JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

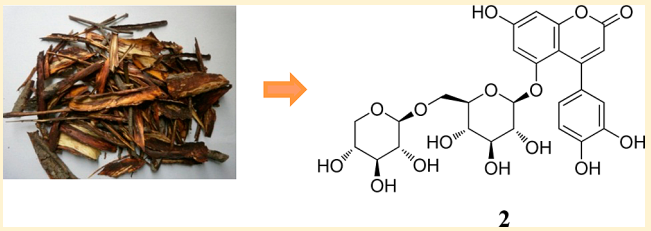
Development of a UHPLC-PDA Method for the Simultaneous Quantification of 4-Phenylcoumarins and Chlorogenic Acid in *Exostema caribaeum* Stem Bark

Araceli Pérez-Vásquez,[†] Erika Castillejos-Ramírez,[†] Sol Cristians, and Rachel Mata*

Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, México D.F., 04510, México

Supporting Information

ABSTRACT: Potential toxic effects in mice of an infusion prepared from the stem bark of *Exostema caribaeum* was assessed by means of the Lorke procedure. The preparation was not found to be toxic, with the LD_{50} value estimated to be more than 5 g/kg. This preparation at 100, 300, and 500 mg/kg also caused a significant hypoglycemic effect and a reduction in the postprandial glycemia peak in both normal and nicotinamide/streptozotocin (NA/STZ)-diabetic mice in an oral sucrose tolerance test. Phytochemical analysis of the infusion revealed that the major active principles are 4-phenylcoumarins (**2**–**8**) and chlorogenic acid (**1**). During this process, a new 4-phenylcoumarin was isolated along with several known analogues. The structure of the new compound was established as 5-*O*-[*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranosyl]-7,3′,4′-trihydroxy-4-phenylcoumarin (**2**) by spectroscopic means. A simple, efficient, fast, and reliable UHPLC-PDA analytical method for quantifying 4-phenylcoumarins and chlorogenic acid (**1**) was developed and validated. Parameters assessed for the method validation were selectivity, linearity, the limits of detection (LOD) and quantification (LOQ), precision, and accuracy. It was found that all calibration curves showed good linearity ($R^2 > 0.9931$), within the range of concentrations tested.



Exostema caribaeum (Jacquin) Roemer & Schultes (Rubiaceae), commonly known as "copalchi" or "copalche", among other names, belongs to the so-called "copalchi" complex, which includes several Rubiaceae species and a few Euphorbiaceae species. The characteristics of these plants are their extremely bitter stem bark and their use as antidiabetic and antimalarial agents. The stem bark of *E. caribaeum* is also valued today in Mexico for treating gastrointestinal ailments and dengue fever.[1−3] Previous phytochemical studies of *E. caribaeum* stem bark resulted in the isolation and characterization of nine 4-phenylcoumarins including compounds **5**, **6**, and **8**.[4−6] Some of these 4-phenylcoumarins as well as the plant stem bark methanol extract showed hypoglycemic activity when tested in rats using well-known pharmacological procedures.[7,8] The organic extract also displayed moderate toxicity to mice.[9] Finally, Noster and Kraus demonstrated that different extracts prepared from *E. caribaeum* stem bark possess moderate in vitro anti-*Plasmodium falciparum* activity.[10]

A major problem with medicinal plants that are commercialized widely in Mexico, including *E caribaeum*, is the adulteration or substitution of herbal drugs with less efficacious if not dangerous products. As a consequence, the prevalence of low-quality raw material reduces the potential for clinical efficacy and raises the risk of adverse reactions. Furthermore, in the context of the global market in medicinal plants, it is very important to establish quality-control procedures for the most widely internationally distributed crude drugs of Mexico. With this in mind, the present study was undertaken to develop a composition test useful for quantifying the active principles of *E. caribaeum* stem bark. For this investigation, an ultra-high-performance liquid chromatography−photodiode array (UHPLC-PDA) analytical method was established and validated for the simultaneous quantification of chlorogenic acid (**1**) and seven 4-phenylcoumarins (**2**–**8**) in two batches of the stem bark of *E. caribaeum*. The potential toxicity and antidiabetic properties of the infusion of this plant part, not previously described, were also assessed.

