JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 5863−5868 **5863**

Phenolic Constituents and Antioxidant Activity of *Wendita calysina* Leaves (Burrito), a Folk Paraguayan Tea

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Burrito tea originates from the leaves of *Wendita calysina*, an indigenous Paraguayan plant, which is commonly consumed in South America and in Western countries. Phytochemical investigation of this species has led to the isolation of 14 constituents, among them 2 new flavanonols, dihydroquercetagetin (**1**) and 3,5,6,7,4′-pentahydroxyflavanonol (**2**), in addition to several known methoxyflavones, methoxyflavonols, phenylethanoid glycosides, and benzoic acid derivatives (**4**−**14**). All structures were elucidated by ESI-MS and NMR spectroscopic methods. Quantitative determination of phenolic constituents from burrito water infusions has been performed by HPLC-UV-DAD. The total antioxidant activity of the tea was measured by the $ABTS^{\bullet+}$ radical cation decolorization and chemiluminescence (CL) assays and compared with the values of other commonly used herbal teas (green and black teas, mate, and rooibos).

KEYWORDS: ***Wendita calysina*; Geraniaceae; burrito; flavanonols and phenylethanoid glycosides; 1D and 2D NMR; HPLC-UV-DAD quantitative determination; teas; antioxidant activities and total phenols**

INTRODUCTION

The consumption of tea, especially green tea (*Camellia sinensis*), has been found to reduce the risk of various cancers in humans (*1*). Interest has now moved to other "teas" such as mate (*Ilex paraguayensis*) (*2*), espinheira-santa (*Maytenus aquifolium*) (*3*, *4*), and rooibos (*Aspalathus linearis*) (*5*), which are believed to have health-giving properties. The health aspects of these teas are mainly linked to their phenolics content and associated antioxidant properties.

The aqueous infusion of the leaves of *Wendita calysina* (Geraniaceae), a Paraguayan herbaceous plant commonly known as "burrito", is used as a beverage in daily life, instead of green tea, and also in traditional medicine as an anti-inflammatory and antirheumatic agent. It is found in the local commerce as powdered, dried, or fresh leaves and as aqueous or aqueous−alcoholic preparations. However, there are no data in the literature concerning the possible pharmacological effects and the chemical constituents of this plant.

Chemical investigation of the methanol extract of the leaves of *W. calysina* led to the isolation of 14 constituents.

HPLC quantitative determination of phenolic constituents in burrito water infusions has been performed to evaluate the contribution of tea to the total dietary polyphenol intake. The total antioxidant activity of burrito tea was determined and compared with the values of other commonly used teas such as green and black teas, mate, and rooibos, using the ABTS radical cation decolorization (TEAC) and chemiluminescence (CL) assays.

The chemical information obtained could be significant not only for understanding folk utilization but also for the future validation of isolated compounds as markers for the quality assessment of burrito leaves and tea.

MATERIALS AND METHODS

General Experimental Procedure. CD measurements were carried out on a J-710 discograph (Jasco, Tokyo, Japan). Optical rotations were determined on a model 192 polarimeter (Perkin-Elmer, Norwalk, CT) equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were recorded on a UV-2101PC, UV−vis scanning spectrophotometer (Shimadzu Italia srl, Milan, Italy). A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and at 150.86 MHz for ^{13}C, using the UXNMR software package was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD, and coupling constants, *J*, are in hertz. DEPT ^{13}C, $^{1}H-^{1}H$ DQF-COSY, $^{1}H-^{13}C$ HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. Electrospray ionization mass spectrometry (ESIMS) was performed using a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Excalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump; the flow rate was 3 μL/min. The capillary voltage was set at 5 V, the spray voltage at 5 kV, and the tube lens offset at 35 V. The capillary temperature was 220 °C. Data were acquired in the MS1 scanning mode (*m*/*z* 150−

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10.1021/jf040100e CCC: $27.50
Published on Web 08/19/2004

