JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 2853–2858 2853

Phenylpropanoid Glycosides from *Tynanthus panurensis*: Characterization and LC-MS Quantitative Analysis

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A phytochemical analysis of the methanol extract of *Tynanthus panurensis* bark led to the isolation of one new phenylpropanoid glycoside, eugenol-*O*-[β-D-xylopyranosyl-(1→5)-*O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranoside], the known verbascoside, isoverbascoside, and leucosceptoside, along with the known flavonoid apigenin 8-*C*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside], namely, katchimoside. Their structures were established by NMR and ESIMS experiments. Additionally, a quantitative study of the phenylpropanoid glycosides fraction of *T. panurensis* bark and of the hydroalcoholic extract prepared according to the traditional recipe was performed by combining high-performance liquid chromatography diode array detection with positive electrospray ionization tandem mass spectrometry. The new eugenol derivate was found to be the most abundant phenylpropanoid glycoside in both dried bark (19.5 mg/g) and hydroalcoholic extract (0.24 mg/mL). The antioxidant activity of all the isolated compounds and of the methanol and hydroalcoholic extract of the bark was determined by measuring the free radical scavenging effects using the Trolox equivalent antioxidant capacity method. The traditional hydroalcoholic extract showed a moderate activity.

KEYWORDS: Phenylpropanoid glycosides; *Tynanthus panurensis*; LC-MS; ESIMS

INTRODUCTION

"Clavo huasca" [*Tynanthus panurensis* (Bur.) Sandw.; Bignoniaceae] is a large woody vine that grows in the Amazon rainforest. In the Peruvian Amazon, *T. panurensis* has been traditionally used as an aphrodisiac, tonic, and energizer as well as an analgesic and in the treatment of rheumatism and diabetes. Nowadays, a liquor prepared by macerating the bark in "aguardiente" (sugar cane liquor) is largely used by the local people (*1*). More recently, due to its fame as an aphrodisiac and energizer, the bark of clavo huasca has become more popular and can be found on the international market as dried powder, tincture, alcoholic, and aqueous−alcoholic preparations. However to the authors' knowledge, apart from a previous study that reported the occurrence of eugenol in the bark oil (*2*), there are no data in the literature concerning the chemical composition and pharmacological properties of *T. panurensis*.

A phytochemical investigation of the methanol extract of the bark of *T. panurensis* led to the isolation of phenylpropanoid glycosides and a flavonoid. The structures of these compounds were elucidated by extensive spectroscopic methods including 1D and 2D NMR experiments as well as ESIMS analysis.

The lack of analytical methods for the quality control of *T. panurensis* bark and its products, and the interesting pharmacological activities of phenylpropanoid glycosides (*3−5*), prompted us to carry out a method based on liquid chromatography (LC) diode array detection (DAD) coupled to positive electrospray ionization (ESI) tandem mass spectrometry (MS/MS) for the separation and identification of the phenylpropanoid fraction of the bark of *T. panurensis*. In particular, this fast, reliable, and accurate method was applied to the quantitative analysis of a hydroalcoholic extract of *T. panurensis* bark prepared according to the traditional recipe.

Moreover, the antioxidant activity of all the isolated compounds, the methanol extract of the bark, and the traditional hydroalcoholic extract was determined by measuring the free radical scavenging effects using the Trolox equivalent antioxidant capacity (TEAC) method.

MATERIALS AND METHODS

Plant Material. *T. panurensis* (Bur) Sandw. (Bignoniaceae) barks were collected in Ucayali, Peru, in Febraury 2001. Herbarium voucher specimens were prepared, identified, and deposited at the Herbarium of the Museum of Natural History of the Universidad Nacional Mayor de San Marcos (Lima, Peru).

Instrumentation. Optical rotations were measured on a Jasco DIP 1000 polarimeter (Jasco Inc., Easton, MD). IR measurements were obtained on a Bruker IFS-48 spectrometer.

NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) at 300 K. All of the 2D NMR spectra were performed on a data matrix 512 × 1024 and acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance; time proportional phase

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10.1021/jf0479867 CCC: $30.25 © 2005 American Chemical Society
Published on Web 03/19/2005

