Phenolic Constituents and Antioxidant Activity of an Extract of *Anthurium versicolor* Leaves

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Fractionation of a methanolic extract of the leaves of *Anthurium versicolor* has resulted in the isolation of two main fractions, I and II. Both the extract and the fractions were assayed for their radical-scavenging activity by means of an in vitro test (bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical) and showed a significant radical-scavenging effect. Subsequent chromatographic fractionation of the most active fraction, II, has led to the isolation and characterization, as major constituents, of four new flavone glycosides, acacetin 6-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside] (**1**), acacetin 6-*C*-[*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside] (**2**), acacetin 6-*C*-[*β*-D-apiofuranosyl-(1→3)-*β*-D-glucopyranoside] (**3**), and acacetin 8-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside] (**4**), as well as vitexin (apigenin-8-*C*-*β*-D-glucopyranoside) and rosmarinic acid. The structures of **1**−**4** were determined using spectroscopic methods.

As a part of our program to develop potential therapeutic agents from South American flora, *Anthurium versicolor* Sodiro, vernacular name "shungopango", was investigated phytochemically and biologically. *Anthurium* is a herbaceous genus of the family Araceae found throughout South America. The flowers of various *Anthurium* species are cultivated intensively for ornamental purposes, and the leaves have traditional uses as an antinflammatory agent.[1−3] So far, only studies on the antinflammatory activity of *A. cerrocampanense* extracts[3] and on the isolation of anthocyanins from the flowers of some *Anthurium* species[2] have been reported. In this paper, we present results on the antioxidant (radical-scavenging) activity of a polar extract obtained from *A. versicolor* leaves in relation to its phenolic content. In fact, preliminary screening of the methanolic extracts of *A. versicolor* leaves revealed the presence of high levels of phenolic compounds (including flavonoid and phenylpropanoid derivatives), whose effectiveness as free-radical-scavenging agents is well known.[5−9] Several studies have reported that specific polyphenols are able to scavenge superoxide and hydroxyl radicals, to reduce lipid peroxyl radicals, and to inhibit lipid peroxidation.[5−9] Plants as sources of antioxidants can be used both for food quality preservation by preventing oxidative deterioration of lipids and for medicinal purposes. Most of the antioxidant capacity of vegetables may be due to their polyphenols possessing wide biological properties and, particularly, their free-radical-scavenging property.[9]

The in vitro antioxidant effect of a polar extract of the leaves of *A. versicolor* was tested employing the DPPH test (the bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical). Also, the total phenolic content of the extract was determined, and the major constituents of the extract were isolated and characterized.

Results and Discussion

The dried leaves of *A. versicolor* were defatted with petroleum ether and chloroform and then extracted with MeOH. The MeOH extract was partitioned between water and *n*-BuOH. Table 1 shows that the total phenolic content for the *n*-BuOH extract, determined by the Folin−Ciocalteau method[10] and expressed as apigenin equivalents, was 190.6 *μ*g/mg. As to the DPPH test,[9,11] the free-radical-scavenging effect elicited by the *n*-BuOH extract of *A. versicolor* was concentration-dependent, so that the EC_{50} value was calculated as 142.6 *μ*g of extract. This extract was subjected to preliminary purification by gel filtration on a Sephadex LH-20 column, giving two main fractions, I and II, which were tested under the same experimental conditions. In comparison to the whole extract, fraction II particularly was more potent in the DPPH test (EC_{50} 47.7 *μ*g) and showed a higher level of total phenols (319.9 *μ*g/mg), suggesting that it contained a higher concentration of the active principles responsible for the observed free-radical-scavenging activity. The activity of fraction II was almost comparable to that of α-tocopherol (EC_{50} 10.1 *μ*g) used as a positive control.