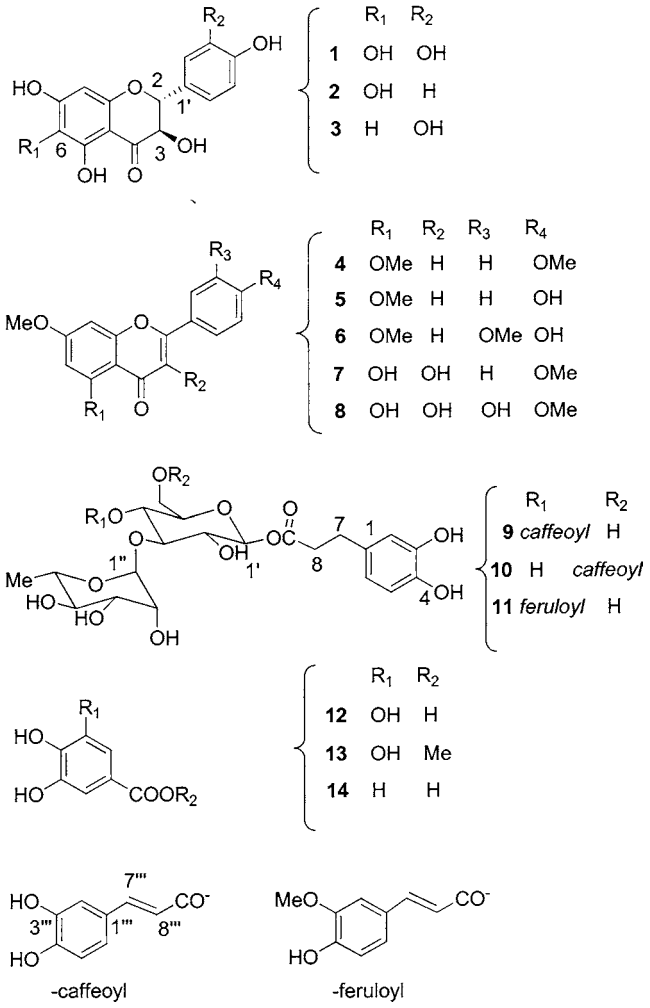

Figure 1. Compounds isolated from *W. calysina* (burrito) leaves: dihydroquercetagetin (**1**), 3,5,6,7,4′-pentahydroxyflavanonol (**2**), dihydroquercetin (**3**), 4′,5,7-trimethoxyapigenin (**4**), 5,7-dimethoxyapigenin (**5**), 4′-hydroxy-3′,5,7-trimethoxyflavone (**6**), kaempferol 7,4′-dimethyl ether (**7**), quercetin 7,4′-dimethyl ether (**8**), verbascoside (**9**), isoverbascoside (**10**), leucosceptoside A (**11**), gallic acid (**12**), methyl gallate (**13**), and 3,4-dihydroxybenzoic acid (**14**).

700). Exact masses were measured by a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument. Electrospray ionization was used in TOF mode at 8.500 resolving power. Samples were dissolved in MeOH, mixed with the internal calibrant, and introduced directly into the ion source by direct infusion. Calibration was performed on the peaks of cesium iodide and synthetic peptide (TOF positive ion calibration solution, Bachem) at *m*/*z* 132.9054 and 829.5398, respectively. Column chromatographies were performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). HPLC separations were performed on a Waters 590 series pumping system equipped with a W R401 refractive index detector and with a 300 × 7.8 mm i.d., 10 μm μ-Bondapak C-18 column and a U6K injector. Quantitative HPLC analyses were performed on an Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector, equipped with a 300 × 4.6 mm i.d., 10 μm μ-Bondapack C-18 column.

Plant Material. The leaves of *W. calysina* Martius were produced by COPROSA Ltda Paraguay (Cooperativa multiactiva de Produccion Servicios publicos, Consumo, Ahorro y Credito San Andres) and furnished by Commercio Altenativo S.r.l. (Ferrara, Italy).

Extraction and Isolation Procedure of Compounds 1−14 (Figure 1). The dried and powdered leaves (1000 g) were defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 19 g of residue.

Table 1. ^{13}C NMR and ^1H NMR Data[a] of Compounds **1** and **2** in CD_3OD

	1		**2**	
position	δ_C	δ_H (J_{HH} in Hz)	δ_C	δ_H (J_{HH} in Hz)
2	85.1	4.96 (d, 11.8)	85.0	4.97 (d, 11.8)
3	73.6	4.54 (d, 11.8)	73.4	4.55 (d, 11.8)
4	198.3		198.5	
5	165.2		164.8	
6	147.0		147.0	
7	169.0		168.6	
8	96.3	5.94 (s)	96.6	5.96 (s)
9	164.4		164.0	
10	101.1		102.1	
1′	129.8		124.1	
2′	115.8	7.02 (d, 2.2)	132.3	7.69 (d, 8.5)
3′	146.3		116.0	6.87 (d, 8.5)
4′	147.1		159.3	
5′	120.9	6.88 (d, 8.5)	116.0	6.87 (d, 8.5)
6′	116.0	6.93 (dd, 2.2, 8.5)	132.3	7.69 (d, 8.5)

[a] Assignments confirmed by 2D COSY, HSQC, HMBC experiments.

The MeOH extract was chromatographed (3 g) on a 1 m × 3 cm i.d. Sephadex LH 20 column with a flow rate of 0.5 mL/min. Ninety fractions of 8 mL were collected. After TLC analysis (Si-gel, *n*-BuOH−AcOH−H_2O, 65:15:25; $CHCl_3$−MeOH−H_2O, 70:30:3), fractions with similar R_f values were combined, giving four major fractions, which were further purified by HPLC on the C-18 μ-Bondpak column at a flow rate of 2.5 mL/min. Fraction I (190 mg) was purified using MeOH−H_2O (35:65) as the eluent to yield pure phenylethanoid glycosides **9** (79 mg, t_R = 25 min), **10** (9 mg, t_R = 24 min), and **11** (42 mg, t_R = 27 min). Fraction II (220 mg) was purified using MeOH−H_2O (70:30) to yield methoxyflavones **4** (18 mg, t_R = 12 min), **5** (17 mg, t_R = 9 min), and **6** (10 mg, t_R = 11 min) and using MeOH−H_2O (55:45) to give methoxyflavonols **7** (13 mg, t_R = 11 min) and **8** (17 mg, t_R = 9 min). Fraction III (203 mg) was purified using 50:50 MeOH−H_2O to yield benzoic acid derivatives **12** (42 mg, t_R = 5 min), **13** (21 mg, t_R = 9 min) and **14** (11 mg, t_R = 6 min). Finally, fraction IV, containing flavanonols, was purified with 70:30 MeOH−H_2O to yield compounds **1** (20 mg, t_R = 6 min), **2** (18 mg, t_R = 8 min), and **3** (20 mg, t_R = 11 min).

