Chemodiversity of Exudate Flavonoids in *Cassinia* and *Ozothamnus* (Asteraceae, Gnaphalieae)

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The lipophilic exudates deposited on aerial parts of four species of *Cassinia* and twelve species of *Ozothamnus* (Asteraceae, Gnaphalieae) have been analyzed for the presence of flavonoid aglycones and some other phenolics. A total of 55 flavonoids were identified, including several rare flavonols. Flavonols are prevailing over flavones, and 8-*O*-substitution is dominant in both groups. *Ozothamnus rosmarinifolius* is exceptional in producing several coumarins. Four dihydrobenzofurans have also been identified from this species. Cluster analysis and principal coordinate analysis of the flavonoid data provide support for combining species of *Cassinia* and *Ozothamnus* into a single genus.

Key words: Cassinia, *Ozothamnus*, Flavonoids

Introduction

Cassinia and *Ozothamnus* are two closely related genera of Asteraceae, tribe Gnaphalieae, that grow in Australia and New Zealand; *Ozothamnus* grows also in New Caledonia. They are represented by some 20 and 50 species, respectively. Taxonomically, they are separated on several poorly correlated character states. *Odixia* is similar to both *Cassinia* and *Ozothamnus* but lacks pappus, otherwise present in these genera. *Cassinia* and *Odixia* species and most *Ozothamnus* species typically bear glandular-hairy leaves and exhibit a more or less obvious lipophilic exudate. In a 1997 paper we already reported on exudate flavonoids of two *Odixia* species and ten *Ozothamnus* species (Wollenweber *et al*., 1997). In the present study, four *Cassinia* species and twelve further *Ozothamnus* species were surveyed for the presence of exudate flavonoids. The objective of this study was to differentiate between species of both genera on the basis of flavonoid patterns using multivariate methods.

Material and Methods

Plant material

Plant material was collected in the field or in the Australian National Botanical Garden, Canberra, and dried in an oven, as reported in a previous paper (Wollenweber *et al*., 1997). Voucher specimens are kept at the Australian National Herbarium in Canberra (CANB) and others.

Collection data and provenances are as follows. Numbers in brackets refer to sample wet weight/sample dry weight/exudate yield in grams.

Cassinia arcuata R. Br.: 40 km from Bridwood towards Nerriga, 35°11′S, 150°04′E, NSW, unknown, CBG 760788. September '95 (–/28.5/4.82).

C. collina C. T. White: Mt. Walsh, 6.5 km S of Biggenden, Queensland, 25°34′S, 152°02′E, 28.5.1977, Telford 5318. May '96 (31.8/13.0/0.95).

C. longifolia R. Br.: near Frith Street, CSIRO Black Mountain Laboratories, ACT, 35°16′S, 149°06′E, 22.3.1995, Puttock 1065. February and May '96 (60.0/24.9/2.67).

C. subtropica F. Muell.: Mt. Warning, 14 km SW of Murwillumbah, NSW, 28°24′S, 153°15′E, 28.9.1973, Telford 3251. May '96 (69.4/30.5/4.26).

Ozothamnus antennaria (DC.) Hook. f.: behind Chalet, Mt. Wellington, Tasmania, 42°54′S, 147°14′E, 28.12.1995, Puttock 1308. January '96 (123.0/60.5/7.52).

O. argophyllus (A. Cunn. ex DC.) A. Anderb.: Careys Peak, Barrington Tops, NSW, 32°04′S, 151°26′E, 12.2.1971, Telford 2738. December '95 (47.6/19.5/2.20).

O. costatifolius (R. V. Sm.) A. Anderb.: Bicheno, Tasmania, 41°53′S, 148°18′E, 25.11.1986, Burns ANBG 1042. December '95 (50.2/20.0/3.82).

O. cuneifolius (Benth.) A. Anderb.: Mt. Elizabeth, summit, ca. 8 km directly ENE of Tambo Crossing, Victoria, 37°29′S, 147°56′E, 12.11.1988, Davies 650, Whinsbury and Donaldson. December '95 (26.0/11.7/1.49).

O. diosmifolius (Vent.) DC.: 2 km along Mayfair road, from Mulgoa-Wallicia road, NSW, 33°51′S, 150°39′E, 20.11.1984, Besley 153, Donaldson and Ollerenshaw. February '96 (18.8/9.7/0.15).

O. ericifolius Hook. f.: Standup Point, Tasmania, 43°11′S, 147°52′E, 30.1.1996, Puttock 1309 (92.9/–/6.68).

O. ferrugineus (Labill.) DC.: 1.2 km S of Copping, Tasmania, 42°49′S, 147°48′E, 30.1.1996, Puttock 1312 (–/24.4/5.15).

O. purpurescens DC.: Huon Road, behind HCC Mt. Park depot, Tasmania, 42°54′S, 147°17′E, 29.12.1995, Buchanan 14050 and Puttock (59.3/33.7/3.74).

O. rodwayi Orchard: near Dead Island, Mt. Wellington, Tasmania, 42°54′S, 147°13′E, 31.1.1996, Puttock 1314. February '96 (99.5/61.0/11.96).

