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Phenolic Compounds from *Cyclopia intermedia* (Honeybush Tea). 1

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The processed leaves and stems of *Cyclopia intermedia* contain 4-hydroxycinnamic acid, the isoflavones formononetin, afrormosin, calycosin, pseudobaptigen, and fujikinetin, the flavanones naringenin, eriodictyol, hesperitin, and hesperidin, the coumestans medicagol, flemichapparin, and sophoracoumestan B, the xanthones mangiferin and isomangiferin, the flavone luteolin, and the inositol (+)-pinitol.

INTRODUCTION

Cyclopia intermedia E. Mey (Fabaceae) is one of the ∼24 *Cyclopia* species of woody legumes endemic to the Cape fynbos (Cape macchia) region of South Africa. The leaves and stems of several species are used to brew a traditional herbal tea with a pleasant taste and a characteristic honey flavor (Watt and Breyer-Brandwijk, 1962). This commodity, known as honeybush tea, is manufactured from mainly two species, *C. intermedia* and *C. subternata* Vogel, both of which are being developed as commercial crop plants (J. H. De Lange, National Botanical Institute, Claremont, South Africa, personal communication, 1996). The only known reports of chemical analyses of the aerial parts of *Cyclopia* species date back to the late 19th century (Watt and Breyer-Brandwijk, 1962); the presence of unidentified compounds was indicated, and an investigation of the major phenolic compounds in the leaves of a variety of species revealed the occurrence of the xanthone *C*-glycoside, mangiferin, and *O*-glycosides of the two flavanones, hesperitin and isosakuranetin (De Nysschen et al., 1996). To support the establishment of the honeybush tea industry as a viable agricultural enterprise, we reinvestigated the phenolic constituents of *C. intermedia*, which is currently the main natural resource for a limited export program (∼25 tons/annum) of this potential health beverage.

EXPERIMENTAL PROCEDURES

^{1}H and ^{13}C NMR were recorded in CDCl$_3$ at 300 and 75 MHz, respectively, with TMS as internal standard. Thin-layer chromatography (TLC) was performed on precoated Merck plastic sheets (silica gel 60 F$_{254}$, 0.25 mm), and the plates were sprayed with sulfuric acid–formaldehyde (40:1) after development. Glass preparative TLC plates (PLC) coated with Merck Kieselgel PF$_{254}$ (1.0 mm) were air-dried and used without prior activation while small scale preparative separations were carried out on Merck precoated glass plates (silica gel PF$_{254}$, 0.25 mm). Compounds were recovered from the adsorbent by elution with acetone. Two-dimensional paper chromatograms were on Whatman No. 1 paper in water-saturated *sec*-butanol and 2% acetic acid/water, respectively, and column chromatography (CC) was on Sephadex LH-20. Acetylations were performed with acetic anhydride/pyridine at ∼25 °C for 12 h, and evaporations were done either under reduced pressure at 50 °C in a rotary evaporator or by freeze-drying of aqueous solutions on a Virtis 12 SL freezemobile.

Extraction and Fractionation. Dried, fermented shoots (2.87 kg) of *C. intermedia,* supplied by Mr. Scheltema Nortje from the farm Nooitgedacht in the Kouga Mountains of the Eastern Cape, were pulverized, and the chlorophyll was extracted consecutively with chloroform (2 × 2.5 L, 24 h each) and acetone (2 × 2.5 L, 24 h each) at ∼25 °C to yield dark green solids (95.0 and 49.8 g, respectively) on evaporation of the solvents. Subsequent extraction with methanol (4 × 2.5 L, 24 h each, ∼25 °C) and 70% acetone/water (4 × 2.5 L, 24 h each, ∼25 °C) gave brown solids following evaporation of the solvents. These were redissolved in water and freeze-dried (190.0 and 134.5 g, respectively). The acetone/water extract is currently under further investigation.

