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ANTIFUNGAL EPICUTICULAR METHYLATED FLAVONOIDS FROM *HELICHRYSUM NITENS*

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Key Word Index—*Helichrysum nitens*; Compositae; antifungal flavonoids; fully methylated flavones; 3,5,6,7,8-pentamethoxyflavone; ecological biochemistry.

Abstract—From the aerial parts of *Helichrysum nitens*, 5,7-dimethoxyflavone, 5,6,7-trimethoxyflavone, 3,5,7-trimethoxyflavone, 5,6,7,8-tetramethoxyflavone, 3,5,6,7-tetramethoxyflavone and 3,5,6,7,8-pentamethoxyflavone have been isolated and identified. In addition, 5-hydroxy-6,7-dimethoxyflavone and 5-hydroxy-6,7,8-trimethoxyflavone have also been found. The antifungal activities of the fully methylated compounds were similar to that of tangeretin (5,6,7,8,4′-pentamethoxyflavone), an antifungal flavonoid isolated from *Citrus* leaves. These compounds have been found externally deposited on the leaf and stem surfaces. 3,5,6,7,8-Pentamethoxyflavone is a new naturally occurring flavonoid.

INTRODUCTION

From the large genus *Helichrysum* (tribe Inuleae) many species have been studied chemically and flavones, chalcones, diterpenes, phloroglucinol and α-pyrone derivatives have been detected [1]. *Helichrysum nitens* Oliv. & Hiern is a plant which was collected on Zomba Plateau (Malawi). In a preliminary study, the compounds present in the rinses obtained by soaking the aerial parts in dichloromethane, showed antifungal activity. The aim of this work is the isolation and identification of the antifungal compounds of this African plant.

RESULTS AND DISCUSSION

The dichloromethane extract of *Helichrysum nitens* was column chromatographed on silica gel starting with petrol–EtOAc (4:1) and increasing the polarity until reaching pure EtOAc. The different fractions were separated on a silica gel Lobar column with chloroform–methanol and petrol–ethyl acetate mixtures and eight (**1–8**) UV-absorbing compounds were isolated. The purity of the different substances was tested by HPLC coupled with a photodiode array detector.

Compounds **1–6** showed fluorescent colours (**1** and **2** bright blue, **3** and **5** pinkish, **4** and **6** dull reddish) under UV light (366 nm). No shifts were observed in their UV spectra in MeOH after addition of NaOMe suggesting the absence of free phenolic groups in these compounds. Their molecular ions in the EIMS analyses indicated that these were fully methylated flavones. The presence of prominent peaks for the $[M-Me]^+$ ions supported their highly methylated character. Significant $[M-H]^+$ peaks were observed in the spectra of compounds **2**, **5** and **6**, suggesting that these were 3-methoxyflavones [2, 3]. The RDA fragmentations indicated the different substitution patterns on A-rings (fragments $[A_1]^+$, $[A_1-Me]^+$, $[A_1-Me-CO]^+$) as well as the presence of unsubstituted B-rings (fragments $[B_1]^+$ 102 *m/z* and $[B_2]^+$ 105 *m/z*) [2]. The presence of a methoxyl at position 3 in compounds **2**, **5** and **6** was also suggested by the presence of $[B_2]^+$ and absence of $[B_1]^+$ peaks in their spectra [2]. The ^{1}HNMR data of these compounds showed multiplets at 8.05–8.15 (2H) and 7.4–7.5 ppm (3H) in compounds **2**, **5** and **6** indicating that they were 3-methoxyflavones with an unsubstituted B-ring [4], and multiplets at 7.8–7.9 (2H) and 7.4–7.4 ppm (3H) in compounds **1**, **3** and **4** indicating that they were flavones with an unsubstituted B-ring [4]. The different methoxyls present in each compound were readily ascertained. The ^{13}CNMR spectra of compounds **2–4** and **6** were also run to confirm their structures. These spectra confirmed the presence of unsubstituted B-rings in the 3-methoxyflavones (128.5 ppm C-2′,6′ and 128.1 ppm C-3′, 5′) and in the flavones (128.9 ppm C-2′, 6′ and 125.9 ppm C-3′, 5′) [5–7]. The C-4 carbonyl signals appeared at 173–177 ppm in accordance to the spectra of 5-methoxyflavones [7]. All these data indicated that compound **1** was 5,7-dimethoxyflavone (chrysin dimethyl ether), **2** was 3,5,7-trimethoxyflavone (galangin trimethyl ether), **3** was 5,6,7-trimethoxyflavone (baicalein trimethyl ether), **4** was 5,6,7,8-tetramethoxyflavone, **5** was 3,5,6,7-tetramethoxyflavone and **6** was 3,5,6,7,8-pentamethoxyflavone.

