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ARTICLE

Chemical Analysis and Screening as Anticancer Agent of Anthocyanin-Rich Extract from Uva Caimarona (*Pourouma cecropiifolia* Mart.) Fruit

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The anthocyanin-rich extract (ARE) of the fruit from *Pourouma cecropiifolia*, a tropical plant native to the Amazon region, showed moderate cytotoxicity toward different cancer cell lines when evaluated by MTT assays. This extract was fractionated using Sephadex LH-20 chromatography to obtain three fractions (F1−F3), the composition of which was analyzed by HPLC-PDA and LC-ESI/MS. F1 was composed primarily of the monomeric anthocyanins delphinidin-3-*O*-β-glucopyranoside, cyanidin-3-*O*-β-glucopyranoside, and cyanidin-3-*O*-(6″-malonyl)glucopyranoside. F2 contained the isomeric flavonols quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-galactopyranoside and quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside, the structures of which were confirmed by ^{1}H and ^{13}C NMR. F3 contained polymeric pigments, which were analyzed using tandem ESI/MS with an ion trap-TOF. The structures of two proanthocyanidin and two flavanol-anthocyanin condensed pigments were suggested on the basis of their MS^{n} fragmentation patterns. After cell viability assays were performed, only fraction F3 showed a cell growth-inhibitory effect similar to the one found for ARE. F3 significantly reduced the viability of HEp-2 larynx, MKN-45 gastric carcinoma, and MCF-7 breast cancer cells; in contrast, the pure compounds did not show promising cytotoxicity toward the cancer cells evaluated.

KEYWORDS: *Pourouma cecropiifolia*; flavonols; condensed pigments; proanthocyanidins; cytotoxic activity; anthocyanins

INTRODUCTION

Pourouma cecropiifolia Mart. (Moraceae), commonly called uva caimarona, uva de monte, caimarón, or caima, is a tropical plant species native to the upper Amazon rainforest (Colombia, Brazil, and Peru). It is a tree that grows to heights of 12−20 m, very similar to *Cecropia*, and grows quickly in rainy climates. The immature fruits are green and turn purple at maturity. The fruits, borne in racemes like grapes, are ovoid to spherical drupes, 2−4 cm in diameter (**Figure 1**), have a sweet, mucilaginous flesh, and contain one seed (*1*). This tropical fruit is eaten fresh or used to prepare jellies, marmalades, and alcoholic beverages; ground seeds are sometimes used as a coffee substitute. The fact that this species is a fast-growing tree makes it a promising agroindustrial raw material for the production of different processed products (*2*). The fruit exhibits a pleasant and soft aroma that was recently characterized (*3*).

Fruit demand around the world has increased due to results obtained from several epidemiological and nutritional studies, which have shown an apparent relationship between increased fruit and vegetable intake and decreased cancer risk (*4*). Key players in this regard are vitamin C, carotenoids, and polyphenolic substances, which due to their radical scavenging activities are able to influence the harmful effects of lipid peroxidation or DNA damage caused by free radicals (*5*). Among polyphenols, the anthocyanins have been clearly shown to be chemopreventive phytochemicals active against oxidative stress-induced cell damage (*6*); however, their bioavailability in humans has been challenged and is controversial (*7*). These natural pigments are responsible for the red and violet to dark blue colors of most of the fruits, flowers, and leaves of angiosperms (*8*, *9*).

The anticancer potential of berry fruits, such as blackberries, raspberries, cranberries, and strawberries, has been highlighted because of their high content of bioactive phytochemicals, including anthocyanins and other polyphenols (*10*). Increased consumption of these fruits has been mainly associated with the decreased risk of cancers of epithelial origin in the digestive system (*11*−*13*). In addition, proanthocyanindin- and/or polyphenol-enriched extracts obtained from fruits have shown cytotoxic effects on tumor cells without having adverse effects on normal cells (*14*−*16*).

Taking into account the increase in cancer death rates worldwide without significant distinction based on age or race, any effort to reduce the incidence of this chronic disease is worthwhile. Thus, as part of our current search for bioactive compounds in tropical fruits, the chemical nature of the constituents of anthocyanin-enriched extract from *P. cecropiifolia* fruit and the inhibition of cancer cell growth of this extract and its fractions toward

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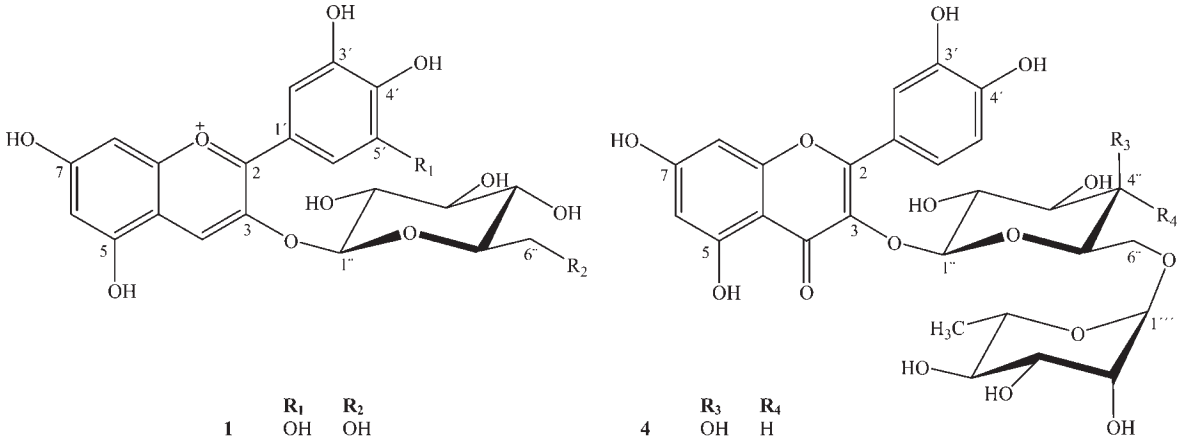
Figure 1. Uva caimarona (*Pourouma cecropiifolia*) fruit.



	R_1	R_2
1	OH	OH
2	H	OH
3	H	malonyl

	R_3	R_4
4	OH	H
5	H	OH

Figure 2. Chemical structures of the anthocyanins and flavonols identified in *Pourouma cecropiifolia* fruit. Anthocyanins: delphinidin-3-*O*-β-glucopyranoside (**1**), cyanidin-3-*O*-β-glucopyranoside (**2**), and cyanidin-3-*O*-(6″-malonyl)glucopyranoside (**3**). Flavonols: quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-galactopyranoside (**4**) and quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside (**5**).

some human cancer cell lines are investigated for the first time in the present work.

MATERIALS AND METHODS

Plant Material. *P. cecropiifolia* fruits were purchased from different local markets in Florencia (Caquetá, Colombia) and transported the same day to Bogotá by air freight for their analysis. Fully ripe fruits, characterized by a pH of 4.7, a sugar-to-water ratio of 11.8 °Brix, and a completely purple peel, were selected. A voucher specimen (COL 507603) was identified by N. R. Salinas and deposited at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia.

Chemicals. All solvents (Merck, analytical grade) were redistilled before use. For LC analyses, acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany), and water was deionized using a Milli-Q water purification system (Milli-Q, Bedford, MA). For LC-MS analyses, acetonitrile, water, and formic acid were purchased from Honeywell Burdick and Jackson (Muskegon, MI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tris acetate-EDTA (TAE) buffer, and other chemicals needed for this assay were purchased from Sigma Chemical Co. (St. Louis, MO).