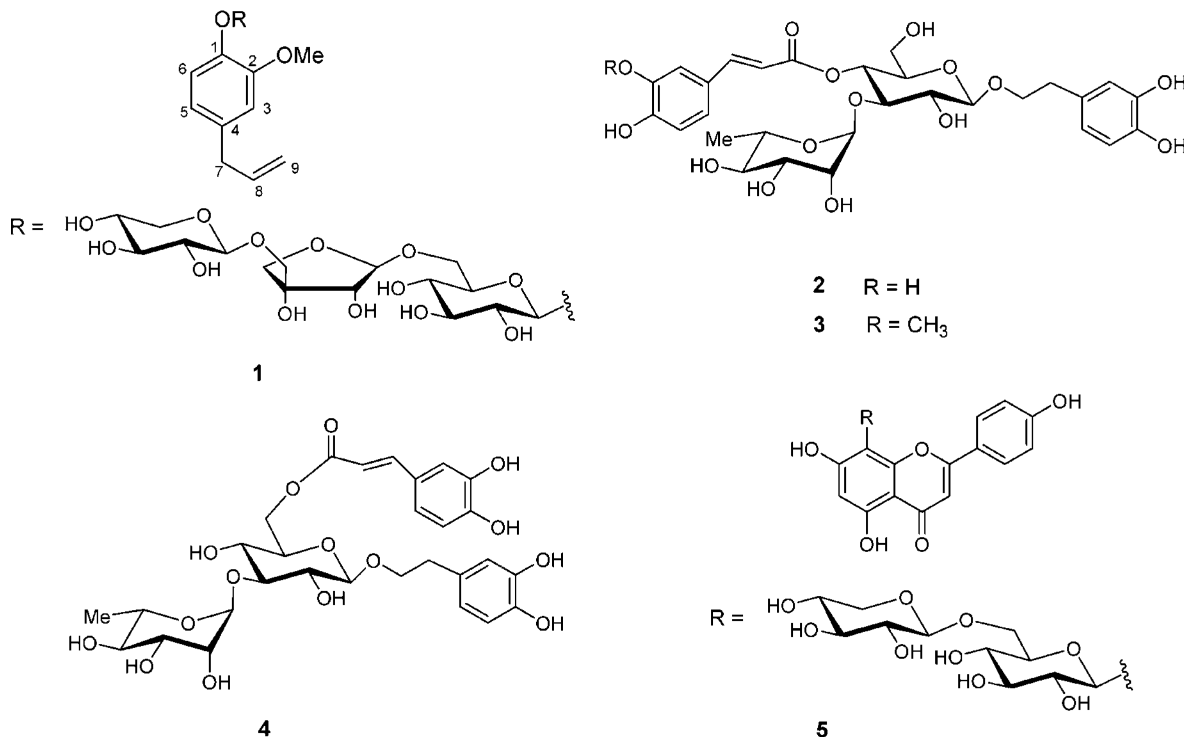

Figure 1. Compounds isolated from the bark of *T. panurensis*.

increment (TPPI) was used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF−COSY, HSQC, and HMBC spectra. 1D TOCSY selective excitation spectra were acquired using waveform generator-based Gauss-shaped pulses, with mixing times ranging from 100 to 120 ms and with an MLEV-17 spin−lock field of 10 kHz preceded by a 2.5 ms trim pulse. The spectra were acquired at 600 MHz. The NMR data were processed using UXNMR software.

ESIMS in the positive ion mode was performed using a Finnigan LCQ Deca ion trap instrument from Thermo Finnigan (San Jose, CA) equipped with Xcalibur 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 5 V, the spray voltage was 46 kV, and the tube lens offset was 55 V. The capillary temperature was 220 °C. Data were acquired in MS1 and MS/MS scanning mode.

LC-ESIMS on-line analyses were performed using the same instrument described above equipped with a Spectra System HPLC (Thermo Finnigan) with a DAD; source conditions were described above. The flow rate was 0.3 mL/min, and the postcolumn split ratio was 9:1; the capillary temperature was 280 °C. Data were acquired in MS1 and MS/MS scanning mode by using the Depending Data Scan, which provided a full MS/MS spectrum for each major mass peak acquired in MS1 mode.

Exact masses were measured by a Voyager DE mass spectrometer (Applied Biosystems, Foster City, CA). Samples were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from adrenocorticotropic hormone (fragments 18−39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard.

HPLC separations were carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector, a G-1322A degasser, and a G-1315A photodiode array detector using a 30 cm × 7.6 mm i.d. μ-Bondapack RP-18 column (Waters Corp., Milford, MA).

Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). All solvents for chromatographic separation were of analytical grade from Carlo Erba (Rodano, Italy). HPLC grade acetonitrile (CH_3CN), trifluoroacetic acid (TFA), MeOH, and H_2O from J. T. Baker (Baker Mallinckrodt, Phillipsburg, NJ) were used for HPLC and LC-MS. Rosmarinic acid and verbascoside used as internal and external standards, respectively, were purchased from Extrasynthese (Lyon, France).