Compounds **7** and **8** showed a dark absorbance under UV light (366 nm) suggesting 5-hydroxyflavonoids. Their UV spectra in MeOH and after the addition of the classical shift reagents clearly showed that the hydroxyl at 5-position was free in both compounds. The EIMS analyses revealed that these compounds were a monohydroxy-dimethoxyflavone (**7**) and a monohydroxytri-

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methoxyflavone (**8**), and the RDA fragments indicated that all the substituents were located on the A-rings. The ^{1}H NMR data confirmed the existence of a free hydroxyl at C-5 (broad singlets at 12.5 ppm), and unsubstituted B-rings (multiplets at 7.92 and 7.55 ppm). The ^{13}C NMR spectrum of compound **8** was also run to confirm the structure and these data coincided with those reported for alnetin (5-hydroxy-6,7,8-trimethoxyflavone) [7]. Compound **7** was identified as 5-hydroxy-6,7-dimethoxyflavone.

5,7-Dimethoxyflavone and 5,6,7,8-tetramethoxyflavone have been previously found in *Helichrysum herbaceum* [8] but the rest of the compounds isolated have not been described in the genus previously. Alnetin (5-hydroxy-6,7,8-trimethoxyflavone was first isolated from the flowers of *Alnus sieboldiana* (Betulaceae) [9], 3,5,7-trimethoxyflavone from the wood of *Aniba riparia* (Lauraceae) [5], 5-hydroxy-6,7-dimethoxyflavone from *Popowia cauliflora* (Annonaceae) [10], 3,5,6,7-tetramethoxyflavone from *Gomphrena martiana* (Compositae) [11] and 5,6,7-trimethoxyflavone from different plants [10], but this is the first time that this compound has been described in the Compositae. 3,5,6,7,8-Pentamethoxyflavone is a new naturally occurring compound. This flavonoid was previously obtained by permethylation of 3,5-dihydroxy-6,7,8-trimethoxyflavone [12]. It is noteworthy that all the flavonoid aglycones identified in this plant bear an unsubstituted B-ring.

The eight flavones isolated are externally deposited on the leaf and stem surfaces since they are the main components of the rinses obtained by soaking the aerial parts in dichloromethane for two min. The antifungal activity of the different compounds was ascertained by a TLC assay, using spores of *Cladosporium cucumerinum* [13]. In this assay, 1 μg of dimethylchrysin (**1**) and trimethylgalangin (**2**), 2 μg of 5,6,7,8-tetramethoxyflavone (**4**), and 5 μg of baicalein trimethyl ether (**3**), 3,5,6,7-tetramethoxyflavone (**5**) and 3,5,6,7,8-pentamethoxyflavone (**6**) were sufficient to prevent growth of the fungus when spotted onto the plate. By contrast, 50 μg of alnetin (**8**) and 5-hydroxy-6,7-dimethoxyflavone (**7**) were inactive. For comparison purposes, the known antifungal flavone tangeretin (5,6,7,8,4′-pentamethoxyflavone) which is responsible in part for the resistance in *Citrus* species to the pathogenic fungus *Deuterophoma tracheiphila* [14], causing the disease known as mal-secco, has also been tested. 2 μg of this substance were necessary to inhibit the growth of *Cladosporium cucumerinum*. Therefore, the fully methylated flavones isolated from *Helichrysum nitens* show an antifungal activity similar to that of tangeretin.

These results coincide with those observed for the antifungal flavones from *Citrus* species [15] since the antifungal activity showed by the fully methylated flavones decreases dramatically when the methyl group at position 5 is removed. It has been suggested that since phenolic substances generally have significant antimicrobial activity, their function in tissues where they accumulate might be to provide chemical barriers to invading micro-organisms [16]. In addition, the methylated, lipophilic flavonoids are especially suitable as protection against fungae and bacteria because of their ease in penetrating membranes [10]. For this reason, the external accumulation of antifungal methylated flavones in *H. nitens* is of ecological significance since it supports the postulated roles for these lipophilic flavonoids.

