Antioxidant Principles from *Bauhinia tarapotensis*

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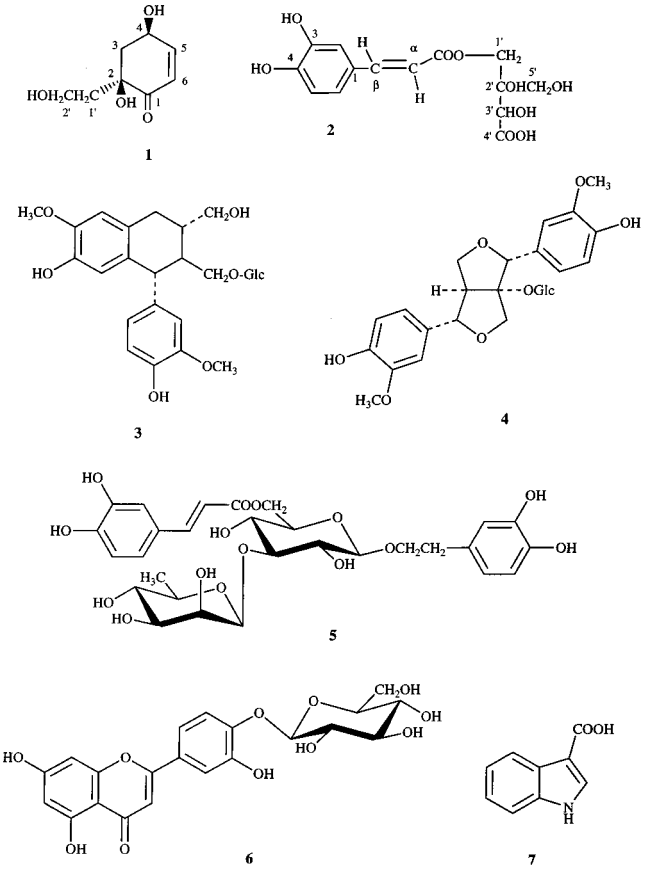
A new cyclohexenone (**1**) and a new caffeoyl ester derivative (**2**), together with the known compounds (−)-isolariciresinol 3-α-*O*-*β*-D-glucopyranoside (**3**), (+)-1-hydroxypinoresinol 1-*O*-*β*-D-glucopyranoside (**4**), isoacteoside (**5**), luteolin 4′-*O*-*β*-D-glucopyranoside (**6**), and indole-3-carboxylic acid (**7**), were isolated from the leaves of *Bauhinia tarapotensis*. The structures of these new compounds were determined by spectroscopic data analysis. The antioxidant activities of **1**−**7** were determined by measuring their free radical scavenging effects, using the 1,1-diphenyl-2-dipicrylhydrazyl free radical (DPPH) and Trolox equivalent antioxidant activity (TEAC) methods, and the coupled oxidation of *β*-carotene and linoleic acid. Compounds **3**−**5** showed good activities in the DPPH and TEAC tests, while compounds **1** and **2** were active in the coupled oxidation of *β*-carotene and linoleic acid bioassay.

Bauhinia tarapotensis Benth. (Leguminosae) is a small tree native to Ecuador, where it is commonly called "pata de vaca". The genus *Bauhinia* includes 250 species, inclusive of shrubs, lianas, and small trees, and is distributed mainly in Africa, Asia, and Latin America.[1] Many plants of the genus are used in traditional medicine for their interesting biological activities such as analgesic, antidiabetic, antiinflammatory, antimicrobial, astringent, and diuretic effects.[2−4] In particular, *B. tarapotensis* leaves are employed as antiinflammatory and decongestant remedies in popular indigenous medicine;[5] the bark has an antidiarrheal property.[6]

As part of our continuing search for bioactive compounds from Latin American medicinal plants, a methanol extract of the leaves of *B. tarapotensis* was found to exhibit significant antioxidant effects, based on the scavenging activity of the stable 1,1-diphenyl-2-dipicrylhydrazyl (DPPH) free radical.[7] Bioassay-guided fractionation of this extract using this antioxidant assay resulted in the isolation of two new compounds, *cis*-2,4-dihydroxy-2-(2-hydroxyethyl)cyclohex-5-en-1-one (**1**) and the caffeoyl ester of apionic acid (**2**), which were purified along with the known derivatives (−)-isolariciresinol 3-α-*O*-*β*-D-glucopyranoside (**3**), (+)-1-hydroxypinoresinol 1-*O*-*β*-D-glucopyranoside (**4**), isoacteoside (**5**), luteolin 4′-*O*-*β*-D-glucopyranoside (**6**), and indole-3-carboxylic acid (**7**), using solvent extraction and repeated column chromatography over Sephadex LH-20 and by droplet countercurrent chromatography (DCCC) and HPLC.