O. rosmarinifolius (Labill.) Sweet: W side of Dee Lagoon, Tasmania, 42°16′S, 146°35′E, 2.11.1986, Burns ANBG 627 (77.5/33.9/2.97).

O. stirlingii (F. Muell.) A. Anderb.: Mt. Franklin road, Brindabella Ra., ACT, 35°25′S, 148°47′E, 6.10.1995, Puttock 1064 and Telford (37.2/13.2/2.40).

O. thyrsoideus DC.: Gudgenby River at Visitors Information Centre, Namadgi National Park, ACT, 35°31′S, 149°00′E, 13.12.1984, Stewart 570, Whigham and Young. November '95 (19.6/7.3/1.22).

Extraction and isolation

Dried plant material was briefly rinsed with acetone and the lipophilic material thus obtained was concentrated *in vacuo*. Resin amounts ranged from 0.8–13.6 % of fresh weight and 1.5–22.6 % of dry weight, respectively (with dry weight ranging from 37.2–59.9 % of fresh weight). The resinous exudates were subject to column chromatography, monitored by TLC on silica gel and on polyamide DC-11. Comparisons with authentic flavonoids, available in E. W.'s laboratory, were done in the same solvent systems, and chromatograms were evaluated as reported previously (Wollenweber *et al.*, 1997). A number of products were isolated either by crystallization from relevant fractions, or by preparative TLC on silica gel. Compounds for NMR analysis were purified to homogeneity by semi-preparative HPLC on a 10 μm Econosil C18 column (10 × 250 mm; Alltech, Deerfield, Illinois, USA) using linear solvent gradients of MeCN in 1% aqueous formic acid at a flow rate of 10 ml/min. The UV trace was monitored at 254 nm and peak fractions were collected manually. Compounds of interest were recovered from the peak fractions by lyophilization.

NMR and MS

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a Bruker DRX 600 spectrometer in DMSO-d_6 at room temperature. DMSO resonances were used as internal shift references. ^{1}H-^{13}C HMQC and HMBC experiments were performed using standard pulse sequences.

Electron impact mass spectra were obtained on a Varian MAT 212 Spectrometer at 70 eV. Atmospheric pressure chemical ionization (APCI)-MS/MS spectra were recorded on a PE Sciex API III Plus triple quadrupole instrument (Sciex, Concord, Canada) as described in Stevens *et al.* (1999). High-resolution FAB-MS experiments were conducted on a Kratos MS50 double focusing magnetic sector instrument using 3-nitrobenzyl alcohol as the matrix.

In the following we report spectral data for some of the isolated flavonols and further phenolics (Fig. 1). Spectral data of other flavonoids are not presented, as they were in accordance with literature data [for references see Valant-Vetschera and Wollenweber (2006)].

5,7,8-Trihydroxy-3,6-dimethoxyflavone (**1**): Orange-yellow needles from toluene, m.p. 181–182 °C. – MS: m/z = 330 $[C_{17}H_{14}O_7]^+$. – ^{1}H NMR: δ_H = 12.15 (s, OH-5), 10.19 (s, OH), 8.93 (s, OH), 8.10 (m, H-2′ and H-6′), 7.59 (m, H3′, H4′, H-5′), 3.81 (s, OMe-3), 3.79 (s, OMe-6). – ^{13}C NMR: δ_C = 178.8 (C-4), 155.0 (C-2), 147.3 (C-7), 144.7

(C-5), 141.1 (C-9), 138.3 (C-3), 131.4 (C-6), 131.0 (C-4′), 130.3 (C-1′), 128.7 (C-3′ and C-5′), 128.3 (C-2′ and C-6′), 125.4 (C-8), 103.6 (C-10), 60.1 (OMe-6), 60.0 (OMe-3).

Gossypetin-3,7,8,4′-tetramethyl ether (**2**): APCI-MS: *m/z* = 375 $[C_{19}H_{19}O_8]^+$. – ^{1}H NMR: δ_H = 12.47 (br s, OH-5), 9.53 (br s, OH-3′), 7.60 (m, 2H, H-2′ and H-6′), 7.15 (d, 1H, *J* = 8.4 Hz, H-5′), 6.59 (s, 1H, H-6), 3.92 (s, 3H, OMe-7), 3.87 (s, 3H, OMe-4′), 3.82 (s, 3H, OMe-8), 3.81 (s, 3H, OMe-3). – ^{13}C NMR: δ_C = 178.4 (C-4), 158.2 (C-7), 156.4 (C-5), 155.5 (C-2), 150.4 (C-4′), 147.8 (C-9), 146.5 (C-3′), 138.0 (C-3), 128.4 (C-8), 122.3 (C-1′), 120.4 (C-6′), 114.9 (C-2′), 112.1 (C-5′), 104.5 (C-10), 95.7 (C-6), 61.1 (OMe-8), 59.7 (OMe-3), 56.5 (OMe-7), 55.6 (OMe-4′).

Gossypetin-3,7,8,3′-tetramethyl ether (**3**): APCI-MS: *m/z* = 375 $[C_{19}H_{19}O_8]^+$. – ^{1}H NMR: δ_H = 12.48 (br s, OH-5), 9.99 (br s, OH-4′), 7.68 (d, 1H, *J* = 2.0 Hz, H-2′), 7.64 (dd, 1H, *J* = 2.0 and 8.4 Hz, H-6′), 7.00 (d, 1H, *J* = 8.4 Hz, H-5′), 6.59 (s, 1H, H-6), 3.92, 3.86, 3.830 and 3.825 (OMe-3, OMe-7, OMe-8 and OMe-3′).