Thus, with the aim to characterize the phytochemical profile of *A. versicolor*, fractions I and II were purified by HPLC. This chromatographic separation gave, as major constituents, ipolamide[12] from fraction I and five flavonols (**1**−**4** and vitexin[13−15]) and rosmarinic acid[16] from fraction II. The structures of the known compounds, ipolamide, vitexin, and rosmarinic acid, were determined by comparison of their spectroscopic data (NMR and MS) with literature values.[12−16] Compounds **1**−**4**, on the other hand, appeared to be new, and their structure identification as acacetin 6-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside] (**1**), acacetin 6-*C*-[*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside] (**2**), acacetin 6-*C*-[*β*-D-apiofuranosyl-(1→3)-*β*-D-glucopyranoside] (**3**), and acacetin 8-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside] (**4**) was based on the evidence outlined below. The new *C*-glycosyl flavones **1** and **2**, as characteristic components of the extract of *A. versicolor*, and vitexin were chosen as markers to be quantified in the extract and fractions. The isolated compounds were

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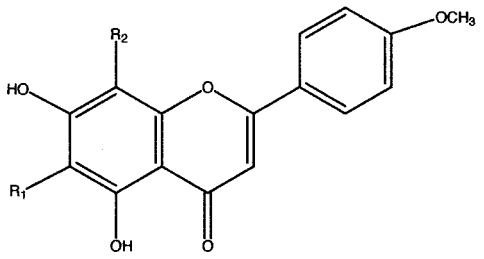
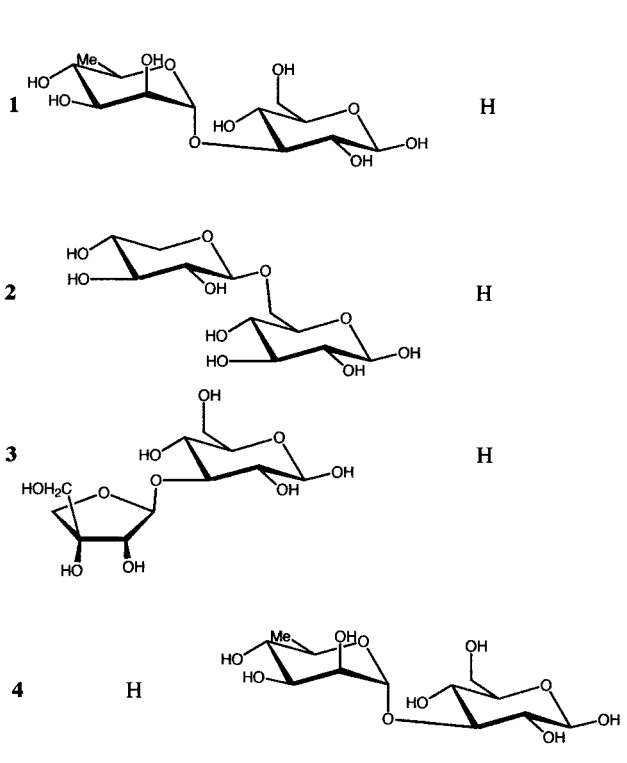
Table 1. Total Phenol Content and Free-Radical-Scavenging Activity of the *n*-BuOH Extract and Fractions I and II from *A. versicolor*

extract and fractions	phenol content[a] (μg/mg extract)[b]	DPPH test [EC_{50} (μg of extract)]
n-BuOH extract	190.6 ± 2.44	142.6 (117.9−172.5)[c]
fraction I	200.0 ± 3.15	100.7 (83.3−122.6)[c]
fraction II	319.9 ± 3.69	47.7 (48.4−56.4)[c]
α-tocopherol[d]		10.1 (8.8−11.4)[c]

[a] Mean ± S.D. of three determinations. [b] Apigenin equivalents. [c] 95% confidence limits. [d] Positive control.

used as standards for quantitative HPLC analysis by a direct calibration method (see Experimental Section).





