High speed centrifugal countercurrent chromatography (HSCCC) isolation and identification by LC-MS analysis of the polar phenolics from Vasconcellea quercifolia

CRISTINA QUISPE*a, EZEQUIEL VIVEROS-VALDEZ*b, JOSÉ A. YARLEQUEc, MARCO R. ARONESd, JUAN C. PANIAGUAe, GUILLERMO SCHMEDA-HIRSCHMANNf

a Universidad de Talca, Instituto de Química de Recursos Naturales, Laboratorio de Química de Productos Naturales, Casilla 747, Talca, Chile.

b Present address: Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Pedro de Alba s/n, Cd. Universitaria, 66450, San Nicolás de los Garza, Nuevo León, México.

c E.F.P. Farmacia y Bioquímica, Facultad de Ciencias Biológicas, Universidad Nacional de San Cristóbal de Huamanga, Ayacucho, Perú.

(Received: April 24, 2013 - Accepted: May 27, 2013)

ABSTRACT

High speed centrifugal countercurrent chromatography (HSCCC) was successfully applied to preparative isolation of the main polar phenolics from the Peruvian Caricaceae Vasconcellea quercifolia A. St.-Hil. High-performance liquid chromatography with diode array (HPLC-DAD) and electrospray ionisation mass spectrometric detection (ESI-MS) was used to carry out a comprehensive characterisation of phenolic compounds from fruits and leaves of this plant. The main phenolics were the quercetin diglycoside rutin and the triglycoside manghaslin. Nine minor flavonoids were tentatively identified as kaempferol, quercetin, isorhamnetin and methoxymyricetin glycosides. HSCCC combined with other chromatographic methods allowed the isolation of the main phenolics from the plant. HPLC-DAD-MS analysis shows that the main phenolic compounds in V. quercifolia are similar to those described for V. pubescens but differs in the identity of the minor constituents.

Keywords: Vasconcellea quercifolia, Caricaceae, Flavonoids, High-speed countercurrent chromatography, High-pressure liquid chromatography

INTRODUCTION

The Caricaceae plant family comprises three genera and 15 species in Peru1, Carica augusti Harms, known under the common name “pati” is one of the endemic species occurring in the Ayacucho Department.2 Taxonomical identification confirmed that the plant formerly classified as C. augusti was Vasconcellea quercifolia A. St.-Hil. At present, most of the species formerly placed in genus Carica have been moved to the genus Vasconcellea, Carica papaya being the only species in genus Carica.2 According to the oral tradition, the fruit of V. quercifolia is said to have been used by the pre-columbian Huari (Wari) culture in practices associated with shamanism. The Huari or Wari is a pre-Incaic culture that developed between 600 and 1,000 BC in the southern Peruvian Andes. The main settlement of this culture was Wari, a few kilometres away from the city of Ayacucho. Archaeologists have pointed out to the fact that the “pati” tree is usually found in archaeological places associated with pre-Columbian cultures.2,3

Thin layer chromatography analysis of the “pati” extracts and colour reactions showed that the plant contain flavonoids. Several chromatographic methods can be used for the isolation of phenolics in plant extracts.6,8 However, irreversible adsorption and/or decomposition of some sample constituents is a disadvantage of solid supports such as silica gel. Low and medium pressure chromatography, including normal and reversed phase supports are solvent and time consuming. High speed counter current chromatography (HSCCC) is a support-free liquid-liquid partition technique in which the stationary phase and the mobile phase are liquids. A long length of tubing wrapped as a coil form a bobbin that act as a “column” which is geared to the main rotor. The hydrodynamic system (HSCCC) rotates about their own axis and also around the centrifugal axis in planetary motion. The method relies on the partition of the sample between two immiscible solvents according to the respective partition coefficients. The bobbin is usually filled with the phase intended to be the stationary phase and the mobile phase is pumped in the end of the column.6,8 High-speed counter current chromatography was successfully applied to the preparative isolation of flavonoid glycosides in medicinal and food plants including Ampelopsis grossedentata10, Paeonia suffruticosa11, Hippophaë rhamnoides12, Crataegus laevigata13 and Artemisia spp.14