Sample Preparation. Fruit epicarps (2 kg, from 8 kg of fruit) were ground in a blender and extracted with methanol/acetic acid (19:1 v/v) overnight at room temperature, in the dark. After the solvent was removed under vacuum, the residue was applied to an 80 × 4 cm Amberlite XAD-7 resin open column (Aldrich Chemical Co., Milwaukee, WI). The column was rinsed with water, and the adsorbed compounds were eluted with 1 L of methanol/acetic acid (19:1, v/v), according to the procedure described by Degenhardt et al. (*17*). The eluate was concentrated under vacuum, and the residue was freeze-dried. The final product was 18.0 g of anthocyanin-rich extract (ARE).

Fractionation of Anthocyanin-Rich Extract. A portion of the freeze-dried powder (450 mg) was applied to the top of a 330 × 31 mm glass column filled with a slurry of Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) for fractionation. Low molecular weight compounds were eluted with methanol/water/TFA (1:4:0.005, v/v/v; 1 L) followed by methanol/water/TFA (1:1:0.002, v/v/v; 1 L) to obtain F1 and F2 fractions, respectively. Next, high molecular weight compounds were eluted with acetone/water/TFA (2:1:0.003, v/v/v; 1 L) at a flow rate of 1.5 mL/min to get F3. After concentration of each fraction under vacuum, the aqueous residues were separately freeze-dried to obtain 113 mg of F1, 110 mg of F2, and 156 mg of F3.

Measurement of Anthocyanins. The concentration of anthocyanins in the ARE and the Sephadex fractions was determined by the spectrophotometric pH-differential method. Dilutions were prepared in 0.025 M potassium chloride and in 0.4 M sodium acetate, adjusted respectively to pH 1.0 and 4.5 with HCl. The absorbance of each dilution was measured at 520 and 700 nm against a distilled water blank using a Genesys 10 UV−visible spectrophotometer (Thermo Electron Corp., Madison, WI). The total monomeric anthocyanin content was calculated as cyanidin-3-glucoside equivalents (in mg) per 100 mg of dry matter (ε value of cyanidin-3-glucoside dissolved in 0.1% HCl in methanol was 26900 L cm^{-1} mol^{-1}, and the molar mass is 449.2 g mol^{-1}) (*18*).

HPLC-PDA. Characterization of the phenolic components present in the ARE and each fraction was done by HPLC-PDA (Merck-Hitachi) using an L-6200A intelligent pump, interface D-6000, and a Merck-Hitachi L-4500 multiwavelength detector. Samples (1 mg/mL) were injected via a Rheodyne injection valve equipped with a 50 μL sample loop. Separations were carried out on a Luna RP-18 5 μm column (250 × 4.6 mm i.d., Phenomenex, Torrance, CA). Two solvents composed of acetonitrile/water/formic acid (A) 3:87:10, v/v/v, and (B) 50/40/10, v/v/v, were used at the flow rate of 0.8 mL/min (*19*). A linear gradient from 6 to

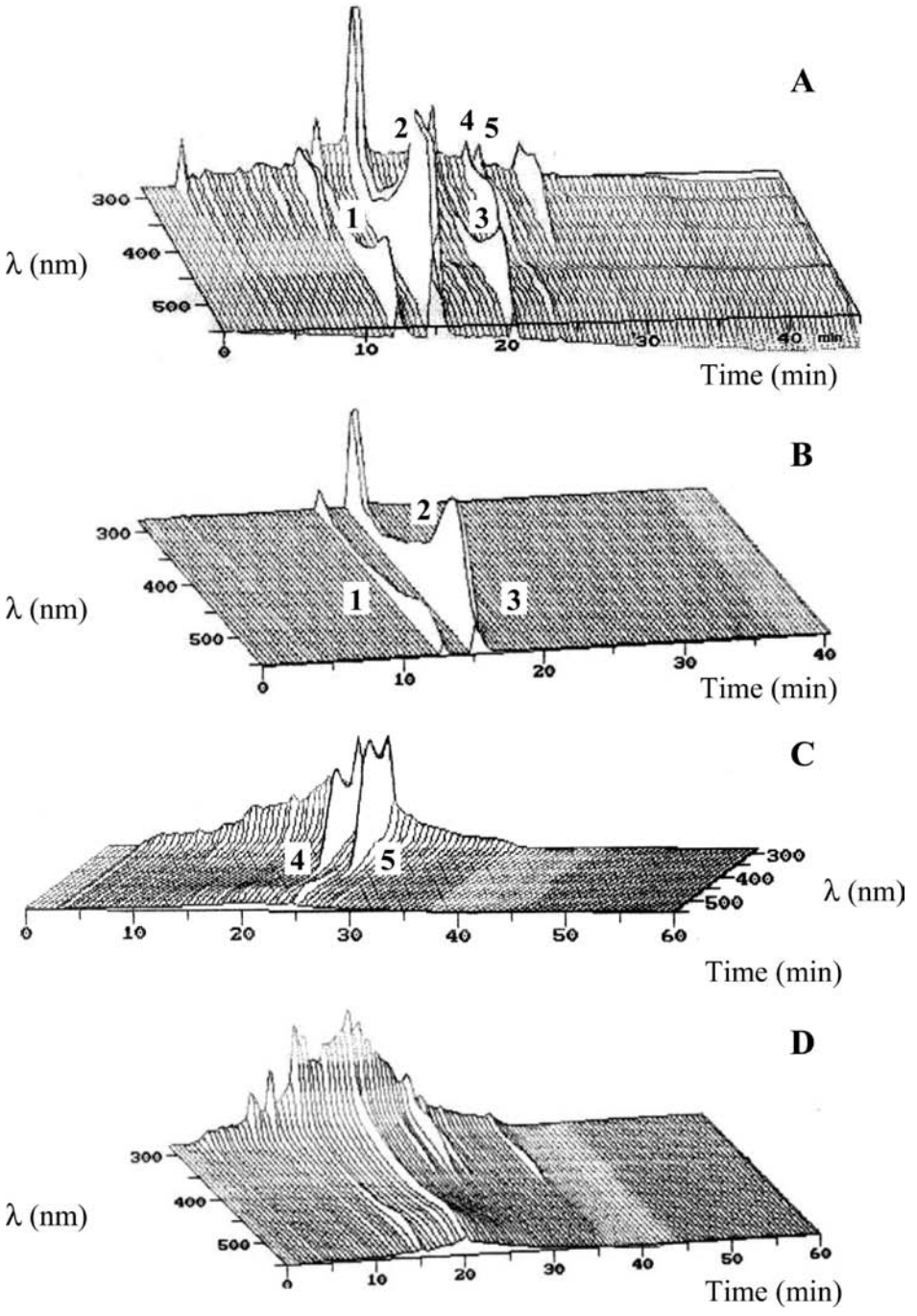


Figure 3. HPLC-PDA analyses of anthocyanin-rich extract of *Pourouma cecropiifolia* (**A**) and the LH-20 fractions F1 (**B**), F2 (**C**), and F3 (**D**).

20% B in 10 min, from 20 to 40% B in 10 min, from 40 to 50% B in 10 min, and from 50 to 6% B in 5 min was used.