2-(5-Acetyl-3-hydroxy-2,3-dihydrobenzofuran-2-yl)allyl acetate (**4**): HR-FAB-MS: *m/z* = 277.10770 ($C_{15}H_{17}O_5^+$, calcd. 277.10760). – ^{1}H NMR: δ_H = 7.99 (d, 1H, *J* = 1.3 Hz, H-4), 7.92 (dd, 1H, *J* = 8.4, 1.5 Hz, H-6), 7.00 (d, 1H, *J* = 8.4 Hz, H-7), 5.35 and 5.33 (each 1H, > C=C<u>H</u>$_2$), 5.24 (d, 1H, *J* = 6.3 Hz, H-3), 5.17 (d, 1H, *J* = 6.3 Hz, H-2), 4.72 and 4.61 (each 1H, d, *J* = 13.8 Hz, -CH_2-), 2.53 (s, 3H, acetyl C<u>H</u>$_3$), 2.07 (s, 3H, ester CO-C<u>H</u>$_3$). – ^{13}C NMR: δ_C = 196.1 (acetyl CO), 170.1 (ester CO), 162.9 (C-7a), 138.9 (> <u>C</u>=CH_2), 131.5 (C-5), 130.7 (C-4), 130.6 (C-3a), 126.8 (C-6) , 114.2 (> C=<u>C</u>H$_2$), 109.8 (C-7), 87.8 (C-2), 70.4 (C-3), 63.7 (-CH_2-), 26.5 (acetyl CH_3), 20.6 (ester CO-CH_3).

2-(5-Acetyl-2,3-dihydrobenzofuran-2-yl)allyl alcohol (**5**): APCI-MS: *m/z* = 219 $[MH]^+$. – ^{1}H NMR: δ_H = 7.82 (br s, 1H, H-4), 7.80 (d, 1H, *J* = 8.4 Hz, H-6), 6.89 (d, 1H, *J* = 8.4 Hz, H-7), 5.41 (t, 1H, *J* = 8.7 Hz, H-2), 5.15 and 5.12 (each 1H, > C=C<u>H</u>$_2$), 4.03 and 3.99 (each 1H, d, *J* = 14.5 Hz, -C<u>H</u>$_2$-OH), 3.44 (dd, 1H, *J* = 16, 9.7 Hz, H-3), 3.12 (dd, 1H, *J* = 16, 7.6 Hz, H-3′), 2.52 (s, 3H, acetyl C<u>H</u>$_3$). – ^{13}C NMR: δ_C = 196.1 (acetyl CO), 163.2 (C-7a), 148.4 (> <u>C</u>=CH_2), 130.3 (C-5), 130.1 (C-6), 127.6 (C-3a), 125.6 (C-4), 109.6 (> C=<u>C</u>H$_2$), 108.6 (C-7), 83.9 (C-2), 60.5 (-<u>C</u>H$_2$-OH), 33.6 (C-3), 26.5 (acetyl <u>C</u>H$_3$).

The dihydrobenzofuran derivatives **6** and **7** were identified by MS and NMR as the acetic acid esters of dihydrobenzofurans **4** and **5**, respectively. Both natural products have been reported in the literature (Pritschow *et al*., 1991; De Lampasona *et al*., 1997).

2-(3-Acetoxy-5-acetyl-2,3-dihydrobenzofuran-2-yl)allyl acetate (**6**): APCI-MS: *m/z* = 319 $[MH]^+$, consistent with the 3-acetic acid ester of compound **4**. – ^{1}H NMR: δ_H = 8.01 (m, 2H, H-4 and H-6), 7.11 (d, 1H, *J* = 9 Hz, H-7), 6.37 (d, 1H, *J* = 6.4 Hz, H-3), 5.45 (d, 1H, *J* = 6.4 Hz, H-2), 5.42 (br s, 2H, > C=C<u>H</u>$_2$), 4.63 and 4.58 (each d, 1H, *J* = 14 Hz, -CH_2-), 2.52 (s, 3H, acetyl C<u>H</u>$_3$), 2.05 and 1.98 (each s, 3H, 2 × ester CO-C<u>H</u>$_3$).