The use of HSCCC for the preparative isolation of main compounds in mixtures and the detection/fingerprinting of extracts constituents by HPLC-ESI-MS are complementary methods for the characterization of constituents in plant extracts.11,12 While the main compounds are isolated by HSCCC and identified by spectroscopic and spectrometric means, the tentative identification of the minor constituents is proposed using HPLC-ESI-MS techniques. Following our studies on South American plants, we examined extracts from the fruits and leaves of “pati” looking selectively for phenolics. Several chromatographic methods were used to isolate the main compounds and to compare the constituents of fruit and leaves. The aim of this work was to disclose the phenolic composition of the species and to compare the constituents with those reported from the edible V. pubescens.16

EXPERIMENTAL

Apparatus

The HSCCC instrument used was a Quattro MK5 Lab Prep (AAECE, S. Wales, U.K.) equipped with four polytetrafluoroethylene multilayer coil of 5 m x 2.16 mm i.d. (SS) tubing of approximately 120 mL of volume capacity each. The solvent was pumped into the column with a SSI Serie II HPLC pump using a constant flow of 3.0 mL/min. A manual sample injection valve with a 10 mL loop was used to introduce the sample into the column. The fraction collector was used in a Gilson, Inc. (Middleton, USA) model FC 203B.

HPLC-DAD analysis was performed using a Merck-Hitachi (Lachrom, Tokio, Japan) equipment consisting of an L-7100 pump, an L-7455 UV diode array detector (DAD), and D-7000 chromatointegrator. The injected volume was 20 μL. Precoated silica gel plates (Merck, Kieselgel 60 F254, 0.25 mm) were used for thin layer chromatography (TLC) analysis of the fractions and extracts.

Plant material

The plant used for the present study was collected at the Ayacucho Department, Peru. A voucher herbarium specimen was identified by the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru on April 7, 2011, as Vasconcellea quercifolia A. St.-Hil. (Certificate No 02-2011).

The fruits and leaves from adult trees were collected in February 2011 in a place clearly related to the Wari culture, in the archeological area of Ayacucho. The leaves were dried at room temperature (25-35°C) for three weeks, powdered and extracted with ethanol. Two different extraction methods were used for the fruits. A representative fresh fruit sample (whole fruit, including flesh and seeds) was washed and homogenized in a blender with ethanol as described below. A second fruit sample was washed, the seeds were separated and the flesh was cut into slices and dried at room temperature for 3 weeks at the shadow. Then, the air-dried material was powdered and extracted with ethanol at room temperature as described. The different extraction protocols for the fruits were aimed to compare possible differences in composition according to the drying process. Also alkaloids were investigated but we did not detected alkaloids in any of the extracts. The whole extracts were lyophilized for a better stability of the extract constituents using a Labeconco Freeze dry system equipment (Kansas City, USA) until a powder was obtained.

Extraction and isolation of Phenolic Compounds

Fruits

The whole dried fruits (0.9 kg) were homogenized in a blender and macerated with 2 L ethanol at room temperature for 24 h with agitation. Then, the fruit was extracted three times for 30 min under reflux with ethanol.
Extracts were filtered, combined and concentrated under reduced pressure to afford a semisolid mass (97.02 g) that after lyophilization afforded 77.35 g of a dark brown powder. The extract was resuspended in 1 L distilled water and extracted with dichloromethane (2 x 500 mL) to afford a dichloromethane-soluble fraction, a water-soluble part and a precipitate in the interphase (245 mg). The unpolar fraction, consisting mainly of waxes and chlorophyll was not further investigated. The water-soluble fraction was extracted with ethyl acetate (3 x 200 mL) to afford an ethyl acetate extract (240 mg). After concentration under reduced pressure, the aqueous phase was passed through an Amberlite XAD-7 column, the column was washed with distilled water and eluted with 300 mL methanol to afford the Amberlite-retained fraction (1.6 g). The Amberlite-retained fraction (1.6 g) was permeated on a Sephadex LH-20 column with methanol. The void volume was 120 mL. Some 40 fractions of 20 mL each were collected and pooled together according to the TLC patterns as follows: 1-7; 8-9; 10-12; 13-23; 24-30 and 31-40.