The negative FABMS of compound **1** showed a $[M - H]^-$ ion at *m/z* 591, consistent with the molecular formula $C_{28}H_{32}O_{14}$, which was also deduced using ^{13}C and DEPT NMR analysis. Major fragments at *m/z* 445 and 429 were assigned to the loss of a deoxyhexose unit with (162 amu) or without (146 amu) the glycosidic oxygen. A 6-*C*- (or 8-*C*)-substituted flavone structure was indicated by ^1H and ^{13}C NMR analysis.[13−15] The 600 MHz ^1H NMR spectrum (Table 2) of **1** showed signals for two anomeric hydrogens (δ 4.93 and 5.22) and a methyl doublet signal (δ 1.31, d, J = 6.0 Hz) in the aliphatic region, suggesting the occurrence of two sugar residues. The other sugar signals were overlapped in the region between δ 3.43 and 4.31. A 6-*C*- (or 8-*C*-)substituted apigenin skeleton was suggested by the appearance in the aromatic region of the ^1H NMR spectrum of a one-proton singlet at δ 6.63 (1H, br s), typical of H-3 of a flavone, two signals at δ 7.10 and 7.93 (each 2H, d, J = 8.0 Hz), the multiplicity of which indicated a 4′-substituted ring B, and another proton singlet at δ 6.50, suggesting that ring A was trisubstituted. From the HMBC spectrum this proton showed correlations with carbons 7 and 9 and was therefore assigned to H-8. The presence of a methoxyl group in the molecule was suggested by a signal at δ_H 3.91 and δ_C 56.1. The position of the −OMe on ring B was indicated by the cross-peaks between the −OMe signal (δ_H 3.91) and C-4′ (δ_C 164.4) and between both H-3′/H5′ (δ_H 7.10) and H-2′/H-6′ (δ_H 7.93) signals and C-4′ in the HMBC spectrum. These data led to the identification of the aglycon as acacetin or 4′-methoxyapigenin. The ^{13}C NMR spectrum of **1** (Experimental Section and Table 2) indicated the presence of 15 aromatic carbon resonances for the aglycon, an −OMe signal, and 12 sugar signals ascribable to one hexose unit and one deoxyhexose unit. The chemical shifts of the aryl carbons and hydrogens were comparable with the corresponding carbons and hydrogens of *C*-6-substituted apigenin derivatives such as isovitexin.[13] The ^{13}C NMR chemical shifts of C-6 (δ_C 109.2) and C-8 (δ_C 95.5) were almost superimposable on those of isovitexin,[13] and different from data reported for 8-*C*-glucosylflavones such as 2″-*O*-rhamnosylvitexin[15] (ca. δ_C 104.1 and 98.2), and diagnostic for the distinction between 6-*C*- and 8-*C*-glycosylflavones.[17−19] The C-6 substitution of **1** was substantiated by correlations seen in the HMBC spectrum, which allowed the assignments of all ^1H and ^{13}C NMR signals, as reported in the Experimental Section.

The sugar residue was determined to be α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside linked to C-6 of the aglycon using 1D TOCSY,[20] 2D DQF-COSY, and HSQC NMR experiments. Two anomeric signals at δ_H 4.93 and 5.22, readily identified in the ^1H NMR spectrum of **1**, correlated to carbons at δ_C 75.3, characteristic of a *C*-glucoside, and δ_C 103.0, respectively, in the HSQC spectrum. In the 1D TOCSY spectrum of **1**, the H-1″ signal at δ 4.93 (d, J = 7.5 Hz) showed connectivities to four methines and a methylene. The COSY spectrum established the proton sequence within this monosaccharide as H-1″ to H_2-6″, of which the multiplicity and coupling constants (Table 2) were typical of a glucopyranosyl substituent.[21] Similar observations on the second sugar residue, obtained by selective excitation of the methyl doublet signal (δ 1.31, d, J = 6.5 Hz), allowed the identification of the H_3-6‴/H-1‴ sequence, and analysis of the correlated ^{13}C NMR signals in the HSQC spectrum led to the identification of a rhamnopyranosyl unit.[21] The β-configuration for the D-glucopyranosyl unit (J_{H1-H2} = 7.5 Hz) and the α-configuration for the L-rhamnopyranosyl (J_{H1-H2} = 1.5 Hz) were deduced by the ^1H NMR data as well as ^{13}C NMR data of key carbons (C-2, C-3, and C-5).[21,22] Moreover, the HSQC spectrum of **1** indicated all of the protonated carbon correlations and thereby led to the assignment of the interglycosydic linkages by comparison of the observed carbon chemical shifts with those of the corresponding methylpyranoside models.[21,22] The absence of any ^{13}C NMR glycosidation shift for the α-L-rhamnopyranosyl moiety suggested that this sugar was the terminal unit. Glycosidation shifts were observed for C-3″ (δ 87.1,

Table 2. ^{13}C NMR and ^{1}H NMR Data of the Sugar Moiety of Compounds **1**−**3** in CD_3OD[a]

