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Polyphenol Characterization in Red Beverages of *Carapa procera* (D.C.) Leaf Extracts

Félix A. Adjé [1,*], Emmanuel N. Koffi [2], Kisselmina Y. Koné [1], Emmanuelle Meudec [3], Augustin A. Adima [1], Paul R. Lozano [4], Yves F. Lozano [4] and Emile M. Gaydou [5]

1 Groupe Chimie de l'Eau et des Substances Naturelles (GCESNA), Groupe de Nutrition, Technologie Alimentaire, Biochimie et Microbiologie (GNTA-BM), Département de Formation et de Recherche en Génie Chimique et Agroalimentaire (DFR-GCAA), Institut National Polytechnique Houphouët-Boigny (INP-HB), Yamoussoukro BP 1093, Côte d'Ivoire; kissel_yu@yahoo.fr (K.Y.K.); amissaaug@gmail.com (A.A.A.)

2 Department of Sciences and Technologies, Advanced Teachers' Training College of Abidjan (ENS), 08 BP 10 Abidjan 08, Côte d'Ivoire; emmanuelkoffi@ymail.com

3 Plateforme polyphenols, UMR 1083 Sciences pour l'œnologie, INRA, 2 Place Pierre Viala, 34060 Montpellier CEDEX 1, France; emmanuelle.meudec@inra.fr

4 UMR GPEB—Génie des Procédés Eau et Bioproduits, CIRAD, TA 40/16, 73 Avenue J.-F. Breton, 34398 Montpellier CEDEX 5, France; paul.lozano@wanadoo.fr (P.R.L.); y.lozano@free.fr (Y.F.L.)

5 Equipe AD2EM (Phytochimie), Institut des Sciences Moléculaires de Marseille, UMR CNRS 6263, Faculté des Sciences et Techniques de Saint-Jerôme, Université Paul Cézanne, Avenue Escadrille Niemen, Case 461, 13397 Marseille CEDEX 20, France; emilegaydou@gmail.com

* Correspondence: felix_adje@yahoo.fr; Tel.: +225-5733-8827

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Abstract: The red aqueous beverages of *Carapa procera* (D.C.) leaf extracts were investigated for their polyphenol contents using HPLC-DAD, HPLC-ESI-MS, and semipreparative HPLC. Polyphenols were extracted, clarified, and concentrated using a multistep process including ultrasound-assisted extraction (UAE), cross-flow microfiltration (CFM), and reverse osmosis (RO). On the basis of analytical and semipreparative chromatographic techniques, 12 phenolic compounds were identified and quantified for the first time: 2 anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside), 5 phenolic acids (protocatechuic, the three caffeoylquinic isomers, and coumaroylquinic acid), and 5 flavonols (quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin aglycone, and kaempferol 3-*O*-rutinoside). From the concentrated extract, it was possible to recover for anthocyanins (28.4 ± 0.3 μmol L^{-1} cyanidin equivalents) the two glycosides of cyanidin, for flavonols (1587 ± 3 μmol L^{-1} quercetin equivalents) the two glycosides of quercetin and kaempferol, and for phenolic acids (3650 ± 10 μmol L^{-1} gallic acid equivalents) chlorogenic and protocatechuic acids.

Keywords: HPLC-DAD; HPLC-ESI-MS; polyphenols; anthocyanins; leaves; *Carapa procera*

1. Introduction

Carapa procera (D.C.) is a medicinal tropical tree which belongs to the *Carapa* genus and the Meliaceae family [1,2]. This plant is usually called "crabwood" in English, "carapa" in French, "andiroba" in Portuguese, and "dona" or "kondou" in Ivorian vernacular languages. The genus *Carapa* includes other species found in Latin America (guianensis), Nicaragua and Costa Rica (nicaraguensis), and East Africa (toucoulouna or grandiflora). In West Africa, only the species *C. procera* grows. Most of the tree parts (bark, pulp, seeds, and leaves) of this species are usually used for their therapeutic properties. Of exploited parts, 57% are composed of the nuts, followed by bark and leaves (12%), and about 7% include the wood and roots [3]. The oil extracted from the seeds is a product of great interest in the cosmetics industry and biological parapharmacy [4]. Extracts obtained from the leaves

