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Anthocyanin Composition of Wild Colombian Fruits and Antioxidant Capacity Measurement by Electron Paramagnetic Resonance Spectroscopy

Liliana Santacruz,[†] José G. Carriazo,[†] Ovidio Almanza,[‡] and Coralia Osorio*[,†]

[†]Departamento de Química and [‡]Departamento de Física, Universidad Nacional de Colombia, AA 14490, Bogotá, Colombia

ABSTRACT: The qualitative and quantitative anthocyanin composition of four wild tropical fruits from Colombia was studied. Compounds of "mora pequeña" (*Rubus megalococcus* Focke.), "uva de árbol" (*Myrciaria aff. cauliflora* O. Berg), coral, and motilón (*Hyeronima macrocarpa* Mull. Arg.) fruits were separately extracted with methanol−acetic acid (95:5, v/v). The anthocyanin-rich extracts (AREs) were obtained by selective adsorption on Amberlite XAD-7. Each extract was analyzed by HPLC−PDA and HPLC−HRESI-MS^n with LCMS-IT-TOF equipment in order to characterize the anthocyanin pigments and the coinjection in HPLC using standards allowed identifying the major constituents in each extract. The antioxidant activity was measured by electron paramagnetic resonance (EPR) and UV−vis spectroscopy, using ABTS and DPPH free radicals. The ARE of motilón (*H. macrocarpa* Müll. Arg) exhibited the highest radical scavenging activity in comparison to the other extracts. A second-order kinetic model was followed in all of the cases. These results suggested that the studied fruits are promising not only as source of natural pigments but also as antioxidant materials for food industry.

KEYWORDS: *tropical fruits, anthocyanins, EPR, antioxidant activity*

INTRODUCTION

Fruit demand around the world has increased due to results obtained from epidemiological and nutritional studies that have shown an apparent relationship between high fruit and vegetable consumption and a decreased incidence of degenerative diseases, such as coronary heart problems, Alzheimeŕs disease, and cancer. Key players in this regard are vitamin C, carotenoids, and polyphenolic substances (anthocyanins, flavonols, and related compounds) which due to their radical scavenging activities are able to neutralize harmful effects of lipid peroxidation or DNA damage caused by free radicals.[1]

Anthocyanins are secondary metabolites of higher plants, responsible for colors from orange to blue in petals, fruit, leaves, and roots. From a chemical point of view, their structure is made of a C_{15} heterocyclic nucleus (2-phenylbenzopyrilium cation or anthocyanidin) bearing at least one and often several sugar residues; these sugars may be esterified by aliphatic and/or aromatic organic acids. Mazza and Miniati[2] have published a book that is a good reference for studying the chemistry, physiology, chemotaxonomy, pharmacology, and technology aspects of the anthocyanins. In this regard, it is also possible to find an extensive list of naturally occurring anthocyanidins or aglycons, among which the six most frequently occurring in plants are cyanidin, delphinidin, pelargonidin, malvidin, peonidin, and petunidin.[3]

Colombia is a country with one of the highest biodiversities in the world; however, there is a considerable lack of knowledge regarding the composition of several tropical fruits. So, knowledge regarding their phenolic composition and antioxidant activity will provide important information needed to open new markets for these products and to promote fruit crop production in Colombian rural areas. Thus, in the present paper the anthocyanin composition and antioxidant activity of four wild tropical fruits were studied.

Rubus megalococcus Focke (Rosaceae) is a wild berry that belongs to the genus *Rubus* that is characterized by a high anthocyanin content, as well as antioxidant activity.[4−6] An ethnobotanical survey has reported that this species is used for treatment of cough and diarrhea.[7] This is a scandent shrub with arching branches to 3 m high; its fruits have a variable size, the largest being around 1.0 cm in diameter, with dark red pulp in the ripe state. This fruit is native to the tropical America and is called "mora pequeña", "mora de páramo", or "mora de gato". According to our knowledge, there is not any chemical study on this species.

Myrciaria aff. cauliflora (Mart.) O. Berg, commonly called guapurú, "uva de árbol", or jaboticaba, belongs to the *Myrtaceae* family. It is a small tree that grows up to 2 m high in the south of Colombia; the dark purple fruits produce the impression of being attached to the stem and are concentrated on the main trunk (3−4 cm in diameter), and the fruit is used to make soft drinks, marmalades, and liquors. From the fruit, a depside named jaboticabin that exhibited antioxidant activity and cytotoxicity against the HT29 colon cancer cell line was identified.[8] Other phenolic compounds, such as cyanidin-3-glucoside, delphinidin-3-glucoside, pyranocyanin B, quercetin, and rutin, were also identified. The antioxidant properties of this fruit have also been reported.[9−11]

The other two fruits, coral and motilón, were both classified as *Hyeronima macrocarpa* Müll. Arg., belonging to the genus *Hyeronima* (*Euphorbiaceae*), which is composed by ca. 15 species distributed in tropical America.[12] The fruits are edible, drupaceous, ellipsoid, and red at maturity, with motilón fruits

