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Phenolic Metabolites from Honeybush Tea (*Cyclopia subternata*)

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Cyclopia subternata is one of the 24 *Cyclopia* species that are used to brew honeybush tea, a unique South African herbal beverage with a pleasant taste and flavor. It contains various antioxidants, very low tannin content, and no caffeine. Many health properties are associated with regular consumption of the tea. Honeybush infusions have been noted as a tonic for colds and influenza, catarrh, and pulmonic tuberculosis and is becoming well-known for its effectiveness in alleviating menopausal symptoms in women. "Unfermented" leaves of *C. subternata* contain pinitol, shikimic acid, *p*-coumaric acid, 4-glucosyltyrosol, epigallocatechin gallate, the isoflavone orobol, the flavanones hesperedin, narirutin, and eriocitrin, a glycosylated flavan, the flavones luteolin, 5-deoxyluteolin, and scolymoside, the xanthone mangiferin, and the flavonol C-6-glucosylkaempferol. The structures were elucidated by spectroscopic and spectrometric analysis.

KEYWORDS: ***Cyclopia subternata*; Fabaceae; honeybush tea; polyphenol; flavonoids; xanthone; glycosides; health beverage**

INTRODUCTION

The genus *Cyclopia* (Fabaceae) consists of about 24 species distributed only in the western and eastern cape coastal regions of South Africa (*1*). *Cyclopia intermedia* and *Cyclopia subternata* are the two species that are usually used to brew honeybush tea (*1*), after being subjected to high-temperature oxidation ("fermentation"), necessary for the formation of the brown color and honey fragrance. The tea is caffeine free and low in tannin content and is used by people of the eastern cape as a medicinal concoction (*2*). *C. intermedia* was previously investigated as part of our ongoing quest for new phenolic metabolites with potential health benefits and has yielded flavonols, flavonones, isoflavones, coumestans, flavones, xanthones, cinnamic acids, and pinitol as a cyclitol (*3*, *4*).

Attempts to grow the tea commercially have had variable success, but most of the tea is still harvested from the wilderness areas and totally unpolluted environment. Studies of the phytochemical composition of the *Cyclopia* species should contribute to the successful commercial establishment of the honeybush tea industry and improve the marketing potential of the tea. We currently report on the isolation and structural determination of compounds from *C. subternata*, which revealed the presence of various flavonoids and xanthones as well as nonphenolic metabolites. Because polyphenols are purported to have significant antioxidant properties (*3*), these results suggest that the tentative claimed health benefits may be concomitant with the presence of these and other polyphenols in the tea.

MATERIALS AND METHODS

Source of Plant Material. Leaves and shoots of *C. subternata* were harvested from two experimental plantations established in the Simondium and Du Toits Kloof areas of the western cape region of South Africa. Drying of the plant material took place in a forced-air-drying tunnel at 40 °C until a moisture content of ∼10% was reached, whereafter it was pulverized. A voucher specimen is being kept in the Chemistry Department of the University of the Free State.

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75 MHz, respectively, on a Bruker AVANCE DPX-300 spectrometer with TMS as internal standard. MS spectra and mass determinations were obtained with a Kratos MS-80 mass spectrometer in the double-focus EI mode.

Authentic Samples. Pinitol was obtained from Merck (Johannesburg, South Africa).

Chromatography and Derivatization. Qualitative thin-layer chromatography (TLC) was performed on 3 × 7 cm Kieselgel 60F_{254}, 0.25 mm, aluminum plates (Merck). Development of the TLC plates in the appropriate solvent was followed by spraying with formaldehyde (40%)/sulfuric acid (2:98) or with anisaldehyde/sulfuric acid/ethanol (5:5:90) and heated to 120 °C. The peracetate derivatives were purified by preparative thin-layer chromatography (PLC) carried out on 20 × 20 cm glass plates coated with 1.0 mm Kieselgel PF_{254} (Merck), which were air-dried and used without prior activation. An application of 10−25 mg per plate was used. Small-scale preparative separations were on 20 × 20 cm precoated silica gel PF_{254}, 0.25 mm, glass plates (Merck).