are locally used to fight against child malnutrition, pulmonary troubles, and stomachic and intestinal diseases [5–7]. These leaves are rich in minerals and are useful for making a fortifying herbal tea, commonly drunk by women after giving birth. They are also recognized to contain tannins and astringents [6]. Their green-reddish color led us to hypothesize that the red coloration may be due to the presence of anthocyanins. According to their medicinal value and the trend of increasing consumption of natural products with bioactive compounds [8], we investigated the polyphenol composition of these aqueous extracts. These compounds are recognized for their antioxidative and coloring properties, and there is growing interest in them. The green-reddish leaves could make *C. procera* a promising source of anthocyanins and other polyphenols. To our knowledge, such compounds have not yet been described from this raw material. Only Gbohaïda et al. [9] have investigated this topic, but they did not describe the chemical structures of intrinsic phenolic compounds. This study aimed, therefore, to evaluate the polyphenols in aqueous extracts obtained by coupling ultrasound-assisted extraction (UAE), cross-flow microfiltration (CFM), and reverse osmosis (RO) processes.

2. Materials and Methods

2.1. Plant Material and Sample Preparation

2.1.1. Plant Material

Fresh leaves of *C. procera* (D.C.) were harvested in the Ivory Coast, near Yamoussoukro, during the bloom season. These leaves were dried in an oven at 40 °C, packed in plastic bags, sealed under vacuum, and shipped to the Agricultural Research Center for International Development (CIRAD). The dried leaves were stored at 4 °C before pilot water extraction.

2.1.2. Pilot Plant Extraction and Concentration

Extracts were obtained according to the method described by Adjé et al. [10] and Koffi et al. [11]. Entire pieces of dried leaves (250 g) were sonicated (119 $mW \cdot g^{-1}$) at room temperature (20 °C) for 1 h with 25 L of deionized tap water and acidified with citric acid (0.01 N) in a stainless-steel container (nominal volume: 30 L) [12]. The extract obtained from this ultrasound process was firstly filtered with a nylon cloth to separate the soaked material from the reddish extract. The red crude extract (CrE) recovered was then used for the following steps of clarification (CFM) and concentration (RO). The CFM pilot plant used was equipped with two stainless-steel positive pumps. One was used to continuously feed the filtration loop (TIA, Bollène, France) with the prefiltered *C. procera* aqueous CrE. The other was used to maintain the constant cross-flow velocity of the extract (CFM retentate) through the membrane during microfiltration. The retentate velocity (4.5 ms^{-1}) was controlled by an electromagnetic flow meter (Krohne, France). The filtration loop was equipped with a ceramic cartridge membrane (P19-60, Membralox, 800 mm long, 0.2 µm average pore size), with a total filtration surface of 0.304 m^2 (Pall-Exekia, Tarbes, France). The CFM process required $\Delta P = 0.6$ bar and a permeate flow average of about 61 $kg \cdot h^{-1} \cdot m^{-2}$ at 20 °C.

The CFM permeate recovered was concentrated through the RO module, which was built on the same stainless-steel frame as that of the microfiltration module. The RO membrane was an industrial type, SW 30–2540 composite polymeric membrane, packed in a spiral-wound configuration (filmtec) with 2 m^2 of filtration surface. The operating pressure was P = 40 bar. The RO retentate was collected. The crude extract (prefiltered water), the CFM permeate, and the RO retentate were filtered through a 0.45 µm membrane before further analyses.

For the confirmation of anthocyanin characterization in *C. procera*, extracts of petals of *Delonix regia* (for which anthocyanins were previously characterized and appeared to be the same) were also carried out in the same conditions [10].

2.2. Standards and Solvents

All reagents and chromatographic solvents (formic acid and acetonitrile) were purchased from VWR-Prolabo (Briare, France). 3,4,5-Trihydroxybenzoic (gallic) acid was purchased from Fluka (Sigma-Aldrich, Germany), and delphinidin, cyanidin, and 3,5,7,3′,4′-pentahydroxyflavone (quercetin) from Extrasynthese (Genay, France).