The occurrence of tangeretin in the leaf wax, rather than within the leaf in the vacuoles, of *Citrus* plants is still only presumptive [17], but in the case of the antifungal flavonoids of *H. nitens* it is clear that they are of external occurrence, supporting a defense role of these compounds in the plant.

EXPERIMENTAL

Plant material. Aerial parts of *Helichrysum nitens* were collected at Zomba Plateau (Malawi) and a voucher specimen is deposited at the Herbarium, University of Malawi, Zomba.

Preliminary antifungal test. Several leaves (*ca* 1 g) were soaked in CH_2Cl_2 for two min. The rinse obtained was TLC chromatographed on silica gel with petrol–ethyl acetate (1:1) and tested for antifungal activity [13].

Extraction and isolation of the antifungal compounds. The air-dried powdered aerial parts (*ca* 130 g) were extracted with CH_2Cl_2 and *ca* 6 g of extract were obtained. 2.83 g of this extract were subjected to flash chromatography on silica gel with petrol–EtOAc (4:1) and increasing the polarity with EtOAc until reaching 100% EtOAc. The different fractions obtained were tested for antifungal activity. The active fractions were purified by Lobar chromatography on a silica gel column with different mixtures of $CHCl_3$–MeOH and petrol–EtOAc. Compounds **1** (33 mg), **2** (35 mg), **3** (19 mg), **4** (34 mg), **5** (4 mg), **6** (7 mg), **7** (42 mg) and **8** (45 mg) were isolated and their purity tested by HPLC on a reversed-phase column (Lichrosorb RP-18, 7 μm, 4.6 × 250 mm) with MeOH–H_2O (3:1) flow 1.5 ml/min, with a photodiode array detector.

Spectral data. UV spectra were measured in MeOH. λ_{max} is in nm. EIMS data are *m/z* values (rel. int.). ^{1}H NMR data are in ppm/TMS measured in $CDCl_3$ (200 MHz). ^{13}C NMR data are in ppm measured in $CDCl_3$ (50 MHz).

1. UV: 305, 263, 235sh; + NaOMe no shifts. EIMS: 282 $[M]^+$ (100), 253 $[M-CHO]^+$ (55), 236 (49), 224 (16), 209 (45), 181 $[A_1+H]^+$ (4), 165 $[A_1-Me]^+$ (8), 152 $[A_1-CO]^+$ (15), 150 $[A_1-Me-Me]^+$ (20), 137 $[A_1-Me-CO]^+$ (13), 105 $[B_2]^+$ (15), 102 $[B_1]^+$ (16), 77 $[Ph]^+$ (18). ^{1}H NMR: 7.85 (2H, *m*, H-2′, H-6′), 7.48 (3H, *m*, H-3′, H-4′, H-5′), 6.70 (1H, *s*, H-3), 6.55 (1H, *d*, J = 2 Hz, H-8), 6.35 (1H, *d*, J = 2 Hz, H-6), 3.94 (3H, *s*, 7-OMe), 3.89 (3H, *s*, 5-OMe).

2. UV: 325, 295, 262, 240sh; + NaOMe no shifts. EIMS: 312 $[M]^+$ (55), 311 $[M-H]^+$ (100), 293 (41), 281 (14), 265 (12), 251 (11), 181 $[A_1+H]^+$ (12), 165 $[A_1-Me]^+$ (7), 152 $[A_1-CO]^+$ (17), 137 $[A_1-CO-Me]^+$ (18), 105 $[B_2]^+$ (42), 77 $[Ph]^+$ (82). ^{1}H NMR: 8.05 (2H, *m*, H-2′, H-6′), 7.48 (3H, *m*, H-3′, H-4′, H-5′), 6.50 (1H, *d*, J = 2 Hz, H-8), 6.32 (1H, *d*, J = 2 Hz, H-6), 3.95 (3H, *s*, 7-OMe), 3.88 (3H-, *s*, 5-OMe), 3.87 (3H-, *s*, 3-OMe). ^{13}C NMR: 174.021 (C-4), 163.922 (C-7), 160.990 (C-5), 158.858 (C-9), 152.518 (C-2), 141.802 (C-3), 130.870 (C-1′), 130.227 (C-4′), 128.416 (C-3′,5′), 128.100 (C-2′, 6′), 109.578 (C-10), 95.834 (C-8), 92.435 (C-6), 60.137 (MeO– at C-3), 56.460, 55.804 (MeO– at C-5,7).