Metabolites from the Methanol Extract. The methanol extract (90.0 g) was subjected to a Craig separation (20 tubes) with water/*n*-butanol/hexane (5:4:1) as mobile phase (200 mL of organic and 200 mL of aqueous phase per tube). Four fractions, A (tubes 1 and 2, 25.5 g), B (tubes 3 and 4, 11.1 g), C (tubes 5−12, 15.4 g), and D (tubes 13−20, 20.8 g), were selected. A white insoluble solid (5.1 g) that precipitated during the separation was filtered off, washed with minimum acetone, and dried. Acetylation of a portion (10 mg) gave octa-*O*-acetylhesperidin (**24**; 9 mg) (Barthe et al., 1988).

Fraction D (20.8 g) was further fractionated by CC on Sephadex LH-20 (5 × 160 cm column, 32.0 mL fractions) with ethanol as eluant to give 15 fractions: D$_1$ (tubes 116−124, 2.05 g), D$_2$ (tubes 125−131, 1.60 g), D$_3$ (tubes 132−144, 1.74 g), D$_4$ (tubes 145−159, 1.72 g), D$_5$ (tubes 160−176, 0.70 g), D$_6$ (tubes 177−186, 0.26 g), D$_7$ (tubes 187−200, 0.26 g), D$_8$ (tubes 201−216, 0.26 g), D$_9$ (tubes 217−234, 1.06 g), D$_{10}$ (tubes 235−269, 1.38 g), D$_{11}$ (tubes 270−318, 0.38 g), D$_{12}$ (tubes 319−350, 0.37 g), D$_{13}$ (tubes 351−396, 0.46 g), D$_{14}$ (tubes 397−446, 0.67 g), and D$_{15}$ (tubes 447−550, 1.55 g). Fractions D$_1$−D$_8$ and D$_{15}$ were respectively composed mainly of chlorophyll and polymeric material and were not further investigated.

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Fraction D$_9$ (100 mg) was acetylated and separated by PLC in hexane/benzene/acetone/methanol (40:40:15:5, ×2). A second purification of the R$_f$ 0.15 band (3.9 mg) by PLC in the same solvent gave 4-*O*-acetylcoumaric acid (**2**; R$_f$ 0.17, 3.1 mg).

Acetylation of fraction D$_{10}$ (100 mg) followed by PLC in chloroform/benzene/acetone (50:45:5, ×2) afforded four bands, D$_{10.1}$ (R$_f$ 0.75, 6.7 mg), D$_{10.2}$ (R$_f$ 0.73, 5.8 mg), D$_{10.3}$ [identified as 7-*O*-acetylfujikinetin (**16**; R$_f$ 0.71, 5.5 mg) (Rao and Srimannarayana, 1984)], and D$_{10.4}$ (R$_f$ 0.50, 4.3 mg). Band D$_{10.1}$ (6.7 mg) was resolved into two compounds (R$_f$ 0.76, 2.6 mg; and R$_f$ 0.74, 3.6 mg) by PLC in chloroform/benzene/acetone (50:45:5, ×2), which, after respective purification by PLC in the same solvent, afforded 7-*O*-acetylformononetin (**8**; R$_f$ 0.76, 2.3 mg) (Bezuidenhoudt et al., 1987) and 7-*O*-acetylafrormosin (**10**; R$_f$ 0.74, 3.4 mg) (Markham and Geiger, 1980). PLC of band D$_{10.2}$ (5.8 mg) in chloroform/benzene/acetone (50:45:5, ×2) yielded 7-*O*-acetylpseudobaptigen (**14**; R$_f$ 0.72, 5.8 mg) (Adinarayana et al., 1982), whereas PLC purification of band D$_{10.4}$ (4.3 mg) in chloroform/benzene/acetone (50:45:5) gave 3′,7-di-*O*-acetylcalycosin (**12**; R$_f$ 0.27, 3.7 mg) (Arisawa et al., 1980).