2.3. Dry Matter

The dry matter content (% DM) was determined according to the norm AFNOR NF V18-109 71/47 version [12]. A 2 mL aliquot of extract was weighed and dried in an oven at 105 °C for 24 h. The dry matter content (%) was then calculated with the following formula:

$$\%DM = \frac{100\ (m_0 - m_1)}{m_0} \quad (1)$$

where m_0 is the weight sample at t = 0 (before drying in the oven), and m_1 is the weight after 24 h. All analyses were done in triplicate.

2.4. Total Polyphenol Analyses by Folin–Ciocalteu (F–C) Method

The F–C method [13–15] was used to determine the total polyphenol contents in *C. procera* aqueous extract samples. Diluted Folin's reagent (1/10, 2.5 mL) was added to 30 µL of the sample. After 2 min of incubation at room temperature, 2 mL of sodium carbonate (75 g L^{-1}) was added. The mixture was maintained at 50 °C for 15 min. The contents, previously cooled, were decanted in spectrophotometric tubes. The calibration was carried out with a gallic acid reference standard. The optical density (OD) was recorded at 760 nm. All assays were repeated three times.

2.5. Total Flavonoid Analyses

Total flavonoid contents were determined using a spectrophotometric method [16,17]. Aliquots of aqueous extract (1 mL) were mixed with 0.3 mL of sodium nitrite (5%, w/v) and 0.3 mL of aluminum chloride (10%, w/v). After incubation at room temperature for 5 min, the reaction mixture was diluted with water to reach 10 mL (final volume) before adding 2 mL of sodium hydroxide (1 N). Then, the absorbance was measured at 510 nm for total flavonoid determination. The calibration was carried out with a delphinidin standard. All measurements were repeated three times.

2.6. Total Monomeric Anthocyanin Analyses

The total monomeric anthocyanin contents (TMACs) in the leaf extracts were determined by a conventional spectrophotometric method [18] as cyanidin 3-*O*-rutinoside equivalents:

$$\mathrm{TMAC}\left(\mathrm{mg{\cdot}L^{-1}}\right) = \frac{A \times MW \times DF \times 10^3}{(\varepsilon \times l)} \quad (2)$$

where $A = (A_{530nm} - A_{700nm})pH_{1,0} - (A_{530nm} - A_{700nm})pH_{4,5}$, MW is the molecular weight of cyanidin rutinoside (595 g·mol^{-1}); DF is the dilution factor (2.5), ε is the molar extinction coefficient (32,800 g·mol^{-1}), and L is the optical length (1 cm).

All measurements were repeated three times.

2.7. Analytical Techniques and Equipment

2.7.1. HPLC-DAD Analyses

HPLC analyses were performed on an Agilent series 1100 HPLC instrument (Agilent, France) equipped with a quaternary pump, a diode array detector, and an autosampler. The samples (20 µL)

were injected on a C18 column (Satisfaction column, 250 mm × 4.6 µm,). The mobile phase consisted of a binary solvent system and was composed of a linear gradient of A (formic acid/water, 0.5/99.5, *v*/*v*) and B (formic acid/acetonitrile 0.5/99.5, *v*/*v*). The linear solvent started from 95% A–5% B up to 60% A–40% B within 60 min at 0.8 mL·min^{-1}. The chromatograms were recorded at 530 nm (anthocyanins), 325 nm (flavonols), and 280 nm (phenolic acids) [12]. Quantitative results were expressed as µmol·L^{-1} of cyanidin equivalents (CE), quercetin equivalents (QE), and gallic acid equivalents (GAE) for anthocyanins, flavonols, and phenolic acids, respectively.