Preparative HPLC. Five compounds (**1**−**5**) were purified by preparative HPLC from fractions F1 and F2. The HPLC instrument used consisted of an L-6200A intelligent pump (Merck-Hitachi) equipped with a Rheodyne injection valve with a 500 μL loop and an L-4250 (Merck-Hitachi) UV−vis detector. The separation was carried out on a Gemini C_{18} 5 μm column (250 × 10 mm i.d., Phenomenex) using water/acetonitrile/TFA (7:3:1, v/v/v) as the mobile phase. The flow rate was 4 mL/min. From F1 pure anthocyanins **1** (6.7 mg), **2** (9.2 mg), and **3** (5.4 mg) were obtained, and from F2 flavonols **4** (7.6 mg) and **5** (5.4 mg) were obtained. Purified compounds were identified on the basis of their LC-MS, ESI-MS^n, and NMR spectra.

HPLC-MS Analysis. LC-MS analyses of fractions were performed using a Shimadzu LCMS-2010 System (Shimadzu, Tokyo, Japan) equipped with a UV−vis detector (SPD-10A) and two pumps (LC-10AD) coupled online with an MS-2010 mass spectrometer. UV and MS data were acquired and processed using Shimadzu LCMS Solution software. The equipment also included an online DGU-14A degasser and a Rheodyne injection valve with a 5 μL loop. A Luna RP-18 5 μm column (150 × 2.0 mm i.d., Phenomenex) was used for the analysis of the constituents present in each fraction. The gradient used was identical to the one described for the analytical HPLC but at a flow rate of 0.2 mL/min. The electrospray ionization (ESI) probe was operated in the positive mode: CDL, 300 °C; block at 240 °C; flow gas (N_2) at 0.5 L/min; CDL voltage, 150.0 kV; Q array voltage RF, 150 V; detector voltage, 1.5 kV; and scan range, m/z 100−800.

For the HRESIMS measurements of pure compounds and analysis of F3, a Shimadzu LCMS-IT-TOF liquid chromatograph mass spectrometer (Kyoto, Japan) was used. Pure compounds (**1**−**5**) were dissolved in acetonitrile/water/formic acid (2:1:0.004, v/v/v), and F3 was dissolved in acetonitrile at a concentration of 8 mg/mL. The sample solutions were injected directly into the system. The MS/MS parameters were as follows: positive mode; CDL temperature, 200 °C; heating block, 200 °C; detector voltage,

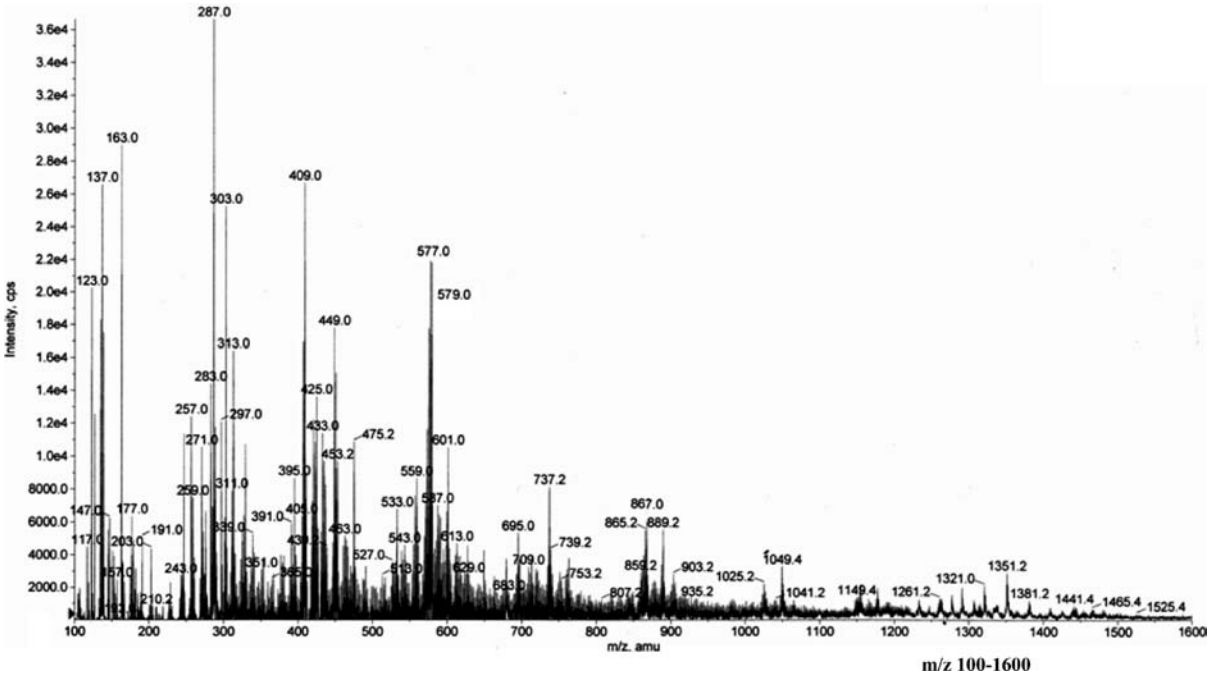


Figure 4. Electrospray mass spectrum of polymeric fraction F3.

Table 1. Major *m*/*z* Fragments of Polyphenols in Fraction F3 by ESI/MS^n Analysis and Their Tentative Identities

identity	precursor ion	MS^2	MS^3
dimeric (epi)catechin **6**	579	579/427/409/291/275/247	–[a]
trimeric (epi)catechin **7**	867	867/579/577	–
(epi)catechin-4→8-cyanidin-3-glucoside **8**	737	737/575	557/449/423
(epi)catechin-(epi)catechin-4→8-cyanidin 3-glucoside **9**	1025	1025/863	863/711/573

[a] –, not recorded.

1.55 kV; flow rate, 1.5 L/min; ion accumulation, 20 ms; and scan range, *m*/*z* 200−1600. The energy of the collision gas (argon) was fixed at 15% for the anthocyanins and at 10% for the flavonols and the polymeric F3 fraction. The LCMS Solution software was used for data collection and analysis.

NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra of anthocyanins and flavonols were measured in CD_3OD/CF_3COOD (19:1, v/v) and DMSO-d_6, respectively, on a Bruker Avance 500 spectrometer at 303 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvents were used as secondary references (for spectra recorded in CD_3OD, these peaks were located at δ 49.0 and 3.40; in DMSO-d_6, the solvent peaks were at δ 39.5 and 2.50). ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (*g*COSY), and ^{13}C resonances were assigned using 2D NMR (*g*HSQC and *g*HMBC) techniques.

Spectroscopic Data. In this section the spectral data of compounds **1**−**5** are reported. Numbering of compounds refers to **Figure 2**.

Delphinidin-3-O-β-glucopyranoside (***1***)*:* HRESIMS 465.1028 [calcd for $C_{21}H_{21}O_{12}$ $(M)^+$ 465.1033]; ESIMS, *m*/*z* 465 $[M]^+$; MS/MS 303 $[M - 162]^+$. ^{1}H and ^{13}C NMR data were in agreement with those published by Azuma et al. (*20*).

Cyanidin-3-O-β-glucopyranoside (***2***)*:* HRESIMS 449.1078 [calcd for $C_{21}H_{21}O_{11}$ $(M)^+$ 449.1084]; ESIMS, *m*/*z* 449 $[M]^+$; MS/MS 287 $[M - 162]^+$. ^{1}H and ^{13}C NMR data were in agreement with those published in the literature previously (*21*).