2-(5-Acetyl-2,3-dihydrobenzofuran-2-yl)allyl acetate (**7**): APCI-MS: *m/z* = 261 $[MH]^+$, consistent with the acetic acid ester of dihydrobenzofuran **5**. – ^{1}H NMR: δ_H = 7.84 (br s, 1H, H-4), 7.81 (dd, 1H, *J* = 8.4, 1.3 Hz, H-6), 6.91 (d, 1H, *J* = 8.4 Hz, H-7), 5.46 (t, 1H, *J* = 8.6 Hz, H-2), 5.30 and 5.23 (each 1H, > C=C<u>H</u>$_2$), 4.65 and 4.59 (each d, 1H, *J* = 14 Hz, -CH_2-), 3.49 (dd, 1H, *J* = 16, 9.8 Hz, H-3), 3.15 (dd, 1H, *J* = 16, 7.5 Hz, H-3′), 2.50 (s, 3H, acetyl C<u>H</u>$_3$), 2.00 (s, 3H, ester CO-C<u>H</u>$_3$). – ^{13}C NMR: δ_C = 196.1 (acetyl CO), 169.9 (ester <u>C</u>O-CH_3, 163.0 (C-7a), 142.8 (> <u>C</u>=CH_2), 130.5 (C-5), 130.1 (C-6), 127.4 (C-3a), 125.6 (C-4), 113.8 (> C=<u>C</u>H$_2$), 108.7 (C-7), 83.6 (C-2), 62.8 (-<u>C</u>H$_2$-OAc), 33.6 (C-3), 26.5 (acetyl <u>C</u>H$_3$), 20.5 (ester CO-<u>C</u>H$_3$).

7-O-Prenylaesculetin (**8**): ^{1}H NMR: δ_H = 9.31 (OH-6), 7.89 (d, 1H, *J* = 9.4 Hz, H-4), 7.02 (s, H-8), 7.01 (s, H-5), 6.23 (d, 1H, *J* = 9.4 Hz, H-3), 5.46 (t, 1H, *J* = 7 Hz, prenyl H-2), 4.63 (d, 2H, *J* = 7 Hz, prenyl H-1), 1.76 and 1.73 (each 3 H, 2× prenyl CH_3).

7-O-Prenylscopoletin (**9**): HR-FAB-MS: *m/z* = 261.11260 ($C_{15}H_{17}O_4^+$, calcd. 261.11214). – APCI-MS: *m/z* = 261 $[MH]^+$. – ^{1}H NMR: δ_H = 7.94 (d, 1H, *J* = 9.5 Hz, H-4), 7.23 (s, H-8), 7.07 (s, H-5), 6.28 (d, 1H, *J* = 9.5 Hz, H-3), 5.45 (t, 1H, *J* = 7 Hz, prenyl H-2), 4.62 (d, 2H, *J* = 7 Hz, prenyl H-1), 3.79 (s, 3H, 6-OMe), 1.76 and 1.72 (each 3 H, 2× prenyl CH_3).

*Compound **10*** was identified as the caffeic acid ester of dihydrocoumaryl alcohol: APCI-MS: *m/z* = 315 $[MH]^+$. – APCI-MS/MS: *m/z* = 163 [caffeic

acid+H–H_2O]$^+$, 135 [163-CO]$^+$, 107 [$HOPhCH_2$]$^+$. – ^{1}H NMR δ_H = 7.05 (d, 1H, J = 2 Hz, H-2), 6.76 (d, 1H, J = 8 Hz, H-5), 7.01 – 6.99 (m, H-6), 7.46 (d, 1H, J = 16 Hz, olefin H-7), 6.27 (d, 1H, J = 16 Hz, olefin H-8), 7.00 (d, 2H, J = 8 Hz, H-2′/6′), 6.67 (d, 2H, J = 8 Hz, H-3′/5′), 2.56 (t, 2H, J = 8 Hz, H-7′), 1.87 (m, 2H, H-8′), 4.08 (t, 2H, J = 7 Hz, H-9′). – ^{13}C NMR: δ_C = 125.5 (C-1), 114.8 (C-2), 145.5 (C-3), 148.4 (C-4), 115.7 (C-5), 121.4 (C-6), 145.0 (C-7), 114.0 (C-8), 166.6 (C-9), 131.1 (C-1′), 129.1 (C-2′/6′), 115.1 (C-3′/5′), 155.4 (C-4′), 30.6 (C-7′), 30.2 (C-8′), 63.1 (C-9′).

Multivariate analysis

The phenetic relationships among species were determined based on flavonoid patterns using clustering and ordination. For each species the presence or absence of each compound was recorded. Trace amounts of flavonoids were treated in the analysis as present. The classification approach used was Ward's method in SAS PROC CLUSTER, with the sorting strategy based on minimizing the semi-partial *R*-squares. A sensitivity analysis was performed to determine if the cluster membership was affected by ties. It was found that species s8 and s14 were as likely to group together as species s8 and s17 (Fig. 2).

A principal coordinate analysis (PCoA) was used to produce an ordination that allows the sets of results to be viewed as points on a set of coordinate axes (Fig. 3). The distance apart in the ordination reflects the relative differences in chemistry of pairs of species. Similarities in flavonoid chemistry among the species were computed using the Jaccard coefficient, as this coefficient does not treat the shared absence of a character as evidence of similarity. Legendre and Legendre (1998) provided a detailed description of the PCoA procedure implemented for these data.