2.7.2. HPLC-ESI-MS Analyses

An ion trap mass spectrometer (LCQ Advantage, Thermo Electron S.A., Courtaboeuf, France) was connected to the HPLC-DAD (Waters-Alliance 2690) equipped with a C18 column (Satisfaction column, 250 mm × 4.6 µm, Cil Cluzeau, France) via an ESI interface for HPLC-MS^2 analyses. The mobile phase consisted of a binary solvent system and was composed of a linear gradient of A (formic acid/water, 0.5/99.5, *v*/*v*) and B (formic acid/acetonitrile 0.5/99.5, *v*/*v*). The linear gradient started from 95% A–5% B to 60% A–40% B within 60 min at 0.8 mL·min^{-1}. UV–vis spectra were recorded from 210 to 650 nm. The mass spectrometer was operated in the positive and negative modes and under the following conditions, respectively, for each mode: the range of *m*/*z* 200–2000 and 100–1500; source voltage: 4 and 3 kV; capillary temperature: 250 °C; sheath gas and auxiliary gas flow: 50–10 and 35–5 units; and collision energy for fragmentation: 30%.

2.7.3. Semipreparative HPLC for Purification

For their HPLC characteristics comparison with their standard, major compounds from concentrated extracts were isolated and purified by semipreparative HPLC using an Agilent series 1200 HPLC instrument (Agilent, France) equipped with a quaternary pump, a diode array detector, and an autosampler. The column was a C 18 AB (250 mm × 10 mm, 5 µm, Cil Cluzeau, France) with a guard column C 18 AB (20 × 8 mm, 5 µm). The mobile phase consisted of a binary solvent system and was composed of a linear gradient of A (formic acid/water, 0.5/99.5, *v*/*v*) and B (formic acid/acetonitrile 0.5/99.5, *v*/*v*). The linear gradient started from 90% A–10% B to 60% A–40% B within 60 min at 2.5 mL·min^{-1}. UV–vis spectra were recorded from 210 to 650 nm. The compound detections were carried out at $\lambda = 280$ nm (phenolic acids), 325 nm (flavonols), and 530 nm (anthocyanins). A fractionated aliquot (0.7 mL) was collected per bottle on the basis of threshold peaks. The column was washed using an isocratic program of a solution (50% acetonitrile/50% water) for 10 min.

2.8. Statistical Analysis

Results are expressed as mean ± standard deviation of three replicates. Data were evaluated by one-way analysis of variance (ANOVA) using Statistica 7.1 (StatSoft, Inc, Tulsa, OK, USA) software. A Newman–Keuls test was performed to determine significance ($p < 0.05$).

3. Results and Discussion

3.1. Global Determination of Polyphenols and Dry Matter of C. procera Pilot Plant Extracts

The results (Table 1) showed that concentration by RO allowed for obtaining a higher content of total phenolic acids, flavonoids, and anthocyanins than that in the clarified macerate (CFM) and CrE. Total phenolic acids in the RO extract was up to 4420 ± 260, while in CFM, the content was 630 ± 44 µmol L^{-1} GAE, and total flavonoids was 1068 ± 2 and 140 ± 1 µmol L^{-1} delphinidin equivalents (DE), respectively. Compared with phenolic and flavonoid values obtained, the anthocyanin contents were low (0.08 ± 0.01 and 1.4 ± 0.2 µmol L^{-1} cyanidin rutinoside equivalents (Cya rut)) in CFM and RO extracts, respectively, showing red colorless extracts. Total phenolic values were 4 times higher than those of total flavonoids. This indicates that other compounds have high importance in the content of the remaining compounds. Further, the process allowed concentrating the anthocyanin content

18 times higher in the clarified macerate and the dry matter content was about 14 times higher in RO (RO: 2.7% ± 0.3%; CFM: 0.20%). At the same time, total phenolic acids and flavonoids were concentrated only 7–8 times higher (Table 1).

Table 1. Polyphenol determinations in *Carapa procera* leaf extracts and dry matter.

Process Coproducts	Extract Volume (L)	Total Phenolic Acids (μmol L^{-1} GAE)	Total Flavonoids (μmol L^{-1} DE)	Anthocyanins (μmol L^{-1} Cya rut)	Dry Matter (%)
Crude extract	244	652 ± 36 [a]	140 ± 1 [a]	0.08 ± 0.01 [a]	0.22 ± 0.01 [a]
Microfiltrate extract	230	630 ± 44 [a]	126 ± 2 [a]	0.06 ± 0.01 [a]	0.20 ± 0.01 [a]
RO concentrate extract	2.4	4420 ± 260 [b]	1068 ± 2 [b]	1.4 ± 0.2 [b]	2.7 ± 0.3 [b]
Concentration factor		7	8	18	13.5

In each column, the averages not followed by the same lowercase letter [a,b] are statistically different at $p < 0.05$. Abbreviations: GAE—gallic acid equivalents; DE—delphinidin equivalents; Cya rut—cyanidin rutinoside equivalents; RO—reverse osmosis.