Fraction D$_{11}$ (100 mg) was acetylated and separated by PLC in chloroform/benzene/acetone (50:45:5, ×2) into 3-*O*-acetyl-sophoracoumestan B (**29**; R$_f$ 0.80, 4.8 mg) (Komatsu et al., 1981) and a second band (R$_f$ 0.84, 3.6 mg), which yielded flemichapparin (**27**; R$_f$ 0.84, 3.1 mg) (Burns et al., 1984) following purification by PLC in chloroform/benzene/acetone (50:45:5, ×2).

The acetylated fraction D$_{12}$ (100 mg) was resolved into two bands, R$_f$ 0.61 (3.5 mg) and 0.57 (3.2 mg) by PLC in hexane/benzene/acetone/methanol (40:40:15:5, ×2). The former band yielded 4′,5,7-tri-*O*-acetylnaringenin (**18**; R$_f$ 0.61, 3.0 mg) (Bohlman et al., 1981) after purification by PLC in hexane/benzene/acetone/methanol (40:40:15:5, ×2), and the latter band gave 3′,5,7-tri-*O*-acetylhesperetin (**22**; R$_f$ 0.57, 2.9 mg) (Arakawa and Nakazaki, 1960) following purification according to the same method.

Acetylation of fraction D$_{13}$ (100 mg) followed by PLC in hexane/benzene/acetone/methanol (40:40:15:5) afforded 3-*O*-acetylmedicagol (**26**; R$_f$ 0.60, 3.6 mg) (Shirataki et al., 1981, 1982) and an impure band (R$_f$ 0.31, 14.5 mg). This band was subjected to PLC in hexane/benzene/acetone/methanol (40:40:15:5) to give 3′,4′,5,7-tetra-*O*-acetylluteolin (**6**; R$_f$ 0.31, 13.8 mg) (Snyckers and Salemi, 1974).

Fraction D$_{14}$ (100 mg) was acetylated and separated by PLC in benzene/acetone/methanol (90:8:2) to afford a single compound (R$_f$ 0.61, 10.8 mg), which was further purified by PLC in the same solvent to yield 3′,4′,5,7-tetra-*O*-acetyleriodictyol (**20**; R$_f$ 0.61, 9.8 mg) (Harborne and Williams, 1983).
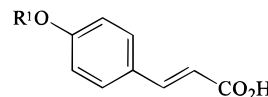
A portion of the methanol extract (10 g) was dissolved in water (300 mL) and extracted with ether (5 × 300 mL). The aqueous layer was freeze-dried to give a crude solid (8.0 g), which was subjected to CC on Sephadex LH-20 (2.5 × 80 cm column, 32.0 mL fractions) in 70% ethanol/water. Two fractions, E$_1$ (tubes 1−15, 5.40 g) and E$_2$ (tubes 16−55, 0.86 g) were selected. Fraction E$_1$ (10 mg) was acetylated and identified as penta-*O*-acetyl-(+)-pinitol (**4**; 9.5 mg).

Acetylation of fraction E$_2$ (10 mg) and PLC in benzene/acetone/methanol (90:8:2, ×2) afforded octa-*O*-acetylisomangiferin (**33**; R$_f$ 0.59, 4.2 mg) and octa-*O*-acetylmangiferin (**31**; R$_f$ 0.20, 3.8 mg) (Aritami et al., 1969).
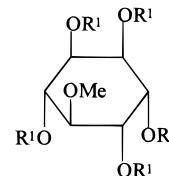
RESULTS AND DISCUSSION