Cyanidin-3-O-(6″-malonyl)glucopyranoside (***3***)*:* HRESIMS 535.1091 [calcd for $C_{24}H_{23}O_{14}$ $(M)^+$ 535.1088]; ESIMS, *m*/*z* 535 $[M]^+$; MS/MS 449 $[M - 86]^+$, 287 $[M - 162 - 86]^+$. ^{1}H and ^{13}C NMR data were in agreement with those published in the literature previously (*21*).

Quercetin 3-O-α-rhamnopyranosyl-(1→6)-β-galactopyranoside (***4***)*:* HRESIMS 611.1606 [calcd for $C_{27}H_{31}O_{16}$ $(M + H)^+$ 611.1613]; ESIMS, *m*/*z* 611 $[M + H]^+$; MS/MS 465 $[M - 146 + H]^+$, 303 $[M - 162 - 146 + H]^+$; ^{1}H NMR [500 MHz, DMSO-d_6] δ 7.55 (1H, br d, J = 7.8 Hz, 6′-H), 7.53 (1H, br s, 2′-H), 6.84 (1H, d, J = 8.2 Hz, 5′-H), 6.38 (1H, br s, 8-H), 6.19 (1H, br s, 6-H), 5.34 (1H, d, J = 7.4 Hz, 1″-H), 4.39 (1H, br s, 1‴-H), 3.71 (1H, d, J = 10.7 Hz, 6″a-H), 3.06−3.29 (9H, m), 0.99 (3H, d, J = 5.4 Hz, 6‴-3H); ^{13}C NMR [125 MHz, DMSO-d_6] δ 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-2, *), 156.1 (C-9), 148.4 (C-4′), 144.8 (C-3′), 133.3 (C-3), 121.5 (C-6′), 121.1 (C-1′), 116.2 (C-2′), 115.2 (C-5′), 104.0 (C-10), 100.9 (C-1″), 100.4 (C-1‴), 98.5 (C-6), 93.4 (C-8), 77.5 (C-2″), 76.4 (C-5″), 74.0 (C-3″), 71.6 (C-4‴), 70.2 (C-4″, *), 70.0 (C-2‴, *), 69.7 (C-3‴), 67.6 (C-5‴), 66.7 (C-6″), 18.0 (C-6‴) (* exchangeable).

Quercetin 3-O-α-rhamnopyranosyl-(1→6)-β-glucopyranoside (***5***)*:* HRESIMS 611.1413 [calcd for $C_{27}H_{31}O_{16}$ $(M + H)^+$ 611.1613]; ESIMS, *m*/*z* 611 $[M + H]^+$; MS/MS 465 $[M - 146 + H]^+$, 303 $[M - 162 - 146 + H]^+$; ^{1}H NMR [500 MHz, DMSO-d_6] δ 7.54 (1H, br d, J = 7.8 Hz, 6′-H), 7.53 (1H, br s, 2′-H), 6.80 (1H, d, J = 8.0 Hz, 5′-H), 6.38 (1H, br s, 8-H), 6.18 (1H, br s, 6-H), 5.45 (1H, d, J = 7.5 Hz, 1″-H), 4.22 (1H, br s, 1‴-H), 3.57 (1H, d, J = 11.5 Hz, 6″a-H), 3.06−3.40 (9H, m), 0.85 (3H, d, J = 5.4 Hz, 6‴-3H).

Cell Culture. Cell lines derived from human larynx (HEp-2), colon (HT-29), gastric (MKN-45), breast (MCF-7), and cervical (HeLa) cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in minimum essential medium (MEM; Sigma) supplemented with 5% fetal bovine serum (Vitacell, ATCC, VA), penicillin/streptomycin (100 UI−100 μg/mL), and 50 μg/mL gentamycin. The cells were grown in 75 mL flasks at 37 °C in a constant humidified atmosphere of 5:95 CO_2/air (*22*).

Cell Viability Assay. Culture flasks in a 90% confluence were trypsinized and counted in a Neubauer chamber using a trypan blue exclusion procedure. Cells were plated into 96-well, flat-bottom plates and allowed to attach for 24 h. Treatments were added and plates incubated for 48 h. Microtitration colorimetric method of MTT reduction was used to determine surviving cells at the end of the treatment period. The basic protocol described by Mosmann (*23*) was used with some modifications (*24*). Percentages of cell survival relative to growth control wells (wells containing only cells and medium) were calculated, and IC_{50}

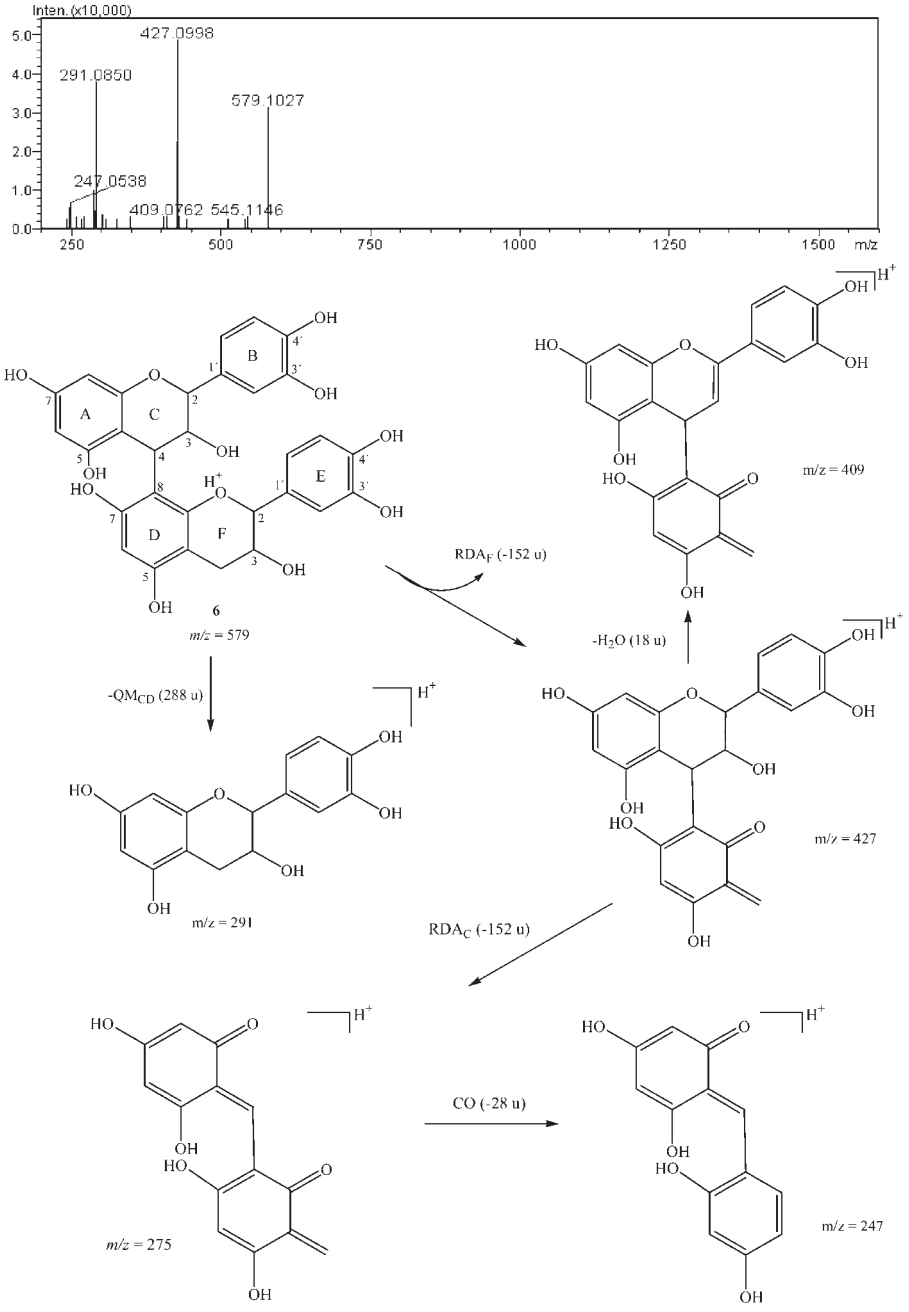


Figure 5. MS^2 spectra of compound **6** (M^+ 579) and scheme of postulated fragmentation.

