; 3,5-dihydroxy-6,7-dimethoxyflavone.

Abstract—From the aerial parts of *Helichrysum decumbens*, three new antifungal phloroglucinol derivatives have been identified. Their structures have been established by UV, IR, EIMS, FAB-MS, ^{1}H NMR and ^{13}C NMR techniques. In addition, the highly methylated flavonoids 3,5-dihydroxy-6,7,8-trimethoxyflavone, 5,7-dihydroxy-3,6,8-trimethoxyflavone and 3,5-dihydroxy-6,7-dimethoxyflavone have been detected, the latter being a new naturally occurring compound. The phloroglucinols prevented the growth of *Cladosporium herbarum* in a bio-assay. These secondary metabolites are externally deposited on the plant surfaces.

INTRODUCTION

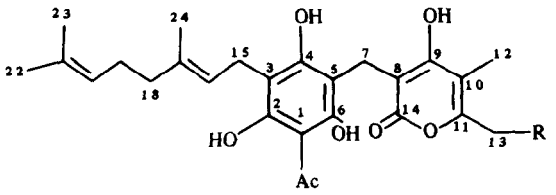
Phytochemical studies of many species of the genus *Helichrysum* (tribe Inuleae) have revealed that this genus produces phloroglucinol and α-pyrone derivatives, flavones, chalcones and diterpenoids [1–3]. In our search for new antifungal compounds from plants growing in semi-arid habitats, we have investigated *Helichrysum decumbens*. This plant grows in maritime areas in south-eastern Spain. In a preliminary study, the compounds present in the rinses obtained by dipping the aerial parts in chloroform, showed antifungal activity. Recently, some antifungal lipophilic flavonoids have been identified in the African species *Helichrysum nitens* [4]. The aim of the present work is the isolation and identification of the antifungal compounds of *Helichrysum decumbens*.

RESULTS AND DISCUSSION

The chloroform extract of *Helichrysum decumbens* was column chromatographed on silica gel with *n*-hexane–EtOAc mixtures The different phloroglucinol derivatives and flavonoids were separated and purified as described in the Experimental. While the phloroglucinol derivatives showed significant antifungal activity against *Cladosporium herbarum*, the flavonoids were inactive.

The phloroglucinol fraction was purified by preparative TLC on silica gel with chloroform. Compounds **1** (80%) and **2** (20%) were then isolated by semipreparative reversed-phase HPLC.

Compound **1** showed a UV spectrum characteristic of phloroglucinol derivatives [5]. The bathochromic shift (21 nm) induced in the UV spectrum by addition of $AlCl_3$ + HCl is indicative of an OH *ortho* to a C=O group. The absence of an acid-labile aluminium complex indicated that no *ortho*-di-hydroxyl system was present. The



	R
1	H
2	Me
3	Et

IR data suggested the presence of an α-pyrone carbonyl (1676 cm^{-1}) and an acetyl carbonyl (1625 cm^{-1}) [2, 6]. The high resolution EI mass spectrum indicated that the molecular weight is 456.2147, corresponding to $C_{26}H_{32}O_7$ and this was confirmed by FABMS in the negative mode which showed a quasi-molecular ion at *m/z* 455 $[M-H]^-$. The EIMS fragmentation of this compound showed clearly the presence of a phloroglucinol ring linked to an α-pyrone ring by a methylene bridge, as well as the occurrence of prenyl groups and the absence of methoxyls (Experimental). The ^{1}H NMR data indicated the existence of a geranyl group [7], one aromatic acetyl, two aromatic methyls and one CH_2 linked to two aromatic rings. The two aromatic methyls showed quite different chemical shifts suggesting that these two were located on the α-pyrone ring. COSY and NOEs experiments confirmed the existence of a geranyl residue in the phloroglucinol ring and two *ortho*-methyl groups on the α-pyrone ring. The ^{13}C NMR spectrum and DEPT experiment confirmed the structure of compound **1**, by comparisons with literature data on other phloroglucinols isolated from African *Helichrysum* species [2, 3].

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Compound **2** showed the same UV characteristics as compound **1**. Its EI and FAB mass spectra indicated that this was 14 mass units higher than compound **1** and that its structure was very closely related. The ^{1}H NMR data showed clearly the presence of an aromatic ethyl instead of the aromatic methyl *ortho* to the α-pyrone group.

The presence of trace amounts of a third phloroglucinol derivative (compound **3**) was detected by HPLC of the original phloroglucinol fraction The UV spectrum was similar to those of the other phloroglucinols and its higher retention time suggested that this was more lipophilic. The EIMS and FABMS analysis of the crude phloroglucinol fraction indicated trace amounts of an additional compound with molecular mass of 484 (14 mass units higher than compound **2**), and the ^{1}H NMR data of this fraction suggested the presence of trace amounts of a compound with a propyl-residue (triplet at 1.00 ppm). These data indicated that the propyl derivative seems to be present as well in the chloroform extracts of *Helichrysum decumbens*