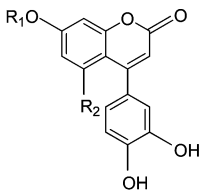
RESULTS AND DISCUSSION

Pharmacological and Toxicological Testing. The potential toxic effects in mice of the *E. caribaeum* stem bark aqueous extract was assessed by means of the Lorke procedure.[11] After 14 days, the treated mice did not exhibit any change in their behavior or body weight or in the macroscopic morphology of the heart, liver, kidney, or lung. The LD_{50} value was more than 5 g/kg. Thus, when compared with the organic-soluble extract (LD_{50} of 0.7 g/kg),[9] the infusion can be regarded as nontoxic according to the Lorke criteria.[11] These results endorsed the preclinical safety testing of this traditional preparation.

Special Issue: Special Issue in Honor of Otto Sticher

Received: September 25, 2013

dx.doi.org/10.1021/np400785z | *J. Nat. Prod.* XXXX, XXX, XXX−XXX



	R_1	R_2
2	H	5-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]
3	H	5-*O*-β-D-glucopyranosyl
4	CH_3	5-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]
5	CH_3	5-*O*-β-D-galactopyranosyl
6	CH_3	5-*O*-β-D-glucopyranosyl
7	H	6"-*O*-acetyl-5-*O*-β-D-glucopyranosyl
8	CH_3	6"-*O*-acetyl-5-*O*-β-D-galactopyranosyl

The antidiabetic activity of the infusion was determined using a known method.[12] As shown in Figure S7 and Table S1 (Supporting Information), the aqueous extract of *E. caribaeum* stem bark (500 mg/kg) caused a significant hypoglycemic effect in nicotinamide/streptozotocin (NA/STZ)-diabetic mice in comparison to the vehicle-treated group ($p < 0.05$). The effect of this aqueous extract (500 mg/kg) was similar to that exerted by glibenclamide (10 mg/kg) when used as the positive control. In the oral sucrose tolerance test, this aqueous extract (100, 300, and 500 mg/kg) caused a significant decrease in the postprandial glycemia peak in both normal and NA/STZ-diabetic mice when compared with the vehicle-treated group ($p < 0.05$) (Figure S8 and Tables S2 and S3, Supporting Information).

Compound Isolation and Identification. For validation of analytical methods, it is mandatory to employ reference standards. However, due to the lack of commercially available analytical 4-phenylcoumarin standards, in this study the isolation of suitable standards from the stem bark of *E. caribaeum* was performed initially. For this purpose, an aqueous extract was subjected to conventional phytochemical isolation. This process led to the purification of chlorogenic acid (**1**), a well-known hypoglycemic agent,[13] and seven 4-phenylcoumarins, including the new natural product **2**. Compounds **1**, **3**, **4**, and **7** are described for the first time from this plant.

The structure of compound **2** was elucidated using spectroscopic and spectrometric methods including one- and two-dimensional NMR techniques (Table 1). The molecular formula was established as $C_{26}H_{28}O_{15}$ by HRESIMS. The NMR spectra (CD_3OD, Table 1) were very similar to those of the 4-phenylcoumarins **3**–**8**. The NMR spectra of compounds **2** and **4** were almost identical, with the only difference between them being the absence of the signals for the methoxy group at C-7 in the spectra of compound **2**. Thus, the aromatic region of the ^1H NMR (Table 1) spectrum of **2** showed signals due to two *meta*-coupled hydrogens (H-6 and H-8), an ABX system formed by H-2, H-5, and H-6, and a typical singlet (δ_H 5.86) for the proton of the α-pyrone (H-3) unit. This spectrum showed also resonances for a disaccharide unit. The ^{13}C NMR resonances (Table 1) of compound **2** were assigned through the analysis of HSQC and HMBC experiments and indicated clearly the presence of one xylopyranosyl unit and one glucopyranosyl moiety in the molecule. The HMBC correlations of the anomeric hydrogen of glucose (H-1″; δ_H 4.73) to C-5 (δ_C 156.0) as well as the upfield shifts of H-1″ and H-2″, due to the protection exerted by the phenyl group at C-4 of the phenylcoumarin core, were consistent with the attachment of the glucose moiety at the C-5 phenolic group. On the other hand, the HMBC correlation between the anomeric hydrogen of xylose (H-1‴; δ_H 4.31) and C-6″ (δ_C 68.2) was in agreement with the 1→6 linkage of the two monosacharides. The nature of the sugars was confirmed by acid hydrolysis of **2** with 2 N HCl to yield an aglycone, together with D-xylose and D-glucose identified by GC-MS analysis of their trimethylsilyl derivatives.[14]