	1			**2**			**3**	
position[b]	δ_C	δ_H (J_{HH} in Hz)[c]	position[b]	δ_C	δ_H (J_{HH} in Hz)[c]	position[b]	δ_C	δ_H (J_{HH} in Hz)[c]
Glc-1″	75.3	4.93 d (7.5)	Glc-1″	75.2	4.92 d (7.5)	Glc-1″	75.2	4.95 d (7.5)
Glc-2″	72.8	4.31 dd (8.5, 7.5)	Glc-2″	73.0	4.16 dd (9.0,7.5)	Glc-2″	72.4	4.37 dd (9.0,7.5)
Glc-3″	87.1	3.62 t (8.5)	Glc-3″	79.9	3.52 t (9.0)	Glc-3″	87.8	3.62 t (9.0)
Glc-4″	70.4	3.56 t (8.5)	Glc-4″	71.0	3.48 t (9.0)	Glc-4″	70.2	3.55 t (9.0)
Glc-5″	82.6	3.46 m	Glc-5″	81.5	3.60 m	Glc-5″	82.8	3.45 m
Glc-6″	62.8	3.78 dd (12.0, 4.5) 3.90 dd (12.0, 3.0)	Glc-6″	70.3	3.79 dd (12.0, 4.5) 3.99 dd (12.0, 3.5)	Glc-6″	62.5	3.77 dd (12.0, 4.5) 3.92 dd (12.0, 3.5)
Rha-1‴	103.0	5.22 d (1.5)	Xy-1‴	105.3	4.28 d (7.5)	Ap-1‴	112.2	5.31 d (2.0)
Rha-2‴	72.2	3.99 dd (1.5, 2.5)	Xy-2‴	74.8	3.22 dd (7.5, 9.0)	Ap-2‴	77.5	4.03 d (2.0)
Rha-3‴	72.4	3.77 dd (2.5, 9.5)	Xy-3‴	77.6	3.31 t (9.0)	Ap-3‴	80.5	
Rha-4‴	74.0	3.43 t (9.5)	Xy-4‴	71.4	3.49 m	Ap-4‴	74.9	4.18 d (10.0) 3.84 d (10.0)
Rha-5‴	70.1	4.07 m	Xy-5‴	66.9	3.20 dd (10.5, 2.0) 3.89 dd (10.5, 5.0)	Ap-5‴	64.9	3.64 br s
Rha-6‴	17.9	1.31 d (6.5)						

[a] Assignments confirmed by 1D TOCSY and 2D COSY, HSQC, and HMBC experiments; the main HMBC correlations are reported in the text. [b]Glc = *β*-D-glucopyranosyl, Ap = *β*-D-apiofuranosyl, Rha = α-L-rhamnopyranosyl, Xy=*β*-D-xylopyranosyl. [c]$^{1}H-^{1}H$ coupling constants in the sugar unit were measured from TOCSY and COSY spectra in Hz.

+7.2 ppm by *β*-effect) of the *β*-D-glucopyranosyl unit, demonstrating the (1→3) linkage between rhamnosyl and glucosyl units in **1**.

The position of the sugar residues in **1** were defined unambiguously by the multiple-bond heteronuclear correlation (HMBC) NMR experiments. The disaccharidic chain was confirmed as being at C-6 on ring A on the basis of correlations due to long-range couplings observed between H-1″ (δ_H 4.93) of the glucosyl unit and C-6 (δ 109.2), C-5 (δ 162.1), and C-7 (δ 165.6) of the aglycon. A cross-peak observed between H-1‴ (δ_H 5.22) and C-3″ (δ 87.1) confirmed that the rhamnopyranosyl substituent was attached to C-3 of the glucopyranosyl unit. All the connectivity information inferred by the HMBC spectrum was compatible only with structure **1**. Accordingly compound **1** was determined as acacetin 6-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside] (**1**).