The present paper deals with the structure elucidation of **1** and **2**, as well as the antioxidant evaluation of all isolated compounds by radical scavenging activity in the free radical DPPH[7] and radical cation 2,2′-azino-bis(3-ethylbenzothiozoline-6-sulfonate) (ABTS) tests[8,9] and the coupled oxidation of *β*-carotene and linoleic acid.[10,11]



Results and Discussion

For the screening of antioxidants from the leaves of *B. tarapotensis*, the antioxidative effects of the extracts and fractions were tested for scavenging activity of DPPH. The dried leaves of *B. tarapotensis* were extracted successively with *n*-hexane, $CHCl_3$, $CHCl_3$−MeOH (9:1), and MeOH. The methanolic extract was partitioned between *n*-BuOH and H_2O, and the *n*-butanol residue was fractionated by a combination of gel filtration chromatography on Sephadex LH-20, DCCC, and reversed-phase HPLC, to yield two new compounds (**1** and **2**) and five known derivatives **3**−**7**. Their structures were elucidated by interpretation of 1D and 2D NMR and ESMS spectra in comparison with literature data.

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Compound **1** was obtained as a pale yellow syrup. Its molecular formula was $C_8H_{12}O_4$, with a $[M]^+$ at *m/z* 172, and it had a UV maximum (MeOH) at 283 nm. The ^{13}C NMR and DEPT spectra indicated that **1** contains two methylenes, two methines, one hydroxymethylene, one hydroxymethine, and two quaternary carbons. The ^1H NMR spectrum of **1** showed two olefinic protons mutually coupled (J = 10.3 Hz) at δ 6.80 and 5.97 (H-5 and H-6, respectively); the first signal showed also an additional coupling (J = 2.0 Hz) with H-4 that resonated at δ 4.15 (1H, ddd) and was therefore attributed to one carbinolic proton. Other ^1H NMR methine signals were at δ 4.00 and 3.88, while four protons assigned to two methylene groups resonated at δ 2.79 and 2.58 and δ 2.29 and 2.22, respectively. Data obtained from 1D-TOCSY and DQF-COSY experiments established the correlations of all protons in compound **1**, showing the sequences H-3−H-6 and H-1′−H-2′. All ^{13}C NMR signals were assigned on the basis of HSQC and HMBC experiments. The definitive structure of **1** was confirmed by analysis of correlation peaks in the HMBC experiment; diagnostic correlations were observed between H-6−C-2, H-6−C-4, H-6−C-3; H-4−C-5, H-4−C-1; H-3−C-2; H-3−C-4, H-3−C-1; H-1′−C-1; H-2′−C-1′, H-2′−C-2. Assigning the stereochemistry of **1** was not trivial, owing to the presence of a quaternary center as well as to some distortion introduced in the six-membered ring by the three unsaturated carbons. The relative configuration of the two hydroxy groups was determined by means of 2D-ROESY NMR spectroscopy. Proton H-4 had rather small coupling constants with both H-3_{ax} and H-3_{eq} (assigned according to their chemical shifts, by analogy with related compounds)[12] and had a strong ROE effect with the same protons; thus it was assigned in a pseudoequatorial position. Furthermore, there was a weak ROE effect between H-3_{eq} and H-1′, located on the hydroxyethyl group, which therefore must be on the same side as H-3_{eq}. These observations led to the conclusion that the two hydroxy groups are *cis* to each other. By minimizing the structure through molecular mechanics,[13] the conformation of the ring appeared twisted, allowing the accommodation of the two bulky substituents at C-2. Intramolecular hydrogen bonding between the hydroxyl at C-2′ and OH-4 or the carbonyl may stabilize the structure proposed. Thus, the structure of **1** was established as *cis*-2,4-dihydroxy-2-(2-hydroxyethyl)cyclohex-5-en-1-one, a new compound.