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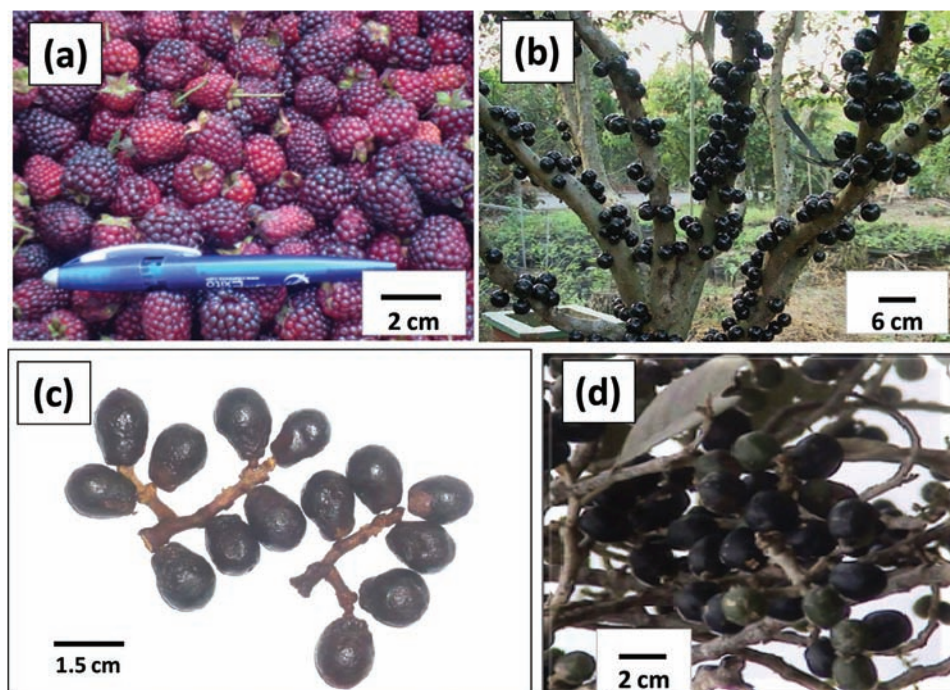


Figure 1. (a) Mora pequeña (*R. megalococcus* Focke), (b) uva de árbol (*M. aff. cauliflora* (Mart.) O. Berg), (c) coral (*H. macrocarpa* Müll. Arg.), and (d) motilón (*H. macrocarpa* Müll. Arg.) fruits.

being up to twice as large as coral ones (Figure 1). Until now, there was not any chemical study regarding these species.

At present, there is a growing trend in the consumption of not only common berries but also exotic "berry-type" fruits (and their foodstuffs) that are found in tropical climates,[13−15] because of their sensory properties as well as their antioxidant activity.[16−18] Antioxidant capacity is related to the compounds capable of protecting a biological system against the potentially harmful effect of processes or reactions involving reactive oxygen and nitrogen species (ROS and RNS). The terms antioxidant activity and antioxidant capacity are often used indistinctly; however, they have different meanings. The "activity" of a chemical would be pointless without specific reaction conditions, such as pressure and temperature. The antioxidant capacity is more independent of specific reactions and usually refers to the results obtained by different assays.[19−21] Thus, selection of the method for measurement of in vitro antioxidant activity is a critical point taking into account that antioxidant compounds may respond in a different manner to different radicals or oxidant sources, and also many factors including the partitioning properties between lipid and aqueous phases and the physical state of the oxidizable substrate usually affect this process. Electron paramagnetic resonance (EPR) is a highly specific and sensitive technique for direct detection and quantification of free radicals generated by ex vivo or in vivo chemical reactions, and it is based on the absorption of microwave radiation by unpaired electrons when they are in a magnetic field.[22,23] This technique has been recently applied to the measurement of antioxidant activity in fruits.[24−26]

As an essential part of our current scientific program on the antioxidant activity of tropical fruits,[26−28] in the present work the qualitative and quantitative anthocyanin composition of four red tropical fruits was determined, as well as their antioxidant capacity by using UV−vis and EPR spectroscopical methods. On the basis of these results, the long-term purpose is to assess whether they have the potential to develop new added-value products (for example, natural food colorants) that can supply the healthy requirements for the modern food industry.

MATERIALS AND METHODS

Plant Material. The fruits used in this study were mora pequeña (*R. megalococcus* Focke) collected in Albania (Santander, Colombia, 1650 m), uva de árbol (*M. aff. cauliflora* (Mart.) O. Berg) and coral (*H. macrocarpa* Müll. Arg.) in Timbio (Cauca, Colombia, 1850 m), and motilón (*H. macrocarpa* Müll. Arg.) collected in La Unión (Nariño, Colombia, 1650 m). A voucher specimen of each species was deposited at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia-Bogotá, under codes COL 531919, COL 531920, COL 531921, and COL 547152, respectively.