Compound **2** was assigned a molecular formula of $C_{27}H_{30}O_{14}$, as deduced by a combination of FABMS and ^{13}C NMR and DEPT analysis. The FABMS and ^{1}H and ^{13}C NMR spectra of **2** indicated the same aglycon as **1**, the presence of an inner glucopyranosyl unit linked through a *C*-glycosidic linkage at C-6 of the aglycon, and the presence of a terminal pentose unit, instead of a terminal rhamnopyranosyl unit (Table 2). The observed downfield shift (+7.5 ppm) of C-6″ (*β*-effect) and the upfield shift of C-5″ (−1.1 ppm) of the glucopyranosyl unit, in comparison with those observed in **1**, were indicative of a glycosidation at C-6″. The terminal sugar unit was identified as a *β*-D-xylopyranosyl unit from the following evidence: the ^{1}H NMR data indicated a *β*-configuration at the anomeric position ($J_{H-1-H-2}$ = 7.5 Hz); a 1D TOCSY subspectrum obtained by irradiating at the well-resolved anomeric proton at δ 4.28 showed a set of coupled protons at δ 3.22, 3.31, 3.49 (all CH) and 3.20 and 3.89 (CH_2); the DQF-COSY spectrum established the proton sequence within this monosaccharide as H-1‴ to H_2-5‴; and analysis of the correlated ^{13}C NMR signals in the HSQC spectrum supported the identification of a xylopyranosyl unit.[21] The interglycosidic linkage of the xylopyranosyl moiety in **2** was established unambiguously by the HMBC spectrum to be at the C-6″ position of the glucopyranosyl unit based on the cross-peak, due to $^{3}J_{C-H}$ long-range coupling, between the anomeric proton (δ 4.28, H-1‴) of the xylopyranosyl unit and C-6″ (δ 70.3) of the glucopyranosyl unit. Correlations due to long-range couplings were also observed between H-1″ (δ_H 4.92) of the glucosyl unit and C-6 (δ 109.3) of the aglycon, confirming that the disaccharidic chain was bonded by a *C*-glycosidic linkage to C-6. Therefore, the structure of **2** was determined as acacetin 6-*C*-[*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside] (**2**).

Compound **3** was assigned a molecular formula of $C_{27}H_{30}O_{14}$, as deduced by a combination of FABMS and ^{13}C NMR and DEPT analysis. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **1** and **2** in the chemical shifts and multiplicities of the signals ascribable to the aglycon and to an inner glucopyranosyl unit. The spectra of **3** showed signals attributable to a terminal apiofuranosyl unit, instead of a rhamnopyranosyl in **1** and a xylopyranosyl moiety in **2** (Table 2). The nature of the terminal sugar unit as *β*-D-apiofuranosyl[22] was deduced by the following evidence: the ^{1}H NMR spectrum indicated an anomeric signal at δ 5.31 (H-1‴, d, J = 2.0 Hz); in the 1D TOCSY experiment, selective excitation of the signal at δ 5.31 led to the enhancement only of H-2‴ (δ 4.03, d, J = 2.0 Hz); and the multiplicity of H-2‴ may be derived only from the presence of a quaternary carbon at C-3‴, characteristic of an apiofuranosyl structure. The ^{13}C NMR spectrum gave 11 carbon signals for the sugar moiety, of which three methylenes were ascribable to C-4‴ (δ 74.9) and C-5‴ (δ 64.9) of an apiofuranosyl unit and to C-6″ (δ 62.5) of a glucopyranosyl unit, respectively. Analysis of the correlated ^{13}C NMR signals in the HSQC spectrum and of the resonances of the quaternary carbon signal (δ 80.5, C-3‴) matched well with a terminal *β*-D-apiofuranosyl linked to an inner *β*-D-glucopyranosyl. C-3″ of the glucopyranosyl unit was shifted downfield (*β*-effect) by 7.9 ppm with respect to **2**, demonstrating the (1→3) linkage between the apiosyl and glucosyl units. Finally, the interglycosidic linkage of apiofuranosyl in compound **3** was confirmed unambiguously to be at C-3″ based on the HMBC cross-peak, due to $^{3}J_{C-H}$ long-range coupling, between the anomeric proton (δ 5.31, H-1‴) of the apiofuranosyl unit and C-3″ (δ 87.8) of the glucopyranosyl unit. Correlations due to long-range HMBC couplings were also observed between H-1″ (δ_H 4.95) of the glucosyl unit and C-6 (δ 109.2) of the aglycon, confirming that the disaccharide chain was bonded by a *C*-glycosidic linkage to C-6. Therefore, the structure of **3** was determined as acacetin 6-*C*-[*β*-D-apiofuranosyl-(1→3)-*β*-D-glucopyranoside] (**3**).