The methanol extract of the fermented leaves and stems of *C. intermedia*, that is, the commercial product, afforded a complex mixture of phenolic compounds which was resolvable only after extensive enrichment and fractionation procedures. Owing to the complexity of the mixture, the fractions had to be derivatized to attain an acceptable level of purity. This invariably led to substantial losses (cf. Experimental Procedures), hence prohibiting reliable quantification of the constituents at this stage. The mixture comprised the hydroxycinnamic acid, 4-coumaric acid (**1**), the inositol, (+)-
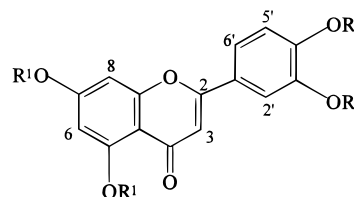
pinitol (**3**), and a variety of C$_6$·C$_3$·C$_6$-type polyphenols as well as two xanthone *C*-glucosides. 4-Coumaric acid (**1**) and (+)-pinitol (**3**) were identified by comparison of the ¹H NMR data of their *O*-acetyl derivatives **2** and **4**

R¹O—[structure] —CH=CH—CO₂H

1, R¹=H
2, R¹=Ac

[structure with OMe]

3, R¹=H
4, R¹=Ac

[flavone structure, positions 2, 3, 5′, 6′, 2′, 8, 6 labeled]

5, R¹=H
6, R¹=Ac

with those of the same derivatives of commercially available reference compounds. Structure elucidation of the single flavone, luteolin **5**, with its known antispasmodic properties (Snyckers and Salemi, 1974) was similarly performed by comparison of the ¹H NMR data of its tetra-*O*-acetyl derivative **6** with those of authentic tetra-*O*-acetylluteolin (Snyckers and Salemi, 1974).

Isoflavones. A single fraction resulting from consecutive enrichment via a Craig countercurrent distribution and column chromatography on Sephadex LH-20 in ethanol afforded the five known isoflavones 7-hydroxy-4′-methoxy- (**7**, formononetin) (Bezuidenhoudt et al., 1987), 7-hydroxy-6,4′-dimethoxy- (**9**, afrormosin) (Markham and Ingham, 1980), 7,3′-dihydroxy-4′-methoxy- (**11**, calycosin) (Arisawa et al., 1980), 7-hydroxy-3′,4′-methylenedioxy- (**13**, pseudobaptigen) (Adinarayana et al., 1982), and 7-hydroxy-6-methoxy-3′,4′-methylenedioxy- (**15**, fujikinetin) (Rao et al., 1984) isoflavone. Identification was performed on the respective *O*-acetyl derivatives **8**, **10**, **12**, **14**, and **16**. Their

[isoflavone structures]
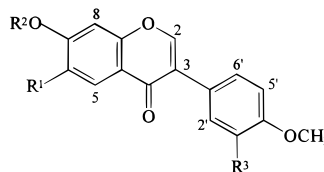
7, R¹=R²=R³=H
8, R¹=R³=H, R²=Ac
9, R¹=OMe, R²=R³=H
10, R¹=OMe, R²=Ac, R³=H
11, R¹=R²=H, R³=OH
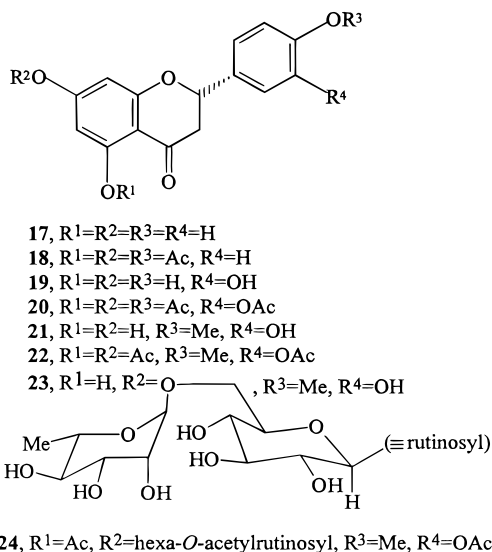**12**, R¹=H, R²=Ac, R³=OAc
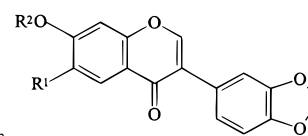
13, R¹=R²=H
14, R¹=H, R²=Ac
15, R¹=OMe, R²=H
16, R¹=OMe, R²=Ac