A second fruit collection (10 kg fresh fruits) was dried at room temperature (25 °C) The plant material was powdered and extracted with ethanol at room temperature for 24 h. The plant/solvent ratio was 0.5 kg/1 L and the extraction was repeated three times. After concentration under reduced pressure and lyophilization, 31.44 g of a crude extract was obtained. Some 31.4 g of the freeze-dried crude extract was resuspended in 2 L hot water and sonicated for 10 min. After filtration, an aqueous extract and an insoluble, lipophilic fraction (4.18 g) were obtained. The water-soluble fraction was passed through an Amberlite XAD-7 column, rinsed with distilled water and desorbed with MeOH (2 x 0.3 L) yielding after concentration under reduced pressure and lyophilization 2.78 g of a dark brown powder. The extract was analyzed for constituents by HPLC-DAD-MS.

**Columns**

- **Preparation of Two-Phase Solvent System and Sample Solutions**
  - The behaviour of the sample was monitored in TLC silica gel. The eluting solvent was the upper phase and lower phase of each of the systems tested, and the detection was UV light at 254 nm. The solvent system selected in the present study was n-butanol:ethyl acetate:water (3:2:5, v/v). The solvents were mixed according to the volume ratios of the two-phase solvent system selected and then thoroughly equilibrated by shaking repeatedly in a separation funnel. Prior to use, the upper phase and the lower phase were separated and degassed by sonication for 25 min. A sample solution of 5 mL was prepared by the dissolution of 540 mg of extract into 2.5 mL of the upper phase and 2.5 mL of the lower phase. The lower phase (aqueous phase) was used as the stationary phase while the mobile phase was the upper layer (organic phase).

**HSCC Separation Procedure**

Sample solutions prepared of the lyophilized Amberlite XAD-7 retained leaf extract fraction were subjected to HSCCC. The separation conditions were as follows. Flow rate: 3.0 mL/min. Temperature: 20°C. Centrifugation speed: 500 rpm. Three out of the four coils were used in the head to tail mode. After hydrodynamic equilibrium, the volume of the stationary phase retained in the coils was 95 mL. The injection volume was 5 mL. Fractions of 8 mL each were collected and pooled together according to the TLC analysis. Fractions 1-7; 8-9; 10-12; 13-23; 24-30 and 31-40 were collected and pooled together according to the TLC patterns as follows.

**LC-DAD and LC-ESI-MS Analysis**

The compounds were monitored at 250 and 320 nm, and UV spectra from 200-600 nm were recorded for peak characterization. For comparative HPLC analyses, detection was set at 250 nm. HPLC-DAD and HPLC-MS analysis were carried out using a 250 mm x 4.60 mm i.d., 5 μm C18-RP Kromasil 100 column (Eka Chemicals, Brewster NY, USA) maintained at 25°C with a linear gradient solvent system consisting of 1% formic acid (A) and MeOH (B) as follows: 90% to 75% A over 20 min; followed by 75% to 40% A from 20 to 45 min, 40% to 25% A from 45 to 50 min, 25% to 40% A from 50 to 55 min, 40% to 75% A from 55 to 60 min and 75% to 90%A from 60 to 75 min at a flow rate of 1.0 mL/min. The injected volume was 20 μL.

HPLC coupled to mass spectrometry was performed using a Squire 4000 plus ion trap spectrometer fitted with electrospray ionization (Bruker Daltonics, Billerica, MA, USA). The capillary voltage was 4000 V. Nitrogen was used as nebulizer gas at 350°C at a flow of 8.0 L per minute, nebulizer pressure was 27.5 psi. Spectra were recorded between m/z 150 and 2000 in positive ion mode. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 v (MS/MS) using helium as the collision gas. The methanol-soluble fraction of the fruit extract as well as the polar leaf extract was dissolved in methanol:water: formic acid (7:3:0.1 % v/v/v, aprox. 1.5 mg/mL) filtered through a 0.45 μm PTFE filter (Waters), and submitted to HPLC-DAD and HPLC-ESI-MS analysis.