3.2. Identification and Quantitation of Polyphenols by Analytical Techniques: HPLC-DAD/LC-MS^2

Identification of polyphenols in *C. procera* aqueous leaf extracts was carried out using chromatographic and spectrometric techniques. Anthocyanins (λ_{max} = 530 nm), flavonols (λ_{max} = 325 nm), and phenolic acids (λ_{max} = 280 nm) were detected on the basis of the comparison of their retention time (RT), elution order, and UV–vis and ESI-MS^2 data with commercial standards or purified compounds from known plant samples.

3.2.1. Anthocyanin Identification

The anthocyanin profiles (Figure 1) showed two peaks: An1 (RT = 27.5 min) and An2 (RT = 29.4 min). Their MS fragmentation in positive mode (Figure 2 and Table 2) indicated for peak An1 a molecular ion in MS^1 at *m/z* 449 and an aglycone of cyanidin at *m/z* 287 corresponding to a loss of 162 amu (one molecule of hexose). MS^2 data of peak An2 showed in full scan a molecular ion at *m/z* 595, as well as in MS^2 fragment at *m/z* 449, and an aglycone of cyanidin at *m/z* 287, corresponding to a loss of 146 u (one molecule of rhamnose), then a loss of 162 u (one molecule of glucose or galactose) [10].

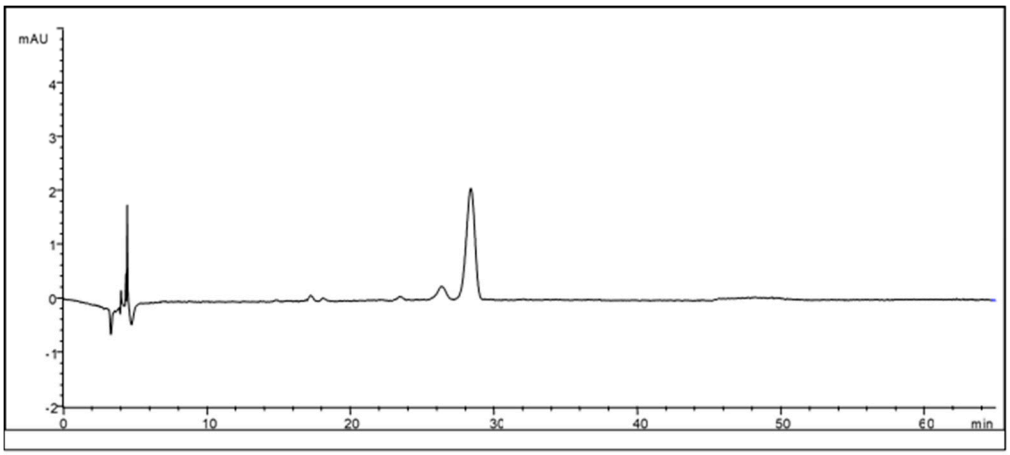


Figure 1. HPLC-DAD of anthocyanins (λ_{max} = 530 nm) of leaf of *C. procera*.