2*R*,3*R*-Dihydroquercetagetin (1) was obtained as a yellow amorphous powder: mp 216−219 °C; $[\alpha]^{25}_D = -63.7°$ (MeOH, *c* 0.1); CD (*c* 0.09% in MeOH) $[\vartheta]_{328}$ 2.9 × 10^3, $[\vartheta]_{295}$ −1.1 × 10^4; $[\vartheta]_{238}$ 3.2 × 10^3; UV (MeOH) λ_{max} (log ϵ) 207 (4.18) and 290 (4.44) nm; HR-ESIMS (*m*/*z*), calcd for $C_{15}H_{12}O_8$, 320.25098, found, 320.05322; ESI-MS, *m*/*z* 321 $[M + H]^+$, 319 $[M - H]^-$, 301 $[M - H^+ - H_2O]^-$; ^1H and ^{13}C NMR data, see **Table 1**.

2*R*,3*R*-3,5,6,7,4′-pentahydroxy-flavanonol (2) was obtained as a yellow amorphous powder: mp 214−218 °C; $[\alpha]^{25}_D = -43.0°$ (MeOH, *c* 0.1); CD (*c* 0.09% in MeOH), $[\vartheta]_{328}$ 2.9 × 10^3, $[\vartheta]_{295}$ −1.1 × 10^4, $[\vartheta]_{238}$ 3.5 × 10^3; UV (MeOH) λ_{max} (log ϵ) 208 (4.21) and 290 (4.40) nm; HR-ESIMS (*m*/*z*), calcd for $C_{15}H_{12}O_7$, 304.25158, found, 304.05831; ESI-MS, *m*/*z* 305 $[M + H]^+$, 303 $[M - H]^-$, 285 $[M - H^+ - H_2O]^-$; ^1H and ^{13}C NMR data, see **Table 1**.

2*R*,3*R*-Dihydroquercetin (3) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 207, 290 nm; CD (*c* 0.01% in MeOH), $[\vartheta]_{332}$ 1.3 × 10^4, $[\vartheta]_{296}$ −5.2 × 10^4, $[\vartheta]_{253}$ 0.8 × 10^4; ESI-MS, *m*/*z* 305 $[M + H]^+$, 303 $[M - H]^-$, 285 $[M - H^+ - H_2O]^-$; ^1H and ^{13}C NMR data were consistent with those previously reported (*6*).

4′,5,7-Trimethoxyapigenin (4): ^1H and ^{13}C NMR data were consistent with those previously reported (*7*); ESI-MS, *m*/*z* 311 $[M - H]^-$.

5,7-Dimethoxyapigenin (5): ^1H and ^{13}C NMR data were consistent with those previously reported (*8*); ESI-MS, *m*/*z* 297 $[M - H]^-$.

4′-Hydroxy-3′,5,7-trimethoxyflavone (6): ^1H and ^{13}C NMR data were consistent with those previously reported (*9*); ESI-MS, *m*/*z* 327 $[M - H]^-$.

Kaempferol 7,4′-dimethyl ether (7): ^1H and ^{13}C NMR data were consistent with those previously reported (*10*); ESI-MS, *m*/*z* 313 $[M - H]^-$.

Quercetin 7,4′-dimethyl ether (8): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*11*); ESI-MS, *m*/*z* 329 [M − H]$^-$.

Verbascoside (9): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*12*); ESI-MS, *m*/*z* 623 [M + H]$^-$.

Isoverbascoside (10): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*13*); ESI-MS, *m*/*z* 623 [M + H]$^-$.

Leucosceptoside A (11): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*14*); ESI-MS, *m*/*z* 637 [M + H]$^-$.

Gallic acid (12): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*15*); ESI-MS, *m*/*z* 169 [M − H]$^-$.

Methyl gallate (13): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*15*); ESI-MS, *m*/*z* 183 [M − H]$^-$.

3,4-Dihydroxybenzoic acid (14): ^{1}H and ^{13}C NMR data were consistent with those previously reported (*15*); ESI-MS, *m*/*z* 153 [M − H]$^-$.

Burrito Infusion Preparation. The aqueous extracts were obtained by pouring 100 mL of boiling distilled water on 3 g of dried leaves and steeping it for 10 min; then the infusions were filtered through filter paper and freeze-dried. The yield of the lyophilized aqueous extract was 374 mg (12.4% of dried leaves). The extractions were performed in triplicate.

HPLC Quantitative Analysis. Quantitative HPLC of the burrito infusion was carried out using a linear gradient with two solvents (A = water−0.01% TFA; B = MeOH−0.01% TFA), where solvent B increased from 20 to 70% over a 30 min period. Detection wavelength was 278 nm, the flow rate was 1 mL/min, and the injection volume was 20 μL. Compounds **9**−**14** were identified by comparing the retention time of the peaks in the aqueous extract with those of the standard compounds previously isolated and characterized by NMR analysis.

Quantification. The lyophilized infusion was diluted to a volume of 10 mL (37.4 mg/mL) in a volumetric flask. Quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standard. Verbascoside (**9**), isoverbascoside (**10**), and leucosceptoside A (**11**) were assayed as verbascoside equivalents. Gallic acid (**12**), methyl gallate (**13**), and 3,4-dihydroxybenzoic acid (**14**) were assayed as gallic acid equivalents.

Linearity. The linearity of responses for verbascoside (**9**) and gallic acid was determined on six levels of concentration with three injections for each level. A linear relationship between peak area versus concentration (0.025−2.5 mg/mL) was observed for the standards. The regression equations were $y = 1255x - 104.73$ ($R = 0.9952$) for verbascoside (**9**) and $y = 31361x - 18.60$ ($R = 0.9919$) for gallic acid (**12**), where y is the peak area and x the concentration used. The minimum detection limit was 0.2 ng, which resulted in a signal-to-noise ratio of 3:1.