Solvents and Reagents. All solvents were of analytical grade (Merck, Darmstadt, Germany). The methanol used during the antioxidant activity assays was of spectrophotometric grade (Merck, Darmstadt, Germany). For LCMS (liquid chromatography−mass spectrometry) analyses, acetonitrile, water, and formic acid were purchased from Honeywell Burdick and Jackson (Muskegon, MI). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), cyanidin-3-*O*-glucoside (Cy-glu), and cyanidin-3-*O*-rutinoside (Cy-rut) were supplied by Sigma-Aldrich (Steinheim, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka Chemie GmbM (Steinheim, Switzerland). Potassium persulfate and L-ascorbic acid were from Merck (Darmstadt, Germany). Delphinidin-3-*O*-glucoside (Dp-glu) and petunidin-3-*O*-rutinoside (Pet-rut) were purchased from TransMit (Marburg, Germany). Delphinidin-3-*O*-rutinoside was isolated and purified from *Solanum betaceum* fruit.[28]

Extraction of Anthocyanins. Whole fruits of mora pequeña (295 g), fruit epicarps of uva de árbol (134 g), and fruits without seeds of coral (123 g) and motilón (98 g) were separately ground in a blender and extracted with methanol−acetic acid (95:5 v/v) overnight in the dark, at room temperature. For each case, 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

Table 1. Qualitative and Quantitative Anthocyanin Composition of Wild Colombian Fruits

peak number[a]	compound[b]	t_R (min) HPLC	molecular weight M^+ exp	molecular weight M^+ calcd	fragment ions MS/MS (m/z)	anthocyanin content[c]
			Coral (*H. macrocarpa* Müll. Arg.)			
1	delphinidin-3-*O*-glucoside	9.1	465.0906	465.1033 ($C_{21}H_{21}O_{12}$)	303 $[M - 162]^{+}$ [d]	0.58 ± 1.30[e]
2	delphinidin-3-*O*-rutinoside	10.6	611.1623	611.1612 ($C_{27}H_{31}O_{16}$)	465 $[M - 146]^+$, 303 $[M - 162 - 146]^{+}$ [d]	12.04 ± 1.30[e]
3	cyanidin-3-*O*-rutinoside	14.4	595.1636	595.1663 ($C_{27}H_{31}O_{15}$)	287 $[M - 162 - 146]^{+}$ [d]	2.07 ± 1.30[e]
4	petunidin-3-*O*-rutinoside	17.9	625.1599	625.1768 ($C_{28}H_{33}O_{16}$)	317 $[M - 162 - 146]^{+}$ [d]	2.49 ± 1.30[e]
			Motilón (*H. macrocarpa* Müll Arg.)			
1	delphinidin-3-*O*-glucoside	8.3	465.0983	465.1033 ($C_{21}H_{21}O_{12}$)	303 $[M - 162]^{+}$ [d]	2.87 ± 0.0[e]
2	delphinidin-3-*O*-rutinoside	9.5	611.1567	611.1612 ($C_{27}H_{31}O_{16}$)	465 $[M - 146]^+$, 303 $[M - 162 - 146]^{+}$ [d]	10.98 ± 0.07[e]
3	cyanidin-3-*O*-rutinoside	13.8	595.1670	595.1663 ($C_{27}H_{31}O_{15}$)	449 $[M - 146]^+$, 287 $[M - 162 - 146]^{+}$ [d]	7.58 ± 0.07[e]
4	petunidin-3-*O*-rutinoside	17.4	625.1765	625.1768 ($C_{28}H_{33}O_{16}$)	317 $[M - 162 - 146]^{+}$ [d]	8.21 ± 0.07[e]
			Mora Pequeña (*R. megalococcus* Focke)			
5	cyanidin-3-*O*-glucoside	12.2	449.1082	449.1084 ($C_{21}H_{21}O_{11}$)	287 $[M - 162]^{+}$ [d]	366.46 ± 2.30[f]
3	cyanidin-3-*O*-rutinoside	14.2	595.1660	595.1663 ($C_{27}H_{31}O_{15}$)	449 $[M - 146]^+$, 287 $[M - 162 - 146]^{+}$ [d]	4.60 ± 2.30[f]
6	cyanidin-3-*O*-(6″-malonyl)-glucoside	23.6	535.1100	535.1087 ($C_{24}H_{23}O_{14}$)	449 $[M - 86]^+$, 287 $[M - 162 - 86]^{+}$ [d]	64.72 ± 2.30[f]
			Uva de Árbol [*M. aff. cauliflora* (Mart) O. Berg]			
1	delphinidin-3-*O* -glucoside	8.7	465.1003	465.1033 ($C_{21}H_{21}O_{12}$)	303 $[M - 162]^{+}$ [d]	26.29 ± 1.10[f]
5	cyanidin-3-*O*-glucoside	11.7	449.1044	449.1084 ($C_{21}H_{21}O_{11}$)	287 $[M - 162]^{+}$ [d]	143.51 ± 1.10[f]