Results and Discussion

Identification of flavonoids

Flavonoids were identified by direct comparison with authentic standards using TLC on silica gel and polyamide DC-11. In a few cases, the TLC data were not conclusive and identity was established by mass spectrometry and NMR spectroscopy. The distinction between 6- and 8-substituted flavonoids was achieved by 2-dimensional

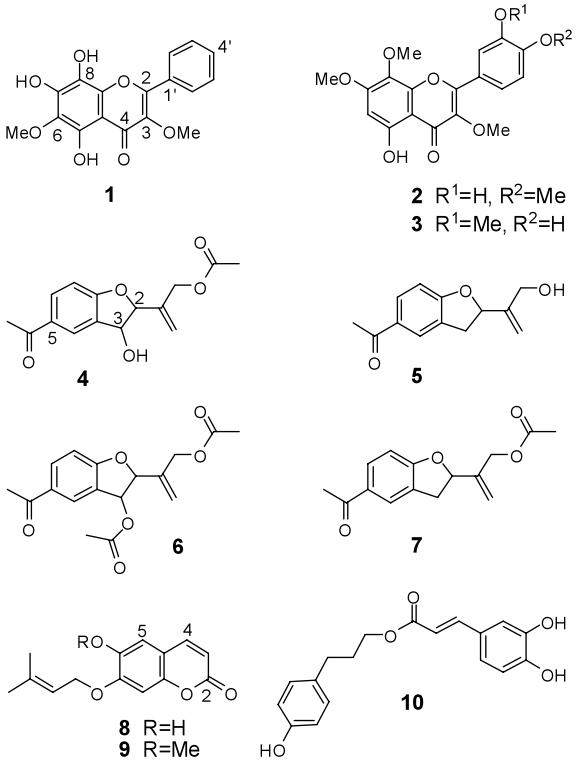

Fig. 1. Chemical structures of flavonoids and other phenolics found in *Cassinia* and *Ozothamnus* species.

NMR using ^{1}H-^{13}C HMBC and HMQC. Fig. 1 shows the chemical structures of compounds discussed here.

The structure of 5,7,8-trihydroxy-3,6-dimethoxyflavone (**1**), isolated from the exudate of *Cassinia arcuata*, was established after examination of its ^{1}H and ^{13}C NMR spectra and by comparison with spectra of flavonol 3-methyl ethers with unsubstituted B-rings (*i.e.* galangin and A-ring-modified galangins). It was clear that the B-ring was unsubstituted like that of galangin. The ^{13}C NMR spectrum of a compound (5,7,8,3′,4′-pentahydroxy-3,6-dimethoxy flavone) isolated from *Gutierrezia microcephala* (Roitman and James, 1985) showed identical A-ring resonances to those of the product in question, and thus its structure was determined to be 5,7,8-trihydroxy-3,6-dimethoxyflavone, previously isolated from *Achyrocline hyperchlora* (Liendro *et al.*, 2007). The corresponding 3,6,8-trimethyl ether (araneol), which we also identified in *C. arcuata* exudate, was known from several Asteraceae before (cf. Valant-Vetschera and Wollenweber, 2006).

Gossypetin-3,7,8,4′-tetramethyl ether (**2**) from *O. ferrugineus* showed a molecular ion $[MH]^+$ with *m/z* 375 in its APCI mass spectrum, consistent with $[C_{19}H_{19}O_8]^+$. MS/MS fragmentation (30 eV) of the $[MH]^+$ ion with *m/z* 375 produced fragment ions with *m/z* = 360 $[MH\text{-}CH_3]^+$, 345 $[MH\text{-}2CH_3]^+$ (100 %), 330 $[MH\text{-}3CH_3]^+$ and 317 $[345\text{-}CO]^+$. In the ^1H NMR spectrum, the low-field resonance at δ_H 12.47 indicated the presence of a free hydroxy group at C-5. This OH proton interacted with C-6 (δ_C 95.7), C-10 (δ_C 104.5) and C-5 (δ_C 156.4) in the ^1H-^{13}C HMBC spectrum. The proton resonance at δ_H 6.59 (H-6) showed cross-peaks with carbon resonances at δ_C 104.5 (C-10), 128.4 (C-8), 156.4 (C-5) and 158.2 (C-7). Carbon atoms 7 and 8 showed correlations with the OMe signals at δ_H 3.92 and 3.82, and therefore it was concluded that the A-ring substitution was 5-OH, 6-H, 7-OMe and 8-OMe. The B-ring proton at δ_H 7.15 (H-5′) showed intense $^3J_{H-C}$ correlations with δ_C 122.3 (C-1′) and 146.5 (C-3′ which did not interact with any of the OMe protons) and a weak $^2J_{H-C}$ correlation with δ_C 150.4 (C-4′). Because C-4′ also correlated with the O-methyl protons resonating at δ_H 3.87 and with the OH-3′ proton (δ_H 9.53), it was concluded that C-4′ carried a methoxy group. The B-ring substitution was determined to be 2′-H, 5′-H, 6′-H, 3′-OH, 4′-OMe. The last OMe group with δ_H 3.81 only interacted with the carbon resonance at δ_C 138.0, which were therefore identified as the OMe-3 and C-3 signals. The flavonoid was thus identified as gossypetin-3,7,8,4′-tetramethyl ether, a flavonol which to our knowledge has been found only twice before, from *Ricinocarpus* leaf (Henrick and Jefferies, 1965) and from aerial parts of *Solanum plaudosum* (Sarmento Silva *et al.*, 2002). NMR data were not reported in these papers. The flavonol was also reported as a constituent of *Helichrysum foetidum* exudate (Wollenweber *et al.*, 1989), but needs to be revised to gossypetin-3,7,8,3′-methyl ether in this case.