Repeatability. The repeatability of the injection integration procedure was determined for compound **9**. The solution was injected 10 times, and the relative standard deviation was 1.39%. Relative standard deviation for retention times was <0.3%.

Preparation of Different Types of Teas. Standard grade green and black teas (*Camellia sinensis*), mate (*Ilex paraguayensis*), and rooibos (*Aspalathus linearis*) were obtained from Commercio Altenativo S.r.l. (Ferrara, Italy). The aqueous extracts were obtained by pouring 100 mL of boiling distilled water on 3 g of dried loose leaves and steeping it for 10 min. The infusions were filtered through filter paper, and the resulting infusion was freeze-dried. The yields of the lyophilized aqueous extracts were 250, 550, 265, and 458 mg for green tea, black tea, rooibos, and mate, respectively, corresponding to 8.3, 18.3, 8.8, and 15.2% of dried leaves, respectively. The extractions were performed in triplicate.

Total Polyphenols Assay. Estimation of the global polyphenols content of different types of teas was performed according to the Folin−Ciocalteu method. Fifteen milligrams of lyophilized infusions obtained from burrito leaves, green and black teas, mate, and unfermented rooibos, respectively, was dissolved in H_2O (1.5 mL) and the extract diluted 10-fold with water. Folin−Ciocalteu reagent (0.5 mL; Merck) was added to the diluted solutions (0.5 mL), and then 0.5 mL of a 100 g/L solution of Na_2CO_3 was added. The absorbance was measured at 720 nm with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances in the calibration curve of gallic acid used as standard phenol. The determination was repeated three times for each sample solution.

ABTS Radical Cation Decolorization Assay. Evaluation of free radical scavenging activity was performed with the Trolox equivalent antioxidant activity (TEAC) assay. TEAC value is based on the ability of the antioxidant to scavenge the preformed radical of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$), with spectrophotometric analysis, according to the method of Re et al. (*16*). Lyophilized infusions were diluted with MeOH to give 0.3, 0.5, 1.0, 1.5, and 2.0 mg/L solutions. The reaction was enhanced by the addiction of 1.0 mL of diluted ABTS$^{\bullet+}$ to 10 μL of each solution of samples or Trolox (standard) or 10 μL of MeOH (control). The determination was repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration as a function of the control's absorbance, 1 min after initial mixing. TEAC values were expressed in millimoles of Trolox per gram of dry weight of plant extracts. All analyses were made in triplicate.

Chemiluminescence Assay. Total antioxidant capacity was assayed by chemiluminescence according to the method of Whitehead et al. (*17*). Enhanced chemiluminescent signal reagent (Amersham) comprising assay buffer and tablets A and B (containing luminol, *p*-iodophenol enhancer, and perborate oxidant) was prepared by adding tablets A and B to the buffer solution. Signal reagent (0.4 mL) was added to distilled water (1 mL) in a glass cuvette containing a magnetic stirrer. The cuvette was placed in a Perkin-Elmer Wallac Victor 2 Chemiluminometer and the reaction commenced by the addition of 25 μL of horseradish peroxidase (4 μg/mL in H_2O). Lyophilized infusions (100 μL of 0.5 mg/mL dissolved in PBS, pH 7.4) were added to the cuvette, and the time for which light output was suppressed was determined. The determination was repeated three times for each sample solution. Comparison was made with a standard curve generated using different concentrations of Trolox in H_2O.

Statistical Analysis. Data are reported as mean ± standard deviation (SD) of triplicate determinations. The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp.).

RESULTS AND DISCUSSION

The MeOH extract of *W. calysina* leaves was fractionated by Sephadex LH-20 in order to investigate its chemical constituents. Fractions were further purified on reversed-phase HPLC to yield pure compounds **1**−**14** (**Figure 1**).

The ESI-MS (100 V, negative ion) mass spectrum of **1** gave as base peak the [M − H]$^-$ ion at *m*/*z* 319. From the mass and ^{13}C and ^{13}C DEPT NMR data, the molecular formula $C_{15}H_{12}O_8$ was deduced for compound **1**. The complete structure of **1** was elucidated by 1D and 2D NMR experiments at 600 MHz. The ^{1}H NMR spectrum of **1** (**Table 1**) displayed a proton signal located at δ 5.94 (1H, s) ascribable to H-8, the only proton of ring A, and proton signals at δ 6.88 (1H, d, $J = 8.5$ Hz, H-5′), 6.93 (1H, dd, $J = 8.5$ and 2 Hz, H-6′), and 7.02 (1H, d, $J = 2$ Hz, H-2′) indicative of a 3′,4′-disubstitution of ring B. A lowfield signal at 198.3 ppm in the ^{13}C NMR spectrum for C=O (C-4) along with the signals at 85.1 (C-2) and 73.6 (C-3) ppm are typical of a flavanonol (dihydroflavonol) structure with a C-5 hydroxyl; the signals for the C=O in C-5 unsubstituted, C-5-OAc and C-5-OCH_3 appear at a significantly higher field (*18*). The flavanonol skeleton of compound **1** was further corroborated by the presence of two characteristic 1H doublets for H-2 an H-3 at δ 4.96 ($J = 11.8$ Hz) and 4.54 ($J = 11.8$ Hz), respectively, in the ^{1}H NMR spectrum. The magnitude of the coupling constants of H-2 and H-3 ($J_{2,3} = 11.8$ Hz) suggests a 2,3-*trans* geometry and the diaxial orientation of the H-2 and H-3 in **1**. The chemical shifts of H-2 (δ 4.96) and of C-2, C-3, and C-4 (85.1, 73.6, and 198.3) are also consistent with its axial orientation (*19*). The absolute stereochemistry was clarified by CD analysis. A positive maximum at ~328 nm and a negative

maximum at ~295 nm are typical for the 2*R*,3*R* configuration (*20*). The HSQC spectrum established all of the correlations between protons and carbons of **1** (**Table 1**), whereas the HMBC spectrum showed connectivities for H-2/C-4, C-9, C-2′ and C-6′, H-3/C-10 and C-1′, and H-8 /C-6 and C-10. Thus, **1** was identified as 2*R*,3*R*-dihydroquercetagetin.