**1H and 13C NMR Analysis**

The structures of purified compounds from fruit and leaves were determined by NMR. 1H and 13C, DEPT, COSY, NOESY, gHMBC, and gHMBC NMR spectra (g = gradient enhanced) were measured on a Bruker Avance spectrometer (Bruker, Rheinstetten, Germany) operating at 400 MHz for 1H and 100 MHz for 13C. All spectra were obtained in CD3OD with chemical shifts expressed in δ (ppm) and coupling constant (J) in Hertz (Hz).

**RESULTS AND DISCUSSION**

**HSCC Purification**

The crude fruit and leaves extracts of C. quercifolia before and after adsorption on Amberlite XAD 7 resin were analyzed by HPLC as described above (Figure 1). The leaves extract (Figure 1 B), shows two compounds that were isolated by HSCCC. The HSCCC fractions of the leaves extract were collected and pooled together according to the TLC analysis. Fractions 1-50 did not contain compounds of interest and were discarded. The main compounds eluted in fractions 51-57 (78 mg, rutin), 58-59 (30 mg, main compound: rutin; minor compound manganisin 3:2), 60-79 (93 mg, main compound: manganisin) and 90-110 (40.4 mg, manganisin and minor flavonoids).

The main fractions of HSCCC were analyzed by HPLC as described above. The chromatograms are presented in Figure 2.

**Figure 1.** HPLC chromatograms of crude *Vasconcellea quercifolia* extracts before (A₁, B₁) and after adsorption (A₂, B₂) on Amberlite XAD 7 resin. A: Fruits; B: Leaves. Detection: UV, 250 nm. Compounds: 2: Manghaslin; 5: Rutin.

**Figure 2.** HPLC chromatograms of selected HSCCC fraction from the leaf extract of *V. quercifolia*. Fraction pools: A: 51-57; B: 60-79. Detection: UV, 250 nm. Compounds: 2: Manghaslin; 5: Rutin.

**LC-DAD and LC-ESI-MS Analysis**

**Leaf Extract**

The HPLC trace of the leaf extract (Figure 1) showed two main constituents and several minor compounds. It could not be completely resolved even using different mixtures of acidified water and methanol as well as acidified water and acetonitrile gradients. The two main phenolics from the leaf extract were isolated by HSCCC (Figure 2), allowing characterization by spectroscopic and spectrometric means. The UV spectrum of compound 2 suggested a flavonol glycoside and the MS gave [M+H]+ at m/z 757, which generated ions at m/z 611, 465, 303, 252, in agreement with a quercetin glycoside bearing two rhamnose and a hexose units (Table 1).

The 1H NMR spectrum of compound 2 showed aromatic signals at δ 7.51 (d, J = 2.2 Hz), δ 7.52 (dd, J = 8.3 and 2.2 Hz) and δ 6.79 ppm (d, J = 8.3), a pair of doublets at δ 6.27 (1H, J = 2.2 Hz) and δ 6.08 (1H, J = 2.2 Hz), three anomeric H signals at δ 5.50 (d, J = 7.6 Hz), δ 5.15 br s and δ 4.43 ppm (brs), two methyl group at δ 0.99 and δ 0.92 ppm (d, J = 6.3 Hz) and overlapped signals in the range 3.20-4.01 ppm, indicating a quercetin triglycoside. The sugar sequence follows from the 13C resonances as well as 1H assigned from COSY, HSQC and HMBC experiments. The 13C NMR data of compound 2 is summarized in Table 2. The compound was identified as quercetin-3-O-(2'-rhamnosyl rutinoside) (manghaslin) (compound 2) in full agreement with the spectroscopic data of the same compound isolated from *Vasconcellea pubescens*.