(concentration that reduces survival of the exposed sample to 50%) values were calculated. The ARE, its fractions, and pure compounds (**1**−**5**) were dissolved in DMSO at 10 mM as a stock solution and conserved at 4 °C. Dilutions with culture media were prepared just prior to addition to test plates with final doses ranging from 0.5 to 50 μg/mL (*25*). At the end of the treatment, the medium was removed and 100 μL of 0.25 mg/mL MTT solution in serum-free medium was added to each well. After 4 h of incubation, the medium was removed and the formazan crystals present in each well were dissolved in 100 μL of DMSO. Plates were mixed for 5 min, and the absorbance at 570 nm was read on a Bio-Rad 550 spectrophotometer. Maximum concentration of DMSO in wells was 0.1% v/v. Doxorubicin-HCl was used as positive control and vehicle (MEM + DMSO) as blank. All treatments were evaluated in triplicates, and three independent experiments were done in different weeks.

Statistical Analyses. Significant differences between treatments were determined by using analysis of variance (ANOVA). The IC_{50} values were obtained by nonlinear regression using GraphPad 4.0-Prism software.

RESULTS AND DISCUSSION

Fractionation and Analysis of Anthocyanin-Rich Extract. The epicarps of *P. cecropiifolia* fruits are rich in anthocyanin pigments (200 ± 44 mg/100 g of epicarp). These anthocyanin pigments were the focus of this study not only because they exhibited cytotoxic activity but also because they could be used in the development of value-added food products. Thus, the ARE of *P. cecropiifolia* obtained by selective retention on Amberlite XAD-7 resin was fractionated on a Sephadex LH-20 column, giving three fractions (F1−F3). The composition of the fractions was analyzed by HPLC-PDA. As can be seen in **Figure 3**, the LH-20 purification effectively fractionated the ARE, as the composition of each fraction was clearly different. The F1 fraction was principally constituted of monomeric anthocyanins (λ_{max} = 520 nm), F2 contained flavonols (λ_{max} = 350 nm), and F3 was a polymeric mixture, with a maximum absorption at 280 nm and some minor absorption at 520 nm. These results were in agreement with the results of the ana-

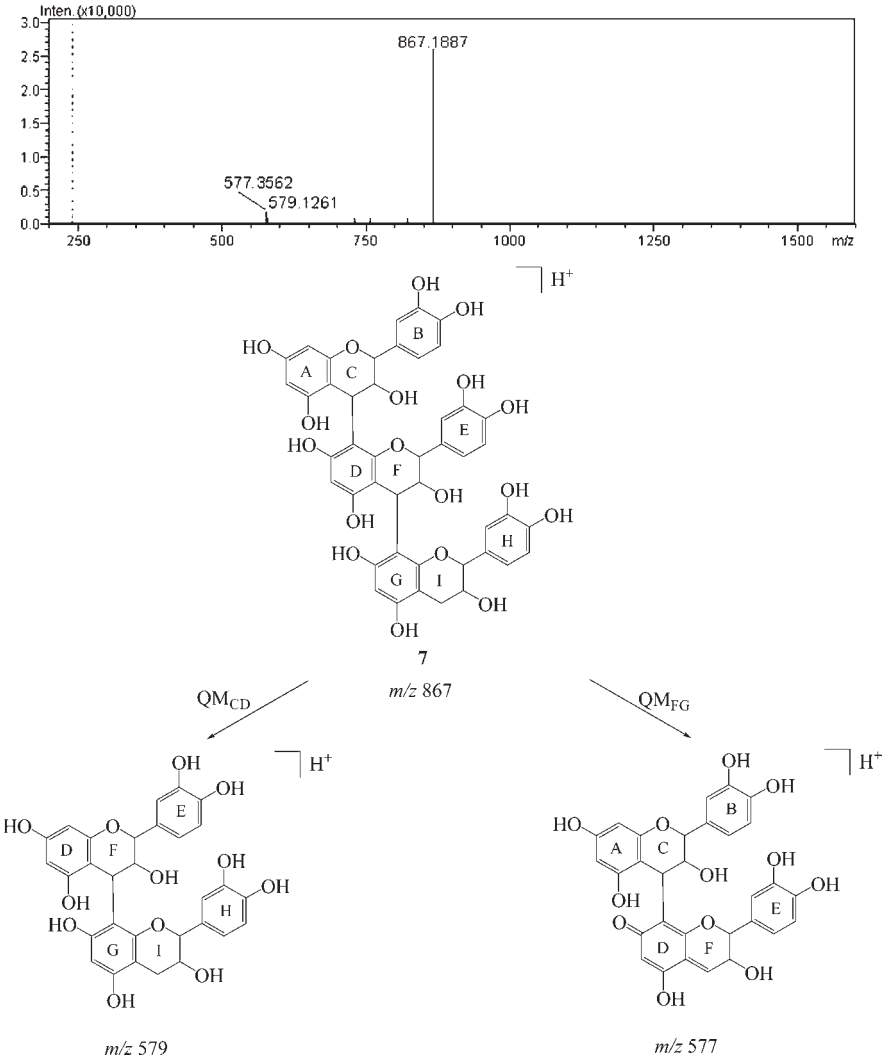


Figure 6. MS^2 spectra of compound **7** (M^+ 867) and scheme of postulated fragmentation.

lysis of the monomeric anthocyanin pigment content, as measured by the pH differential method, which showed a considerable difference in pigment content between the three fractions. These values were 95.3 ± 5.6, 24.2 ± 3.2, and 2.6 ± 0.8 mg of cy-3-glu equiv/100 mg extract for F1, F2, and F3, respectively.

The structures of the three major anthocyanins (**1**−**3**) present in F1 were identified as delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-malonyl-glucoside, respectively, by analysis of their MS, 1D NMR, and 2D NMR spectra. With regard to the mass spectrometry data, anthocyanin peaks 1−3 showed molecular ions at (*m*/*z*) 465, 449, and 535, respectively. During MS/MS analysis, molecular ions of compounds **1** and **2** produced fragment ions corresponding to each anthocyanidin at 303 (delphinidin) and 287 (cyanidin) by loss of glucosyl moiety (−162 u). The fragment ion at 449 in the spectrum of compound **3** indicates that molecular ion lost 86 *m*/*z*, corresponding to a malonyl group, and further loss of a glucosyl group (162 *m*/*z*) yielded the common anthocyanidin aglycone cation at 287 (cyanidin). Compounds **1** and **2** are the most common anthocyanins found in fruits and vegetables that exhibit an effective antioxidant activity due to their hydroxyl ortho-substitution on the B ring (*26*). Cyanidin-3-*O*-malonyl-glucoside has been isolated from red onions (*27*) and the juice of blood oranges (*28*), among others. These three anthocyanins were isolated and identified from *P. cecropiifolia* fruit for the first time in this study.