Table 1. NMR Spectroscopic Data (125 MHz ^{13}C, 500 MHz ^1H, CD_3OD) of the 4-Phenylcoumarin 2

position	δ_C	δ_H (*J* in Hz)	HMBC
2	161.9		
3	111.0	5.86 (s)	C-2, C-4a, C-1′
4	157.0		
4a	103.1		
5	156.0		
6	99.4	6.64 (d, 1.8)	C-4a, C-5, C-7, C-8
7	162.2		
8	97.3	6.47 (d, 1.8)	C-4a, C-6, C-7, C-8a
8a	156.8		
1′	131.6		
2′	114.9	6.80 (d, 1.8)	C-4, C-1′, C-3′, C-4′
3′	144.2		
4′	145.6		
5′	114.3	6.80 (d, 8.0)	C-1′, C-3′
6′	119.7	6.71 (dd, 7.8, 2.2)	C-4, C-4′
1″	100.2	4.73 (d, 7.5)	C-5
2″	73.3	2.71 (dd, 8.6, 7.4)	C-3″, C-1″
3″	76.1	3.26	
4″	69.6	3.21 (m)	C-3″
5″	76.4	3.50 (m)	
6″	68.2	4.05 (brd, 10.9)	
		3.73 (dd, 11.3, 6.5)	
1‴	103.8	4.31 (d, 7.4)	C-6″
2‴	73.7	3.21 (m)	C-1‴
3‴	76.2	3.26	C-4‴, C-2‴
4‴	69.8	3.46 (ddd, 10.1, 9.9, 5.3)	
5‴	65.5	3.82 (dd, 11.5, 5.3)	
		3.08 (dd, 11.5, 10.3)	

The remaining 4-phenylcoumarins were identified by comparison of their spectroscopic data with those previously reported,[5,6,15−17] and chlorogenic acid (**1**) was identified by comparison with an authentic sample obtained commercially.

Optimization of Extraction Time. For the plant sample investigated, an infusion was chosen as the extraction method by considering the mode in which the crude drug is used in traditional medicine. In addition, no attempt was made to use an organic solvent, because it was demonstrated previously that such extracts are toxic to mice.[9] Although the infusion is usually prepared by treatment with boiling water for 30 min, in this study the influence of time on the extraction efficiency was monitored at 10, 20, 30, and 40 min. In general, the results indicated that the active principles are extracted with the best yield within 30 min (Figure 1).

UHPLC Method Validation and Application. Ultra-high-performance liquid chromatography was selected for the quality control of *E. caribaeum* stem bark, because this relatively new technique offers a potential decrease in time of analysis and reduced solvent consumption.[18] Several mobile phases and two

dx.doi.org/10.1021/np400785z | J. Nat. Prod. XXXX, XXX, XXX−XXX

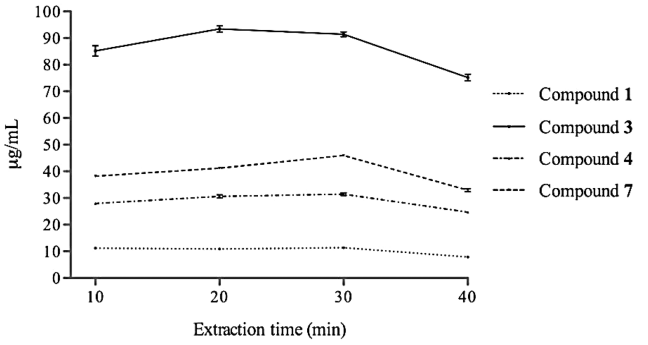


Figure 1. Influence of time on the extraction efficiency of 4-phenylcoumarins **3**, **4**, and **7** and chlorogenic acid (**1**).