The 1H NMR spectrum of compound 5 showed aromatic signals at δ 7.76 (d, J = 2.2 Hz), δ 7.72 (dd, J = 8.3 and 2.2 Hz) and δ 6.97 ppm (d, J = 8.3), a pair of doublets at δ 6.47 (1H, J = 2.2 Hz) and δ 6.28 (1H, J = 2.2 Hz) as well as two anomeric H signals at δ 5.19 (d, J = 7.6 Hz) and δ 4.62 ppm (brs), a methyl group at δ 1.22 (d, J = 6.3 Hz) and overlapped signals in the range 3.20-3.90 ppm, indicating a diglycoside of quercetin. The compound was unambiguously identified as rutin by its 13C NMR data, correlation spectra and comparison with a reference sample. The 13C NMR data of compound 5 are presented in Table 2. Full scan ESI and MS mass spectra of compounds 2 and 5 are shown in Figure 4.

**Table 1.** Tentative identification of phenolic compounds in *Vasconcellea quercifolia* fruit and leaf extract by LC-DAD, LC-MS and MS/MS data. For minor compounds, no UV data can be given.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>λ Max (nm)</th>
<th>MW</th>
<th>[M+H]+ and MS/MS ions</th>
<th>Tentative identification</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.78</td>
<td>352, 300 sh, 260 sh, 255</td>
<td>756</td>
<td>757, 611, 465, 303, 252</td>
<td>Manghaslin* (M)</td>
<td>2</td>
</tr>
<tr>
<td>35.10</td>
<td>-</td>
<td>784</td>
<td>-</td>
<td>Kaempferol rhamnoside diglucuronide</td>
<td>8</td>
</tr>
<tr>
<td>37.20</td>
<td>346, 300 sh, 263</td>
<td>740</td>
<td>741, 595, 449, 287</td>
<td>Kaempferol 3-(2'-rhamnosyl rutinoside)</td>
<td>1</td>
</tr>
<tr>
<td>37.86</td>
<td>352, 255</td>
<td>770</td>
<td>771, 625, 479, 317</td>
<td>Isorhamnetin 3-(2'-rhamnosyl rutinoside)</td>
<td>3</td>
</tr>
<tr>
<td>37.89</td>
<td>-</td>
<td>517</td>
<td>-</td>
<td>Quercetin glucuronide K salt</td>
<td>9</td>
</tr>
<tr>
<td>38.00</td>
<td>355, 300 sh, 260 sh, 255</td>
<td>778</td>
<td>779, 633, 477, 331</td>
<td>Dimethoxyquercetin-dirhamnoside-156</td>
<td>Unknown</td>
</tr>
<tr>
<td>39.38</td>
<td>-</td>
<td>547</td>
<td>-</td>
<td>Methoxymyricetin glucuronide K salt</td>
<td>11</td>
</tr>
<tr>
<td>39.62</td>
<td>355, 300 sh, 260 sh, 255</td>
<td>610</td>
<td>611, 465, 303</td>
<td>Rutin* (M)</td>
<td>5</td>
</tr>
<tr>
<td>40.64</td>
<td>350, 300 sh, 255</td>
<td>624</td>
<td>625, 479, 317</td>
<td>Isorhamnetin rutinoside</td>
<td>6</td>
</tr>
<tr>
<td>42.43</td>
<td>-</td>
<td>501</td>
<td>-</td>
<td>Kaempferol glucuronide K salt</td>
<td>7</td>
</tr>
<tr>
<td>44.04</td>
<td>348, 300 sh, 264</td>
<td>594</td>
<td>595, 285</td>
<td>Kaempferol rutinoside</td>
<td>4</td>
</tr>
<tr>
<td>48.54</td>
<td>-</td>
<td>531</td>
<td>-</td>
<td>Isorhamnetin glucuronide K salt</td>
<td>10</td>
</tr>
</tbody>
</table>

*Identified by NMR, MS, UV spectra and comparison with a standard sample. M: main compounds.
Table 2. ¹³C NMR data of manghaslin (compound 2) and rutin (compound 5) isolated from Vasconcellea quercifolia fruits (100 MHz, CD₂OD, δ-values).