The ESI-MS (100 V, negative ion) mass spectrum of **2** gave as base peak the $[M - H]^-$ ion at *m*/*z* 303. From the mass and ^{13}C and ^{13}C DEPT NMR data, the molecular formula $C_{15}H_{12}O_7$ was deduced for compound **2**. In the ^1H NMR spectrum a 3,5,6,7,4′-substituted flavanonol skeleton was suggested by the appearance in the aromatic region of two doublet signals at δ 6.87 (2H, d, $J = 8.5$ Hz) and 7.69 (2H, d, $J = 8.5$ Hz) assigned to H-3′,H-5′ and H-2′,H-6′ respectively, indicative of a 4′-substitution on ring B, by the presence, as in **1**, of a one-proton singlet at δ 5.96 (1H, br s) typical of H-8 on a 5,6,7-trihydroxy-substituted ring A and by two characteristic 1H doublets for H-2 an H-3 at δ 4.97 ($J = 11.8$ Hz) and 4.55 ($J = 11.8$ Hz), respectively. In the ^{13}C NMR spectrum C-2, C-3, and C-4 resonances appear at δ 85.0, 73.4, and 198.5, respectively, as expected in 2,3-*trans* flavanonols with aryl and hydroxyl substituents at C-2 and C-3 equatorially oriented (*21*). The absolute configuration at C-2 and C-3 was 2*R*,3*R* as determined from the CD spectrum. The structure of **2** was confirmed by correlations seen in the HSQC and HMBC spectra. From these data **2** was determined to be 2*R*,3*R*-3,5,6,7,4′-pentahydroxyflavanonol.

Compound **3** was identified as the known flavanonol dihydroquercetin by ESI-MS and NMR spectroscopic methods (*6*). Methoxyflavones 4′,5,7-trimethoxyapigenin (**4**), 5,7-dimethoxyapigenin (**5**), and 4′-hydroxy-3′,5,7-trimethoxyflavone (**6**) and methoxyflavonols kaempferol 7,4′-dimethyl ether (**7**) and quercetin 7,4′-dimethyl ether (**8**) were identified by comparison of their ^1H and ^{13}C NMR, DFQ-COSY, HSQC, and HMBC spectra with literature data (*7*−*11*). They also showed the same ESI-MS, $[\alpha]_D$, and UV data as values in the literature. The presence of methoxyl groups and their location were established on the basis of the ^{13}C NMR spectra and HMBC data with respect to unmethylated models.

Compound **9** was identified as verbascoside by HPLC comparison with authentic standard and according to its ^1H, ^{13}C NMR, and ESI-MS data (*12*). The structure of isoverbascoside (**10**) was confirmed using ^1H and ^{13}C NMR. The ^1H NMR spectrum of isoverbascoside was similar to that of verbascoside, except for differences in the chemical shifts of H-4′ (verbascoside, δ 4.81; isoverbascoside, δ 3.41) and 2H−6 (verbascoside, δ 3.63 and 3.84; isoverbascoside, δ 4.34 and 4.50) in their glucosyl moiety. The ^{13}C NMR chemical shifts of isoverbascoside were close to those of verbascoside, but slight differences were observed in the shifts at C-3′, C-4′, and C-6′ (verbascoside, δ 81.66, 70.69, 62.49; isoverbascoside, δ 84.45, 70.94, 65.20). Compound **11** showed ESI-MS and ^1H and ^{13}C NMR data superimposable with those reported in the literature for leucoceptoside A (*14*). Comparison of its NMR data with those of verbascoside indicated the presence in **11** of a methoxyl group. The substitution at the C-3‴ position of the caffeoyl moiety by this methoxy group was deduced from the resonance of C-3‴ shifted downfield by 1.3 ppm and of C-2‴ and C-4‴ shifted upfield by ca. 3.0 and 3.6 ppm with respect to unmethoxylated verbascoside. Compound **12** was identified as gallic acid from its ^1H NMR spectrum and further confirmed by comparison of its chromatographic behavior with that of an authentic sample. The NMR spectra of compound **13** also showed the presence of a gallic acid moiety, but in addition there was a methyl ester signal; thus, compound **13** was identified as methyl gallate. Compound **14** showed spectroscopic data and chromatographic behavior identical with those of 3,4-dihydroxybenzoic acid.