Compound **2** was isolated as a red syrup. Its molecular formula, $C_{14}H_{16}O_9$, was deduced from the ESIMS and ^{13}C NMR spectra data. The UV spectrum showed absorption bands at 240 and 330 nm. In the ^1H NMR spectrum, the signals for three aromatic protons at δ 7.09 (^1H, d, J = 1.8 Hz), 7.00 (^1H, dd, J = 8.0, 1.8 Hz), and 6.81 (^1H, d, J = 8.0 Hz), corresponding to a typical 1,2,4-trisubstituted aromatic ring, were observed. In addition, the ^1H NMR spectrum also showed the signals for two *trans* olefinic protons at δ 7.64 (1H, d, J = 16.0 Hz) and 6.34 (1H, d, J = 16.0 Hz), suggesting the presence of a *trans*-caffeoyl moiety in compound **2**. This was supported by the ^{13}C NMR spectrum, which at low field showed signals for a caffeoyl moiety (see Experimental Section). In addition, the ^{13}C NMR spectrum showed five other carbon signals arising from a polyoxygenated structure. This was substantiated in the ^1H NMR spectrum, from the signals at δ 4.54 (1H, s), 4.40 (1H, d, J = 12.0 Hz), 4.33 (1H, d, J = 10.0 Hz), 4.30 (1H, d, J = 10.0 Hz), and 4.25 (1H, d, J = 12.0 Hz), which correlated in the HSQC spectrum with ^{13}C NMR signals at δ 71.4, 73.9, and 65.5, respectively. The assignments of all protons and carbons of **2** were based on the results of the 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments. Finally, the structure of **2** was confirmed by a series of diagnostic HMBC correlations: H-α−C-1′; H-β−COO, H-β−C-2; H-1′−C-α, H-1′−C-3′, H-1′−C-5′; H-3′−C-2′, H-3′−C-4′; H-5′−C-1′, H-5′−C-4′. Compound **2** was therefore identified as the 3-(3,4-dihydroxyphenyl)prop-2-enoyl ester of 2,3,4-trihydroxy-3-hydroxymethylbutyric acid or the caffeoyl ester of apionic acid.[14]

Table 1. Antioxidant Activities of Compounds **1**−**7** in the TEAC and Auto-oxidation Assay[a]

		auto-oxidation assay	
compound	TEAC	t = 60 min	t = 120 min
1	0.076	43.0	9.7
2	0.484	25.2	20.5
3	0.826	−10.0	−6.2
4	0.935	10.7	8.5
5	1.228	24.6	7.2
6	0.445	16.2	10.0
7	0.092	−10.1	−6.5
rutin	2.42		
caffeic acid	1.26		
BHT[b]		63.9	62.5

[a] For protocols used, see Experimental Section. [b] BHT = 2,6-di-*tert*-butyl-4-methoxyphenol; standard control substance.

Five known compounds were also isolated in this investigation and were identified as (−)-isolariciresinol 3-α-*O*-β-D-glucopyranoside (**3**),[15] (+)-1-hydroxypinoresinol 1-*O*-β-D-glucopyranoside (**4**),[16] isoacteoside (**5**),[17] luteolin 4′-*O*-β-D-glucopyranoside (**6**),[18] and indole-3-carboxylic acid (**7**),[19] by comparison with published physical and spectral data.

The antioxidant activity of extracts, fractions, and pure compounds **1**−**7** was studied in the DPPH free-radical scavenging test: the methanolic extract and major fractions 7, 8, and 12 derived from Sephadex LH-20 of the *n*-butanolic residue exhibited activity in this test (see Experimental Section) compared with all the other extracts and fractions. Compound **5** showed the most potent antioxidant activity with IC_{50} 1.3 μM, while **3** and **4** exhibited moderate potencies with IC_{50} values of 10.5 and 20.3 μM, respectively. The free-radical scavenging activity of pure compounds was confirmed in the TEAC assay. This method measures the relative ability of antioxidant substances to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonate) ($ABTS^{\bullet+}$) as compared to a standard amount of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analogue.[8] The activity of the tested compounds was expressed as TEAC (Trolox equivalent antioxidant activity) values, with a 1.0 mM concentration of standard Trolox solution having an antioxidant capacity equivalent to 1.0 mM concentration solution of the compound under investigation. The results (Table 1) confirmed that compound **5** exhibited free-radical scavenging activity in comparative potency to reference antioxidant compound caffeic acid,[20] while **3** and **4** had more moderate activities. Finally, the antioxidative effect of pure compounds **1**−**7** on the auto-oxidation of linoleic acid was examined. Membrane lipids are abundant in unsaturated fatty acids that are most susceptible to oxidative processes. In particular, linoleic acid is the target of lipid peroxidation.[21] Compounds **1**−**7** were assessed in this assay, and the values of AA (antioxidant activity), measured at t = 60 and t = 120 min employing the bleaching of β-carotene as a model system, are reported in Table 1. The data show that all compounds tested have moderate AA, which in the case of **1** and **2** were only slightly less potent than the standard phenolic antioxidant 2,6-di-*tert*-butyl-4-methoxyphenol (BHT).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda 11 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C, using the UXNMR software package and a Varian VXR-300 NMR spectrometer operating at 300 MHz for ^{1}H were used for NMR experiments; chemical shifts are expressed in δ (ppm) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. ^{13}C-DEPT, 1D-TOCSY, ^{1}H−^{1}H DQF-COSY, 2D-ROESY, ^{1}H−^{13}C HSQC, and HMBC experiments were carried out using the conventional pulse sequences as described in the literature.[22] EIMS were recorded with a VG-ZAB instrument. ESIMS (positive mode) were obtained from a Hewlett-Packard 1090L instrument with a diode array detector, managed by a HP 9000 workstation interfaced with a HP 1100 MSD API-electrospray unit. Column chromatography was performed over Sephadex LH-20 (Pharmacia); droplet countercurrent chromatography (DCCC) was performed on an apparatus manufactured by Büchi, equipped with 300 tubes; HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column and Shimadzu injector.