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>157.04 s</td>
<td>157.07 s</td>
</tr>
<tr>
<td>3</td>
<td>133.05 s</td>
<td>134.27 s</td>
</tr>
<tr>
<td>4</td>
<td>177.87 s</td>
<td>177.90 s</td>
</tr>
<tr>
<td>5</td>
<td>160.32 s</td>
<td>161.52 s</td>
</tr>
<tr>
<td>6</td>
<td>98.43 d</td>
<td>98.60 d</td>
</tr>
<tr>
<td>7</td>
<td>164.35 s</td>
<td>164.57 s</td>
</tr>
<tr>
<td>8</td>
<td>93.36 d</td>
<td>93.53 d</td>
</tr>
<tr>
<td>9</td>
<td>157.53 s</td>
<td>157.92 s</td>
</tr>
<tr>
<td>10</td>
<td>104.48 s</td>
<td>104.34 s</td>
</tr>
<tr>
<td>1'</td>
<td>122.07 d</td>
<td>121.75 s</td>
</tr>
<tr>
<td>2'</td>
<td>114.69 d</td>
<td>114.71 d</td>
</tr>
<tr>
<td>3'</td>
<td>144.51 s</td>
<td>144.42 s</td>
</tr>
<tr>
<td>4'</td>
<td>148.15 s</td>
<td>148.40 s</td>
</tr>
<tr>
<td>5'</td>
<td>116.04 d</td>
<td>116.37 d</td>
</tr>
<tr>
<td>6'</td>
<td>122.16 s</td>
<td>122.22 d</td>
</tr>
</tbody>
</table>

Glucose

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.10 d</td>
</tr>
<tr>
<td>2</td>
<td>78.66 d</td>
</tr>
<tr>
<td>3</td>
<td>77.51 d</td>
</tr>
<tr>
<td>4</td>
<td>70.88 d</td>
</tr>
<tr>
<td>5</td>
<td>75.66 d</td>
</tr>
<tr>
<td>6</td>
<td>66.89 t</td>
</tr>
</tbody>
</table>

Rhamnose

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>101.23 d</td>
</tr>
<tr>
<td>2</td>
<td>71.00 d</td>
</tr>
<tr>
<td>3</td>
<td>70.74 d</td>
</tr>
<tr>
<td>4</td>
<td>72.67 d</td>
</tr>
<tr>
<td>5</td>
<td>68.57 d</td>
</tr>
<tr>
<td>6</td>
<td>16.42 q</td>
</tr>
</tbody>
</table>

Rutin

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.85 d</td>
</tr>
<tr>
<td>2</td>
<td>70.92 d</td>
</tr>
<tr>
<td>3</td>
<td>70.48 d</td>
</tr>
<tr>
<td>4</td>
<td>72.49 d</td>
</tr>
<tr>
<td>5</td>
<td>68.32 d</td>
</tr>
<tr>
<td>6</td>
<td>16.12 q</td>
</tr>
</tbody>
</table>

The ¹H NMR spectrum of the pooled fractions 80-110 from the HSCCC separation showed a triglycoside (compound 3) with the same substitution pattern as compound 2 but differing in the presence of a methoxyl group at δ 4.03 ppm. The ¹H NMR spectrum of compound 3 showed aromatic signals at δ 7.62 (d, J=2.2 Hz), δ 7.63 (dd, J=8.3 and 2.2 Hz) and δ 6.90 ppm (d, J=8.3), a pair of doublets at δ 6.39 (1H, J=2.2 Hz) and δ 6.20 ppm (1H, J=2.2 Hz), three anomic H signals at δ 5.60 (d, J=7.6 Hz), δ 5.25 (brs) and δ 4.53 (br s) ppm, a methoy group at δ 4.03, two methyl group at δ 1.02 (d, J=6.4 Hz) and δ 1.10 (d, J=6.4 Hz) and overlapped signals in the range 3.20-3.90 ppm, pointing out to aisorhamnetin triglycoside. The mass spectrum of compound 3 showed a [M+H]⁺ ion at m/z 771 and consecutive loss of two rhamnoses and one hexose units support the identification of 3 as isorhamnetin 3-(2”-rhamnosyl rutinoside).