Compound **4** had the same molecular formula of **1** and similar NMR spectral data. The hydrogen and carbon signals of rings B and C and of the sugar chain were superimposable on those of compound **1**. A *C*-8-substituted

acacetin structure was suggested by the ^{1}H (δ 6.37, s, H-6) and ^{13}C NMR (δ 105.1 for C-8, 99.2 for C-6) signals characteristic for C-8-glycosylated flavones.[15] The C-8-substitution was confirmed by the cross-peaks observed between H-1″ (δ 4.95, 1H, d, J = 7.5 Hz) of the glucopyranosyl unit and C-8, C-7 (δ 165.9), and C-9 (δ 157.5) of the aglycon in the HMBC spectrum. Therefore, **4** was assigned as acacetin 8-*C*-[α-L-rhamnopyranosyl-(1→3)-*β*-D-glucopyranoside].

Flavonoids represent a large group of metabolites found as natural constituents in a number of plant families, and several recent reviews have dealt with their structure, properties, and biosynthesis. They proved to be able to elicit various biological effects such as on capillary fragility and permeability[23] and on inflammation.[24] Many researchers have demonstrated the in vitro antioxidant/free-radical-scavenging activity of flavonols.[5,7,9,25] Also the antioxidant effects of the phenyl propanoid rosmarinic acid are well recognized.[26]

The findings obtained in the present paper clearly demonstrate that the *n*-BuOH extract of *A. versicolor* leaves as a whole possess antioxidant/free-radical-scavenging effectiveness (EC_{50} 142.6 μg), which seems to be correlated to its total phenolic content (190.6 μg/mg). Furthermore, the extract appeared to contain, as major components, a series of characteristic *C*-glycosyl flavones, such as compounds **1**−**4** and vitexin, and of other phenolic compounds, such as rosmarinic acid. The antioxidant activity of the complex phenolic pool contained in the whole *n*-BuOH extract of *A. versicolor* was potentiated when the extract was fractionated to give fraction II, which showed a lower EC_{50} (47.7 μg) correlatable to a higher total phenol level (319.9 μg/mg). Also the concentration of *C*-glycosyl flavones **1** and **2** and vitexin, used as markers to characterize the flavone content of the extract and fractions, was higher in fraction II than in the whole extract (7.69, 11.8, and 6.82% w/w in fraction II with respect to 1.00, 2.03, 1.56% w/w in the extract), as determined by analytical HPLC.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. UV spectra were obtained with a Perkin-Elmer 550 SE spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm in MeOH solutions. For NMR experiments in CD_3OD, a Bruker DRX-600 spectrometer was used, operating at 599.2 MHz for ^{1}H and 150.9 for ^{13}C and using the UXNMR software package; DEPT, ^{1}H−^{1}H DFQ-COSY (double-quantum filtered COSY), ^{1}H−^{13}C HSQC, and HMBC experiments were obtained using conventional pulse sequences. 1D TOCSY[20] (selective excitation spectra) were acquired using waveform generator-based GAUSS-shaped pulses, with mixing times ranging from 100 to 120 ms, and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. Chemical shifts are expressed in δ (ppm) referring to the following solvent peaks: δ_H 3.34 and δ_C 40.0 for CD_3OD. The FABMS were recorded in a glycerol matrix in the negative-ion mode on a VG ZAB instrument (XE atoms of energy 2−6 kV). Semi-preparative HPLC separations were carried out on a Waters Model 6000A pump equipped with a U6K injector and a Model 401 refractive index detector. Quantitative HPLC analysis was performed with a Shimadzu LC-10AD system equipped with a Model SPD-10AV UV−vis detector and a Rheodyne Model 7725 injector (Millipore, Boston, MA), loop 20 μL. Peak areas were calculated with a Shimadzu Chromatopac C-R6A integrator. TLC analysis was performed on Si gel SiF_{254} (Merck) and visualized with the spray reagents cerium sulfate in H_2SO_4 or vanillin (3 g of vanillin, 4 mL of HCl, 100 mL of MeOH). 1,1-Diphenyl-2-picrylhydrazyl radical, apigenin, and α-tocopherol were purchased from Sigma-Aldrich (Milan, Italy).

Plant Material. The leaves of *A. versicolor* Sodiro were collected near Riobamba, Ecuador, in February 1996 and identified by Dr. M. Tapia, ESPOCH. A specimen of the plant (A.V. 1, 1996) used in this study has been deposited at the Herbarium of ESPOCH, Riobamba, Ecuador.