Plant Material. Leaves of *B. tarapotensis* Benth. were collected from the Pastaza region, Ecuador, in July 1995. The plant material was identified by Dr. Medardo Tapia, Escuela Superior Politecnico de Chimborazo, Ecuador, where a voucher specimen was deposited.

Extraction and Isolation. The air-dried powdered leaves of *B. tarapotensis* (400 g) were defatted with *n*-hexane and successively extracted for 48 h with $CHCl_3$, $CHCl_3$−MeOH (9:1), and MeOH, by exhaustive maceration (3 × 2 L), to give 2.4, 14.0, 8.5, and 19.0 g of the respective residues. The methanolic extract, when tested for antioxidant potency in the DPPH assay, exhibited an IC_{50} value of 19 μg/mL and was therefore partitioned between *n*-BuOH and H_2O to give a butanol residue, which was chromatographed on Sephadex LH-20, using MeOH as eluent, to obtain 210 fractions of 8 mL, which were pooled into 16 major fractions. Fraction 7 demonstrated antioxidant activity (IC_{50} 28 μg/mL) and was submitted to DCCC with $CHCl_3$−MeOH−H_2O (7:13:8) in which the stationary phase consisted of the upper phase (descending mode, flow 15 mL/h), to obtain pure compound **1** (20 mg) together with 27 fractions. Fraction 22 was finally purified by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 mL min^{-1}) with MeOH−H_2O (40:60) to yield (−)-isolariciresinol 3-α-*O*-*β*-D-glucopyranoside **3** (t_R = 18 min, 12 mg).[15] Fractions 8, 12, and 15 from the initial purification over Sephadex LH-20 showed DPPH antioxidant activity (IC_{50} 20, 10.6, and 85 μg/mL, respectively) and were therefore fractionated over RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 mL min^{-1}) with MeOH−H_2O (55:45) (fractions 8 and 15) and with MeOH−H_2O (40:60) (fraction 12), to afford, respectively, (+)-1-hydroxypinoresinol 1-*O*-*β*-D-glucopyranoside **4** (t_R = 13 min, 8 mg) from fraction 8,[16] compound **2** (t_R = 7 min, 7 mg) and isoacteoside **5** (t_R = 30 min, 25 mg) from fraction 12,[17] and luteolin 4′-*O*-*β*-D-glucopyranoside **6** (t_R = 12 min, 6 mg) from fraction 15.[18] Crystallization from methanol of fraction 16 afforded pure compound indole-3-carboxylic acid **7** (10 mg).[19]

2,4-Dihydroxy-2-(2-hydroxyethyl)cyclohex-5-en-1-one (1): pale yellow syrup; $[\alpha]_D^{25}$ +13° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 283 (4.08), 328 (1.62) (sh) nm; ^{1}H NMR (600 MHz, CD_3OD), δ 2.22 (1H, m, H-1′a), 2.29 (1H, m, H-1′b), 2.58 (1H, dd, J = 12.2, 4.9 Hz, H-3$_{ax}$), 2.79 (1H, dd, J = 12.2, 4.4 Hz, H-3$_{eq}$), 3.88 (1H, m, H-2′a), 4.00 (1H, m, H-2′b), 4.15 (1H, ddd, J = 4.9, 4.4, 2.0 Hz, H-4), 5.97 (1H, d, J = 10.3 Hz, H-6), 6.80 (1H, dd, J = 10.3, 2.0 Hz, H-5); ^{13}C NMR (200 MHz, CD_3OD), δ 40.5 (t, C-3), 40.7 (t, C-1′), 67.1 (t, C-2′), 75.3 (s, C-2), 82.3 (d, C-4), 128.8 (d, C-6), 150.7 (d, C-5), 199.0 (s, C-1); EIMS *m*/*z* 172 [M]$^+$, 110 (40), 82 (100), 68 (39); *anal.* C 55.78%, H 7.04%, O 37.18%, calcd for $C_8H_{12}O_4$, C 55.81%, H 7.02%, O 37.17%.