Extraction and Isolation Procedures. Dried and powdered bark (400 g) of *T. panurensis* was extracted for a week, three times, at room temperature using solvents of increasing polarity, namely, petroleum ether (1.5 L), chloroform (1.5 L), and methanol (1.5 L), to afford 1.54, 5.84, and 7.28 g, respectively. The extractive solutions were dried in a rotavapor at 40 °C. Part of the methanol extract (1.75 g) was fractionated initially on a 100 × 5.0 cm Sephadex LH-20 column, using MeOH as mobile phase, and 90 fractions (8 mL each) were obtained. Fraction 29 (61.4 mg) was chromatographed by HPLC on a 300 × 7.6 mm i.d. μ-Bondapack RP-18 (Waters) column using MeOH/H_2O (7:18) as eluent at a flow rate of 2.5 mL/min, to afford compound **1** (4.9 mg, t_R = 36 min) (**Figure 1**). Fractions 32−33 (64.0 mg) were chromatographed by HPLC/DAD on the same column using H_2O as eluent A and CH_3CN as eluent B at a flow rate of 2.5 mL/min, to afford compounds **2** (7.6 mg, t_R = 35.3 min) and **3** (2.9 mg, t_R = 41.9 min). Fractions 34−36 (64.7 mg) were chromatographed under the same conditions to afford compound **4** (2.9 mg, t_R = 27.9 min). In both cases, the elution program started with 15% of eluent B and remained isocratic for 10 min, and then a linear gradient was performed to 30% B in 30 min. The detection wavelength was 330 nm. The methanol extract of *T. panurensis* bark was chromatographed by HPLC/DAD on a 300 × 10 mm i.d. Atlantis RP-D-C_{18} column (Waters) affording compound **5** (1.0 mg, t_R = 24.4 min). Gradient elution was performed with H_2O/0.05% TFA (solvent A) and CH_3CN/0.05% TFA (solvent B) at a constant flow rate of 3.0 mL/min. An increasing linear gradient of solvent B was used, starting at 10% of B up to 30% in 30 min. Detection was carried out at 365 nm.

Eugenol-*O*-[β-D-xylopyranosyl-(1→5)-*O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranoside] (**1**) was obtained as an amorphous powder: $[\alpha]^{24}_D$ − 81.3° (*c* 0.2, MeOH); IR (KBr) ν_{max} 3450, 1515, 1262, 1220, 1070 cm^{-1}; UV (MeOH) λ_{max} 275 nm; ^{1}H NMR (CD_3OD, 600 MHz), see **Table 1**; ^{13}C NMR (CD_3OD, 150 MHz), see **Table 1**; ESIMS, *m/z* 613 [M + Na]$^+$, 481 [M + Na − 132]$^+$, 449 [M + Na − 132 − 32]$^+$, 317 [M + Na − 132 − 132 − 32]$^+$; HRMALDIMS, *m/z* 613.2116 [M + Na]$^+$, calcd for $C_{26}H_{15}O_{38}Na$, 613.2108.

Verbascoside (**2**) ^{1}H and ^{13}C NMR data were consistent with previously published data (*6*); ESIMS, *m/z* 647 [M + Na]$^+$.

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

position	δ_C	δ_H (J in Hz)
1	145.4	
2	149.8	
3	113.1	6.87 d (1.7)
4	134.9	
5	121.5	6.79 dd (8.3, 1.7)
6	117.8	7.11 d (8.3)
7	40.6	3.36 d (6.8)
		3.36 d (6.8)
8	138.4	5.99 ddt (16.9, 10.0, 6.8)
9	114.1	5.07 dd (10.0, 2.0)
		5.10 dd (16.9, 2.0)
OMe	56.0	3.84 s
Glc		
1′	101.9	4.82 d (7.5)
2′	74.9	3.50 dd (9.0, 7.5)
3′	77.7	3.44 dd (9.0, 9.0)
4′	71.2	3.38 dd (9.0, 9.0)
5′	76.5	3.55 ddd (9.0, 4.5, 2.5)
6′	68.1	3.63 dd (12.0, 2.5)
		4.00 dd (12.0, 4.5)
Api		
1″	110.6	4.97 d (2.4)
2″	78.0	4.00 d (2.4)
3″	80.0	
4″	74.9	3.92 d (9.5)
		3.74 d (9.5)
5″	72.8	3.58 d (9.9)
		3.95 d (9.9)
Xyl		
1‴	105.3	4.25 d (7.5)
2‴	74.7	3.24 dd (7.5, 9.0)
3‴	77.4	3.33 dd (9.0, 9.0)
4‴	71.0	3.49 ddd (11.0, 9.0, 4.5)
5‴	66.5	3.20 t (11.0)
		3.90 dd (11.0, 4.5)

[a] Assignments were confirmed by 1D TOCSY, DQF–COSY, HSQC, and HMBC experiments.