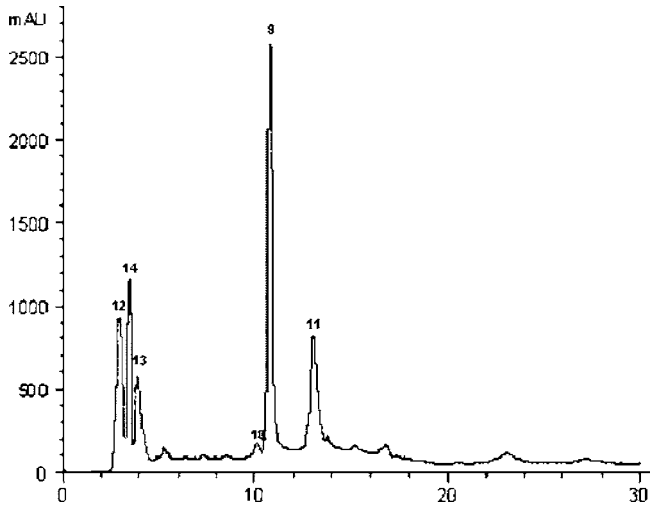

Figure 2. HPLC-DAD chromatogram of burrito tea. Peaks (from left to right): gallic acid (**12**), 3,4-dihydroxybenzoic acid (**14**), methyl gallate (**13**), isoverbascoside (**10**), verbascoside (**9**), and leucosceptoside A (**11**). Detector was set at 278 nm.

Dihydroflavonols (flavanonols) appear to be rare in plants and food and have been shown to be intermediates in the biosynthesis of other flavonoid classes. Dihydroquercetagetin (**1**) and 3,5,6,7,4′-pentahydroxyflavanonol (**2**) have never been isolated from any other natural source; the novelty of these compounds resides in the 6-OH substitution of ring A of known dihydroquercetin and dihydrokaempferol, respectively. Dihydroquercetin (**3**) is the most common member of this family. It occurs in nature as free phenol, as glycoside, and in the form of free and glycosylated phenol ethers. Neoastilbin, a dihydroquercetin 3-*O*-rhamnoside, is responsible for the sweetness of the herb *Engelhardtia chrysolepis* (*22*); 4′-methoxydihydroquercetin, 3-acetyldihydroquercetin, and 3-acetyl-4′-methoxydihydroquercetin, respectively, were rated by Kinghorn et al. (*23*) to be 40, 80, and 400 times sweeter than sucrose, and many flavanonols of similar structures are known to be sweet principles.

The quantitative analysis of the phenolic compounds from burrito water infusion was performed by HPLC-UV-DAD to evaluate the contribution of tea to the dietary polyphenols ingestion. Water-soluble natural products such as phenylethanoid glycosides **9**−**11** and benzoic acid derivatives **12**−**14** were found to be the main components of tea, whereas flavanonols **1**−**3**, methoxyflavones **4**−**6**, and methoxyflavonols **7** and **8** were undetectable by our analytical methods, indicating that in the infusion, obtained according to folk information by pouring 100 mL of boiling water on 3 g of dried burrito leaves, the concentration of these compounds is very low.

The t_R values for compounds **9**−**14** were 11.14, 10.50, 13.21, 2.56, 2.92, and 2.70 min, respectively (**Figure 2**). The concentrations of each compound in the aqueous extract (374 mg for a single cup of burrito tea), calculated from the experimental peak areas by interpolation to standard calibration curves, were 2.90% for compound **9**, 0.13% for **10**, 1.00% for **11**, 1.38% for **12**, 1.52% for **13**, and 0.64% for **14**, corresponding to 10.84, 0.48, 3.74, 5.15, 5.69, and 2.39 mg/100 mL of burrito infusion, respectively. Relative standard deviations were in the range of 3.31−4.13% calculated as the mean of three replications,

Table 2. Total Polyphenols (TP) and Antioxidant Activities of Different Types of Teas

tea	TP[a] (mg/g)	TEAC assay[a] (mmol of Trolox/g)	CL assay[a] (mmol of Trolox/g)
burrito	87.5 ± 2.6	2.4 ± 0.6	2.2 ± 0.4
green	213.2 ± 5.9	4.5 ± 0.55	5.9 ± 0.6
black	141.4 ± 3.9	3.9 ± 0.4	3.8 ± 0.4
mate	112.1 ± 4.1	2.8 ± 0.7	3.1 ± 0.6
rooibos	167.5 ± 4.7	1.8 ± 0.3	2.1 ± 0.3

[a] Mean ± SD of three determinations.

Table 3. Total Polyphenols (TP) and Antioxidant Activity of 1 Cup of Different Types of Teas

tea	solid intake[a]	TP[a] (mg/cup)	TEAC assay[a] (mmol of Trolox/cup)	CL assay[a] (mmol of Trolox/cup)
burrito	374 ± 9	32.7 ± 1.0	0.9 ± 0.2	0.8 ± 0.3
green	250 ± 6	53.2 ± 1.9	1.1 ± 0.4	1.5 ± 0.5
black	550 ± 14	77.7 ± 2.8	2.1 ± 0.5	2.1 ± 0.5
mate	459 ± 12	51.3 ± 1.5	1.3 ± 0.4	1.4 ± 0.4
rooibos	265 ± 8	44.2 ± 1.3	0.5 ± 0.3	0.6 ± 0.2

[a] Mean ± SD of three determinations.

whereas those for retention times were <0.3%. Phenylethanoid glycosides intake (15.1 mg/100 mL of burrito infusion, corresponding to a single cup of tea) appears to be very remarkable in view of their well-known biological activity. They have been reported to have antibiotic activities (*24*), to inhibit platelet aggregation (*25*), and to inhibit leukotriene B4 formation (*26*). Moreover, phenylethanoid glycosides can function as chain-breaking antioxidants to inhibit the peroxidation of mouse liver microsomes (*27*) and the autoxidation of linoleic acid in micelles (*28*).