Gossypetin-3,7,8,3′-tetramethyl ether (ternatin, **3**), isolated from *O. rosmarinifolius*, showed APCI-MS and MS/MS spectra identical to gossypetin-3,7,8,4′-tetramethyl ether. The ^1H signals arising from the A-ring were also identical for both compounds. The gossypetin tetramethyl ether from *O. rosmarinifolius* revealed an ABX spin system with slightly different δ_H values. It was therefore concluded that this flavonoid had the alternative B-ring substitution pattern with the methoxy group at C-3′. Its identity was confirmed by co-TLC with an authentic sample of gossypetin-3,7,8,3′-tetramethyl ether. This gossypetin derivative has been found several times before in members of several plant families.

Other phenolics

In addition to the highly methylated flavonols, four dihydrobenzofuran derivatives were isolated from the leaf exudate of *O. rosmarinifolius*. The elemental composition of the first dihydrobenzofuran derivative **4** was determined to be $C_{15}H_{16}O_5$ by high-resolution FAB-MS. APCI-MS/MS fragmentation of the $[MH]^+$ ion with *m/z* 277 gave rise to a base peak at *m/z* 217, which represents $[MH\text{-}(O{=}C{=}CH_2)\text{-}(H_2O)]^+$. Its ^1H NMR spectrum revealed the presence of an acetyl group (δ_H 2.53), an acetoxy group (δ_H 2.07), three aromatic protons belonging to an ABX spin system, an allylic moiety, and two protons attributable to a 2,3-substituted dihydrobenzofuran moiety. In the HMBC spectrum, the OAc methyl protons interacted with the acetyl CO (δ_C 170.1) and methylene carbon atoms (δ_C 63.7). The methylene carbon atoms showed cross-peaks with the olefin protons and H-2, which was taken as evidence for the presence of an acetylated allyl alcohol moiety positioned at C-2 of the 2,3-dihydrobenzofuran nucleus. The other acetyl group was determined to be positioned at C-5 because its acetyl protons (δ_H 2.53) interacted with the aromatic carbon atoms at δ_C 130.7 (C-4) and δ_C 126.8 (C-6) in the HMBC spectrum. In order to account for the number of oxygen atoms and the 2,3-dihydrobenzofuran doublets that integrated for one proton each, an OH-group had to be present at C-3. Further analysis of the HMBC and HMQC spectra allowed assignment of all proton and carbon resonances to the dihydrobenzofuran derivative **4**, 2-(5-acetyl-3-hydroxy-2,3-dihydro-benzofuran-2-yl)-allyl acetate. This compound was previously isolated from *Helichrysum italicum* ssp. *microphyllum* (Appendino *et al.*, 2007).

Dihydrobenzofuran **5** differed from derivative **4** by the absence of an acetic acid ester and a hydroxy group at C-3, consistent with its molecular formula $C_{13}H_{14}O_3$. The two protons at C-3 each resonated as a double doublet due to coupling with its geminal proton and with H-2. Detailed analysis of the HMBC and HMQC spectra allowed identification of this compound as 2-(5-acetyl-2,3-dihydrobenzofuran-2-yl)allyl alcohol, previously isolated from *Ophryosporus charua*

(Asteraceae) (De Lampasona *et al.*, 1997) and from *Helichrysum italicum* ssp. *microphyllum* (Appendino *et al.*, 2007).

The dihydrobenzofuran derivatives **6** and **7** were identified by MS and NMR as the acetic acid esters of dihydrobenzofurans **4** and **5**, respectively. Thus compound **6** is 2-(3-acetoxy-5-acetyl-2,3-dihydrobenzofuran-2-yl)allyl acetate and compound **7** is 2-(5-acetyl-2,3-dihydrobenzofuran-2-yl)allyl acetate. Both natural products have been reported in the literature (Pritschow *et al.*, 1991; De Lampasona *et al.*, 1997).

Two prenyl coumarins isolated from *O. rosmarinifolius* were identified as 7-*O*-prenylaesculetin (**8**) and 7-*O*-prenylscopoletin (**9**). Finally, the caffeic acid ester of dihydrocoumaryl alcohol, compound **10**, was isolated from *O. thyrsoideus*. This natural product has not been reported previously.

Flavonoid distribution

All flavonoids detected in the lipophilic exudates of the *Cassinia* and *Ozothamnus* species studied are listed in Table I. Some of them, in particular the 8-*O*-substituted products, are not very common in nature. For previous reports on their occurrence see Valant-Vetschera and Wollenweber (2006).