Leucosceptoside (**3**) ^{1}H and ^{13}C NMR data were consistent with previously published data (*7*); ESIMS, *m/z* 639 [M + Na]$^+$.

Isoverbascoside (**4**) ^{1}H and ^{13}C NMR data were consistent with previously published data (*6*); ESIMS, *m/z* 647 [M + Na]$^+$.

Katchimoside (**5**): ^{1}H NMR (CD_3OD, 600 MHz) aglycon moiety δ 6.94 (2H, d, J = 8.0 Hz, H-2′, H-6′), 6.60 (1H, s, H-3), 6.30 (1H, s, H-6); ^{13}C NMR (CD_3OD, 150 MHz) aglycon moiety δ 164.0 (C-2), 102.6 (C-3), 181.9 (C-4), 155.8 (C-5), 98.9 (C-6), 162.5 (C-7), 104.2 (C-8), 160.6 (C-9), 104.2 (C-10), 121.8 (C-1′), 128.5 (C-2′), 116.0 (C-3′), 160.9 (C-4′), 116.0 (C-5′), 128.5 (C-6′); ^{1}H NMR (CD_3OD, 600 MHz) sugar portion δ 4.92 (1H, d, J = 7.5 Hz, H-1_{glc}), 4.16 (1H, dd, J = 9.0, 7.5 Hz, H-2_{glc}) 3.52 (1H, t, J = 9.0 Hz, H-3_{glc}) 3.48 (1H, t, J = 9.0 Hz, H-4_{glc}), 3.60 (1H, m, H-5_{glc}), 3.79 (1H, dd, J = 12.0, 4.5 Hz, H-$6a_{glc}$), 3.99 (1H, dd, J = 12.0, 3.5 Hz, H-$6b_{glc}$), 4.28 (1H, d, J = 7.5 Hz, H-1_{xyl}), 3.22 (1H, dd, J = 9.0, 7.5 Hz, H-2_{xyl}), 3.31 (1H, t, J = 9.0 Hz, H-3_{xyl}), 3.49 (1H, m, H-4_{xyl}), 3.20 (1H, dd, J = 10.5, 2.0 Hz, H-$5a_{xyl}$), 3.89 (1H, dd, J = 10.5, 5.0 Hz, H-$5b_{xyl}$); ^{13}C NMR (CD_3-OD, 150 MHz) sugar portion δ 75.2 (C-1_{glc}), 73.0 (C-2_{glc}), 79.9 (C-3_{glc}), 71.0 (C-4_{glc}), 81.5 (C-5_{glc}), 70.3 (C-6_{glc}), 105.3 (C-1_{xyl}), 74.8 (C-2_{xyl}), 77.6 (C-3_{xyl}), 71.4 (C-4_{xyl}), 66.9 (C-5_{xyl}) (*8*); ESIMS, *m/z* 565 [M + H]$^+$.

Preparation of Phenylpropanoid Glycoside Standards. A stock solution of verbascoside (1 mg/mL) was prepared by dissolving in MeOH. Four different solutions, containing, respectively 5, 10, 25, and 50 μg/mL of verbascoside and 20 μg/mL of rosmarinic acid, were prepared in MeOH and used for method development.

Sample Preparation for Quantitative Analysis. *Methanol Extract*. Air-dried and powdered bark of *T. panurensis* (20.5 g) was extracted in methanol (3 × 60 mL) on an Accelerated Solvent Extraction (Dionex, Sunnyvale, CA) system, working at 40 °C and 10 atm for 7 min. The extraction yield was 9.24%.

The traditional extract was prepared by macerating 20 g of dried powdered bark of *T. panurensis* in 100 mL of ethanol/H_2O (1:1) for 24 h.

LC-MS Analysis. For quantitative purposes the extracts were analyzed by LC-ESIMS "on-line" using the same instruments and parameters described above. Analyses were performed on a 150 × 2.1 mm i.d. Atlantis RP-D-C_{18} column (Waters) at a flow rate of 0.3 mL/min. Gradient elution was performed with water/0.05% TFA (solvent A) and CH_3CN/0.05% TFA (solvent B). An increasing linear gradient of solvent B was used, starting at 10% B up to 30% in 30 min. Data were acquired in MS1 and MS/MS scanning mode by using the Depending Data Scan. In MS1 the mass range was 200−800 amu.

Calibration and Quantification. The quantitative determination of phenylethanoid glycosides was performed directly by HPLC-MS. A standard curve for verbascoside was prepared over a concentration range of 5−50 μg/mL with four different concentration levels (5, 10, 25, and 50 μg/mL) and triplicate injections at each level. Peak area ratios between the area of verbascoside and that of rosmarinic acid (20 μg/mL), used as internal standard, were calculated and plotted against the corresponding standard concentration using weighed linear regression to generate standard curves. Five aliquots of the methanol and traditional hydroalcoholic extract of *T. panurensis* were analyzed to quantify their phenylpropanoid contents. Internal standard (20 μg/mL) was introduced into both extracts and standards before the extraction.