The sum of all phenolic compound concentrations obtained by quantitative HPLC (75.64 mg/g of lyophilized aqueous extract and 28.29 mg/100 mL cup) was compared with the results obtained from the Folin−Ciocalteu assays, generally considered as the method of choice to estimate total phenol contents in plant extracts (*29*). The total polyphenols in burrito tea, determined according to the Folin−Ciocalteu method and expressed as gallic acid equivalents, was 87.5 mg/g of dry weight, corresponding to 32.72 mg/100 mL cup of tea.

The free radical scavenging activity of the burrito infusion was evaluated and compared with the values of commonly used herbal teas (green and black teas, mate, and rooibos), in the antioxidant (TEAC) and chemiluminescence (CL) assays. The first test measures the relative ability of antioxidant substances to scavenge the radical cation $ABTS^{\bullet+}$ as compared to a standard amount of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The CL assay measures the inhibition of iodophenol-enhanced chemiluminescence by a horseradish peroxidase/perborate/luminol system. Trolox was used as reference antioxidant compound. A similar trend for the antioxidant activity of these teas using both TEAC and CL assays was found (**Tables 2** and **3**).

The comparison between the two methods of measuring antioxidant power was conducted using two approaches: first, a comparison was made by means of Student's *t* test applied to pairs of values, producing the result that there were no significant differences at a 95% confidence level ($t = 1.5157 < t_{95} = 2.2621$). Second, a regression curve was constructed between the values obtained by each method; in this case, the values obtained are shown in **Figure 3**. As can be seen by the coefficient of regression obtained ($R^2 = 0.9362$), the two methods give similar results. In addition, from the slope of the curve (1.1735), it can be concluded that the sensitivity of the two methods is similar, although rather better for the CL method.

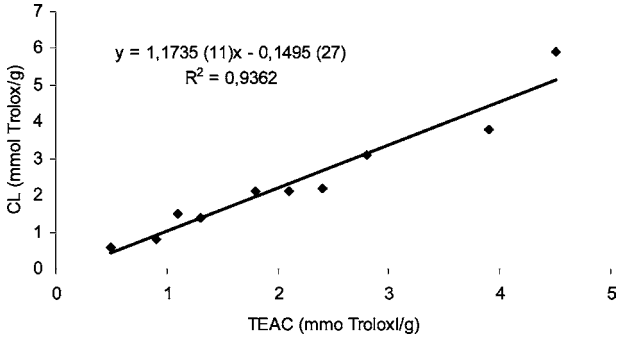

Figure 3. Regression curve for the values of antioxidant power obtained by TEAC and CL methods.

At the same time, the total polyphenols in green and black teas, mate, and rooibos were measured in accordance with the method of Folin−Ciocalteau, to enable a comparison between the measured values of antioxidant activity with the content of the samples in polyphenols.

As shown in **Table 2**, burrito tea showed an antioxidant activity higher than that of the rooibos and provided antioxidant activity similar to mate infusion in both TEAC and CL assays. When compared with different water infusions of *C. sinensis* (green and black teas) obtained in the same conditions, the antioxidant values were 46 and 38% lower in the TEAC assay and 63 and 42% lower in the CL assay, respectively. In any case, the values of antioxidant activity were in good agreement with the total polyphenols content of the examined infusions.

We also compared the total polyphenols contents by Folin and the antioxidant activity of 1 cup of burrito to that of 1 cup of green tea, black tea, mate, and rooibos for evaluate the real antioxidant power offered by drinking a single cup of these teas (**Table 3**). Each beverage was prepared by pouring 100 mL of boiling distilled water on 3 g of dry loose leaves for the same brew time (10 min). The results showed that the antioxidant activity of examined infusions was clearly related to in-cup concentrations of tea components and polyphenols.

The relationship between total phenolic content and antioxidant activity of teas was calculated. The results indicated that when all teas were included in the statistical analysis, there was a positive relationship: TEAC = 0.0312(10) TP − 0.4363(55) ($R^2 = 0.7567$) and CL = 0.0327(0.8) TP − 0.4143(46) ($R^2 = 0.8203$). These data confirmed the role played by the family of phenolic compounds as antioxidants.

From all data reported herein we can state that burrito leaves are rich in antioxidants and that regular intake of its infusion may improve antioxidant status and possibly reduce the risk of chronic disease associated with oxidative stress. Further studies are necessary to confirm if the evident potential of this new tea as an important dietary source of antioxidant power offers a real and useful in vivo improvement of the antioxidant status.

LITERATURE CITED

(1) Benzie, I. F.; Szeto, Y. T.; Strain, J. J.; Tomlinson, B. Consumption of green tea causes rapid increase in plasma antioxidant power in humans. *Nutr. Cancer* **1999**, *34*, 83−87.

(2) Mazzafera, P. Mate drinking: Caffeine and phenolic acid intake. *Food Chem.* **1997**, *60*, 67−71.

(3) Vilegas, W.; Sanommiya, M.; Rastrelli, L.; Pizza, C. Isolation and structure elucidation of two new flavonoid glycosides from the infusion of *Maytenus aquifolium* leaves. Evaluation of the antiulcer activity of the infusion. *J. Agric. Food Chem.* **1999**, *47*, 403−406.

(4) Leite, J. P. V.; Rastrelli, L.; Romussi, G.; Oliveira, A. B.; Vilegas, J. H. Y.; Vilegas, W.; Pizza, C. Isolation and HPLC quantitative analysis of flavonoid glycosides from brazilian beverages (*Maytenus ilicifolia* and *M. aquifolium*). *J. Agric. Food Chem.* **2001**, *49*, 3796−3801.