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Compounds were located by UV light (254 nm) and eluted from the adsorbent with acetone. Two-dimensional paper chromatography (PC) was done on 28.5 × 46 cm Whatman no. 1 paper in water-saturated *sec*-butanol and acetic acid/water (2:98), respectively, and the chromatograms were sprayed with bisdiazotized benzidine. Column chromatography (CC) was performed on Sephadex LH-20, and compounds were eluted at 0.5 mL/min, collecting fractions of 12 mL. Solvents were evaporated under reduced pressure at 40 °C in a rotary evaporator or by freeze-drying of aqueous solutions on a Virtis 12 SL freezemobile. Acetylations were performed with acetic anhydride/pyridine at 40 °C for 12 h.

Extraction and Fractionation. To remove chlorophyll, the pulverized plant material (6.0 kg) was extracted consecutively with chloroform (2 × 6.0 L, 24 h each) and acetone (2 × 5.0 L, 24 h each) at ~25 °C to yield dark green solids (155.5 and 86.8 g, respectively) on evaporation of solvents. Ensuing extractions with methanol (4 × 5.0 L, 24 h each, ~25 °C) and subsequently 70% acetone/water (5 × 5.0 L, 24 h each, ~25 °C) afforded brown solids following evaporation of the solvents. These were redissolved in water and freeze-dried to give 495.5 and 702.5 g of extract, respectively.

Metabolites from the Acetone and Methanol Extracts. Due to the complexity of the mixtures of the metabolites in the acetone and methanol extracts, the compounds were purified and identified as peracetate derivatives (**Chart 1**). This required several steps of separation, which did not allow accurate quantification of the compounds. Characterization of the glycosides by hydrolysis was not possible due to the very low masses of the compounds isolated. Identification of all compounds is based on NMR spectroscopic methods. The acetone (21.0 g) and methanol (20.0 g) extracts were respectively fractionated by Sephadex LH-20 column chromatography (5 × 60 cm, flow rate of 1 mL/min, 32.0 mL fractions) with ethanol as the eluant. Following TLC analysis in hexane/acetone/methanol (5:4:1), the collected fractions were combined into eight fractions for the acetone extract, A1−8 (A1, tubes 0−48, 3.23 g; A2, tubes 49−106, 4.05 g; A3, tubes 107−128, 4.12 g; A4, tubes 129−204, 14.64 g; A5, tubes 205−270, 2.56 g; A6, tubes 271−350, 4.98 g; A7, tubes 351−470, 4.83 g; A8, tubes, 471−520, 3.74 g), and into five fractions for the methanol extract, B1−5 (B1, tubes 0−30, 1.28 g; B2, tubes 31−86, 4.21 g; B3, tubes 87−130, 4.94 g; B4, tubes 131−230, 4.52 g; B5, tubes 231−290, 4.8 g). Fractions A1, A2, B1, and B5 did not contain compounds pertaining to this investigation and were not further investigated.

RESULTS AND DISCUSSION

Investigation of the derivatized fractions of the extracts of *C. subternata* revealed the presence of a varied range of phenolic and nonphenolic compounds as well as glycosides that were characterized as flavanones **1**, **3**, and **5**, flavones **7**, **9**, and **11**, a flavonol **13**, an isoflavone **15**, epigallocatechin gallate **17**, a flavan **19**, a xanthone **21**, 4-glucosyltyrosol **23**, *p*-coumaric acid **25**, (+)-pinitol **27**, and (±)-shikimic acid **29**.

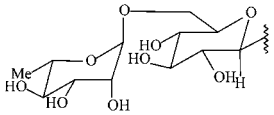
A precipitate (100 mg) from the initial acetone extract was acetylated and purified by PLC in toluene/acetone (8:2) to give three bands at R_f 0.43 (10.5 mg), 0.35 (17.0 mg), and 0.30 (13.0 mg). Further purification of these bands in the same solvent yielded the *O*-acetyl derivatives **2**, **28**, and **10** of the known hesperidin **1** (9.0 mg) (*3*), (+)-pinitol **27** (15.0 mg) (*3*, *5*), and scolymoside **9** (11.0 mg) (*6*), respectively.

Acetylation of A3 (50 mg) and PLC in toluene/acetone (8:2) afforded the peracetate derivative **22** of mangiferin **21** (R_f 0.45, 5.0 mg) (*3*).

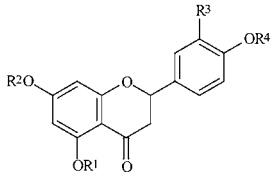
Fraction A4 (50 mg) was acetylated and separated in toluene/acetone (7:3) to give one band, which on subsequent separation in the same solvent yielded the per-*O*-acetyl derivative **24** of 4-glucosyltyrosol **23** (R_f 0.21, 3.3 mg) (*3*).