Fruit extract

Selected TIC and HPLC chromatograms of Sephadex LH-20 fractions from the fruit extract are shown in Figure 3. Full scan ESI mass spectra and fragmentation of manghaslin (Figure 4a) and rutin (Figure 4b) are shown. The proposed fragmentation of compounds 6, 3 and 1 is presented in Figure 5. Table 1 shows the identification of the phenolic compounds labeled as peaks 1-11 following the elution/isolation order. Table 2 shows the ¹³C NMR data of manghaslin (compound 2) and rutin (compound 5). The proposed structures of flavonol derivatives tentatively identified in C. quercifolia is presented in Figure 6.

From the whole dried fruits, TLC and HPLC analysis of the pooled fractions 1-7, 24-30 and 31-40 did not showed compounds of interest and were discarded. The fraction pool 8-9 presented a main product identified as manghaslin (compound 2) and two minor constituents (compounds 6 and 8). The main constituent in the fraction 10-12 (compound 5) was identified as quercetin-3-O-α-L-rhamnopyanosyl-(1→6)-β-D-glucopyranoside (rutin) based on the ¹H, ¹³C and HMBC data. p-Hydroxybenzoic acid was also present. The fraction pool 13-23 contained rutin as well as minor glycosides. TOF-MS-ESI analysis of the hydrolysates products revealed the presence of the aglycones kaempferol, quercetin and isorhamnetin. After TLC analysis and HPLC fingerprinting, the interphase precipitate (245 mg) and the EtOAc-soluble fraction (240 mg), presenting similar composition, were pooled and analyzed by HPLC-DAD-MS. From the Amberlite-retained fraction of pati fruit extract, a main compound was isolated and identified as rutin (compound 5) by its UV, NMR, mass spectra and Rt comparison with a standard sample. In the methanol-soluble precipitate formed in the dichloromethane:water interphase, rutin was also obtained as a main constituent. Other minor compounds were identified in the Sephadex fractions of the Amberlite-retained extract. The main compound in the HPLC chromatogram of fractions 8-9 was the quercetin triglycoside manghaslin. Two minor constituents were also detected, differing in the aglycone and sugar moieties. While the UV spectrum of the compound found at t=40.64 min is in agreement with a 3-O-flavonol glycoside, the loss of a rhamnose and an hexose bound to an aglycone of [M+H]⁻ at m/z 317 suggest the presence of isorhamnetin rutinoside (compound 6). The compound 8 eluting at t=35.10 min showed an aglycone at [M+H]⁺ at m/z 287, in agreement with kaempferol. The sugars were identified as a rhamnose (146 amu) [M+H+Rhamnose]⁻: 639 and two glucuronic acid units (loss of 352 amu). The structure was tentatively assigned to kaempferol rhamnosyl diglucuronate. The fragmentation found for the compound 1 with a [M+H]⁺ ion at m/z 741, suggest loss of two rhamnose and one hexose attached to the aglycone kaempferol. The structure proposed is in agreement with a flavonol triglycoside differing from manghaslin (compound 2) by the presence of only one hydroxy function in the flavonol B-ring. The HPLC chromatogram of the fraction XAD 10-12 shows a main compound at 39.62 min whose MS fully agree with that of rutin (compound 5). Minor constituents at 42.43 and 48.54 min showed a mass of 502 and 532 amu, respectively and a loss of 215 amu, compatible with a glucuronic acid unit (m/z 176) and K. The genines are in agreement with kaempferol [M+H]⁺: 287 and isorhamnetin [M+H]⁺: 317, respectively. The compounds are tentatively identified as the potassium adduct of glucuronic acid and kaempferol (compound 7) or isorhamnetin (compound 10). Two additional minor flavonoid diglycosides were detected. The mass spectra of the compounds showed the loss of a (rhamnose-hexose) unit leading to the aglycone kaempferol and isorhamnetin. The structures were tentatively identified as rhamnohexoses of kaempferol (compound 4) and isorhamnetin (compound 6), respectively. The last group of fractions from the Sephadex column (XAD 13-23) contained lower molecular weight compounds. In the TOF-MS two main compounds were detected at t = 37.89 and 39.38 min, presenting characteristic loss of 215 amu, compatible with K and glucuronic acid. The genines were tentatively identified as quercetin and methoxymyricetin, respectively (compounds 9 and 11).
Figure 3. TIC (upper) and HPLC chromatogram (lower) of selected LH-20 chromatography fractions from *Vasconcellea quercifolia* fruit extracts. A: Fractions 8-9; B: Fractions 13-23. Detection: UV, 250 nm. Compounds: 2: Manghaslin; 5: Rutin.