(5) Bramati, L.; Aquilano, F.; Pietta, P. Unfermented rooibos tea: quantitative characterization of flavonoids by HPLC-UV and determination of the total antioxidant activity. *J. Agric. Food Chem.* **2003**, *51*, 7472−7474.

(6) Baderschneider, B.; Winterhalter, P. Isolation and Characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788−2798.

(7) Machida, K.; Osawa, K. On the flavonoid constituents from the peels of *Citrus hassaku* Hort. ex Tanaka. *Chem. Pharm. Bull.* **1989**, *37*, 1092−1094.

(8) Kaneko, T.; Sakamoto, M.; Ohtani, K.; Ito, A.; Kasai, R.; Yamasaki, K.; Padorina, W. G. Secoiridoid and flavonoid glycosides from *Gonocaryum calleryanum*. *Phytochemistry* **1995**, *39*, 115−120.

(9) Borai, P.; Dayal, R. A flavone glycoside from *Dalbergia stipulacea* leaves. *Phytochemistry* **1993**, *33*, 731−732.

(10) Amaro-Luis, J. M.; Herrera, R.; Luis, J. G. Abietane diterpenoids from *Salvia chinopeplica*. *Phytochemistry* **1998**, *47*, 895−897.

(11) Haraguchi, H.; Hashimoto, K.; Yagi, A. Antioxidative substances in leaves of *Polygonum hydropiper*. *J. Agric. Food Chem.* **1992**, *40*, 1349−1351.

(12) Sticher, O.; Lahloub, M. F. Phenolic glycosides of *Paulownia tomentosa* bark. *Planta Med.* **1982**, *46*, 145−148.

(13) Kawada, T.; Asano, R.; Makino, K.; Sakuno, T. Synthesis of isoacteoside, a dihydroxyphenylethyl glycoside. *J. Wood Sci.* **2002**, *48*, 512−515.

(14) Miyase, T.; Koizumi, A.; Ueno, A.; Noro, T.; Kuroyanagi, M.; Fukushima, S.; Akiyama, Y.; Takemoto, T. Studies on the acyl glycosides from *Leucoseptrum japonicum* (Miq.) Kitamura et Murata. *Chem. Pharm. Bull*. **1982**, *30*, 2732−2737.

(15) Nunez Selles, A.; Velez Castro, H.; Aguero-Aguero, J.; Gonzalez-Gonzalez, J.; Naddeo, F.; De Simone, F.; Rastrelli, L. Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indica* L.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *J. Agric. Food Chem.* **2002**, *50*, 762−766.

(16) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(17) Whitehead T. P.; Thorpe, C. H. G.; Maxwell, S. R. J. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal. Chim. Acta* **1992**, *266*, 265−277.

(18) Agrawal, P. K.; Thakur, R. S.; Mansal, M. C. *C-13 NMR of Flavonoids*; Agrawal, P. K., Ed.; Elsevier: Amsterdam, The Netherlands, 1989; p 95.

(19) Foo, L. Y. Configurationand conformation of dihydroflavonols from *Acacia melanoxylon*. *Phytochemistry* **1987**, *26*, 813−817.

(20) Gaffield, W.; Waiss, A. C. Structural relationships and interconversions of isomeric astilbins. *J. Org. Chem.* **1995**, *40*, 1057−1061.

(21) Souquet, J.-M.; Labarbe, B.; Le Guerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(22) Kasai, R.; Hirono, S.; Chou, W.-H.; Tanaka, O.; Chen, F. Sweet dihydroflavonol rhamnoside from leaves of *Engelhardtia chrysolepis*, a Chinese folk medicine, Hung-qi. *Chem. Pharm. Bull.* **1988**, *36*, 4167−4170.

(23) Nanayakkara, N. P. D.; Hussain, R. A.; Pezzuto, J. M.; Soejarto, D. D.; Kinghorn, A. D. Potential sweetening agents of plant origin. 13. An intensely sweet dihydroflavonol derivative based on a natural product lead compound. *J. Med. Chem.* **1988**, *31*, 1250−1253.

(24) Shoyama, Y.; Matsumoto, M.; Nishioka, I. Four caffeoyl glycosides from callus tissue of *Rehmannia glutinosa*. *Phytochemistry* **1986**, *25*, 1633−1636.

(25) Cano, E.; Veiga, M.; Jimenez, C.; Riguera, R. Pharmacological effects of three phenylpropanoid glycosides from *Mussatia*. *Planta Med.* **1990**, *56*, 24−26.

(26) Kimura, Y.; Okuda, H.; Nishibe, S.; Arichi, S. Effects of caffeoylglycosides on arachidonate metabolism in leukocytes. *Planta Med.* **1987**, *53*, 148−153.

(27) Li, J.; Zheng, R. L.; Liu, Z. M.; Jia, Z. J. Scavenging effects of phenylpropanoid glycosides on superoxide and its antioxidation effect. *Acta Pharmacol. Sin*. **1992**, *13*, 427−430.

(28) Zheng, R. L.; Wang, P. F.; Li, J.; Liu, Z. M.; Jia, Z. J. Inhibition of the autoxidation of linoleic acid by phenylpropanoid glycosides from *Pedicularis* in micelles. *Chem. Phys. Lipids* **1993**, *65*, 151−154.

(29) Scalbert, A. Quantitative methods for the estimation of tannins in plant tissues. In *Plant Polyphenols. Synthesis, Properties and Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: London, U.K., 1992; p 269.

Received for review March 1, 2004. Revised manuscript received June 18, 2004. Accepted June 22, 2004.

JF040100E