stationary phases (UHPLC BEH Shield-C_{18} and UHPLC-BEH C_8) were examined. The former stationary phase was regarded as the more appropriate. Acetonitrile and water were selected for the mobile phase. Since chromatographic separation of analytes was much more efficient after acidification of the mobile phase, two different acids were tested: trifluoroacetic acid (TFA) and formic acid. The optimal UHPLC separation conditions were achieved with a mixture of water containing 0.1% formic acid and acetonitrile. To achieve good sensitivity and accuracy for quantification, different UV λ_{max} values of the four standards were considered. Thus, the PDA detection wavelength (λ) was set at 327 nm for the 4-phenylcoumarins and 248 nm for chlorogenic acid (**1**). Under these optimized conditions, effective baseline resolution was achieved for compounds **1**–**8**, and coelution was detected only for 4-phenylcoumarins **5** and **6**, while the rest of components were separated appropriately. A representative chromatogram of the major components in the *E. caribaeum* stem bark infusion is illustrated in Figure 2. Compounds **1**–**8** were selected for

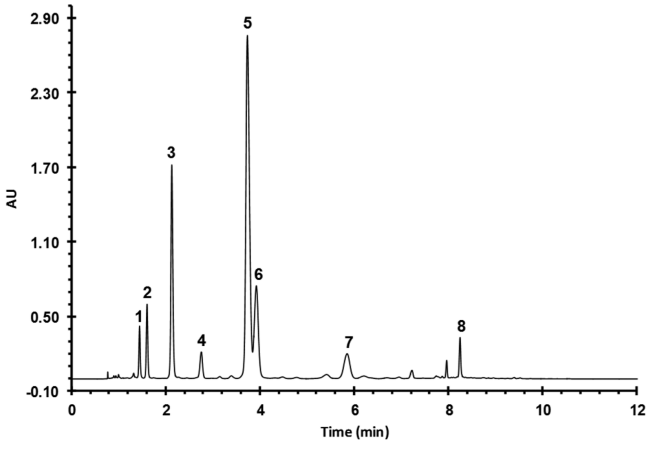


Figure 2. UHPLC-PDA chromatogram of *E. caribaeum* stem bark aqueous extract (EC-2) under optimized conditions; detection wavelength 327 nm. For chromatographic conditions, see the Experimental Section.

quantitative determination because some of these possess hypoglycemic effects,[8] and several were obtained in good yields from *E. caribaeum* and other species.[5,6,8]

For assay validation,[18] compounds **1**, **3**, **4**, and **7** were included as standards and were selected due to the fact that they are soluble in the mobile phase employed and were obtained in good yields. The curve for compound **3** was employed to quantify compounds **5** and **6** simultaneously, the curve of 4-phenylcoumarin **7** was used for **2**, and the curve of compound **4** was employed to quantify **8** considering that they have similar structural properties and their UV absorption profiles are identical. Calibration curves were constructed by plotting the peak areas (y) against the corresponding standard concentration (x, μg/mL), using a linear least-squares fit regression model. The data in Table 2 indicate that the calibration curves showed good linearity with a high correlation coefficient within the concentration ranges tested: 10 to 150 μg/mL for **1** and **7**, 50 to 350 μg/mL for **3**, and 10 to 300 μg/mL for **4**. In all cases, correlation coefficient (R^2) values were found to exceed 0.99.

The limit of detection and limit of quantification values were 0.13 μg/mL and 0.38 μg/mL for compound **1**, 0.34 μg/mL and 1.05 μg/mL for compound **3**, 0.03 μg/mL and 0.09 μg/mL for compound **4**, and 0.08 μg/mL and 0.23 μg/mL for compound **7**. The precision of the method was determined, and the relative standard deviations (%RSD) for intraday and interday repeatability values were less than 1.86% and 1.91%, respectively (Table 2). The recovery rates of all analytes ranged from 100.51% to 99.94%, which demonstrated sufficient accuracy of the developed method. The results of the stability study indicated that the samples are very stable in water solution and the % RSDs of the four analytes were all less than 2.0%.

The validated method was applied successfully for the quantification of 4-phenylcoumarins (**2**–**8**) and chlorogenic acid (**1**) in two different geographical batches (EC-1 and EC-2) of the crude drug. The results are presented in Table 3 and show that the content of marker active compounds was similar in the two batches. In both cases, the 4-phenylcoumarins **3**, **5**, and **6** were found in higher amounts, whereas the concentrations of chlorogenic acid (**1**) and 4-phenylcoumarins **2**, **4**, **7**, and **8** were lower.