Figure 4. Full scan ESI mass spectra and fragmentation (MS²) of the quercetin derivatives manghaslin (compound 2) (Figure 4 a) and rutin (compound 5) (Figure 4 b) unambiguously identified in *Vasconcellea quercifolia* by NMR spectroscopy.

Figure 5. Proposed fragmentation of the flavonol glycosides (m/z 625, 771, 741) (compounds 6, 3 and 1) tentatively identified in *Vasconcellea quercifolia*.
The chromatographic fingerprints of *V. quercifolia* and *V. pubescens* proved to be a suitable method for the fast isolation of the main flavonoids occurring in both fruit and leaf extracts from *V. quercifolia*. The main flavonoids occurring in both fruit and leaf “pati” extracts proved to be the quercetin diglycoside rutin and the corresponding glucuronides as well as diglycosides from kaempferol and identified for the first time. The main compounds were the quercetin flavonol B-ring.

The MS of the compound with pseudomolecular [M+H]⁺ ion at m/z 779 shows the consecutive loss of a rhamnose, a fragment accounting for 156 amu and a second rhamnose unit. The molecular weight of the aglycone is in agreement with dimethoxyquercetin and the glycoside is a dirhamnoside derivative of the flavonol bearing an additional, not identified unit of 156 amu.

Excluding rutin, all other flavonoids are reported for the first time for this species. Triglycosides related to those found in this species were reported from Bidens parviflora and Bidens andicola. Kaempferol-3-O-[α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] was previously isolated from Humulus lupulus flowers. The phenolic profile of *V. quercifolia* shows the same main flavonoids as those found in the edible fruits of *V. pubescens*. However, the minor constituents of *V. pubescens* were ferulic or isoferulic acid glucosides, different from the kaempferol andisorhamnetin glycosides occurring in the Peruvian *V. quercifolia*. The main flavonoids occurring in both fruit and leaf “pati” extracts proved to be the quercetin diglycoside rutin and the corresponding glucuronides as well as diglycosides from kaempferol andisorhamnetin, among others.

**CONCLUSION**

HSCCC proved to be a suitable method for the fast isolation of the flavonoid glycosides from *V. quercifolia*, allowing further unambiguous characterization of the main compounds by NMR spectroscopy and giving support to the tentative identification of additional constituents. From the aerial parts and fruits of *V. quercifolia*, several flavonoid glycosides were isolated and identified for the first time. The main compounds were the quercetin triglycoside manghasil and the diglycoside rutin. Other flavonoids differing in the hydroxylation pattern of the B ring were tentatively identified based on the UV and MS data. The genin included kaempferol, quercetin,isorhamnetin, and the corresponding glucuronides as well as diglycosides from kaempferol andisorhamnetin, among others.

The chromatographic fingerprints of *V. quercifolia* phenolics can be used for comparison with other related species and suggest a trend in the chemistry of Caricaceae fruits, where rutin and manghasil appear to be the main phenolics. The phenolics occurring in *Vasconcellea quercifolia* have been reported as free radical scavengers and antioxidant compounds. While the fruits of this Peruvian cariaceae are not edible, there are several other wild-growing related species with potential as food. The data presented in this report can be used as a starting point for further food chemistry studies on South American Caricaceae.

**ACKNOWLEDGMENTS**

Financial support by Programa de Investigacion en Productos Bioactivos, Universidad de Talca is kindly acknowledged. CQ and EVV thank the PBCT Program, PSD-50 for a postdoctoral grant.

**REFERENCES**