Antioxidant Activity. The in vitro antioxidant activities of the isolated compounds and of the methanol and hydroalcoholic extracts of the bark were determined by the Trolox equivalent antioxidant capacity (TEAC) assay (*9*) as previously reported (*10*). The TEAC value is based on the ability of the antioxidant to scavenge the radical cation $ABTS^+$ with spectrophotometric analysis. The antioxidant activities of the isolated compounds and the methanol and hydroalcoholic extracts are expressed as TEAC values. The TEAC value is defined as the concentration of a standard Trolox (Aldrich) solution with the same antioxidant capacity as a 1 mM concentration of the tested compound. In the case of the extracts the TEAC value is defined as the concentration of a standard Trolox solution with the same antioxidant capacity as a 1 mg/mL of the tested extract.

RESULTS AND DISCUSSION

Isolation and Structure Determination of Compounds 1−5. The dried bark of *T. panurensis* was submitted to sequential extraction with petroleum ether, chloroform, and methanol. The methanol extract was fractionated on Sephadex LH-20. Repeated column chromatography of the fractions on reversed-phase HPLC yielded one new compound **1**, along with four known compounds, **2−5** (**Figure 1**).

Compound **1** showed a major ion peak at *m/z* 613 [M + Na]$^+$ and significant fragment ion peaks at *m/z* 481 [M + Na − 132]$^+$, *m/z* 449 [M + Na − 132 − 32]$^+$, and *m/z* 317 [M + Na − 132 − 132 − 32]$^+$ in the positive ESIMS. In conjunction with the analysis of the ^{13}C NMR and HSQC spectrum, its molecular formula was deduced to be $C_{26}H_{15}O_{38}$ by HRMALDI mass spectrometry. The ^{13}C NMR spectrum showed 26 signals, 10 of them corresponding to the aglycon moiety (**Table 1**). The ^{1}H NMR spectrum of **1** (**Table 1**) showed three signals corresponding to aromatic protons at δ 7.11 (1H, d, J = 8.3 Hz), 6.87 (1H, d, J = 1.7 Hz), and 6.79 (1H, dd, J = 8.3, 1.7 Hz), three signals for olefinic protons at δ 5.99 (1H, ddt, J = 16.9, 10.0, 6.8 Hz), 5.10 (1H, dd, J = 16.9, 2.0 Hz), and 5.07 (1H, dd, J = 10.0, 2.0 Hz), one signal corresponding to a methylene group at δ 3.36 (2H, d, J = 6.8 Hz), and one signal for one methoxy group at δ 3.84 (3H, s) suggesting the presence of an eugenol moiety as the structure of the aglycon portion (*11*, *12*). Additionally, resonances of anomeric protons were observed in the ^{1}H NMR spectra at δ 4.97 (1H, d, J = 2.4 Hz), 4.82 (1H, d, J = 7.5 Hz), and 4.25 (1H, d, J = 7.5 Hz). 1D−TOCSY, DQF−COSY, and HSQC NMR experiments showed

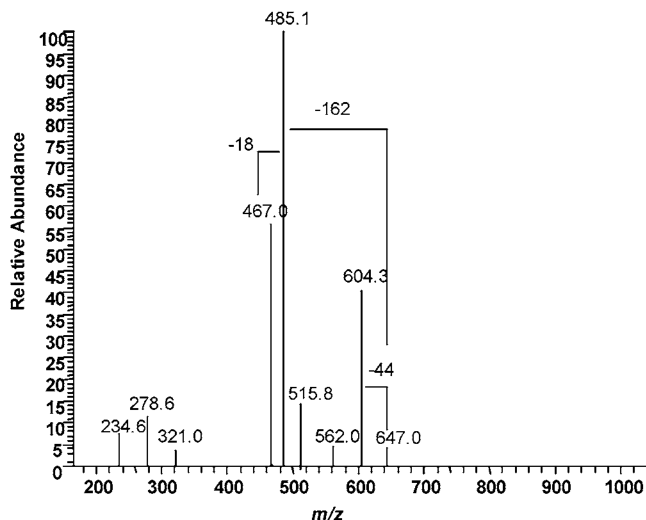

Figure 2. ESIMS/MS spectrum of verbascoside.