In summary, phytochemical analysis of an infusion of *E caribaeum* stem bark led to the isolation of a new 4-phenylcoumarin (**2**). A simple, efficient, fast, and reliable UHPLC-PDA analytical method for quantifying 4-phenylcoumarins and chlorogenic acid (**1**) was developed and validated. This method was applied successfully to quantify these components in two different batches of the plant.

EXPERIMENTAL SECTION

General Experimental Procedures. Infrared spectra were obtained in the range 4000–400 cm^{-1} using a Perkin-Elmer Spectrum 400 FT-IR spectrometer (Perkin-Elmer, San Jose, CA, USA). ^1H and ^{13}C NMR spectra were acquired on a JEOL ECA-500 NMR spectrometer (JEOL, Tokyo, Japan) operating at 500 MHz for ^1H and 125 MHz for ^{13}C, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as internal standard. Mass spectra were recorded on a Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Rochester, NY, USA) using electrospray ionization (ESI).

Reagents and solvents were analytical or HPLC grade; chlorogenic acid (**1**), acarbose, glybenclamide, nicotinamide, streptozotocin, sucrose, and Tween 80 were purchased from Sigma-Aldrich (Toluca, Mexico); HPLC grade water, methanol, acetonitrile, formic acid (88.0%), and ethyl acetate were purchased from Tecrom (Mexico City, Mexico). The purity of the isolated compounds used as standards were determined by UHPLC analysis using a normalization method and were calculated to be ≥97%. Open column chromatography was carried out on silica gel 60 (Merck, 70–230 mesh; Darmstadt, Germany) or Sephadex LH-20 (Sigma-Aldrich Quimica, Toluca, México). All separations with semipreparative RP-HPLC were

dx.doi.org/10.1021/np400785z | J. Nat. Prod. XXXX, XXX, XXX–XXX

Table 2. Validation Report of the Method for Simultaneous Determination of Selected Constituents in the Stem Bark of *E. caribaeum*

compound	linear range (μg/mL)	calibration equation	R^{2a}	LOD (μg/mL)	LOQ (μg/mL)	precision		recovery (% mean)	stability (% RSD)
						intraday (% RSD)	interday (% RSD)		
1	10−150	$y = 58\,101x + 228\,029$	0.9955	0.13	0.38	0.63	1.20	99.98	0.60
3	50−350	$y = 43\,610x + 747\,088$	0.9931	0.34	1.05	0.36	0.62	99.94	0.13
4	10−300	$y = 27\,482x + 7413.7$	0.9998	0.03	0.09	1.86	1.91	100.07	0.32
7	10−150	$y = 40\,569x + 18\,862$	0.9987	0.08	0.23	0.63	1.46	100.51	0.11

[a] R^2 correlation coefficient for five data points in the calibration curves ($n = 6$).

Table 3. Contents (mg/g) of Eight Major Compounds in Two Batches of the Stem Bark of *E. caribaeum* ($n = 3$)

compound	t_R (min)	content (mg/g) (mean ± sd)	
		EC-1	EC-2
1	1.45	11.46 ± 0.29	8.82 ± 0.07
2[a]	1.61	16.62 ± 0.35	27.5 ± 0.78
3	2.12	100.24 ± 1.63	83.88 ± 1.39
4	2.76	21.09 ± 0.17	28.25 ± 0.27
5 + **6**[b]	3.74/3.80	359.41 ± 5.58	350.64 ± 5.33
7	5.85	43.37 ± 1.12	35.75 ± 0.73
8[c]	8.25	27.70 ± 0.59	25.67 ± 0.47

[a] Quantified as **7**. [b] Quantified as **3**. [c] Quantified as **4**.

performed using a Waters Dual HPLC system equipped with a quaternary pump (Waters, model 600), a UV/vis dual detector (model 2487), and a Phenomenex Nucleosil C_{18} column (250 × 10 mm, i.d., 5 μm). Elution was conducted in the isocratic mode with acetonitrile−water (80:20) at a flow rate of 4.0 mL/min. The UV detector was set at a monitoring wavelength of 327 nm.

Quantitative analysis was performed on a Waters Acquity UHPLC-H class system (Waters Co., Milford, MA, USA) equipped with a photodiode array detector, sample manager, and quaternary solvent manager. System control, data collection, and data processing were accomplished using Waters Empower 3 chromatography software.