[a]Numbers refer to compound numbers to those given in the text. [b]Identified by coinjection with the corresponding standard. [c]Values are expressed as means ± SD (n = 3). [d]Obtained by MS/MS from molecular ion. [e]Expressed as milligrams of dp-3-rut/g fruit (FW, fresh weight). [f]Expressed as milligrams of cy-3-glu/g fruit (FW).

compounds were eluted with 1 L of methanol−acetic acid (95:5, v/v), according to the procedure described by Degenhardt et al.[29] Finally, 2.1, 1.8, 2.0, and 3.0 g of anthocyanin-rich extracts (AREs) from mora pequeña, uva de árbol, coral, and motilón were obtained, respectively.

One gram each of coral and motilón AREs were separately fractionated over Toyopearl HW-40F (150 g, Tosoh Bioscience with methanol/water/TFA (1:4:0.005, v/v/v, 1 L) to obtain 34 fractions for coral and 71 for motilón of 10 mL each. The fractions that contained the anthocyanins were selected for further analyses: fractions 12−16 (351 mg) for coral and fractions 31−37 (324 mg) for motilón.

High-Performance Liquid Chromatography−Photodiode Array Detector (HPLC−PDA). Characterization of the phenolic components present in the AREs was done by HPLC−PDA (Agilent Technologies series 1200). Samples (1 mg/mL) were injected via a Rheodyne injection valve equipped with a 50 μL sample loop. Separations were carried out on a Zorbax-SB C18 column (250 × 4.6 mm i.d., 5 μm, Phenomenex). Two solvents composed of acetonitrile/water/formic acid (A) 3:87:10 and (B) 50:40:10 (v/v/v) were used with a flow rate of 0.8 mL/min.[30] A sequence of linear gradient from 6 to 20% of B in 10 min, from 20 to 40% of B in 10 min, from 40 to 50% of B in 10 min, and from 50 to 6% of B in 5 min was used.

The quantification of anthocyanins was carried out with respect to external standards in a five-point calibration curve (5.0−80.0 mg/mL) of cyanidin-3-*O*-β-D-glucoside (r^2 = 0.994) and delphinidin-3-*O*-β-D-rutinoside (r^2 = 0.999). The obtained results were expressed as mean values ± standard deviations (SD) of three independent experiments.

High-Performance Liquid Chromatography−Electrospray Ionization Multiple Mass Spectrometry (HPLC−ESI-MS^n). The AREs of each fruit were analyzed by HPLC−ESI-MS/MS in order to characterize the anthocyanins and other phenolic compounds. This technique provides valuable information on the structural elucidation of anthocyanins, whose molecular ion is easily obtained because they are ionized in solution. Through MS^n experiments it is possible to get information not only about the anthocyanidins but also on other moieties, such as sugars or acyl groups, that are present as constituents of these compounds. Thus, HR-ESIMS measurements of AREs were performed in a Shimadzu liquid chromatograph-ion trap-time of flight mass spectrometer (LCMS−IT-TOF, Kyoto, Japan). The samples were dissolved in methanol at a concentration of 1 mg/mL. The equipment also included an online DGU-14A degasser and a Rheodyne injection valve with a 5 μL loop. A LUNA RP-18 column (150 × 2.0 mm i.d., 5 μm, Phenomenex) was used for the analysis of the components in each extract. The gradient used was identical to that described for the analytical HPLC but with a flow rate of 0.2 mL/min. The electrospray ionization (ESI) probe was operated in the positive mode: CDL, 300 °C; block at 200 °C; flow gas (N_2) at 1.5 L/min; CDL voltage, 1.8 kV; ion accumulation, 20 ms; and scan range m/z, 100−900 u. The energy of the collision gas (argon) was fixed at 50%. LCMS Solution software was used for data collection and analysis. The MS/MS parameters were optimized for each substance.

Determination of Antioxidant Activity by UV−Vis Spectrophotometry. The extraction method published by Vasco et al.[18] was used. Briefly, 0.5 g of freeze-dried fruits was subsequently extracted with 20 mL of methanol:water (1:1, v/v) and then 20 mL of acetone:water (7:3, v/v) with centrifugation. For each fruit, the supernatants were pooled in 50 mL volumetric flasks and the volumes were made up with distilled water. Extractions were performed in triplicate. The radical scavenging activity of fruit extracts against ABTS and DPPH free radicals was measured using a Thermo Scientific evolution 300 UV−vis spectrometer.