3. UV: 305, 264, 240sh; + NaOMe no shifts. EIMS: 312 $[M]^+$ (15), 297 $[M-Me]^+$ (100), 269 $[M-Me-Co]^+$ (11), 254 $[M-Me-CO-Me]^+$ (16), 210 $[A_1]^+$ (2), 195 $[A_1-Me]^+$ (3), 180 $[A_1-Me-Me]^+$ (2), 167 $[A_1-Me-CO]^+$ (14), 152 $[A_1-Me-CO-Me]^+$ (5), 102 $[B_1]^+$ (8), 77 $[Ph]^+$ (9). ^{1}H NMR: 7.87 (2H, *m*, H-2′, H6′), 7.50 (3H, *m*, H-3′, H-4′, H-5′), 6.82 (1H, *s*, H-3), 6.69 (1H, *s*, H-8), 3.99 (6H, *s*, 5-OMe, 7-OMe), 3.91 (3H, *s*, 6-OMe). ^{13}C NMR: 177.16 (C-4), 161.106 (C-2), 157.778 (C-7), 154.525 (C-9), 152.529 (C-5), 140.419 (C-6), 131.572 (C-1′), 131.241 (C-4′), 128.941 (C-3′, 5′), 125.966 (C-2′, 6′), 108.376 (C-3, 10), 96.296 (C-8), 62.1 (MeO– C-5), 61.5 (MeO– C-6), 56.3 (MeO– C-7).

4. UV: 305, 270, 250sh; + NaOMe no shifts. EIMS: 342 $[M]^+$ (20), 327 $[M-Me]^+$ (100), 313 $[M-CHO]^+$ (5), 299 [M – Me

$-CO]^+$ (15), 284 $[M-Me-CO-Me]^+$ (24), 266 (17), 225 $[A_1-Me]^+$ (5), 197 $[A_1-Me-CO]^+$ (23), 182 $[A_1-Me-CO-Me]^+$ (17), 102 $[B_1]^+$ (19), 77 $[Ph]^+$ (8). ^1H NMR: 7.92 (2H, *m*, H-2′, H-6′), 7.49 (3H, *m*, H-3′, H-4′, H-5′), 6.69 (1H, *s*, H-3), 4.09 (3H, *s*, 7-OMe), 4.01 (3H, *s*, 8-OMe), 3.93 (6H, *s*, 5-OMe, 6-OMe). ^{13}C NMR: 177.152 (C-4), 161.004 (C-2), 151.416 (C-7), 148.248 (C-9), 147.682 (C-5), 144.056 (C-6), 138.031 (C-8), 131.474 (C-1′), 131.318 (C-4′), 128.962 (C-3′, 5′), 125.899 (C-2′, 6′), 114.856 (C-10), 107.955 (C-3), 62.206, 62.005, 61.772, 61.604 (MeO– at C-5,6,7,8).

5. UV: 312, 260, 245sh; +NaOMe no shifts. EIMS: 342 $[M]^+$ (48), 341 $[M-H]^+$ (51), 327 $[M-Me]^+$ (100), 323 $[M-19]^+$ (15), 297 $[M-46]^+$ (9), 284 $[M-58]^+$ (21), 210 $[A_1]^+$ (5), 195 $[A_1-Me]^+$ (16), 181 $[A_1-CHO]^+$ (4), 167 $[A_1-Me-CO]^+$ (27), 105 $[B_2]^+$ (29), 77 $[Ph]^+$ (24). ^1H NMR: 8.05 (2H, *m*, H-2′, H-6′), 7.49 (3H, *m*, H-3′, H-4′, H-5′), 6.75 (1H, *s*, H-8), 4.00 (3H, *s*, 7-OMe), 3.95 (3H, *s*, 5-OMe), 3.91 (3H, *s*, 6-OMe), 3.86 (3H, *s*, 3-OMe).