Plant Material. A first batch of the stem bark of *E. caribaeum* was collected at Tuzantlan (EC-1), and a second was collected at Temalac (EC-2), Atenango del Río Municipality, Guerrero State, Mexico, both in July 2010. The plant specimens were authenticated by Dr. Sol Cristians and deposited at the Faculty of Sciences Herbarium UNAM (vouchers: FCME 131339 and 131340, respectively). Batch 2 was used to validate the analytical method and for isolating chlorogenic acid (**1**) and seven 4-phenylcoumarins (**2**−**8**).

Extraction and Isolation. Thirty grams of fine powder (particle size <2000 μm, mesh size, 2 mm) of the dried stem bark of *E. caribaeum* was extracted with boiled distilled water (5000 mL) for 30 min. After extraction, the resulting infusion was filtered through filter paper (Whatman No. 1) to remove macroparticles and was concentrated under reduced pressure to obtain 10.74 g of dried aqueous extract.

In order to isolate the 4-phenylcoumarins, 7.11 g of the aqueous extract was dissolved in 30 mL of MeOH, and the soluble fraction was filtered, evaporated (6.41 g), and separated by silica gel column chromatography, eluting with hexane−EtOAc (100:0 → 0:100) followed by EtOAc−MeOH (100:0 → 50:50), to obtain nine fractions (F1−F9). Fraction F2 (74 mg) was submitted to repeated washing with MeOH to give 5-*O*-(6″-acetyl-β-D-galactopyranosyl)-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**8**, 18 mg). The remaining solution generated was filtered and evaporated to obtain 52 mg of a methanol fraction (F2-M), which was chromatographed on a Sephadex LH-20 column (CH_2Cl_2−MeOH, 5:95), to yield eight fractions (F2-M1−F2-M8). From fraction F2-M6, 9 mg of 5-*O*-(6″-acetyl-β-D-glucopyranosyl)-7,3′,4′-trihydroxy-4-phenylcoumarin (**7**) was precipitated. Fraction F4 (106 mg) was washed with MeOH to obtain 5-*O*-β-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**6**, 61 mg) as a residual solid; the MeOH-soluble fraction was purified using semipreparative RP-HPLC to obtain 5-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxy-4-phenylcoumarin (**3**, 16 mg). From fraction F6 (132 mg) precipitated 72 mg of 5-*O*-β-D-galactopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**5**). Fraction F8 (58 mg) was subjected to semipreparative RP-HPLC to obtain 5-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]-7,3′,4′-trihydroxy-4-phenylcoumarin (**2**, 15 mg) and 5-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**4**, 16 mg). Finally, RP-HPLC of fraction F9 yielded 16 mg of chlorogenic acid (**1**).

Compound ***2****:* brown solid; mp >320 °C; $[\alpha]^{25}_D$ −49 (*c* 1.0, MeOH); UV λ_{max} (log ε) 356 (3.98), 328 (4.05) nm; IR ν_{max} 3279, 2906, 1684, 1598, 1357, 1037 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive-ion mode) *m*/*z* 581.1482 $[M + H]^+$ (calcd for $C_{26}H_{28}O_{15}$, 581.1497).

Acid Hydrolysis of Compound 2 and Sugar Analysis. A 4 mg amount of **2** in 5 mL of 2 N HCl was refluxed for 1 h. Then, the reaction mixture was extracted with EtOAc (3 × 8 mL). The aqueous phase was neutralized with 1 N KOH, extracted with *n*-butanol (8 mL), washed with H_2O (2 × 4 mL), and concentrated to yield a colorless residue. The mixture was treated with chlorotrimethylsilane (Sigma Sil-A) and then analyzed by GC-MS,[14] using the following conditions: HP-5 MS column (30 m × 0.25 mm × 0.25 μm); He at a constant flow rate of 1.2 mL/min; the GC column temperature program applied was 65 °C isothermal for 2 min, linear gradient to 150 at 6 °C/min for 5 min, followed by an isothermal hold at 300 °C for 15 min. The retention times for TMS derivatives of common sugars were used as standards for GC identification; only D-xylose (t_R 33 min) and D-glucose (t_R 45.0) were detected.

Preparation of Standard and Sample Solutions. The accurately weighed powder (12 mg) of the dried aqueous extract of *E. caribaeum* stem bark was redissolved in 10 mL of water, and then the solution was filtered through a 0.2 μm membrane filter before being injected into a UHPLC setup for analysis. For the specific calibration plot, a stock solution of 1.0 mg/mL of each standard was prepared in H_2O, stored at 4 °C, and brought to room temperature before use. Five working standard solutions were prepared by serial dilution of the stock solution with H_2O, in appropriate concentrations.