Following the method published by Re et al.,[31] an ABTS$^{\bullet+}$ solution was prepared by dissolving 38.4 mg of ABTS (7.0 mM) and 6.6 mg of potassium persulfate (2.45 mM) in 10 mL of demineralized water. The stock solution was diluted with methanol until the absorbance of the solution was finally at 0.7 at λ = 734 nm (0.18 mM). An aliquot of 10 μL of each sample was added to 1 mL of the above-mentioned ABTS solution, and the absorbance was measured spectrophotometrically at 734 nm after exactly 6 min. The activities of the extracts were estimated within the range of the dose−response curve of Trolox (0.5 to 2.0 mM) and expressed as the "Trolox-equivalent antioxidant capacity" (TEAC). The antioxidant activity of the extracts was determined after dilution (depending on their activity) and expressed as millimole of Trolox/g of fruit (dry weight). All experiments were done in triplicate and solvent blanks were run before each assay. Ascorbic acid was used as standard.

For the case of DPPH free radical, the radical scavenging activity of each extract was estimated according to the method of Brand-Williams et al.[32] and expressed as DPPH percentage per g of fruit (dry weight). In this assay, reaction mixtures containing 3.9 mL of a methanolic DPPH solution (0.27 mM, 10.8 mg/100 mL MeOH) and 100 μL of test solutions (samples) were mixed, and the decrease in absorbance was determined at 514 nm after 0, 5, 10, and 15 min, and every 15 min thereafter until the reaction reached a steady state (absorbance below 10% of the initial value). The concentration of DPPH in the reaction medium was calculated according to a calibration curve at 514 nm.

Determination of Antioxidant Activity by EPR. EPR analysis was performed at room temperature in a Bruker ESP 300 spectrometer (Bruker Instruments). EPR measurements were conducted using a radiation of 9.44 GHz (X band) with a modulation frequency of 100 kHz, a sweep width of 100 G, modulation amplitude of 0.49 G, scan time of 41.94 s, and microwave power of 20 mW. The integral intensities of EPR spectra were obtained by evaluating their double integrals (DI_{EPR}). The relative concentration (C_{rel}) of free radicals for the individual reaction times (t) was calculated as

$$C_{rel} = [DI_{EPR}/DI_{EPR}^{ref}]_t$$

where DI_{EPR} and DI_{EPR}^{ref} represent double integrals determined for the samples (antioxidant plus free radical) and for the reference (free radical in methanol), respectively.[33]

Reactive free radical solutions were immediately prepared before measurements, as was above-described for UV−vis spectrophotometry analyses, and their stability was confirmed by EPR.[26] The same free radical solutions with a proportion of radical to sample equal to those described above for UV−vis analyses were used. All measurements were started precisely 2 min after mixing the radical solutions with samples, and then the EPR spectra were recorded during 30 min. In both cases, solvent and sample blanks were separately run in each assay in order to be sure that no absorption along EPR measurements occurred.

Kinetic Analysis. Taking into account previous studies,[34,35] a second-order kinetic model was assumed for the free radical scavenging reaction of ARE against DPPH and ABTS radicals. To determine the rate constant (k), the expression $1/C_t - 1/C_o$ was plotted against time (minutes), and a linear regression analysis was used, following the equation

$$(1/C_t) - (1/C_o) = kt$$

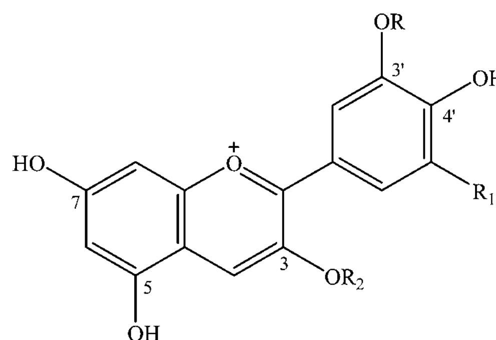
where k is the slope, C_o is the initial free radical amount, C_t is the free radical amount at a specific time, and t is the reaction time (minutes).

Statistical Analyses. Analysis of variance (ANOVA) and Tukey test were performed using the MINITAB version 16 software. Differences at $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Anthocyanin Composition of Wild Colombian Fruits. Four Colombian tropical fruits were selected as target for their chemical study because they were considered as promising source of anthocyanin pigments. The major anthocyanins were characterized by their typical fragmentation in the ESI-MS spectrum as well as the coinjection with standards (Table 1).