¹H NMR spectra in CDCl$_3$ invariably displayed the one-proton singlet at δ 7.98−8.02 reminiscent of the vinylic H-2 resonance of isoflavones (Markham and Geiger, 1993), whereas those of the pseudobaptigen and fujikinetin derivatives **14** and **16**, respectively, additionally displayed a two-proton singlet at δ 6.02, characteristic of the protons of the methylenedioxy functionality. The oxygenation patterns were simply established from the multiplicities and chemical shifts of the aromatic spin systems, the positions of the *O*-methyl and −O−CH$_2$−O

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substituents then being confirmed by the appropriate correlation ^{1}H NMR spectrometric experiments, for example, nuclear Overhauser effect spectrometry (NOE) or correlation spectrometry (COSY).

In addition to their significant natural functions in plants, for example, as phytoalexins in the defense against fungal infection (Laks et al., 1989), the isoflavones also have important physiological effects in humans, that is, anticancer (Adlercreutz et al., 1986), estrogenic (Smolenski et al., 1981), and antimicrobial (Perrin and Cruickshank, 1969) activities.

Flavanones. Four known flavanones, 5,7,4′-trihydroxy- (**17**, naringenin) (Bohlman et al., 1981), 5,7,3′,4′-tetrahydroxy- (**19**, eriodictyol) (Harborne and Williams, 1983), 5,7,3′-trihydroxy-4′-methoxy- (**21**, hesperitin) (Arakawa and Nakazaki, 1960), and 5,3′-dihydroxy-4′-methoxy-7-*O*-rutinosyl- (**23**, hesperidin) (Barthe et al., 1988) flavanone were obtained in a relatively pure state.

17, R[1]=R[2]=R[3]=R[4]=H
18, R[1]=R[2]=R[3]=Ac, R[4]=H
19, R[1]=R[2]=R[3]=H, R[4]=OH
20, R[1]=R[2]=R[3]=Ac, R[4]=OAc
21, R[1]=R[2]=H, R[3]=Me, R[4]=OH
22, R[1]=R[2]=Ac, R[3]=Me, R[4]=OAc
23, R[1]=H, R[2]=O—, R[3]=Me, R[4]=OH

24, R[1]=Ac, R[2]=hexa-*O*-acetylrutinosyl, R[3]=Me, R[4]=OAc

These compounds were identified by comparison of the ^{1}H NMR data of their *O*-acetyl derivatives **18**, **20**, **22**, and **24** with those of authentic samples from our collection of reference compounds. Their flavanone character was immediately apparent from the typical three-spin system of the protons of the heterocyclic ring, for example, H-2(C), δ 5.50 (dd, *J* = 2.5, 8.5 Hz), H-3β-(C), δ 2.81 (dd, *J* = 2.5, 12.0 Hz), and H-3α(C), δ 3.02 (dd, *J* = 8.5, 12.0 Hz) for tetra-*O*-acetyleriodictyol (**20**). The circular dichroism (CD) spectra of these derivatives exhibited the anticipated synchronous Cotton effects (negative for the π → π* transition at ∼290 nm and positive for the n → π* transition at ∼340 nm) that were compatible with flavanones possessing 2*S* absolute configuration (Gaffield, 1970).

Interesting physiological properties have been attributed to the flavanones, for example, the vitamin-like activity of citrin (a mixture of eriodictyol and hesperidin) (Hughes and Wilson 1977; Middleton and Kandaswami, 1993) and antimicrobial, antiviral, and anti-inflammatory (Middleton and Kandaswami, 1993), properties.