Extraction and Isolation. The powdered, dried leaves (195 g) were defatted at room temperature with petroleum ether and $CHCl_3$ and then extracted with MeOH to give 13.26 g of residue. This was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH-soluble portion (4.06 g). An aliquot (2.0 g) of the *n*-BuOH extract was chromatographed over a Sephadex LH-20 column (100 × 5 cm) using MeOH as eluent. Fractions (9 mL) were collected and checked by TLC [Si gel, *n*-BuOH−HOAc−H_2O (60:15:25)]. Fractions 9−25 (I) (118 mg), containing an iridoid and phenolic mixture revealed by TLC, were submitted to RP-HPLC on a C_{18} μ-Bondapack column (30 cm × 7.8 mm, flow rate 2.5 mL min^{-1}) using MeOH−H_2O (4:6) as the eluent to yield ipolamide[12] (5 mg, t_R 3.8 min). Fractions 26−60 (317 mg), containing a phenolic mixture as indicated by TLC, were separated using MeOH−H_2O (1:1) as the eluent, giving rosmarinic acid[16] (6 mg; t_R 2.6 min), compounds **1** (20 mg; t_R 26.0 min), **2** (10 mg; t_R 17.1 min), **3** (6 mg; t_R 29.8 min), and **4** (4 mg; t_R 24.5 min], and vitexin[13−15] (apigenin-8-*C*-*β*-D-glucopyranoside, 8 mg; t_R15 min).

Compound 1: amorphous powder; mp 210−212 °C; $[\alpha]^{20}_D$ −79.5° (*c* 1, MeOH); UV (MeOH) λ_{max} (log ϵ) 329 (4.40), 273 (4.30) nm; ^{1}H NMR δ 3.91 (3H, s, OMe), 6.50 (1H, s, H-8), 6.63 (1H, s, H-3), 7.10 (2H, d, J = 8.0 Hz, H-3′ and H-5′), 7.93 (2H, d, J = 8.0 Hz, H-2′ and H-6′); ^{13}C NMR δ 183.8 (C-4), 165.6 (C-2 and C-7), 164.4 (C-4′), 162.1 (C-5), 158.7 (C-9), 129.2 (C-6′ and C-2′), 124.4 (C-1′), 115.6 (C-3′ and C-5′), 109.2 (C-6), 105.0 (C-10), 104.3 (C-3), 95.5 (C-8), 56.1 (OMe); ^{1}H and ^{13}C NMR for the sugar chain, see Table 2; FABMS *m/z* 591 [M − H]$^-$; 445 [(M − H) − 146]$^-$, 429 [(M − H) − 162]$^-$; *anal.* C 56.82%, H 5.40%, calcd for $C_{28}H_{32}O_{14}$, C 56.76%, H 5.44%.

Compound 2: amorphous powder; mp 200−201 °C; $[\alpha]^{20}_D$ −58.2° (*c* 0.5, MeOH); UV (MeOH) λ_{max} 336 (4.44), 276 (4.30) nm; ^{1}H and ^{13}C NMR data for the aglycon were almost superimposable on those of compound **1**; ^{1}H and ^{13}C NMR for the sugar chain, see Table 2; FABMS *m/z* 577 [M − H]$^-$; 445 [(M − H) − 132]$^-$, 429 [(M − H) − 148]$^-$; *anal.* C 56.10%, H 5.19%, calcd for $C_{27}H_{30}O_{14}$, C 56.03%, H 5.23%.

Compound 3: amorphous powder; mp 208−210 °C; $[\alpha]^{20}_D$ −63.1° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 328 (4.38), 272 (4.32) nm; ^{1}H and ^{13}C NMR data for the aglycon were almost superimposable on those of compound **1**; ^{1}H and ^{13}C NMR for the sugar chain, see Table 2; FABMS *m/z* 577 [M − H]$^-$; 445 [(M − H) − 132]$^-$, 429 [(M − H) − 148]$^-$; *anal.* C 56.05%, H 5.20%, calcd for $C_{27}H_{30}O_{14}$, C 56.03%, H 5.23%.