Chromatographic Conditions for UHPLC-PDA Analysis. Separations were performed at 35 °C using an Acquity UHPLC BEH Shield-RP18 column (100 × 2.1 mm, 1.7 μm) at a flow rate of 0.4 mL/min. The mobile phase consisted of acetonitrile (A) and water containing 0.1% (v/v) formic acid (B). The following gradient elution program was used: 16% A for 0−5 min, 16−30% A for 5−7 min, 30% A for 7−10 min, and 30−16% A for 10−12 min. The injection volume was 10 μL. The UV detector was set at a monitoring wavelength of 327 nm.

Method Validation. The UHPLC-PDA method was validated according to the International Conference on Harmonization (ICH) Guidelines and included a determination of selectivity, linearity, precision, accuracy, LOD, and LOQ.[19] The determination of peak purity to improve the selectivity was assessed using PDA detection wavelengths of 327 nm for 4-phenylcoumarins **3**, **4**, and **7** and 248 nm for **1**. LOD and LOQ were determined based on the standard deviation (σ) of the response and the slope (*S*) [LOD = 3.3 × σ/*S* and LOQ = 10 × σ/*S*, respectively]. The precision was evaluated using

dx.doi.org/10.1021/np400785z | J. Nat. Prod. XXXX, XXX, XXX−XXX

repeatability (intraday) and intermediate precision (interday). Intraday and interday variations were established using six replicates within the same day and three consecutive days (a total of 18 determinations), respectively. Recovery experiments were performed in order to study the accuracy of the method. The samples were spiked with known amounts of the standards, at different concentrations levels (low, medium, and high): **1** (10, 50, and 150 μg/mL), **3** (50, 150, and 350 μg/mL), **4** (10, 100, and 300 μg/mL), and **7** (10, 60, and 150 μg/mL). The average recoveries were calculated by the following formula: recovery (%) = [(amount found − original amount)/amount spiked] × 100%. The stability was tested by analyzing the sample solution at different time points (0, 12, 24, and 48 h), and peak areas of all standards were recorded and compared. The % RSD ≤ 2.0 was taken as a measure of precision and stability.

Acute Toxicity. The toxicity studies and antidiabetic assays were performed on male ICR mice (body weight range, 20−25 g). The animals were purchased from Centro UNAM-Harlan (Mexico City, Mexico). Mice were housed in a climate- and light-controlled room with a 12 h light/dark cycle and maintained on a standard pellet diet and water ad libitum. Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. The Institutional Committee for Care and Use of Laboratory Animals (FQ/CICUAL/021/11) of Facultad de Química, UNAM, approved the experimental protocols. The test was conducted in two phases, as described previously.[11]

Acute Hypoglycemic and Oral Sucrose Tolerance Tests. The aqueous extracts at different doses (100, 300, and 500 mg/kg bw) and control drugs (glibenclamide 10 mg/kg bw; acarbose, 5 mg/kg bw) were suspended in the vehicle (Tween 80, NaCl 0.9%). Sucrose (2 g/kg) was used for the carbohydrate tolerance test. The control mice group received only the vehicle. Treatments were orally given in all cases. Blood samples were collected from caudal vein by means of a small incision in the end of the tail. Blood glucose levels (mg/dL) were estimated by a commercial glucometer (One Touch Ultra, Milpitas, CA, USA). The hypoglycemic test and the oral sucrose tolerance test were conducted as previously described.[12] Percentage variation of glycemia for each group was calculated with respect to initial (0 h) level according to the following: % variation of glycemia = $[(G_i - G_t)/G_i] \times 100\%$, where G_i is initial glycemia values and G_t is the glycemia value after sample administration. Both studies were performed in normoglycemic and NA/STZ-diabetic mice.[12]

Diabetes was induced by a single ip (intraperitoneal) injection of freshly prepared STZ (120 mg/kg bw) as previously described.[20] Mice with blood glucose levels higher than 200 mg/dL were considered diabetic and included in the study.[12]

Statistical Analysis. Data are expressed as the means ± SEM for the number of animals in each group (n = 6). Analysis of variance (ANOVA) was used to analyze the changes in blood glucose and other parameters. Dunnett range posthoc comparisons were used to determine the source of significant differences where appropriate; $p < 0.05$ was considered statistically significant. Prisma Graph-Pad (version 4.0) software was used for statistics.