Coumestans. The three coumestans, representative of the fully oxidized state of the heterocyclic C-ring of isoflavonoids, comprised 3-hydroxy-8,9-methylenedioxy-(**25**, medicagol) (Shirataki et al., 1981, 1982), 3-methoxy-8,9-methylenedioxy- (**27**, flemichapparin) (Burns et al., 1984), and 3-hydroxy-4-methoxy-8,9-methylenedioxy-

Table 1. ^{13}C NMR of the Coumestan Derivatives 26, 27, and 29 in CDCl₃ at 23 °C

carbon	26	27	29
C-1	122.4	122.5	119.8
C-2	119.2	114.0	116.0
C-3	153.0	156.0	146.0
C-4	111.5	102.0	108.0
C-4a	153.9	155.5	145.9
C-6	158.0	163.0	158.0
C-6a	117.0	116.5	117.2
C-6b	107.0	105.0	106.0
C-7	100.0	100.5	100.7
C-8	146.5	147.0	146.8
C-9	148.5	147.5	148.5
C-10	94.5	96.5	94.5
C-10a	152.0	151.0	151.5
C-11a	158.5	161.0	159.5
C-11b	111.0	107.0	112.6
O*C*H₃		57.0	62.0
O*C*H₂O	102.5	102.5	102.5
*C*H₃CO	21.6		21.1
CH₃*C*O	169.2		170.0

(**28**, sophoracoumestan B) (Komatsu et al., 1981) coumestan. Flemichapparin C **27** and the *O*-acetyl derivatives **26** and **29** of medicagol **25** and sophoracoumestan B **28** all displayed the intense bright blue fluorescence on TLC under UV irradiation characteristic of coumestans (Ingham et al., 1988).

Despite the relatively simple spin systems displayed in the ^{1}H NMR spectra (CDCl₃) of compounds **26**, **27**, and **29**, the absence of protons associated with heterocyclic rings rendered structure elucidation more complicated than for the previous compounds. Crucial
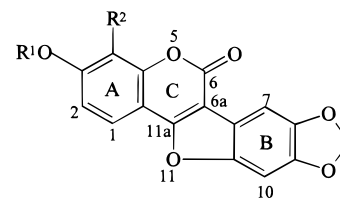

25, R[1]=R[2]=H
26, R[1]=Ac, R[2]=H
27, R[1]=Me, R[2]=H
28, R[1]=H, R[2]=OMe
29, R[1]=Ac, R[2]=OMe

aspects relevant to definition of structure are hence briefly discussed for the medicagol derivative **26**. The molecular formula, $C_{18}H_{10}O_7$, was confirmed by MS analysis (M⁺, *m*/*z* 326). The ^{1}H NMR spectrum (CDCl₃) displayed an aromatic ABX system (H-1, δ 7.98, d, *J* = 8.5 Hz; H-2, δ 7.20 dd, *J* = 2.5, 8.5 Hz; H-4, δ 7.30, d, *J* = 2.5 Hz), two aromatic singlets (H-7, δ 7.51; H-10, δ 7.17), the two-proton singlet (δ 6.12) reminiscent of the methylenedioxy functionality, and a single aromatic *O*-acetyl resonance (δ 2.39). A carbon resonance at δ 158.0 in the ^{13}C NMR spectrum of derivative **26** (Table 1) strongly suggested a lactone-type carbonyl group (Kalinowski et al., 1984) and thus the coumarin arrangement typical of coumestans. Such an assumption was supported by the conspicuous deshielded C-11a signal (158.5 ppm) resulting from the inherent electron deficiency at the β-carbon of α,β-unsaturated esters and which is enhanced by the 11a-O function in the coumestan framework. This resonance was then utilized as the reference signal to access the spin system and hence substitution pattern of the A-ring via the appropriate C−H correlation experiments, for example,

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HMBC and HMQC. These techniques similarly also facilitated definition of the 8,9-methylenedioxy-substituted B-ring. A comparable approach also led to full structural assignment for compounds **27** and **29** (cf. Table 1).