The flavonol mikanin (6-hydroxykaempferol-6,7,4′-trimethyl ether) had been encountered as an aglycone in the course of work on terpenoids of three *Cassinia* species, the flavone pectolinarigenin (scutellarein-6,4′-dimethyl ether) in *C. uncata*, the dihydroflavonol pinobanksin and its 3-*O*-acetate in *C. arcuata* (for references see Reid and Bohm, 1994). Their localization in or on the plants had not been considered. Pinobanksin and its 3-*O*-acetate were also found in *C. quinquefaria*, together with pinocembrin, galangin, 6-hydroxygalangin and 6-methoxygalangin (Wollenweber *et al.*, 1993). The authors stressed the flavonoids' localization in the lipophilic exudate of aerial parts. Reid and Bohm (1994) reported on leaf exudate flavonoids of four additional species of *Cassinia*. Some of these flavonoids were also found in *O. leptophyllus* (Wood *et al.*, 1999). Wollenweber *et al.* (1997) reported on exudate flavonoids of two *Odixia* and ten *Ozothamnus* species. They found a total of 52 more or less lipophilic flavonoids, including several rare methyl ethers of flavons and flavonols. *O. lycopodioides* leaf resin yielded four highly methoxylated flavones, two of them being methylendioxyflavones (Rumbero *et al.*, 2000). A methylendioxychalcone had been reported earlier from *O. aggregatus* (cited as *Helichrysum glomeratum*, see Bohm and Stuessy, 2001).

The present study reports on exudate flavonoids of four species of *Cassinia* and tewelve species of *Ozothamnus*. As in the preceeding paper on *Odixia* and *Ozothamnus* exudate flavonoids (Wollenweber *et al.*, 1997), more than 50 flavonoid aglycones were identified. Again they include several rare flavonols, all bearing 8-*O*-substitution. It may be worth mentioning that 6-*O*-substitution alone is almost missing (only one methyl ether each of 6-hydroxygalangin and of 6-hydroxykaempferol, no quercetagetin derivative), while it co-occurs with 8-*O*-substitution in two flavonols and three flavones. 8-*O*-Substitution is clearly dominating, as was seen already in the preceeding survey (Wollenweber *et al.*, 1997).

Among the species now studied, *O. rosmarinifolius* deserves special mention insofar as it exhibits a quite unusual pattern of lipophilic exudate components. While most of the material consists of triterpenes, such as betulin, betulinic acid, ursolic acid and morolic acid, flavonoids occur only in trace amounts. Prominent TLC spots with blue or whitish-blue fluorescence are due to the coumarins aesculetin, 7-*O*-prenyl aesculetin, scopoletin, and 7-*O*-prenyl-scopoletin. These coumarins were identified by their MS and NMR data and confirmed by direct comparison with authentic samples. In addition, the exudate contains small amounts of several dihydrobenzofurans, four of which have been identified. A closely related prenylated dihydrobenzofuran, supposed to be derived from 3,5-diprenyl *p*-coumaric acid, has earlier been isolated from *Odixia angusta* and was called odixia acid (Zdero *et al.*, 1991).

Taxonomic significance of flavonoid variation

The presence/absence of flavonoids in each of the species (Table I) was analyzed by cluster analysis (Fig. 2) and principal coordinate analysis (Fig. 3) to determine relative closeness between the species.

In the cluster analysis (Fig. 2), the initial split is into two major clusters of sizes 7 and 9 species with two of the *Cassinia* species (s2 and s3) in one major cluster and the other two *Cassinia* species (s4 and s5) in the other. The two *Cassinia* species in the first major cluster form initial pairs with

Table I. Flavonoid distribution in *Ozothamnus* and *Cassinia*.

	Species*															
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Galangin	+	+			+	+		+		+		+			+	
Gal-3-Me	+	+			+	+		+		+		+			+	
Gal-7-Me					+	+										
Gal-3,7-diMe					+	+										
6-OH-Gal-3,6-diMe	+															
8-OH-Gal-3-Me	+							+		+						
8-OH-Gal-7-Me												+				
8-OH-Gal-8-Me												+				
8-OH-Gal-3,8-diMe										+						
Kaempferol		+		+			+				+	+	+		+	+
Kae-3-Me		+			+	+	+				+	+				
Kae-7-Me																+
Kae-3,7-diMe					+		+				+					+
Kae-3,4′-diMe							+									
Kae-7,4′-diMe					+											
Kae-3,7,4′-triMe					+						+					
6-OH-Kae-6-Me				+												
Herbacetin-3-Me								+				+				
Herb-3,8-diMe							+					+				
Herb-7,8-diMe				+												
Herb-3,7,8-triMe							+						+			
Herb-7,8,4′-triMe				+												
Herb-3,7,8,4′-tetraMe											+		+			
5,7,8-triOH-3,6-diOMe	+															
5,7-diOH-3,6,8-triOMe	+															
Quercetin		+				+	+				+	+	+		+	+
Qu-3-Me					+		+					+	+		+	+
Qu-7-Me						+										+
Qu-3′-Me						+			+							+
Qu-3,7-diMe					+		+				+					+
Qu-3,3′-diMe															+	
Qu-7,3′-diMe							+									
Qu-3,7,3′-triMe					+											+
Gossypetin-8-Me													+			
Goss-3,8-diMe													+			
Goss-3,7,8-triMe							+						+	+		
Goss-3,7,8,3′-tetraMe													+	+		
Goss-3,7,8,4′-tetraMe											+					
Goss-3,7,8,3′,4′-pentaMe											+		+			
Apigenin		+			+			+			+			+		
Ap-7-Me					+			+			+					
Ap-7,4′-diMe					+						+					
Scutellarein-6-Me			+													
Isoscutellarein								+								
Isoscut-4′-Me								+								
Isoscut-7,8,4′-triMe											+					
Luteolin									+		+			+		
Lut-7-Me								+			+					
Lut-3′-Me											+					
5,7,4′-triOH-6,8-diOMe-flavon		+														
5,4′-diOH-6,7,8-triOMe-flavon		+														
5,7-diOH-6,8,4′-triOMe-flavon		+														
Pinocembrin						+				+		+			+	
Pinoc-7-Me						+										
Pinobanksin		+								+		+			+	
Other phenolics														+		+