The HPLC profiles of AREs were obtained at 520 (anthocyanins) and 370 nm (flavonols), showing that the anthocyanin composition of coral and motilón was similar, which is in agreement with the fact that the two fruits were classified under the same scientific name. Four anthocyanins were identified, the major constituent being delphinidin-3-*O*-rutinoside (**2**). The mass spectrometry data indicated that this compound contains a unit of delphinidin linked to one rhamnose moiety (*m/z* 465) and one hexose moiety (*m/z* 303). Additionally, the molecular ion obtained by HRMS at *m/z* 611.1623 for coral and *m/z* 611.1567 for motilón were in agreement with a molecular formula of $C_{27}H_{31}O_{16}$ (calcd 611.1612). Finally, the structure of this compound was determined by comparison with an authentic sample. The other constituents were identified in the same way as described above as delphinidin-3-*O*-glucoside (**1**), cyanidin-3-*O*-rutinoside (**3**), and petunidin-3-*O*-rutinoside (**4**) (Figure 2). The gel



	R	R_1	R_2
1	H	OH	glu
2	H	OH	rha-(1→6)-glu
3	H	H	rha-(1→6)-glu
4	CH_3	OH	rha-(1→6)-glu
5	H	H	glu
6	H	H	mal-6-glu

Figure 2. Chemical structures for the anthocyanins identified in wild tropical Colombian fruits: delphinidin-3-*O*-β-D-glucoside (**1**), delphinidin-3-*O*-rutinoside (**2**), cyanidin-3-*O*-rutinoside (**3**), petunidin-3-*O*-rutinoside (**4**), cyanidin-3-*O*-glucoside (**5**), cyanidin-3-*O*-(6″-malonyl)-glucoside (**6**) (glu = glucose; rha = rhamnose; mal = malonyl residue).

permeation chromatography of motilón ARE allowed isolation of those fractions (fractions 46−51) enriched with the flavonol at retention time of 23.2 min (λ 371 nm). HRMS spectra of this compound showed a pseudomolecular ion at *m/z* 611.1657 ($[M + H]^+$) and also an adduct ion at *m/z* 633.1468 ($[M + Na]^+$), which suggested a molecular weight of 610 for this flavonol. During MS/MS analysis, the fragment ions at *m/z* 465 ($[M - 146 + H]^+$) and 303 ($[M - 146{-}162 + H]^+$) were obtained from the pseudomolecular ion, thus indicating the

presence of a quercetin linked to one hexose and one pentose. On the basis of the HPLC analysis for this compound coinjected with an authentic sample isolated from *Sicana odorifera* fruit,[27] this flavonol was identified as quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside. The main differences between coral and motilón are their size and also the total anthocyanin content, which was higher in the motilón (29.64 mg/g fruit) than in the coral (17.18 mg/g fruit). It is noteworthy that a deep botanical study is needed to differentiate if they are either botanical varieties or ecotypes. This is the first time that the anthocyanin composition of these fruits is reported in the literature.

The structures of the three major anthocyanins contained in the ARE of *R. megalococcus* were identified as cyanidin-3-*O*-glucoside (**5**), cyanidin-3-*O*-(6″-malonyl)glucoside (**6**), and cyanidin-3-*O*-rutinoside (**3**). The fragment ion at m/z 449 in the spectrum of compound **6** indicates that molecular ion lost m/z 86, corresponding to a malonyl group, and further loss of a glucosyl group (m/z 162) yielded the common cyanidin aglycon cation at m/z 287. This identification was confirmed by coinjection with an authentic sample isolated from *Bactris guineensis*.[36] It is important to point out that neither flavonols nor polymeric anthocyanin were detected in the ARE of *R. megalococcus*. These results are in agreement with those found in the literature for other *Rubus* species, since cyanidin-3-*O*-glucopyranoside and other cyanidin derivatives have been reported as the major anthocyanin-type pigments of these fruits.[5,6,37,38]

The analysis of ARE of *M. aff. cauliflora* showed the presence of cyanidin-3-*O*-glucoside (**1**) and delphinidin-3-*O*-glucoside (**5**), which is consistent with the results previously published.[8,9]

Antioxidant Activity. The anthocyanin-rich extracts of all fruits were subjected to ABTS and DPPH assays in order to evaluate their in vitro antioxidant activity. Several analytical strategies are possible for the end point measurement of this reaction; these include either measurement at a fixed time point (antioxidant capacity in micromole of radical degraded per micromole of antioxidant) or measurement of reaction rate (antioxidant potential in micromole of radical degraded per minute).[23] Table 2 summarizes the antioxidant activities of fruit extracts determined by UV−vis spectrophotometry. Under two assays, the extract of motilón (*H. macrocarpa* Müll Arg.) exhibited the highest values for antioxidant activity, followed by the extract of uva de árbol (*M. aff. cauliflora*), mora pequeña (*R. megalococcus*), and coral (*H. macrocarpa* Müll Arg.). The fact that the antioxidant values for coral were lower than those for motilón could be attributable to the differences in the anthocyanin content of two fruits, which supports the hypothesis that they are different varieties. Villareal et al.[39] reported an antioxidant value of 39.34 μmol Trolox/g fruit for the pulp extract of motilón. This value is lower than that reported in Table 2 because they processed only the pulp of the fruit.

