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 factions, 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, I, 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-xylpyranosyl-(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.

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–Ciocalteu method and expressed as apigenin equivalents, was 190.6 μg/mg. As to the DPPH test, the free-radical-scavenging effect elicited by the n-BuOH extract of A. versicolor was concentration-dependent, so that the EC₅₀ value was calculated as 142.6 μg 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 (EC₅₀ 47.7 μg) and showed a higher level of total phenols (319.9 μg/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 (EC₅₀ 10.1 μg) 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 from fraction I and five flavonols (1–4 and vitexin) and rosmarinic acid 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. 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-xylpyranosyl-(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...
The negative FABMS of compound \( \textbf{1} \) 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 \(^{1}H\) and \(^{13}C\) NMR analysis.\(^{13-15}\) The \( 600 \) MHz \(^1H\) NMR spectrum (Table 2) of \( \textbf{1} \) showed signals for two anomeric hydrogens (\( \delta \) 4.93 and 5.22) and a methyl doublet signal (\( \delta \) 1.31, \( J = 6.0 \) Hz) in the aliphatic region, suggesting the occurrence of two sugar residues. The other sugar signals were overlapped in the region between \( \delta \) 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 \( \delta \) 6.63 (1H, br s), typical of H-3 of a flavone, two signals at \( \delta \) 7.10 and 7.93 (each 2H, \( J = 8.0 \) Hz), the multiplicity of which indicated a 4'-substituted ring B, and another proton singlet at \( \delta \) 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 \( \delta \) 3.91 and \( \delta \) 56.1. The position of the \(-\text{OME}\) on ring B was indicated by the cross-peaks between the \(-\text{OME}\) signal (\( \delta \) 3.91) and C-4' (\( \delta \) 164.4) and between both H-3'/H5' (\( \delta \) 7.10) and H-2'/H-6' (\( \delta \) 7.93) signals and C-4' in the HMBC spectrum. These data led to the identification of the aglycon as acacetin or 4'-methoxypapigenin. The \(^{13}C\) NMR spectrum of \( \textbf{1} \) (Experimental Section and Table 2) indicated the presence of 15 aromatic carbon resonances for the aglycon, an \(-\text{OME}\) 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 (\( \delta \) 109.2) and C-8 (\( \delta \) 95.5) were almost superimposable on those of isovitexin,\(^{13}\) and different from data reported for 8-C-glucosylflavones such as 2'-O-rhamnopyritylvin (\( \delta \) 104.1 and 98.2), and diagnostic for the distinction between 6-C- and 8-C-glycosylflavones.\(^{17-19}\) The C-6 substitution of \( \textbf{1} \) was substantiated by correlations seen in the HMBC spectrum, which allowed the assignments of all \(^{1}H\) and \(^{13}C\) NMR signals, as reported in the Experimental Section.

The sugar residue was determined to be \( \alpha-L\)-rhamnopyranosyl-(1\(\rightarrow\)3)-\(\beta\)-D-glucopyranoside linked to C-6 of the aglycon using 1D TOCSY,\(^{20}\) 2D DQF-COSY, and HSQC NMR experiments. Two anomeric signals at \( \delta \) 4.93 and 5.22, readily identified in the \(^1H\) NMR spectrum of \( \textbf{1} \), correlated to carbons at \( \delta \)C 75.3, characteristic of a C-glucoside, and \( \delta \)C 103.0, respectively, in the HSQC spectrum. In the 1D TOCSY spectrum of \( \textbf{1} \), the H-1” signal at \( \delta \) 4.93 (\( J = 7.5 \) Hz) showed correlations to four methines and a methylene. The COSY spectrum established the proton sequence within this monosaccharide as H-1” to H-2’’, 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 (\( \delta \) 1.31, \( J = 6.5 \) Hz), allowed the identification of the H-3’’/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 \( \beta \)-configuration for the D-glucopyranosyl unit (\( J_{H1-H2} = 7.5 \) Hz) and the \( \alpha \)-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 \( \textbf{1} \) indicated all of the protonated carbon correlations and thereby led to the assignment of the interglycosidic 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 \( \alpha-L\)-rhamnopyranosyl moiety suggested that this sugar was the terminal unit. Glycosidation shifts were observed for C-3’” (\( \delta \) 87.1, 87.5,

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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