Three highly methylated flavonols (**4–6**) were isolated from the flavonoid fraction. Compounds **4** and **5** both had the same M_r (*m/z* 344) as for dihydroxytrimethoxyflavones, and compound **6** (*m/z* 314) as for a dihydroxydimethoxyflavone. Their UV spectra in methanol and after addition of the classical shift reagents suggested that these compounds had unsubstituted B-rings and free hydroxyls in the C-5 position. The UV spectra after addition of sodium acetate indicated that compounds **4** and **6** had a substituted 7-hydroxyl while compound **5** had a free hydroxyl in the C-7 position. These UV spectra indicated that compounds **4** and **6** had a free hydroxyl in the C-3 position while compound **5** had a substituted hydroxyl The EIMS analyses confirmed the presence of unsubstituted B-rings (fragment *m/z* 105) and the substitution pattern on the A and C rings (RDA fragments). Thus, compound **4** is 3,5-dihydroxy-6,7,8-trimethoxyflavone, previously reported from *Helichrysum graveolens* [8], compound **5** is 5,7-dihydroxy-3,6,8-trimethoxyflavone previously reported from *Anaphalis araneosa* [9] and compound **6** is 3,5-dihydroxy-6,7-dimethoxyflavone, a new naturally occurring compound These compounds had no antifungal activity against *Cladosporium herbarum* at the concentrations assayed, but their permethylated derivatives had significant antifungal activity against *Cladosporium cucumerinum* as reported recently [4]. The fact that *Helichrysum* species (both *Helichrysum decumbens* and *H nitens* [4]) produce biochemical defences against fungi, is of some interest, especially when considering that the antifungal compounds were phloroglucinol derivatives in the former and flavonoids in the latter, since these compounds are biosynthesized, at least partly, by two different metabolic pathways.

EXPERIMENTAL

Plant material. Helichrysum decumbens Cambess. aerial parts were collected at flowering in La Manga del Mar Menor (Murcia, Spain) A voucher specimen was deposited in the herbarium, Department of Botany, Murcia University (Spain)

Preliminary antifungal test Several flowering stems (*ca* 5 g) were soaked in $CHCl_3$ for 2 min The rinse obtained was TLC chromatographed with petrol–EtOAc (1.1) and tested for antifungal activity against *Cladosporium herbarum* [10]

Extraction and isolation of antifungal phloroglucinols and flavonoids. Whole fresh plant material (*ca* 500 g) was dipped in $CHCl_3$ for 10 min. and the rinses obtained were taken to dryness at room temp. This extract was fractionated by 'flash' CC on silica gel (G-60) with mixtures of *n*-hexane and EtOAc, starting with pure *n*-hexane and finishing with pure EtOAc A flavonoid fraction and a phloroglucinol fraction were obtained The flavonoid fraction was submitted to prep TLC on silica gel with $CHCl_3$, and the isolated flavonoids (**4–6**) purified by passing through Sephadex LH-20 with MeOH The phloroglucinol fraction was purified by passing through a Sephadex LH-20 column with MeOH and the main component partly purified by prep TLC on silica gel with $CHCl_3$ When this fraction was tested for purity by HPLC on HD-Sil-18 column (10 μm) with MeOH–Pi buffer 0.1 M pH 6 5 (65 35) (flow 1 5 ml/min), three compounds [**1**, **2** and **3** (this in trace amounts)] were separated (R_t = 7.24, 9 64 and 11 35 respectively) and their phloroglucinol nature was demonstrated by their UV spectrum recorded with a photodiode array detector This fraction (*ca* 20 mg) yielded compounds **1** and **2** when separated in semipreparative HPLC with MeOH–Pi buffer 0 1 M pH 6 5 (13 7 and 4 1 after 1 hr) on an HD-sil-18-20-60 column (238 × 18 5 mm) with a solvent flow 4 ml/min Detection at 277 nm

Spectral data UV spectra were measured in MeOH λ_{max} is in nm IR· Nujol cm^{-1} EIMS data are *m/z* values (rel int) FABMS (negative mode) data are *m/z* values, and samples were dissolved in nitrobenzyl-alcohol ^{1}H NMR. ppm/TMS measured in MeOD (400 MHz) ^{13}C NMR ppm measured in MeOD (100 MHz)