Caffeoyl ester of apionic acid (2): red syrup; $[\alpha]_D^{25}$ +50° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (1.45) (sh), 330 (2.33) nm; ^{1}H NMR (600 MHz, CD_3OD), δ 4.25 (1H, d, J = 12.0 Hz, H-5′a), 4.30 (1H, d, J = 10.0 Hz, H-1′a), 4.33 (1H, d, J = 10.0 Hz, H-1′b), 4.40 (1H, d, J = 12.0 Hz, H-5′b), 4.54 (1H, s, H-3′), 6.34 (1H, d, J = 16.0 Hz, H-α), 6.81 (1H, d, J = 8.0 Hz, H-5), 7.00 (1H, dd, J = 8.0, 1.8 Hz, H-6), 7.09 (1H, d, J = 1.8 Hz, H-2), 7.64 (1H, d, J = 16.0 Hz, H-*β*); ^{13}C NMR (200 MHz, CD_3OD), δ 65.5 (t, C-1′), 71.4 (d, C-3′), 73.9 (t, C-5′), 77.0 (s, C-2′), 114.5 (d, C-α), 115.2 (d, C-2), 116.5 (d, C-5), 123.2 (d, C-6), 127.6 (s, C-1), 146.9 (s, C-3), 147.8 (d, C-*β*), 149.9 (C-4), 168.6 (COO), 177.7 (C-4′); ESIMS (positive-ion mode) *m*/*z* 351 [M + Na]$^+$, 163, 114; *anal.* C 51.18%, H 4.93%, O 43.89%, calcd for $C_{14}H_{16}O_9$, C 51.22%, H 4.92%, O 43.86%.

Scavenging Activity of DPPH Radicals. The potential antioxidant activity of plant extracts and fractions was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Aliquots of 30 μL of a methanolic solution containing each pure compound were added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated.[7] IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

TEAC Test. Pure compounds were tested by using the Trolox equivalent antioxidant activity (TEAC) assay. The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonate) (ABTS$^{\bullet+}$) with spectrophotometric analysis.[8] The ABTS$^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The ABTS$^{\bullet+}$ solution was then diluted with PBS (pH = 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Samples were diluted with methanol to produce solutions of 0.3, 0.5, 1, 1.5, and 2 mM concentration. The reaction was enhanced by the addition of 1 mL of diluted ABTS to 10 μL of each sample solution. Determinations were repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of concentration of compound or standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co., Gillingham, Kent, U.K.). The antioxidant activities of compounds **1**−**7** are expressed as TEAC (Trolox Equivalent Antioxidant Activity) values in comparison with TEAC activity of reported reference compounds, rutin and caffeic acid.[23] TEAC value is definite as the concentration of standard Trolox solution with equivalent inhibition to 1 mM concentration solution of the compound under investigation.

Auto-oxidation of *β*-Carotene. Oxidation of linolenic acid was measured by the method described by Pratt.[11] Quantities of linolenic acid (20 mg) and Tween 20 (200 mg) were placed in a flask, and a solution of 2 mg of *β*-carotene in 10 mL of $CHCl_3$ was added. After removal of $CHCl_3$, 50 mL of distilled water saturated with oxygen for 30 min was added. Aliquots (200 μL) of each compound, dissolved in ethanol to a 15 μg/mL solution, were added to each flask with shaking. Samples without test compounds were used as blanks, and a sample with 2,6-di-*tert*-butyl-4-methoxyphenol (BHT, Aldrich Chemical Co., Gillingham, Kent, U.K.) was used as a control substance. Samples were subjected to oxidation by placing in an oven at 50 °C for 3 h. The absorbance was read at 470 nm at regular intervals. The antioxidant activity was expressed as AA and calculated with the equation

$$AA = 100\left[1 - \frac{A_0 - A_t}{A_{00} - A_{0t}}\right]$$

A_0 = absorbance at the beginning of the incubation, with compound; A_t = absorbance at the time t, with compound; A_{00}

= absorbance at beginning of the incubation, without compound; A_{0t} = absorbance at the time *t*, without compound

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