Acetylation of A5 (50 mg) and PLC separation in toluene/acetone (8:2) afforded the peracetate derivative **20** of 6-*O*-glucosylkaempferol **19** (R_f 0.42, 5.5 mg) (*3*).

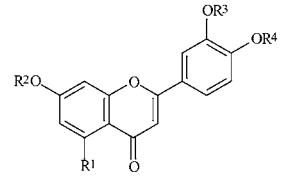
Chart 1. Structures of Compounds Isolated from *C. subternata* and Their Acetate Derviatives



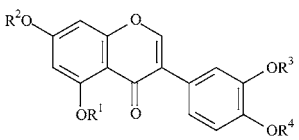
Rutinosyl moiety



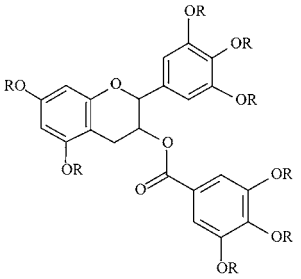
1, R^1=H; R^3=OH; R^4=Me; R^2= rutinosyl
2, R^1=Ac, R^3=OAc, R^4=Me,
R^2= hexa-*O*-acetylrutinosyl
3, R^1=R^3=R^4=H, R^2=rutinosyl
4, R^1=R^4=Ac, R^3=H,
R^2=hexa-*O*-acetylrutinosyl
5, R^1=R^4=H, R^3=OH, R^2= rutinosyl
6, R^1=R^4=Ac, R^3=OAc,
R^2= hexa-*O*-acetylrutinosyl



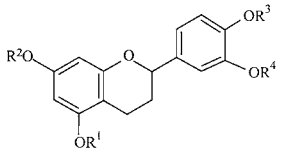
7, R^1=R^2=R^3=R^4=H
8, R^1=H, R^2=R^3=R^4=Ac
9, R^1=OH, R^2=R^3=R^4=H
10, R^1=OAc, R^2=R^3=R^4=Ac
11, R^1=OH, R^3=R^4=Ac;
R^2= rutinosyl
12, R^1=OAc, R^3=R^4=Ac
R^2= hexa-*O*-acetylrutinosyl



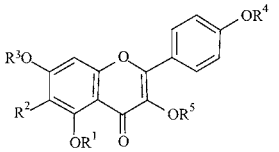
13, R^1=R^2=R^3=R^4=H
14, R^1=R^2=R^3=R^4=Ac



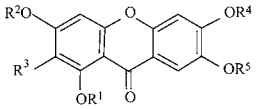
15, R=H
16, R=Ac



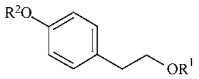
17, R^2=R^3=R^4=H,
R^1=2-β-*D*-glucopyranosyl
18, R^2=R^3=R^4=Oac
R^1=tetra -*O*-acetyl-2-β-*D*-glucopyranosyl



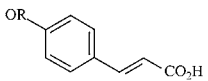
19, R^1=R^3=R^4=R^5=H,
R^2=2-β-*D*-glucopyranosyl
20, R^1=R^3=R^4=R^5=Ac, R^2=tetra-*O*-acetyl-2-β-*D*-glucopyranosyl



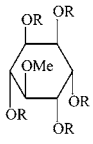
21, R^1=R^2=R^4=R^5=H,
R^3=2-β-*D*-glucopyranosyl
22, R^1=R^2=R^4=R^6=Ac,
R^3=tetra-*O*-acetyl-2-β-*D*-glucopyranosyl



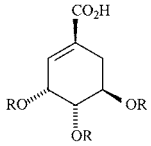
23, R^1=H, R^2=*O*-β-*D*-glucopyranosyl
24, R^1=Ac, R^2=tetra-*O*-acetyl-β-*D*-glucopyranosyl



25, R=H
26, R=Ac



27, R=H
28, R=Ac



29, R=H
30, R=Ac

Fraction A6 (100 mg) was acetylated and separated by PLC (toluene/acetone, 8:2) to give two bands at R_f 0.66 (13.4 mg) and 0.46 (5.5 mg). Purification of the bands in the same solvent

gave the peracetate derivatives **8** and **10** of 5-deoxyluteolin **7** (R_f 0.66, 12.5 mg) (*3*) and luteolin **9** (R_f 0.46, 4.5 mg) (*3*), respectively.