Table 2. Antioxidant Activity of Extracts of Wild Tropical Colombian Fruits[a]

sample (ARE)	antioxidant activity: ABTS (mmol Trolox/g fruit)	antioxidant activity: % DPPH remained/g fruit
motilón (*H. macrocarpa* Müll. Arg.)	0.426 ± 0.004[a]	23.72 ± 1.08[a]
uva de árbol (*M. aff. cauliflora*)	0.345 ± 0.036[b]	25.72 ± 0.77[ab]
mora pequeña (*R. megalococcus* Focke)	0.223 ± 0.022[c]	26.84 ± 0.78[b]
coral (*H. macrocarpa* Müll Arg.)	0.136 ± 0.036[d]	27.13 ± 1.36[b]

[a]Data are given as means ± standard deviation (n = 3). Values followed by the same letter within the same column are not significantly different ($p < 0.05$) according to Tukey's multiple range test.

For the evaluation of antioxidant activity by EPR spectroscopy, the same four fruit extracts as well as the above-mentioned free-radical solutions were used. This technique allowed a direct assessment of the reduction of the free-radical concentration due to their reaction with the antioxidant compounds. Before the analyses, the stability of free radicals solutions (ABTS or DPPH) was verified by the continuous measurements of EPR spectra during 30 min. Stable concentrations along this time were observed, thus indicating a reliable procedure for the next assays in the presence of antioxidant species. Figure 3 shows some spectra for the two

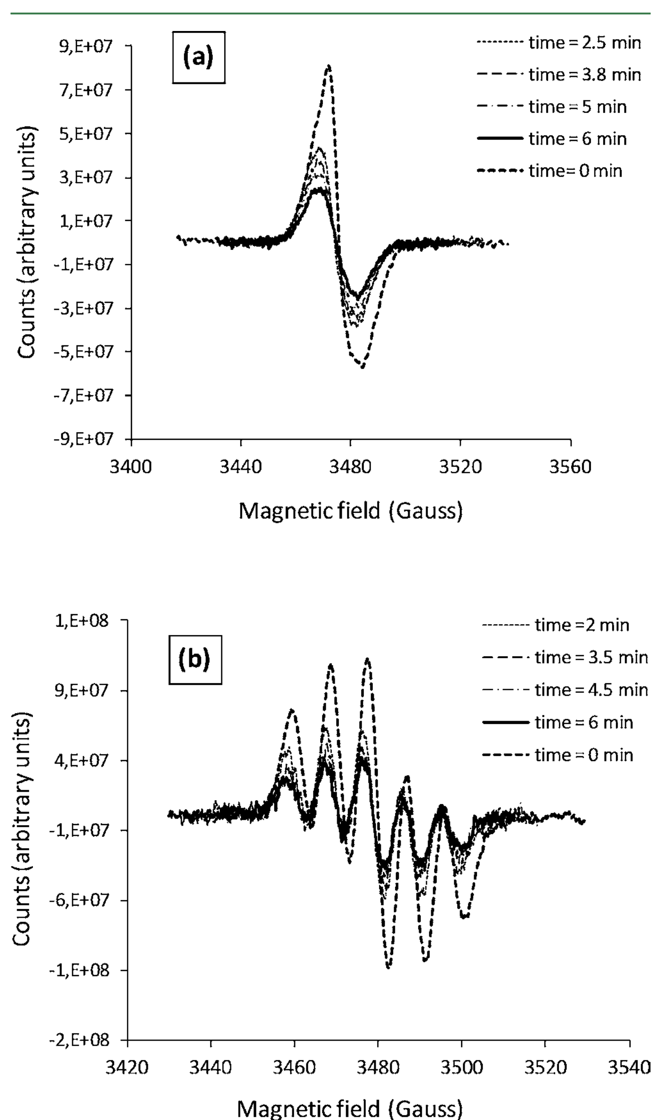


Figure 3. Set of individual EPR spectra of motilón (*H. macrocarpa* Müll Arg.) ARE monitored at several times of reaction with (a) ABTS and (b) DPPH free radicals.

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free radicals under effect of motilón extract, revealing an intensity decrease due to the scavenging of free radicals as a consequence of antioxidant activity of compounds. Different shapes are observed for the two spectra due to the differences in chemical structure of each radical (ABTS or DPPH). The characteristic g values (2.008 in a field of 3479 G for ABTS, and 2.011 in a field of 3473 G for DPPH) were in agreement with those reported in the literature.[26] Figure 4 verifies a clear decrease of the under-curve area of spectra through time due to a continuous reduction in the concentration of free radicals by the effect of the antioxidant compounds in all fruit extracts.