<table>
<thead>
<tr>
<th>extract and fractions</th>
<th>phenol content (^{a}) (( \mu g/mg ) extract)</th>
<th>DPPH test ( [EC_{50} \ (\mu g \ of \ extract)] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-BuOH extract</td>
<td>190.6 ± 2.44</td>
<td>142.6 (117.9–172.5) (^{c})</td>
</tr>
<tr>
<td>fraction I</td>
<td>200.0 ± 3.15</td>
<td>100.7 (83.3–122.6) (^{d})</td>
</tr>
<tr>
<td>fraction II</td>
<td>319.9 ± 3.69</td>
<td>47.7 (48.4–56.4) (^{d})</td>
</tr>
<tr>
<td>( \alpha )-tocopherol(^{d})</td>
<td>10.1</td>
<td>10.1 (8.8–11.4) (^{d})</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean \pm S.D. of three determinations. \(^{b}\) Apigenin equivalents. \(^{c}\) 95% confidence limits. \(^{d}\) Positive control.
The position of the sugar residues in 1 were defined unambiguously by the multiple-bond heteronuclear correlation (HMBC) NMR experiments. The disaccharidic unit was established unambiguously by the HMBC spectrum to be C-6 of the aglycon, and the presence of a terminal pentose unit, instead of a terminal rhamnopyranosyl in 2, was confirmed (Table 2). The nature of the terminal sugar unit as \( \beta \)-d-apiofuranosyl was deduced by the following evidence: the \( \text{\(^1\)}\)H NMR spectrum indicated an anomeric signal at \( \delta \) 5.31 (H-1\( \beta \), d, \( J = 2.0 \) Hz); in the 1D TOCSY experiment, selective excitation of the signal at \( \delta \) 5.31 led to the enhancement only of H-2\( \beta \) (d, \( J = 2.0 \) Hz); and the multiplicity of H-2\( \beta \) was derived only from the presence of a quaternary carbon at C-3\( \beta \), characteristic of an apiofuranosyl structure. The \( \text{\(^{13}\)}\)C NMR spectrum gave 11 carbon signals for the sugar moiety, of which three methylenes were ascribable to C-4\( \alpha \) (\( \delta \) 109.2), C-3\( \alpha \) (\( \delta \) 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-\([\beta\text{-d-xylopyranosyl-(1→3)\-d-glucopyranoside}]\) (2).

Compound 3 was assigned a molecular formula of \( \text{C}_{26}\text{H}_{30}\text{O}_{14} \), as deduced by a combination of FABMS and \( \text{\(^{13}\)}\)C NMR and DEPT analysis. The \( \text{\(^1\)}\)H and \( \text{\(^{13}\)}\)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 \( \beta \)-d-apiofuranosyl was deduced by the following evidence: the \( \text{\(^1\)}\)H NMR spectrum indicated an anomeric signal at \( \delta \) 5.31 (H-1\( \beta \), d, \( J = 2.0 \) Hz); in the 1D TOCSY experiment, selective excitation of the signal at \( \delta \) 5.31 led to the enhancement only of H-2\( \beta \) (d, \( J = 2.0 \) Hz); and the multiplicity of H-2\( \beta \) was derived only from the presence of a quaternary carbon at C-3\( \beta \), characteristic of an apiofuranosyl structure. The \( \text{\(^{13}\)}\)C NMR spectrum gave 11 carbon signals for the sugar moiety, of which three methylenes were ascribable to C-4\( \alpha \) (\( \delta \) 109.2), C-3\( \alpha \) (\( \delta \) 109.3) of the aglycon, confirming that the disaccharidic chain was bonded by a C-glycosidic linkage to C-6. Therefore, the structure of 3 was determined as acacetin 6-\([\beta\text{-d-xylopyranosyl-(1→3)\-d-glucopyranoside}]\) (3).
acacetin structure was suggested by the H1 (δ 6.37, s, H-6) and 13C 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 permeability23 and on inflammation.24 Many researchers have demonstrated the in vitro antioxidant/free-radical-scavenging activity of flavonoids.5,7,25 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 (EC50 142.6 mg/mL), which seems to be correlated to its total phenolic content (190.6 mg/g). 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 antioxidative 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 EC50 (47.7 μg/mL) correlatable to a higher total phenol level (319.9 μg/mL). 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, and 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 on a Perkin-Elmer 141 polarimeter using a sodium lamp uncotted. UV spectra were obtained with a Perkin-Elmer the extract), as determined by analytical HPLC.

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 CHCl3 and then extracted with MeOH to give 12.12 g of residue. This was partitioned between n-BuOH and H2O (1:1) 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–H2O (60:15:25)]. Fractions 9–25 (118 mg), containing an iridoid and phenolic mixture revealed by TLC, were submitted to RP-HPLC on a C18 μ-Bondapack column (30 cm × 7.8 mm, flow rate 2.5 mL min−1) using MeOH–H2O (4: 6) as the eluent to yield 1-palmitolein (5 mg, tR 3.8 min). Fractions 26–60 (317 mg), containing a phenolic mixture as indicated by TLC, were separated using MeOH–H2O (1:1) as the eluent, giving rosmarinic acid (6 mg, tR 2.6 min, compounds 1 (20 mg; tR 26.0 min), 2 (10 mg; tR 17.1 min), 3 (6 mg; tR 29.8 min), and 4 (4 mg; tR 24.5 min), and vitexin (11–15 mg). Further characterization of compound 4 was performed with a Shimadzu LC-10AD system equipped with a U6K injector and a Model 6000A pump equipped with a Waters 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 Spectrolyzer 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 Silica (Merck) and visualized with the spray reagents cerium sulfate in H2SO4 or vanillin (3 g of vanillin, 4 mL of HCl, 100 mL of MeOH).
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 analytical interpolation in standard calibration lines as used for compounds A. -BuOH extract or of fractions I and II from A. versicolor the MeOH solution containing different amounts of the tication were determined using the stable 1,1-diphenyl-2-tocopherol as a positive control. All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated by using the Litchfield & Wilcoxon\(^2\) test. Results are reported in Table 1.

**References and Notes**