6. UV: 335sh, 310, 265, 250sh; +NaOMe no shifts. EIMS, 372 $[M]^+$ (42), 371 $[M-H]^+$ (30), 357 $[M-Me]^+$ (100), 353 $[M-19]^+$ (9), 341 $[M-31]^+$ (14), 329 $[M-Me-CO]^+$ (13), 313 (17), 299 (8), 225 $[A_1-Me]^+$ (7), 197 $[A_1-Me-CO]^+$ (18), 105 $[B_2]^+$ (26), 77 $[Ph]^+$ (19). ^1H NMR: 8.15 (2H, *m*, H-2′, H-6′), 7.5 (3H, *m*, H-3′, H-4′, H-5′), 4.00 (3H, *s*, 7-OMe), 3.99 (3H, *s*, 8-OMe), 3.97 (3H, *s*, 5-OMe), 3.94 (3H, *s*, 6-OMe), 3.88 (3H, *s*, 3-OMe). ^{13}C NMR: 173.855 (C-4), 150.825 (C-7), 148.091 (C-9), 146.785 (C-5), 143.840 (C-6), 141.309 (C-2), 137.816 (C-8), 133.770 (C-3), 130.921 (C-1′), 130.322 (C-4′), 128.595 (C-3′, 5′), 128.187 (C-2′, 6′), 115.120 (C-10), 62.291, 62.047, 61.849, 61.677 (MeO– C-5,6,7,8), 60.066 (MeO– C-3).

7. UV: 315, 272, 250sh; +NaOMe, 370, 284; $+AlCl_3$, 390sh, 338, 283, 254; $+AlCl_3+HCl$, 385sh, 333, 281, 252; +NaOAc, 312sh, 272. EIMS, 298 $[M]^+$ (86), 283 $[M-Me]^+$ (83), 269 $[M-CHO]^+$ (36), 255 $[M-Me-CO]^+$ (68), 181 $[A_1-Me]^+$ (18), 153 $[A_1-Me-CO]^+$ (100), 102 $[B_1]^+$ (18), 77 $[Ph]^+$ (27). ^1H NMR: 12.561 (1H, *br*, OH-5), 7.89 (2H, *m*, H-2′, H-6′), 7.53 (3H, *m*, H-3′, H-4′, H-5′), 6.68 (1H, *s*, H-3), 6.57 (1H, *s*, H-8), 3.97 (3H, *s*, 7-OMe), 3.92 (3H, *s*, 6-OMe).

8. UV: 350sh, 320sh, 280; +NaOMe, 400, 282; $+AlCl_3$, 390, 335, 296; $+AlCl_3+HCl$, 400, 330sh, 295; +NaOAc, 350sh, 320sh, 280. EIMS: 328 $[M]^+$ (66), 313 $[M-Me]^+$ (100), 298 $[M-Me-Me]^+$ (10), 285 $[M-Me-CO]^+$ (8), 270 $[M-Me-CO-Me]^+$ (10), 227 $[A_1+H]^+$ (2), 211 $[A_1-Me]^+$ (9), 199 $[A_1+H-CO]^+$ (13), 183 $[A_1-Me-CO]^+$ (15), 102 $[B_1]^+$ (9), 77 $[Ph]^+$ (6). ^1H NMR: 12.43 (1H, *br*, OH-5), 7.92 (2H, *m*, H-2′, H-6′), 7.55 (3H, *m*, H-3′, H-4′, H-5′), 6.69 (1H, *s*, H-3), 4.12 (3H, *s*, 7-OMe), 3.97 (3H, *s*, 8-OMe), 3.95 (3H, *s*, 6-OMe). ^{13}C NMR: 183.006 (C-4), 163.937 (C-2), 153.038 (C-7), 149.463 (C-9), 145.622 (C-5), 136.420 (C-6), 133.083 (C-8), 131.975 (C-4′), 131.259 (C-1′), 129.150 (C-3′,5′) 126.276 (C-2′, 6′), 107.133 (C-10), 105.271 (C-3), 62.204, 61.754, 61.171 (MeO– at C-6, 7,8).

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