Compound **1** UV λ_{max} nm· 350sh, 292, +NaOMe, no shifts, +$AlCl_3$, 362sh, 315, +$AlCl_3$ + HCl, 362sh, 313; +NaOAc, no shifts. IR ν_{max} cm^{-1}· 1676, 1625, 1574, 1314, 1172, 1127, 1070, 725 EIMS *m/z* (rel. int.) 456 2147 ($C_{26}H_{32}O_7$, calcd mass 456 2147) (8), 387 (2) $[M-C_5H_9]^+$, 371 (11) $[M-C_6H_{13}]^+$, 347 (3), 333 (49) $[M-C_9H_{15}]^+$, 320 (23), 303 (23) [M − α-pyrone ring, M − 153]$^+$, 290 (14), 247 (67) $[303-C_4H_8]^+$, 235 (94) $[303-C_5H_8]^+$, 181 (83) $[333-C_9H_{14}]^+$, 153 (54) [α-pyrone ring $+CH_2]^+$, 141 (75) [α-pyrone ring +2H]$^+$, 69 (100), 55 (29) FABMS 455 $[M-H]^-$. ^{1}H NMR δ 5.1359 (2H, *br t*, *J* = 7 Hz, H-16 and H-20, CH=CMe_2), 3 6059 (2H, *br s*, H-7, φ–CH_2–φ), 3 3001 (2H, *br d*, *J* = 7 Hz, H-15, φ–CH_2–CH=C), 2 6636 (3H, *s*, Me–CO), 2 4979 (3H, *s*, H-13, Me–φ), 2 1951 (2H, *d*, *J* = 7 Hz, H-18), 2.0796 (2H, *dd*, *J* = 7 Hz, H-19), 1 9582 (3H, *s*, H-12, Me–φ), 1.6587 (3H, *br s*, H-22, Me–CMe=CH–), 1.6436 (3H, *br s*, H-23, Me–), 1 6132 (3H, *br s*, H-24, Me–) ^{13}C NMR 205 6 (*s*, C =O–Me), 170 6 (*s*, C-14), 161 5 (*s*, C-4), 161 3 (*s*, C-6 and C-2), 159.4 (*s*, C-11), 158 1 (*s*, C-9), 136 0 (*s*, C-21), 132 0 (*s*, C-17), 125 0 (*d*, C-16), 124.0 (*d*, C-20), 110 4 (*s*, C-8), 109 7 (*s*, C-5), 107 0 (*s*, C-3), 106 9 (*s*, C-1), 102.7 (*s*, C-10), 33 0 (*q*, C-25), 32.9 (*t*, C-15), 27 6 (*t*, C-18), 25 8 (*q*, C-24), 23.6 (*q*, C-23), 22 4 (*t*, C-19), 18 8 (*t*, C-7), 17 7 (*q*, C-22), 17 2 (*q*, C-13), 10 5 (*q*, C-12)

Compound **2** UV λ_{max} nm 350 sh, 292. EIMS· 470 FABMS. *m/z* 469 $[M-H]^-$. ^{1}H NMR: δ 5.1428 (2H, *br t*, *J* = 7 Hz, H-16 and H-20), 3 6136 (2H, *s*, H-7, φ–CH_2–φ), 3 2750 (2H, *d*, *J* = 7 Hz, H-15), 2 6667 (3H, *s*, Me–CO, H-25), 2.5962 (2H, *q*, *J* = 7 5 Hz, φ–CH_2–Me), 2.1968 (2H, *d*, *J* = 7 Hz, H-18), 2 0798 (2H, *dd*, *J* = 7 Hz, H-19), 1.9696 (3H, *s*, H-12, Me–φ), 1 6560 (3H, *s*, Me–CMe=CH–), 1 6438 (3H, *s*, Me–CMe=CH–), 1 6113 (3H, *s*, Me–CMe=CH–), 1.881 (3H, *t*, *J* = 7 5 Hz, Me–CH_2–φ).

Compound **4**. UV λ_{max} nm 379, 317, 276; +NaOMe, 432, 353, 257, +$AlCl_3$, 429, 356, 284, +$AlCl_3$ + HCl, 428, 355, 284, +NaOAc, no shifts. Permethylation with CH_2N_2 yielded 3,5,6,7,8-pentamethoxyflavone identified by TLC comparisons with an authentic sample isolated from *Helichrysum nitens* [4] EIMS *m/z* (rel int)· 344 (51) $[M]^+$, 329 (54) $[M-Me]^+$, 326 (4), 315 (3), 301 (22) $[M-MeCO]^+$, 243 (10) $[A_1]^+$, 227 (2), 215 (9), 105 (100) $[B_2]^+$, 77 $[B_2-CO]^+$ (59), 51 (22)

Compound **5**. UV λ_{max} nm: 278; +NaOMe, 367, 283; +$AlCl_3$, 292; +$AlCl_3$+HCl, 292; +NaOAc, 375 sh, 282 Permethylation with CH_2N_2 yielded 3,5,6,7,8-pentamethoxyflavone. EIMS *m/z* (rel. int.): 344 (41) $[M]^+$, 329 (75) $[M-Me]^+$, 301 (10) $[M-MeCO]^+$, 243 (7) $[A_1]^+$, 215 (10), 105 $[B_2]^+$ (100), 77 (98), 51 (53).

Compound **6**. UV λ_{max} nm: 318, 269; +NaOMe, 366, 271; +$AlCl_3$, 344, 282, 253; +$AlCl_3$+HCl, 340, 280; +NaOAc, 370, 271. Permethylation with CH_2N_2 yielded 3,5,6,7-tetramethoxyflavone identified by TLC comparisons with an authentic sample isolated from *Helichrysum nitens* [4], EIMS *m/z* (rel. int.): 314 (42) $[M]^+$, 299 (59) $[M-Me]^+$, 296 (15), 285 (8), 271 (37) $[M-MeCO]^+$, 211 (12) $[A_1]^+$, 197 (6), 183 (10), 105 (100) $[B_2]^+$, 77 (98), 51 (65)

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