The coumestans share many of the physiological properties of the isoflavones (vide supra), with arguably the most important their phytoestrogenic characteristics (Bickhoff et al., 1969; Martin et al., 1978).

Xanthones. Two known xanthones, 2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy- (**30**, mangiferin) (Aritami et al., 1969) and 4-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy- (**32**, isomangiferin) (Aritami et al., 1969) 9*H*-
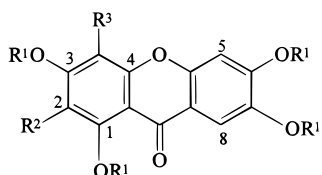
30 R^1=R^3=H, R^2=2-β-*D*-glucopyranosyl
31 R^1=Ac, R^3=H, R^2=tetra-*O*-acetyl-2-β-*D*-glucopyranosyl
32 R^1=R^2=H, R^3=2-β-*D*-glucopyranosyl
33 R^1=Ac, R^2=H, R^2=tetra-*O*-acetyl-2-β-*D*-glucopyranosyl

xanthen-9-one, were purified and identified as their per-*O*-acetyl derivatives **31** and **33**, respectively. At ambient temperature (23 °C) the spectra of both **31** and **33** displayed the typical adverse effects of dynamic rotational isomerism about the xanthenyl–glucosyl bond. The relatively low energy barrier to free rotation was overcome at 60 °C, at which sharp resonances were evident in both the ^{1}H and ^{13}C NMR spectra. Both compounds **31** and **33** displayed relatively simple splitting patterns, that is, three one-proton singlets [δ 6.88 (H-4), 7.50 (H-5), 8.05 (H-8); δ 7.28 (H-2), 7.40 (H-5), 8.03 (H-8) for **31** and **33**, respectively] in the aromatic region, four three-proton singlets for the aromatic *O*-acetyl resonances, and four three-proton singlets reminiscent of the acetoxy signals of a C–C linked β-D-glucopyranosyl moiety [δ 5.27, 4.90 (both d, both *J* = 6.0 Hz), anomeric protons for **31** and **33**, respectively]. The substitution patterns of the aromatic rings of each compound were unequivocally established by the extensive utilization of heteronuclear multiple quantum correlation (HMQC) for direct C–H couplings and heteronuclear multiple bond correlation (HMBC) for couplings over two to four bonds. When taken in conjunction with the characteristic xanthone carbonyl chemical shifts (Kalinowski et al., 1984) (δ 174.0 for both compounds **31** and **33**) (Table 2), these data confirmed the structures of the xanthone derivatives as the per-*O*-acetates of mangiferin **30** and isomangiferin **32**, respectively.

Xanthones exhibit a variety of pharmacological properties, for example, the antiviral, antifungal, antiinflammatory, and lysosomal membrane stabilization effects of mangiferin (Beecher et al., 1990).

The methanol extract of the commercial product from which honeybush tea is brewed thus contains a substantial number of phenolic compounds with pharmacological properties that may contribute toward its appeal as a health beverage. Our current efforts aimed at unraveling the phenolic profile in the metabolic pool of *C. intermedia* hence also focus on the phenomenon of the natural sweetness of honeybush tea, as well as

Table 2. ^{13}C NMR of the Aglycon Moieties of Xanthone Derivatives 31 and 33 in CDCl$_3$ at 23 °C

carbon	31	33
C-1	157.5	158.0
C-2	155.8	112.1
C-3	151.6	154.9
C-4	113.2	152.0
C-4a	152.8	150.0
C-4b	155.1	153.0
C-5	116.2	113.0
C-6	148.1	148.0
C-7	139.9	139.7
C-8	121.2	121.2
C-8a	114.5	118.5
C-9	174.0	174.0
C-9a	115.4	120.5

the potential of the various phenolic compounds to act as antioxidants and to exhibit alternative health-promoting properties in the human diet.

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