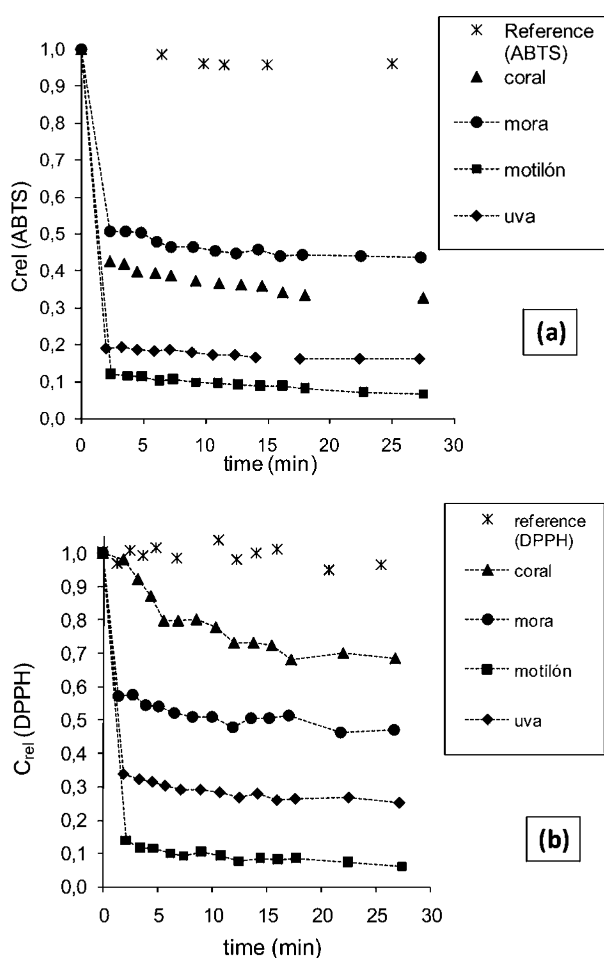

Figure 4. Relative concentrations of (a) ABTS and (b) DPPH free radicals in the presence of AREs.

Table 3 shows the values for rate constants of each extract with ABTS or DPPH free-radical solution. The comparison of these values allows drawing conclusions about the ability of fruit extracts to scavenge the free radicals. The extract of motilón (*H. macrocarpa* Müll Arg.) exhibited the highest rate constant values, in agreement with the highest antioxidant activity values obtained by UV–vis spectroscopy. This fact could be attributable to the presence of delphinidin-3-rutinoside and delphinidin-3-glucoside in the extract of motilón. In contrast, the extract of mora pequeña exhibited the lowest scavenging activity against ABTS and DPPH free radicals, noting that its extract does not have any delphinidin derivative. In a recent study,[28] it was shown that delphinidin rutinoside showed the highest antioxidant value in comparison with cyanidin-3-rutinoside and pelargonidin-3-rutinoside, due to the presence of a higher number of hydroxyl groups in the ortho position on the anthocyanidin B-ring, thus increasing the efficiency in scavenging of free radicals.

Table 3. Free-Radical Scavenging Activity Rate Constants of Extracts against ABTS and DPPH

sample	K (L mol^{-1} min^{-1})	correlation coefficient (r)
ABTS		
motilón (*H. macrocarpa* Müll Arg.)	1.54×10^7	0.993
uva de árbol (*M. aff. cauliflora*)	1.93×10^6	0.940
coral (*H. macrocarpa* Müll Arg.)	1.73×10^6	0.957
mora pequeña (*R. megalococcus* Focke)	7.71×10^5	0.879
DPPH		
motilón (*H. macrocarpa* Müll Arg.)	1.17×10^7	0.951
uva de árbol (*M. aff. cauliflora*)	1.36×10^6	0.911
coral (*H. macrocarpa* Müll Arg.)	5.85×10^5	0.905
mora pequeña (*R. megalococcus* Focke)	5.85×10^5	0.872

The kinetic data (rate constant values) are most precious[35] since the antioxidant potentiality of a compound should be applied as protection against oxidative stress provoked for radical species in the cellular media, which involves dynamic competitions between scavenging radicals and their harmful reactions in the human tissues. In this kinetic competition the radical structures not only should be (in theory) totally abated but also consumed before they can react with other cellular chemical structures, so the reaction rate of consumption of free radicals for the AREs is very important for this process.

It is important to point out that TEAC and DPPH assays are usually applied to evaluate and rank antioxidants without taking into account their limitations. For example, there are indications that the TEAC value does not exactly correlate with the antioxidant activity due to the contribution of byproducts to the overall absorbance at 714 nm (the reaction does not stop after 6 min).[40] In the case of DPPH assay, it has been proved that radical scavenging activity is influenced by the polarity of the solvent, chemical structure of radical scavenger, and the pH of the reaction mixture,[35,41] and additionally for the case of anthocyanins, their maximum absorption wavelength (520 nm) is quite close to that of free radical (515 nm). In this sense, EPR measurements applied to determine antioxidant activity is a useful technique, because it avoids many troubles derived from using UV–vis spectroscopy and allow obtaining more accurate results. Finally, on the basis of the above-mentioned results, the studied fruits are promising not only as a source of natural pigments but also as antioxidant materials for food industry.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +57-1-3165000, ext. 14472. Fax: +57-1-3165220. E-mail: cosorior@unal.edu.co.

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