* 2, *Cassinia arcuata*; 3, *C. collina*; 4, *C. longifolia*; 5, *C. subtropica*; 6, *Ozothamnus antennaria*; 7, *O. argophyllus*; 8, *O. costatifolius*; 9, *O. cuneifolius*; 10, *O. diosmifolius*; 11, *O. ericifolius*; 12, *O. ferrugineus*; 13, *O. purpurescens*; 14, *O. rodwayi*; 15, *O. rosmarinifolius*; 16, *O. stirlingii*; 17, *O. thyrsoideus.*

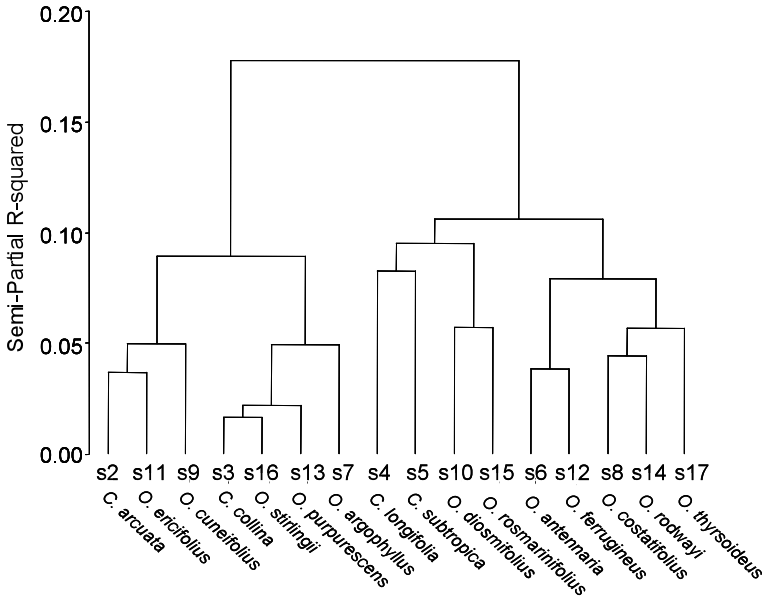

Fig. 2. Cluster analysis based on Jaccard coefficient distance calculated using the differences in flavonoid chemistry.

members of the *Ozothamnus* genus (s11 and s16, respectively) and these initial pairs are low in the dendogram (semi-partial R-squared < 0.04) indicating relative closeness to these members of the *Ozothamnus* genus. Although species s4 and s5 form their first cluster pair with each other, it is higher in the dendogram (semi-partial R-squared > 0.08) as the last pair of species to form a cluster with another species indicating a relatively larger distance from all of the other species.

In the ordination, the four *Cassinia* species are well-separated with each located in a different quadrant of the space formed by the first two axes from the principal coordinate analysis (Fig. 3). In this space each of the four *Cassinia* species are closer to a member of *Ozothamnus* (sometimes much closer) than they are to any member of the *Cassinia* genus. For example, *C. collina* (s3) is closest to *O. stirlingii* (s16) and *O. purpurescens* (s13) in both the cluster analysis and the ordination. Of the flavonoids present in either *C. collina* (s3) or *O. purpurescens* (s13), half of them (5/10) were present in both species. Of the flavonoids present in either *C. collina* (s3) or *O. stirlingii*, nearly half of them (6/13) were present in both species. In contrast, *C. collina* (s3) shares less than 20% of the detected flavonoids with any of the other members of *Cassinia* (2/11, 0/11 and 1/10 with s2, s4 and s5, respectively).

Ward and Breitwieser (1998) concluded, based on published data and their own morphological studies, that the two genera, *Cassinia* and *Ozothamnus*, should be lumped together to form a single genus. Both the cluster analysis and the ordination of the flavonoid presence-absence data provide support for the single genus viewpoint.

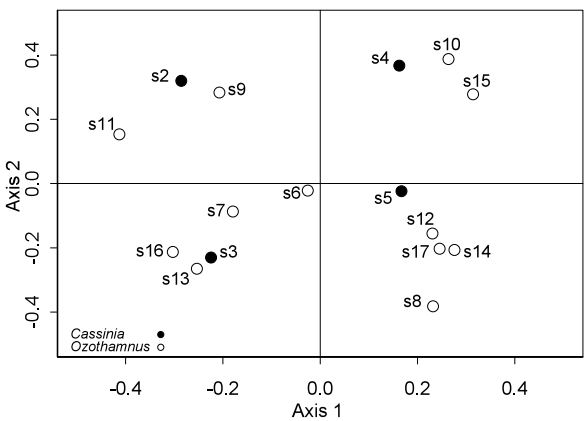

Fig. 3. Principal coordinate analysis results summarizing the differences in flavonoid chemistry among species in two dimensions (first two axes). *Cassinia* species are represented by closed circles and *Ozothamnus* species are represented by open circles.

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