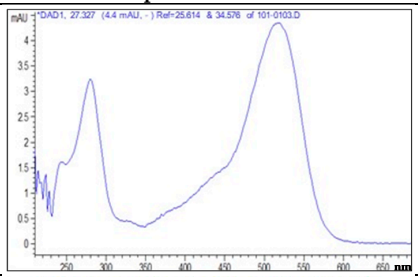
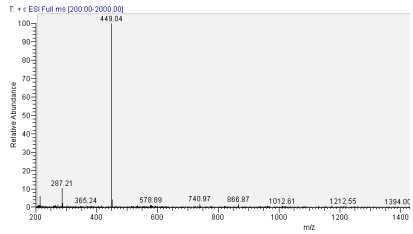
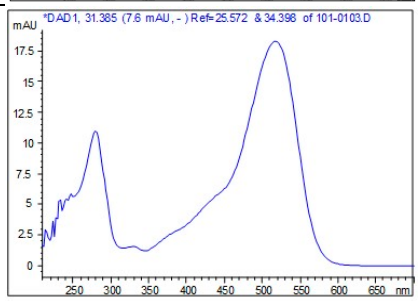
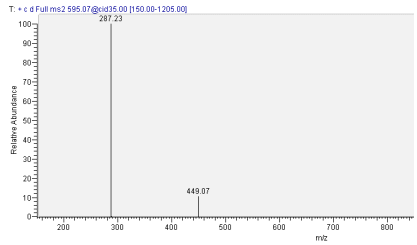
Identified Anthocyanins	HPLC-DAD spectra UV–vis	Fragmentations HPLC-ESI-MS	
cyanidin 3-*O*-glucoside			MS^1
cyanidin 3-*O*-rutinoside			MS^2

Figure 2. UV–vis spectra and HPLC-ESI-MS of *C. procera* leaves.

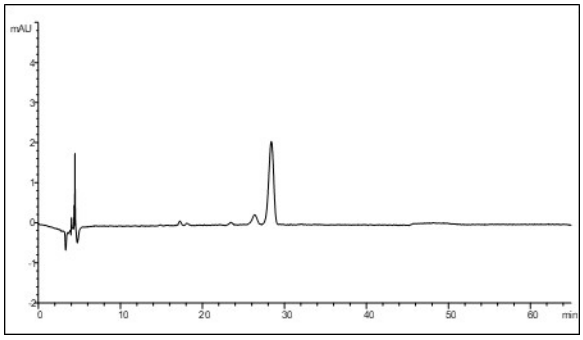
Table 2. LC-ESI-MS of anthocyanins of leaves of *C. procera*.

Peak n°	T_R (min)	λ_{max} (nm)	$[M + H]^+$ *m/z*	$[M - X]^+$ *m/z*	Identified Anthocyanins	%
An1	27.5	516	449	287 [M-162]	cyanidin 3-*O*-glucoside	19
An2	29.4	516	595	449 [M-146] 287 [M-162]	cyanidin 3-*O*-rutinoside	81

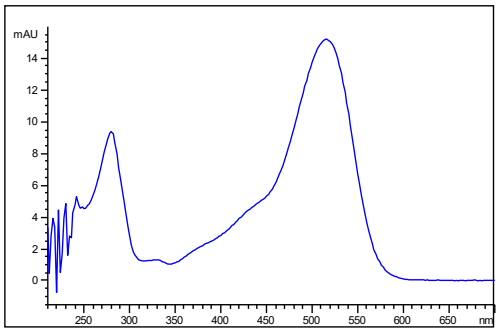
The UV–vis maximal absorption was in both cases 516 nm (Figure 2 and Table 2), confirming the presence of cyanidin aglycone. Peaks An1 and An2 corresponded to compounds containing an aglycone of cyanidin linked to one hexose and two molecules of sugar (one rhamnose and one hexose), respectively.

These retention time, fragmentation, and UV–vis data were the same as those of the main compounds of the petals of *D. regia* Bojer ex Hook: cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside [10]. Therefore, we suggested for peaks An1 and An2 the structures of cyanidin 3-*O*-glucoside (19% of total peak area) and cyanidin 3-*O*-rutinoside (81% of total peak area), respectively (Table 2).

The nature of sugar moieties were confirmed using cochromatography of diluted extracts of both plant samples (Figure 3a–e) of *C. procera* and *D. regia* [10].