the presence of one β-D-apiofuranosyl unit (δ 4.97), one β-D-glucopyranosyl unit (δ 4.82), and one β-D-xylopyranosyl unit (δ 4.25) (**Table 1**). The HSQC spectrum also showed glycosylation shifts for C-6′ (δ 68.1) of the glucopyranosyl unit and C-5″ (δ 72.8) of the apiofuranosyl unit, suggesting that β-D-xylopyranosyl was a terminal unit. An unambiguous determination of the sequence and linkage sites was obtained from the HMBC correlations. Key correlation peaks were observed in the HMBC spectrum of **1** between H-1′ of the glucopyranosyl unit at δ 4.82 and C-1 (δ 145.4) of the aglycon, between H-1″ of the apiofuranosyl unit at δ 4.97 and C-6′ (δ 68.1) of the glucopyranosyl unit, and finally between H-1‴ of the terminal xylopyranosyl unit at δ 4.25 and C-5″ (δ 72.8) of the apiofuranosyl unit. On the basis of this evidence, the new compound **1** was established as eugenol-*O*-[β-D-xylopyranosyl-(1→5)-*O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranoside].

Verbascoside (**2**), leucosceptoside (**3**), and isoverbascoside (**4**) were identified by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature (*6*, *7*). Compound **5** was identified as apigenin 8-*C*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside], namely, katchimoside, previously isolated by Darmograi et al. from *Gypsophila paniculata* (*8*).

ESIMS and ESIMS/MS Analysis of Verbascoside. To obtain mass spectrometric-based information to evaluate the presence of different phenylpropanoid glycosides, direct introduction experiments of verbascoside were initially performed. Positive ionization was selected due to the absence of phenolic functions in compound **1**.

$[M + Na]^+$ ions were observed when the data were acquired in MS1 scanning mode (*m/z* range = 150−1300) The fragmentation pattern observed for verbascoside was in complete agreement with its structure. The daughter ion spectrum of the major ion at *m/z* 647.0 $[M + Na]^+$ (**Figure 2**) of verbascoside displayed three principal fragment ions at *m/z* 603.3 $[M + Na - 44]^+$ due to the loss of a CO_2 molecule, at *m/z* 485.1 $[M + Na - 162]^+$ corresponding to the loss of a caffeoyl unit, and at *m/z* 467.0 $[M + Na - 162 - 18]^+$ ascribable to a further loss of water. Additionally, a minor fragment was observed at *m/z* 321.0 $[M + Na - 162 - 18 - 146]^+$ corresponding to the loss of a deoxyhexose unit from the ion at *m/z* 467.0. Other minor fragments were observed at *m/z* 278.6 $[M + Na - 162 - 18 - 146 - 44]^+$, corresponding to the subsequent loss of a CO_2 unit, and at *m/z* 234.6, ascribable to the loss of a further CO_2 unit.

LC-MS and LC-MS/MS Analysis of the Methanol Extract of *T. panurensis*. Using positive ion electrospray LC-MS, all four phenylpropanoid glycosides (**1**−**4**) were identified in the methanol extract by comparing their retention times and MS data to those of authenticated standards. The best separation of the phenylpropanoid glycosides fraction in the total ion current (TIC) chromatogram was obtained by using an Atlantis D-C18 column. To improve the separation and identification of the single compounds, reconstructed ion chromatograms (RICs) were obtained for *m/z* 591 (**1**), 617 (**3**), and 625 (**2**, **4**). The TIC profile and RICs of the methanol extract of *T. panurensis* are shown in **Figure 3**. It is worthwhile to note that in LC-MS the phenylpropanoid glycosides displayed the pseudomolecular ion $[M + H]^+$, whereas in direct introduction analysis we observed only the adduct ion $[M + Na]^+$.

Moreover, LC-MS analysis of the methanol extract of *T. panurensis* revealed the presence of one peak with a retention time of 14.36 min at *m/z* 565 corresponding to a compound not detected during the isolation step (**Figure 3**). LC-MS/MS product ion scan analysis was applied to achieve further information on this compound. The TIC chromatogram relative to the LC-MS/MS product ion scan experiment on the methanol extract of *T. panurensis* is shown in **Figure 4**. In particular,