Peaks 4 and 5 from F2 have the same mass spectrometry profile but differ in retention time (23.0 and 25.7, respectively). The mass spectrometry data indicated that these two compounds contain quercetin linked to one rhamnose moiety (*m*/*z* 465) and one hexose moiety (*m*/*z* 303). On the basis of the analysis of NMR spectra for these two compounds and the co-injection of authentic samples isolated from *Sicana odorifera* (*29*) for HPLC analysis, compounds **4** and **5** were identified as quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-*β*-galactopyranoside and quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-*β*-glucopyranoside (rutin), respectively. The identity of the sugar moieties of compounds **4** and **5** isolated from *S. odorifera* was established by the analysis of NMR proton signals of the acetylated compounds (*30*). For compound **4**, the hexose was galactose (*31*), and for compound **5**, it was glucose (*32*). In both cases, the presence of rhamnose was confirmed. Rutin (compound **5**) is a common natural compound that has been detected in different vegetal species (*9*). In contrast, compound **4** is less abundant in nature than rutin and has been identified in few fruit sources, among which could be mentioned *Aronia melanocarpa* (*32*), *Ziziphus jujuba* (*33*), and *S. odorifera* (*29*).

The HPLC-PDA analysis of F3 (**Figure 3D**) suggested the presence of a mixture of polymeric proanthocyanidins. With the aim of a qualitative characterization of these compounds, unfractionated F3 was injected directly in the ESI-MS instrument, yielding a complex mass spectrum ranging from *m*/*z* 100 to 1600

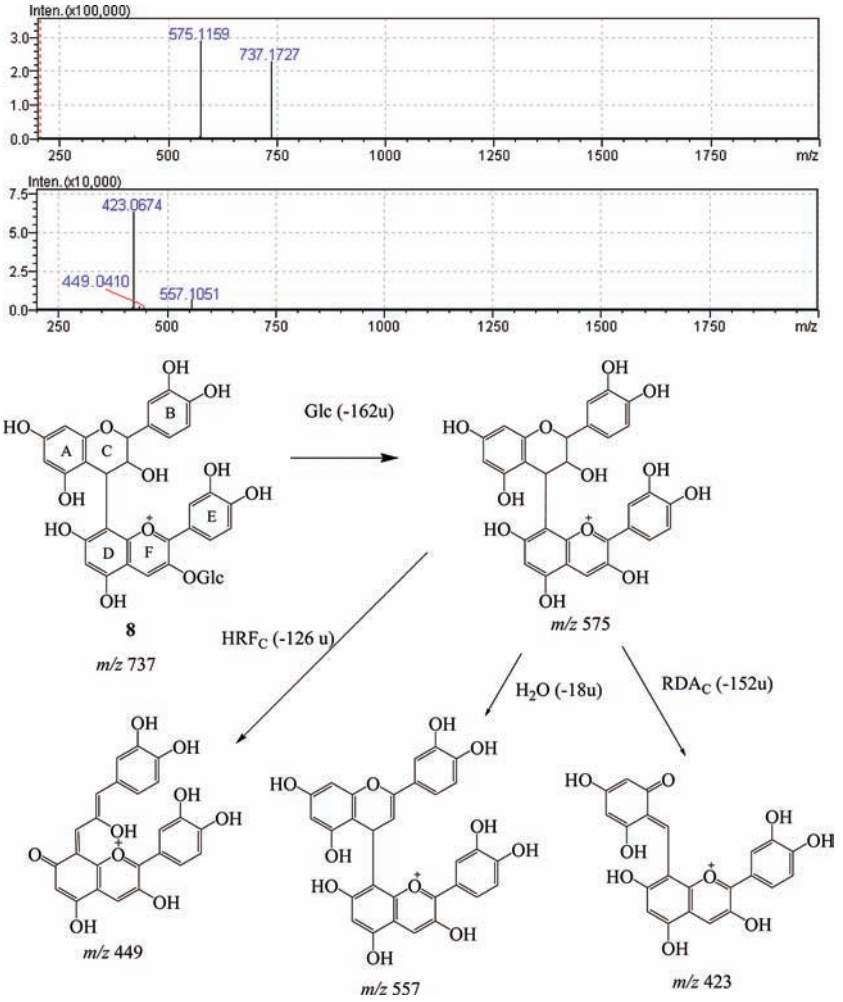


Figure 7. MS^2 and MS^3 spectra of compound **8** (M^+ 737) and scheme of postulated fragmentation.

(**Figure 4**). The most abundant ion, *m*/*z* 287, is consistent with a monomer of cyanidin, and another notable ion at *m*/*z* 303 could correspond to a monomer of delphinidin or to protonated quercetin. In the higher mass range, some ions (*m*/*z* 579, 737, 867, and 1025) were observed that were consistent with molecular masses of dimeric and trimeric proanthocyanidins. After the analysis of the fragment ions obtained in the ESI-MS spectra of F3 and on the basis of the structural mechanisms reported in the literature (*34*, *35*), the presence of procyanidin dimer **6** and trimer **7** and two flavanol−anthocyanin condensed pigments (**8** and **9**) was proposed. This proposal was confirmed by tandem mass spectrometry (MS^n) with an ion trap, which was used to select the singly charged ions *m*/*z* 1025, 737, 867, and 579 as precursors. The fragment ions obtained are summarized in **Table 1**.

The molecular ion of compound **6** at *m*/*z* 579 was in agreement with the structure of a procyanidin homodimer composed of two (epi)catechin units (the chirality at C-3 of the flavan-3-ols cannot be differentiated by mass spectrometry). The fragment ion at *m*/*z* 427 can be explained by a retro-Diels−Alder (RDA) fission of one of the flavan-3-ol monomers at ring F. The ion at *m*/*z* 409 was likely produced by loss of water from the fragment ion at *m*/*z* 427. The ion at *m*/*z* 291, which corresponds to a catechin monomer, could be considered as a diagnostic ion produced by quinone methide (QM) fission of proanthocyanidins (*34*) as depicted in **Figure 5**. The presence of other minor fragment ions at *m*/*z* 275 and 247 could be explained by RDA fission of ring C of the *m*/*z* 427 ion and subsequent neutral loss of CO, respectively. The interflavonoid linkage was proposed to be C4→C8, as this type of linkage is more common than a C4→C6 linkage (*36*). This type of procyanidin has been isolated from a diverse range of plant species (*37*).

The structure proposed for compound **7** was a homotrimer of (epi)catechin because the MS^2 spectrum of the precursor ion with *m*/*z* 867 showed ions at *m*/*z* 579 and 577 as major product ions, which can be explained by QM fission of interflavanoid linkages C−D and F−G, respectively (**Figure 6**).

Compound **8** was revealed to be a heterodimer with a(n) (epi)-catechin in the upper unit and cyanidin-3-glucoside in the lower unit. This proposal was supported because the MS^2 spectrum of the ion at *m*/*z* 737 showed a major product ion with *m*/*z* 575, which resulted from the loss of a hexose unit (−162 u) from the monomeric anthocyanin. This fragment ion was isolated and fragmented in a third stage to produce ions at *m*/*z* 557 and 423, which can be derived from the precursor ion by loss of water and RDA fission of ring C, respectively (**Figure 7**). The small peak at *m*/*z* 449 observed in the MS^3 spectrum may be formed after the elimination of ring A from the (epi)catechin unit by heterocyclic ring fission (HRF) of ring C. This process is characteristic of an upper flavanol-3-ol unit (*35*, *36*). The (epi)catechin−cyanidin-3-glucoside has been tentatively identified as a minor constituent of red runner beans (*36*, *38*), purple corn (*39*), and black soybeans (*40*).