Fraction A7 (100 mg) was acetylated and purified by TLC in toluene/acetone (8:2) to give two bands R_f 0.50 (9.4 mg) and 0.42 (16.0 mg). PLC purification of the R_f 0.50 band in the same solvent yielded the glucosylated flavan **17** as the per-*O*-acetyl derivative **18**: ^{1}H NMR ($CDCl_3$) (δ_H) 7.33 (1H, d, J = 2.5 Hz, H-2′), 7.15 (1H, d, J = 8.5 Hz, H-5′), 7.04 (1H, dd, J = 2.5, 8.5 Hz, H-6′), 6.87 (1H, d, J = 2.5 Hz, H-8), 6.79 (1H, d, J = 2.5 Hz, H-6); C-ring, 4.58 (1H, dd, J = 3.0, 9.0 Hz, H-2), 2.50−2.69 (2H, m, H-4), 1.55−1.70 (2H, m, H-3); glucose, 5.69 (1H, dd, J = 8.0, 9.0 Hz, H-2″), 5.50 (1H, t, J = 9.0 Hz, H-3″), 5.34 (1 H, dd, J = 9.0, 10.0 Hz, H-4″), 4.87 (1H, d, J = 8.0 Hz, H-1″), 4.28 (1H, dd, J = 2.0, 12.5 Hz, H-6″), 4.10 (1H, dd, J = 5.5, 12.5 Hz, H-6″), 3.25, (1H, m, H-5″); aromatic *O*Ac, 1.89 (3H, s), 1.87 (3H, s), 1.86 (3H, s); aliphatic *O*Ac, 1.86 (6H, s), 1.81 (3H, s), 1.76 (3H, s); MS, *m*/*z* (%) 1073 (M^+, 0), 599 (42), 137 (42), 123 (55), 109 (100).

Purification of the R_f 0.42 band by PLC in toluene/acetone (8:2) afforded the peracetate derivative **6** of the flavanone eriocitrin **5** (5.5 mg) (*7*).

Acetylation of fraction A8 (50 mg) and PLC in toluene/acetone (8:2) gave the peracetate derivative **14** of the isoflavone orobol **13** as a single band (R_f 0.76, 3.6 mg) (*8*, *9*).

Fraction B2 (100 mg) was acetylated and separated by PLC in toluene/acetone (8:2) to give two bands, which on subsequent separation in the same solvent yielded the per-*O*-acetyl derivatives **26** and **30** of *p*-coumaric acid **25** (R_f 0.62, 4.6 mg) (*3*) and (±)-shikimic acid **29** (R_f 0.66, 32.6 mg) (*11*), respectively.

Fraction B3 (50 mg) was acetylated and purified by PLC in toluene/acetone (8:2) to give the peracetate derivative **4** of the known flavanone narirutin **3** (R_f 0.33, 8.0 mg) (*7*).

Acetylation of fraction B4 (100 mg) and subsequent PLC in toluene/acetone (8:2) yielded the peracetate derivative **16** of epigallocatechin gallate **15** as a single band (R_f 0.27, 33.6 mg) (*10*) in substantial amounts.

Flavonoids. The highly insoluble hesperidin **1** (*3*), narirutin **3** (*7*), and eriocitrin **5** (*7*) were the flavanones isolated and characterized as the peracetate derivatives **2**, **4**, and **6**, respectively. Hesperidin, the observed major flavonoid constituent of the plant, was isolated in relatively high quantities as a precipitate from an ethanolic solution of the acetone extract. The ^{1}H NMR of the peracetate derivatives of **2**, **4**, and **6** showed resonances at δ ~2.75 and ~3.0 (H-3) and δ ~5.5 (H-2), diagnostic of the flavanone nucleus. In addition, each of the three flavanones displayed two meta-coupled doublets integrating for one proton each in the aromatic region, indicative of resorcinol A-rings. The AA′BB′ system exhibited by the peracetate **4** indicates a para-substituted B-ring, which is confirmed by association of 2-H(C) with 2′,6′-H(B) in the NOESY spectrum. Similar association of 2′- and 6′-H(B) with 2-H(C) confirmed an ABX system for the B-rings of *O*-acetyl derivatives **2** and **6**. Nuclear Overhauser effect (NOE) indicating association of the single O*Me* with 5-H(B) confirmed the 4′-OMe substitution in **1**. A sugar moiety is revealed by the COSY spectrum. The linkages, rhamnosyl (1‴→6″) glucosyl and C-7−O−C-1″ of the glycosyl to ring A in flavanones **2**, **4**, and **6**, are confirmed by NOE association of H-6″ with H-1‴ and of H-6 and H-8 with H-1″, respectively. The circular dichroism (CD) spectra of these derivatives exhibited the synchronous Cotton effects (negative for the $\pi \rightarrow \pi^*$ transition at ~300 nm and positive for the $n \rightarrow \pi^*$ transition at ~340 nm), which are compatible with flavanones possessing a 2*S* absolute configuration (*12*). Compound **1** was confirmed to be hesperedin by comparison of ^{1}H NMR data of its *O*-acetyl derivative **2** with that of an authentic sample. Similarly, the structures of narirutin **3** and eriocitrin **5** were confirmed by comparison of the ^{1}H NMR data of their peracetates with that in the literature (*7*).