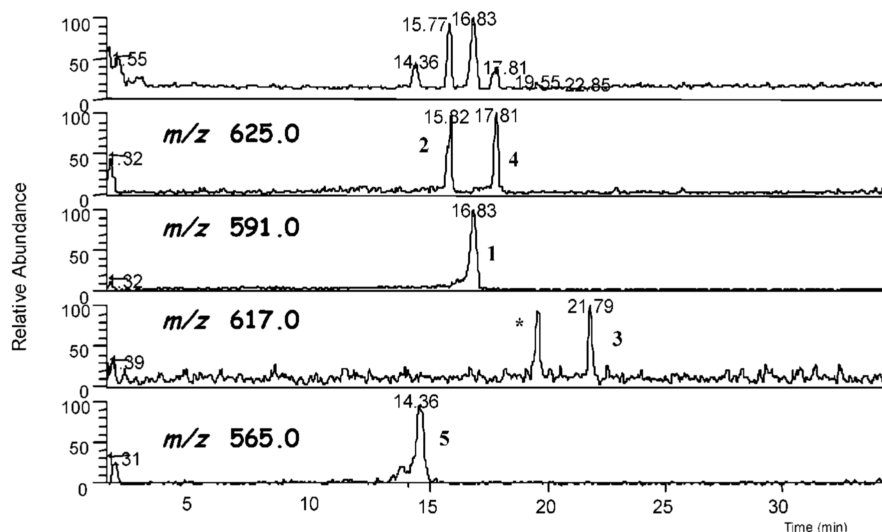

Figure 3. LC-MS analysis of the methanol extract of *T. panurensis* bark: **1**, eugenol-*O*-[β-D-xylopyranosyl-(1→5)-*O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranoside]; **2**, verbascoside; **3**, leucosceptoside; **4**, isoverbascoside; **5**, katchimoside; *, unknown peak.

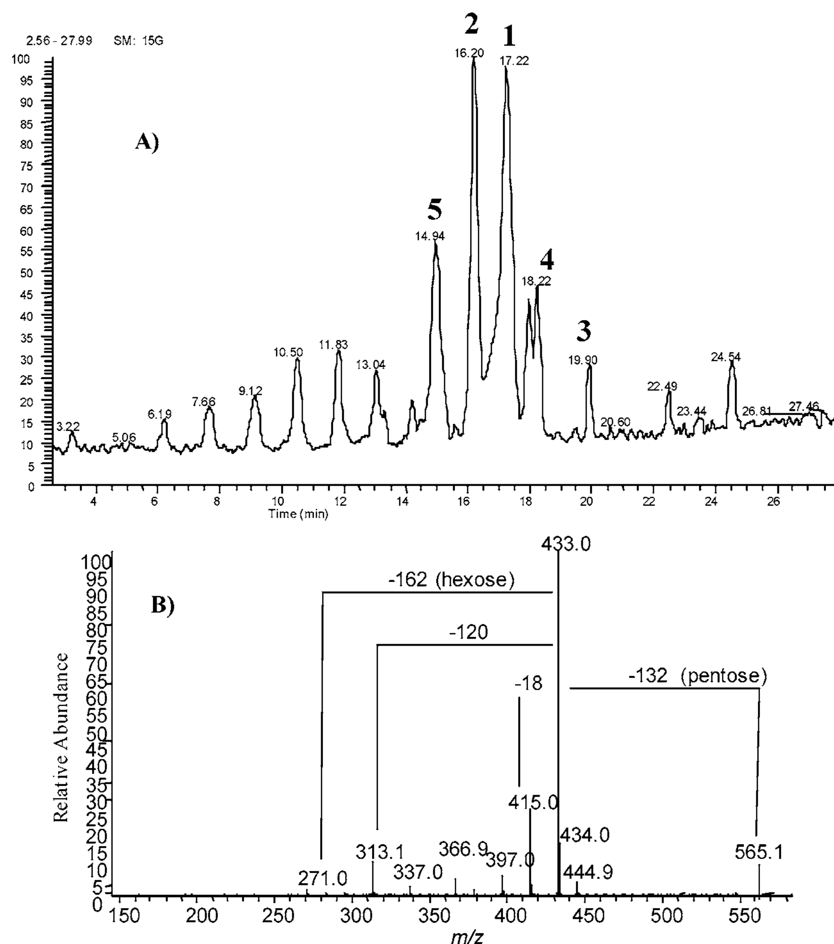

Figure 4. (**A**) LC-MS/MS analysis of the methanol extract of *T. panurensis* bark: **1**, eugenol-*O*-[β-D-xylopyranosyl-(1→5)-*O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranoside]; **2**, verbascoside; **3**, leucosceptoside; **4**, isoverbascoside; **5**, katchimoside. (**B**) On-line MS/MS spectrum of katchimoside (**5**) using the depending data scan.

the tandem mass spectrum produced by the fragmentation of the peak at *m/z* 565.0 with a retention time of 14.94 min showed a major fragment ion at *m/z* 433.0 corresponding to the loss of a pentose unit and a subsequent ion at *m/z* 313 $[M + H - 132 - 120]^+$, 120 amu lower, in agreement with the fragmentation pattern described in the literature for *C*-glycoside flavonoids. (*13*, *14*). The aglycon peak at *m/z* 271 allowed us to hypothesize apigenin as the aglycon moiety. This compound was further isolated by HPLC, and its structure was established by extensive 1D and 2D NMR experiments as apigenin 8-*C*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside], namely, katchimoside (**5**).