The structure of compound **9** was proposed to be a hetero-trimer resulting from the condensation of two monomers of (epi)catechin and one unit of cyanidin-3-glucoside. The MS^2 studies on the precursor ion at *m*/*z* 1025 indicated that the

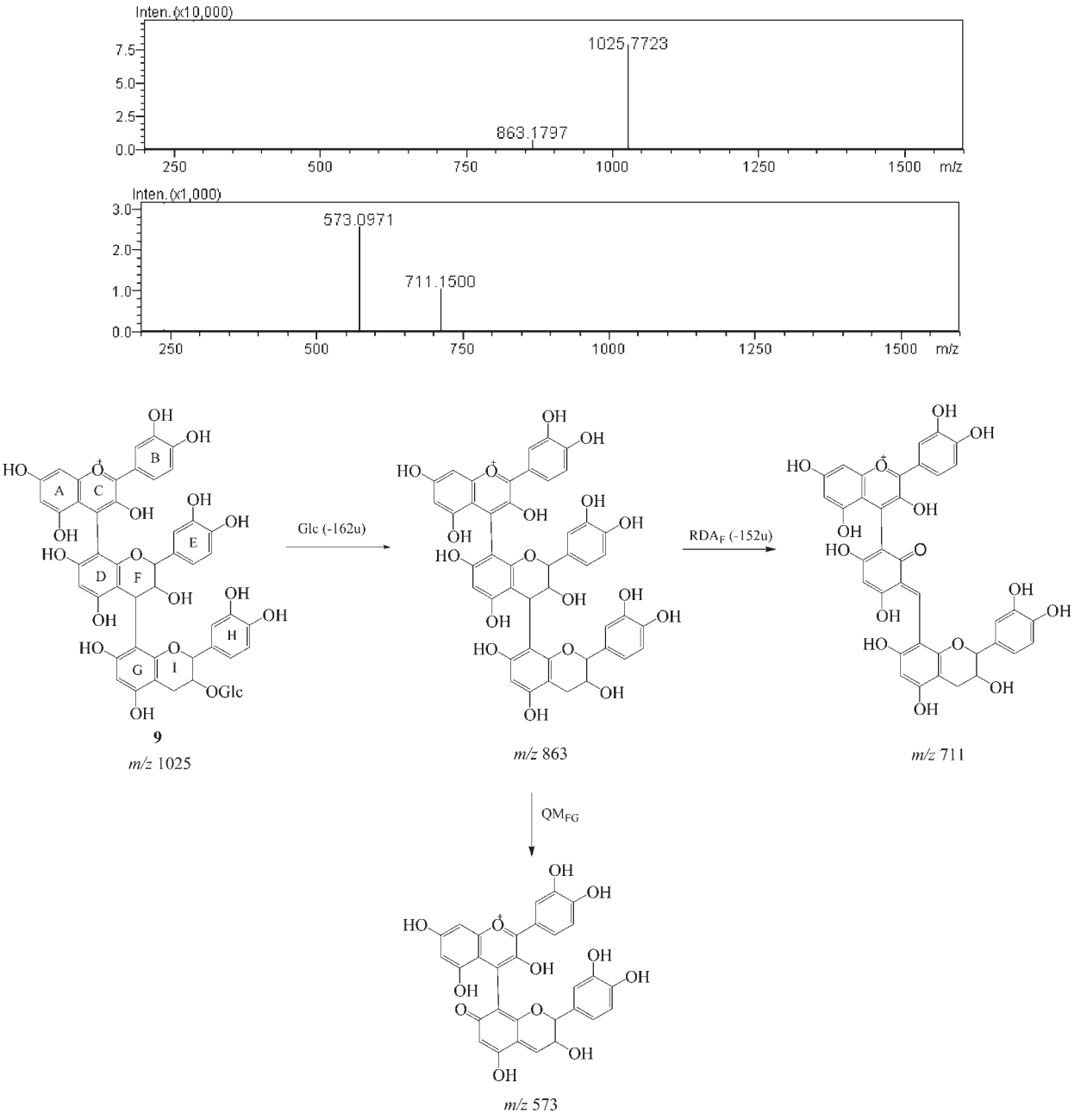


Figure 8. MS^2 and MS^3 spectra of compound **9** (M^+ 1025) and scheme of postulated fragmentation.

fragment ion at *m*/*z* 863 can be formed after loss of hexose from the anthocyanin moiety. The ions with *m*/*z* 573 and 711 from the ion at *m*/*z* 863 were likely produced by QM fission of the F−G linkage and RDA of ring F, respectively (**Figure 8**). The formation of the ion at *m*/*z* 573 indicates that cyanidin was the upper terminal unit of compound **9**.

Proanthocyanidins are oligomeric and polymeric end products of the flavonoid biosynthetic pathway, which has been found in many natural sources. Among proanthocyanidins, the flavanol−anthocyanin condensed pigments have been the subject of recent studies because they are formed during the maturation and aging of red wines and during the storage and processing of plant-derived foods. Flavanol-3-ol−anthocyanin condensed pigments also play a significant role in the color and quality of these processed food products. As far as we know, this is the first time that the trimer (epi)catechin-(epi)catechin-4→8-cyanidin 3-glucoside has been proposed to be a constituent of a natural source, supporting the conclusion of González-Paramás et al. (*36*) that these condensed pigments may occur naturally in plants and that they are not exclusively formed during the processing of foods.

Cytotoxic Activity. The ARE from *P. cecropiifolia* fruit showed moderate cytotoxic effects on HEp-2, MKN-45, and MCF-7 cancer cell lines (< 50 μg/mL). After fractionation of ARE on a Sephadex LH-20 column, each fraction was analyzed using a cell viability assay. It was found that fraction F3 showed a cell growth inhibitory effect similar to that found for ARE (**Table 2**). In contrast, the F1 and F2 fractions did not show any significant effects on these cancer cell lines. Sephadex LH-20-based fractionation of ARE was used not only because it was able to efficiently separate monomeric from polymeric pigments in the ARE but also because this fractionation does not affect the bioactivity of the compounds (*41*). The cytotoxicity of pure compounds **1**−**5** was also evaluated (**Table 2**), and the flavonols **4** and **5** exhibited some selectivity toward HEp-2 and MKN-45 cancer cell lines. In general, none of the compounds examined showed significant cytotoxic activity for the cancer cell lines

Table 2. IC_{50} of Anthocyanin-Rich Extract and Fractions Isolated from *Pourouma cecropiifolia* Fruit

	IC_{50}[a] (μg/mL) with cancer cell line				
fraction/compound	HEp-2	HT-29	MKN-45	MCF-7	HeLa
anthocyanin-rich extract (ARE)	19.09 ± 1.20	>50	21.76 ± 1.76	34.85 ± 2.02	>50
F1	>50	>50	>50	>50	>50
F2	>50	>50	>50	>50	>50
F3	18.0 ± 2.0[b]	34.9 ± 1.50[c]	15.4 ± 1.21[b]	24.6 ± 1.74[b,c]	>50
compound **1**	>50	>50	>50	>50	—[d]
compound **2**	33.25 ± 5.52	>50	43.23 ± 9.09	>50	—
compound **3**	>50	>50	>50	>50	—
compound **4**	28.33 ± 1.45	>50	27.88 ± 6.14	>50	—
compound **5**	29.21 ± 4.71	>50	32.57 ± 6.93	>50	—
doxorrubicin-HCl[e]	0.12 ± 0.11	0.20 ± 0.04	0.10 ± 0.03	0.09 ± 0.05	0.28 ± 0.16

[a] Fifty percent inhibitory concentration (IC_{50}) was measured by MTT assay after 48 h of incubation, and values are expressed as means ± SE (n = 3). Values with different letter([b,c]) in a row are statistically different (P < 0.05) by ANOVA. [d] —, not determined. [e] Positive control.