Compound 4: amorphous powder; mp 207−209 °C; $[\alpha]^{20}_D$ −90.4° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 331 (4.42), 270 (4.30) nm; ^{1}H NMR δ 3.90 (3H, s, OMe), 6.37 (1H, s, H-6), 6.61 (1H, s, H-3), 7.12 (2H, d, J = 8.0 Hz, H-3′ and H-5′), 7.99 (2H, d, J = 8.0 Hz, H-2′ and H-6′); ^{13}C NMR δ 183.6 (C-4), 165.9 (C-7), 165.2 (C-2), 164.4 (C-4′), 162.1 (C-5), 157.5 (C-9), 129.3 (C-6′ and C-2′), 124.4 (C-1′), 115.8 (C-3′ and C-5′), 105.1 (C-8), 104.8 (C-10), 104.3 (C-3), 99.2 (C-6), 56.2 (OMe); ^{1}H and ^{13}C NMR data for the sugar chain were almost superimposable on those of compound **1**; FABMS *m/z* 591 [M − H]$^-$; 445 [(M − H) − 146]$^-$; *anal.* C 56.75%, H 5.40%, calcd for $C_{28}H_{32}O_{14}$, C 56.76%, H 5.44%.

Quantitative HPLC Analysis. Preparation of Standard Solutions. To prepare a standard solution containing compounds **1** and **2** and vitexin, accurately weighed amounts of each compound were dissolved in MeOH. Serial concentrations with a range 0.90−3.00 μg/mL for **1**, 3.50−8.75 μg/mL for **2**, and 4.75−9.50 μg/mL for vitexin were prepared.

Calibration. Quantitative HPLC was conducted using a C-18 μ-Bondapack column (150 × 3.9 mm i.d.). The mobile phase was a linear solvent gradient starting from 30% to 100% MeOH (solvent B) in 0.01 M phosphate buffer, pH 5.0 (solvent A) over 45 min. The elution gradient was 30−40% of B in 5

min, 40−65% from 5 to 30 min, and 65−100% from 30 to 45 min. The analyses were carried out in triplicate, the flow-rate was 1 mL/min, the absorbance was monitored with a UV detector set at λ 270 nm, and the injection volume was 20 μL. Calibration graphs were plotted showing a linear relationship between concentration versus peak areas for all compounds. The regression equations were $y = 232933x + 1331.8$ ($R = 0.9979$) for **1**, $y = 108854x + 76.429$ ($R = 0.9995$) for **2**, and $y = 125239x - 1672.7$ ($R = 0.9964$) for vitexin, where y is the peak area and x is the concentration used.

Quantitative Analysis of the Extract and Fractions. Accurately weighed amounts of the *n*-BuOH extract of *A. versicolor* and fraction I and fraction II were dissolved in MeOH and analyzed at the same chromatographic conditions as used for compounds **1**, **2**, and vitexin. The attribution of the chromatographic peak was based on the retention times of the single compounds and confirmed by analysis in comparison with the isolated standards. The concentrations of each compound were calculated from the experimental peak areas by analytical interpolation in standard calibration lines as 1.00%, 7.69%, 0.35% for compound **1**, 2.03%, 11.18%, 1.27% for compound **2**, and 1.56%, 6.82%, 0.13% for vitexin, respectively in the extract, in fraction II, and in fraction I.

Quantitative Determination of Total Phenols. The *A. versicolor* dried *n*-BuOH extract and fractions I and II, dissolved in MeOH, were analyzed for their total phenolic content according to the Folin−Ciocalteu colorimetric method.[8,10] Total phenols were expressed as apigenin equivalents (μg/mg extract). Results are reported in Table 1.

Bleaching of the Free-Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test). The antiradical activities of the *A. versicolor* extract and fractions and α-tocopherol under investigation were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^\bullet$) and the procedures described by Rapisarda et al.[8] and Saija et al.[11] In its radical form, DPPH$^\bullet$ has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 μL) of the MeOH solution containing different amounts of the *n*-BuOH extract or of fractions I and II from *A. versicolor* was added to 1.5 mL of daily prepared DPPH$^\bullet$ solution (0.025 g/L in methanol); the maximum concentration employed was 200 μg/mL. An equal volume (37.5 μL) of the vehicle alone was added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV−visible spectrophotometer 20 min after starting the reaction. The DPPH$^\bullet$ concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH$^\bullet$ (% DPPH$^\bullet_{REM}$) was calculated as follows:

$$\% \text{ DPPH}^\bullet_{REM} = [\text{DPPH}^\bullet]_T/[\text{DPPH}^\bullet]_0 \times 100$$

where T is the experimental duration time (20 min). α-Tocopherol was used as a positive control in the test. All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated by using the Litchfield & Wilcoxon[27] test. Results are reported in Table 1.

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