Hesperidin is renowned for its vitamin C like activity (*13*) and anti-inflamatory, antimicrobial, and antiviral properties (*13*). Hesperidin also produces an analgesic and exertes mild antipyresis (*14*). Indications that hesperidin reduces aggregation of blood cells (erythrocytes) and abnormal capillary permeability and fragility and its protection against various traumas and stress have been reported (*15*).

The flavone luteolin **9** (*3*) and its 5-deoxy **7** (*3*) and 7-*O*-rutinosyl (scolymoside) **11** (*6*) analogues were isolated as amorphous peracetate derivatives **10**, **8**, and **12**, respectively. The ^{1}H NMR spectra of **8**, **10**, and **12** invariably displayed the one-proton singlet between δ 6.5 and 6.8 reminiscent of the H-3 resonance of the flavones (*16*). The connectivities, rhamnosyl (1‴→6″) glucosyl and C-7−O−C-1″ of the glycosyl moiety to the A-ring in **12** were confirmed by NOE associations of H-6 and H-8 with H-1‴ and H-6″ with H-1‴, respectively. All the glycosidic resonances in the ^{1}H NMR spectrum were assigned from the COSY data. The sequential connectivities, chemical shift parameters, and coupling constant data confirmed that the rutinosyl unit was an *O*-linked glycosyl with β-configuration (*17*). Luteolin is known for its antispasmodic (*18*) and antioxidant properties (*19*). The antioxidant (*19*, *20*), diuretic (*16*), antiviral (*20*), and antispasmodic (*16*) properties and cardioprotective effects (*21*) of flavones are well established. Luteolin and its glycosides have particularly been found to induce antihypertensive activity even in excess of the reference drug, papaverine (*22*).

A single isoflavone, **13**, displayed a singlet at δ 8.0 in the ^{1}H NMR spectrum of the peracetate derivative **14**, which is characteristic of the single proton at C-2 in isoflavones. The ^{1}H NMR also exhibited the AB and ABX systems corresponding to the tetrasubstituted A-ring and trisubstituted B-ring, respectively. Compound **B** was confirmed to be orobol (*8*−*10*). The immunosuppressive and anti-inflammatory effects of orobol are well-established (*10*).

Epigallocatechin gallate **15**, a predominant flavonoid found in different types of teas (*23*), was isolated and characterized as its peracetate derivative **16** by comparison of its ^{1}H NMR data with an authentic sample. In the ^{1}H NMR spectrum of the *O*-acetyl derivative of epigallocatechin gallate **16**, the aliphatic protons H-2, H-3, H-4_{ax}, and H-4_{eq} are typically displayed at δ 5.65 (br, m), δ 5.20 (br, s), δ 3.08 (dd, J = 5.0 and 16.9 Hz), and δ 3.0 (dd, J = 3.0 and 16.9 Hz), respectively. This was confirmed by an NOE association of H-2 with the 2′,6′ singlet of ring B. H-2, -3, and -4 were confirmed by correlations in the COSY spectrum. Correlation in an HMBC spectrum from the carbonyl carbon to the singlet (H-2″,6″) distinguished the gallate from H-2′,6′ of the ring B (singlet). The two meta-coupled doublets in the aromatic region suggest a resorcinol-type hydroxylation pattern on ring A.