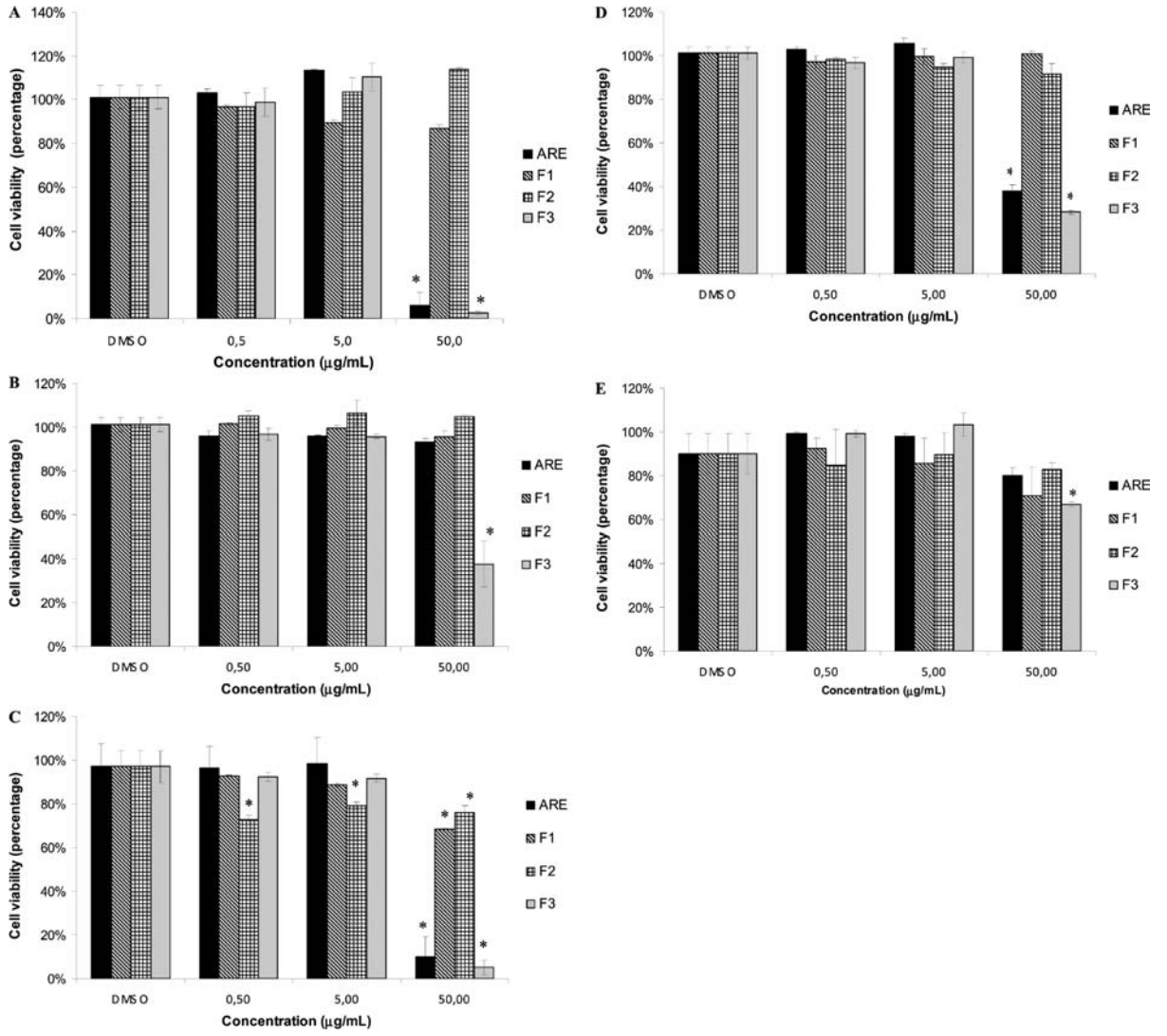


Figure 9. Cytotoxic activity (%) of anthocyanin-rich extract (ARE) and its fractions in a dose-dependent pattern (0.5−50 μg/mL) for the (**A**) HEp-2, (**B**) HT-29, (**C**) MKN-45, (**D**) MCF-7, and (**E**) HeLa cancer cell lines. Values are expressed as means ± standard error (SE) of three independent experiments in triplicate. ∗, significantly different compared with control (P < 0.05).

evaluated (the reference value recommended by the U.S. National Cancer Institute for promising cytotoxic compounds is <4 μg/mL) (*42*, *43*). These results are in agreement with previous studies which demonstrated that the in vitro cytotoxic and antiproliferative activities toward some cancer cell lines are less effective in anthocyanin-rich fractions than the original extracts (*15*). This fact has been explained as a synergistic or additive effect of anthocyanins with other active components in the extracts (*16*).

Although the bioactivity of F3 varied with different cancer cell lines (**Figure 9**), a concentration-dependent effect on cell viability at a concentration >5 μg/mL was seen in all cases. The lowest IC_{50} for F3 was observed against HEp-2 (larynx), MKN-45

(gastric), and MCF-7 (breast) cancer cell lines, which suggests selective activity toward these lines. A remarkable activity of F3 against HT-29 colon cancer cell line was also found: exposure of these cells to 50 μg/mL of F3 resulted in a growth inhibition of approximately 60%. For the anthocyanin fraction F1 at a concentration of 50 μg/mL, the highest bioactivities were found against the HEP-2, MKN-45, and HeLa cancer cell lines, with growth inhibition values close to 20, 30, and 30%, respectively. In the flavonol fraction (F2), the lowest cell viability was seen for the MKN-45 cell line when a concentration of 50 μg/mL was used.

Some studies have also shown a relationship between proanthocyanidin mixtures and cytotoxic (*44*) and antitumor activities (*45*). Their mechanisms of action in cancer cells remain unclear; however, Mantena et al. (*14*) suggested that the grape seed proanthocyanidins induce apoptosis and inhibit tumor growth and metastasis of breast cancer cells through disruption of mitochondrial pathways. Grape seed proanthocyanidins have been shown to have a cytotoxic effect on tumor cells without having an adverse effect on normal cells (*46*); in contrast, strawberry polyphenols were found to be equally cytotoxic to tumor cell lines, lymphocyte, or normal human breast and prostate cell lines (*47*). For the case of *P. cecropiifolia* polymeric fraction F3, experiments to evaluate its growth inhibitory effect on normal cells need to be done.

In conclusion, we identified fractions of *P. cecropiifolia* fruit extract that have promising cytotoxic effects on larynx, gastric, and breast cancer cell lines. The cytotoxic activities are promising because the calculated IC_{50} values were < 100 μg/mL, which is the reference value recommended by NCI for extracts or fractions (*42*). The presence of cytotoxic compounds in *P. cecropiifolia* fruit highlights the potential for use of this Amazonian natural resource as an anticancer natural agent; however, further research is required to prove these results in vivo. These findings are also important because the epicarp of the fruit is usually considered to be a waste material.

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