■ ASSOCIATED CONTENT

Supporting Information

UV and selected NMR spectra for compound **2**. Full tables and figures of hypoglycemic and antihyperglycemic effects of the infusion of the plant material. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 525-556225289. Fax: 525-556225329. E-mail: rachel@unam.mx.

Author Contributions

†A. Pérez-Vásquez and E. Castillejos-Ramírez contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from Consejo Nacional de Ciencia y Tecnología (CONACyT, Proyecto99395) and DGAPA-UNAM PAPIIT *IN*212913. The technical assistance of I. Rivero-Cruz, R. I. del Villar, M. Gutiérrez, G. Duarte-Lisci, and R. del Carmen-Lezama is also recognized. E.C. and S.C. acknowledge scholarships awarded by CONACyT to carry out graduate studies.

■ DEDICATION

Dedicated to Prof. Dr. Otto Sticher, of ETH-Zurich, Zurich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry.

■ REFERENCES

(1) Andrade-Cetto, A.; Heinrich, M. *J. Ethnopharmacol.* **2005**, *99*, 325−348.
(2) Martínez, M. *Las Plantas Medicinales de México*; Ediciones Botas: México City, 1989; p 87.
(3) Linares, E.; Bye, R. A. *J. Ethnopharmacol.* **1987**, *19*, 153−183.
(4) Sánchez-Viesca, F. *Phytochemistry* **1969**, *8*, 1821−1823.
(5) Mata, R.; Calzada, F.; García, M.; Reguero, M. *J. Nat. Prod.* **1987**, *50*, 866−870.
(6) Mata, R.; Calzada, F.; García, M. *J. Nat. Prod.* **1988**, *51*, 851−856.
(7) Pinto, A.; Capasso, A.; Sorrentino, L. *Arzneimittelforschung* **1997**, *47*, 829−833.
(8) Guerrero-Analco, J.; Medina-Campos, O.; Brindis, F.; Bye, R.; Pedraza-Chaverri, J.; Navarrete, A.; Mata, R. *Phytochemistry* **2007**, *68*, 2087−2095.
(9) Déciga-Campos, M.; Guerrero-Analco, J. A.; Quijano, L.; Mata, R. *Pharmacol., Biochem. Behav.* **2006**, *83*, 342−348.
(10) Noster, S.; Kraus, L. J. *Planta Med.* **1990**, *56*, 63−65.
(11) Lorke, D. *Arch. Toxicol.* **1983**, *54*, 275−287.
(12) Escandón-Rivera, S.; González-Andrade, M.; Bye, R.; Linares, E.; Navarrete, A.; Mata, R. *J. Nat. Prod.* **2012**, *75*, 968−974.
(13) Rodriguez de Sotillo, D. V.; Hadley, M. *J. Nutr. Biochem.* **2002**, *13*, 717−726.
(14) Cruz-Morales, S.; Castañeda-Gómez, J.; Figueroa-González, G.; Mendoza-García, A. D.; Lorence, A.; Pereda-Miranda, R. *J. Nat. Prod.* **2012**, *75*, 1603−1611.
(15) Aquino, R.; D'Agosino, M.; De Simone, F.; Pizza, C. *Phytochemistry* **1988**, *27*, 1827−1830.
(16) Mata, R.; Del Rayo-Camacho, M.; Mendoza, S.; Cruz, M. C. *Phytochemistry* **1992**, *31*, 3199−3201.
(17) Cristians, S.; Guerrero-Analco, J. A.; Pérez-Vásquez, A.; Palacios-Espinosa, F.; Ciangherotti, C.; Bye, R.; Mata, R. *J. Nat. Prod.* **2009**, *72*, 408−413.
(18) Nováková, L.; Matysová, L.; Solich, P. *Talanta* **2006**, *68*, 908−918.
(19) ICH. Harmonized Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1). Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (accessed on 03.06.2012).
(20) Masiello, P.; Broca, C.; Gross, R.; Roye, M.; Manteghetti, M.; Hillaire-Buys, D.; Novelli, M.; Ribes, G. *Diabetes* **1998**, *47*, 224−229.