Quantitative Analysis. The calibration curve obtained by plotting the area ratio between the external (verbascoside) and internal (rosmarinic acid) standards versus the known concentration of verbascoside was linear in the range of 5−50 μg/mL with r^2 values of >0.99 for all compounds (**1**−**5**). The limit of detection was 8.25 ng/mL. LC/MS analysis of a mixture of both internal and external standards is shown in **Figure 5**.

As shown in **Table 2**, either in the bark or in the traditional hydroalocholic extract, the major compound was compound **1** followed by verbascoside (**2**) and katchimoside (**5**). Results have also shown that this analytical method was precise and reliable. Precision was studied by intra- and interday assays at four concentration levels for each compound. By analyzing replicate standard concentration in the same day and day-to-day, it was shown that standard deviation was not higher than ±2.00%. Because all of the compounds are present as major constituents in *T. panurensis*, the proposed LC-MS method may be

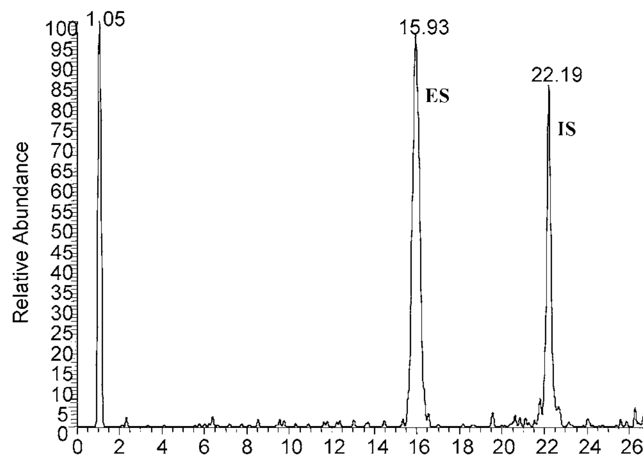

Figure 5. LC-MS analysis of external standard added with internal standard at one point of the calibration curve. IS, internal standard (rosmarinic acid); ES, external standard (verbascoside).

considered as suitable for routine quantitative determination either of the plant material or its derived products, and thus to be the starting point of quality control protocols.

The pharmacological activities of phenylpropanoids have been extensively reviewed (*3*−*5*). In particular, verbascoside has been shown to possess an interesting analgesic activity and protective effect against decrease of sex and learning behaviors in mice (*3*, *4*). All of this evidence and the high amounts of phenylpropanoid glycosides found in the bark and in the traditional

Table 2. Quantitative Content of Compounds **1**–**5** in Dried Bark and in the Traditional Hydroalcoholic Extract of *T. panurensis*[a]

compd	dried bark (mg/g)	hydroalcholic extract (mg/mL)
1	19.512 ± 1.689	0.2380 ± 0.0049
2	4.236 ± 0,832	0.0524 ± 0.0022
3	0.581 ± 0.361	0.0076 ± 0.0003
4	1.154 ± 0,120	0.0203 ± 0.0028
5	1.504 ± 0,046	0.0149 ± 0.0017

[a] $n = 3$.

Table 3. Free Radical Scavenging Activities of Compounds **1**–**5**, Methanol Extract, and Hydroalcoholic Traditional Extract in the TEAC Assay

compd	TEAC value[a] (mM ± SD)
1	0.342 ± 0.023
2	0.691 ± 0.081
3	0.287 ± 0.012
4	0.345 ± 0.020
5	0.855 ± 0.033
quercetin	2.120 ± 0.011

extract	TEAC value[a] (mg/mL ± SD)
methanol extract	1.051 ± 0.054
hydroalcoholic traditional extract	0.650 ± 0.032

[a] $n = 3$.

extract are in agreement with the traditional use of *T. panurensis* as an aphrodisiac.

Free Radical Scavenging Activity. Because previous studies have found that many phenylpropanoid glycosides including verbascoside showed a strong antioxidant activity (*15*−*18*), the antioxidant activities of compounds **1**−**5** were evaluated and compared to that of quercetin in the TEAC assay (**Table 3**). Among the isolated compounds **1**−**5**, katchimoside (**5**) displayed the highest TEAC value, near 1 (the same antioxidant activity of Trolox in the used system), followed by verbascoside (**2**), which compared to quercetin was 3-fold lower. The TEAC values of the methanol and hydroalcoholic extracts were also obtained. As shown in **Table 3**, only the methanol extract displayed a TEAC value >1.

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Received for review December 1, 2004. Revised manuscript received February 15, 2005. Accepted February 15, 2005.

JF0479867