Galloyl esters of catechins are more active as cancer preventatives than nongalloylated catechins due to their lower redox potentials (*24*). They have the highest activity as antioxidants and are the most effective inhibitors of lipid peroxidation (*25*, *26*). The antibacterial and deodorizing effects of catechins slow tooth decay (anticaries) and improve breath freshness (*27*). Epigallocatechin gallate is capable of suppressing angiogenesis, a key process of blood vessel growth required for solid tumor development and metastasis (*28*, *29*).

The ^{1}H NMR spectrum of the peracetate derivative **17** of flavan **18** indicated the presence of two methylene multiplets (δ 2.60, H-4$_{a,b}$) and (δ 1.65, H-3$_{a,b}$) consistent with ring C of the flavan moiety. The presence of aliphatic protons in the region between δ 3.2 and 5.4 was consistent with a glucosyl moiety. The glucosyl was established from the COSY data. The connectivity of the glucosyl moiety to the aglycon was designated by NOE association of only H-6 of ring A with the H-1′ proton of the glucosyl unit. The ^{1}H NMR spectrum also showed the presence of an ABX system for ring B and two meta-coupled proton signals for ring A. Conspicuous absence of a low-field resonance at ~200 ppm in the ^{13}C NMR spectrum confirmed the absence of a carbonyl at C-4 in the flavan C-ring.

A *C*-6-linked glucosylated derivative of kaempferol **19** was isolated as a peracetate derivative **20**. Absence of the C-ring protons in the ^{1}H NMR spectrum in the peracetate derivative **20** was consistent with a flavonol skeleton. The glucosyl moiety was established from the COSY data. The structure was confirmed by comparison of the ^{1}H NMR data with that in the literature (*4*).

Xanthones. Mangiferin **21**, 1,3,6,7-tetrahydroxyxanthone-C-2-β-D-glucoside, was isolated as the peracetate derivative **2** and its structure elucidated by comparison of its ^{1}H NMR data with that in the literature (*3*). Mangiferin is a common constituent of folk medicines and has potential as an antioxidant and an antiviral agent and is used for melancholia (*30*). Recently it has been reported to be a potent scavenger of free radicals (*31*), to be a potential cure for diabetes mellitus (*32*), and to act as an agent for lowering body weight (*33*). Generally, xanthones are reported to possess antitumor (*34*), antileukemic, antiulcer, antimicrobial, antihepatotoxic, and CNS-depressant activities (*35*). Bioactivities including cytotoxic, anti-inflammatory, and antifungal activities, enhancement of choline acetyltransferase activity, and inhibition of lipid peroxidase have been described (*36*). Xanthones and their derivatives have also been shown to be effective as allergy inhibitors and bronchodilators in the treatment of asthma (*37*). Structurally related 1,3,5,6-, 1,3,6,7-, 2,3,6,7-, and 3,4,6,7-tetraoxygenated xanthones have been reported to possess antiplatelet effects, the mechanism of 1,3,6,7-tetraoxygenated xanthones being due to both inhibition of thromboxane formation and phosphoinositide (*38, 39*).

4-Glucosyltyrosol **23** and *p*-coumaric acid **25**, the monoaryls isolated from the tea, were confirmed by comparison of ^{1}H NMR data of their peracetate derivatives **24** and **26** with that in the literature (*3*). 4-Hydroxytyrosol is a potent antioxidant (*40*). The antihepatotoxic properties of *p*-coumaric acid are well-established (*41*).

Nonphenolics. Pinitol **27**, a well-established expectorant (*41*), was identified by comparison of the ^{1}H NMR data of its *O*-acetyl derivative **28** with those of the commercially available reference compound. ^{1}H NMR data of **30**, the *O*-acetyl derivative of known shikimic acid **29**, were in agreement with the data in the literature (*11*).

As a result of our continued in-depth investigations of the *Cyclopia* species from which honeybush tea is brewed, the "unfermented" leaves and shoots of *C. intermedia* have revealed phenolic metabolites with diverse physiological properties that have potential benefits for human health. Flavonoids (especially hesperidin), mangiferin (a xanthone), and (+)-pinitol dominate in the tea. The results from this study may in part justify some of the claimed properties such as use of the tonic for colds and influenza and its effectiveness in alleviating menopausal symptoms in women. Honeybush tea is thus becoming an increasingly popular health-promoting beverage with low caffeine content and antioxidant properties.

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