(**a**)



(**b**)

Figure 3. *Cont.*

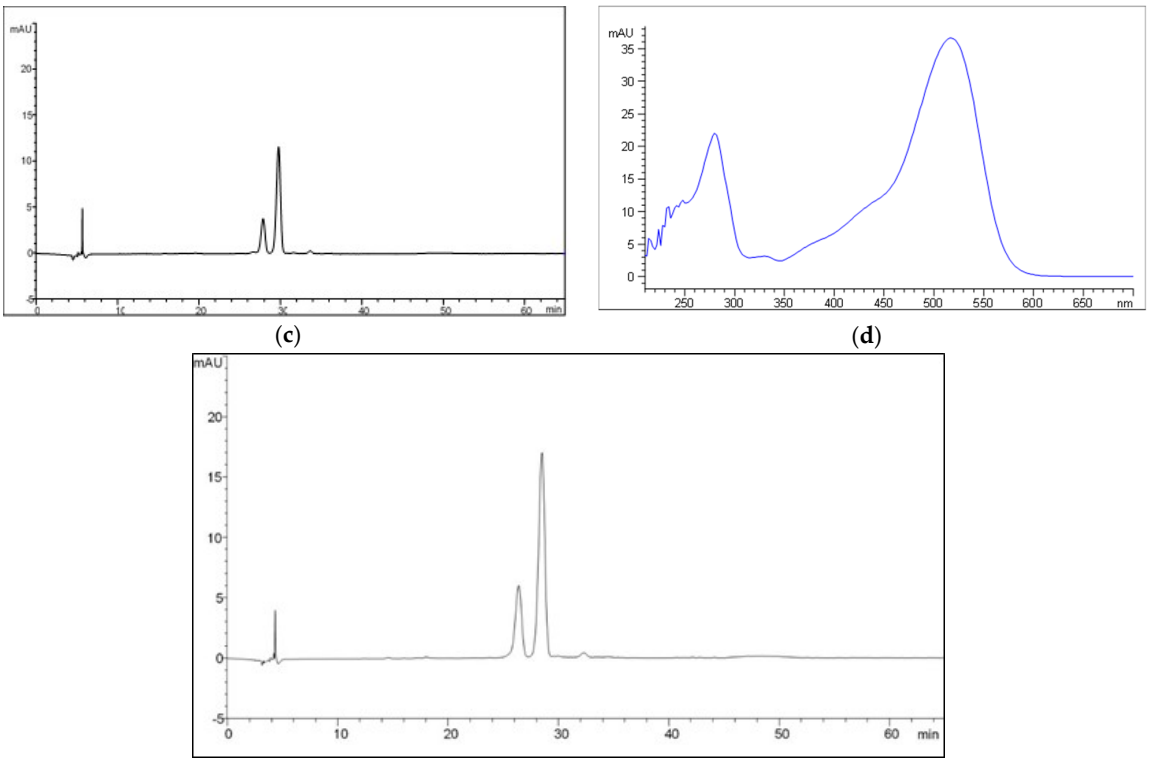


Figure 3. Cochromatography of anthocyanins from *C. procera* (leaf) and *D. regia* (petal) extracts: (**a**) HPLC-DAD of *C. procera*, (**b**) UV–vis peak 1, (**c**) HPLC-DAD of *D. regia*, (**d**) UV–vis peak 2, and (**e**) cochromatography HPLC-DAD of *C. procera* and *D. regia* anthocyanins.

3.2.2. Flavonol Identification

Flavonol compounds were identified from *C. procera* leaf extracts at λ_{max} = 325 nm. Among the 10 peaks considered on the chromatographic profile (Figures 4 and 5), 5 were flavonols (F1–F5). Table 3 shows the positive LC-ESI-MS^2 data of these five compounds (peaks F1–F5). F1 and F2 were identified as the two major flavonols (27% of the total of detected peaks).

Table 3. LC-ESI-MS of flavonols extracted from *C. procera* leaves.

Peak n°	RT (min)	λ_{max} (nm)	$[M + H]^+$ *m/z*	$[M - X]^+$ *m/z*	Identified Flavonols	%
F1	46.5	356	611	465 [M-146] 303 [M-162]	quercetin 3-*O*-rutinoside	15
F2	47.2	356	465	303 [M-162]	quercetin 3-*O*-galactoside	12
F3	47.7	356	465	303 [M-162]	quercetin 3-*O*-glucoside	a
F4	49.0	348	595	449 [M-146] 287 [M-162]	kaempferol rutinoside	a
F5	58.6	368	303		quercetin	a

a: less than 2%. Abbreviation: RT—retention time.

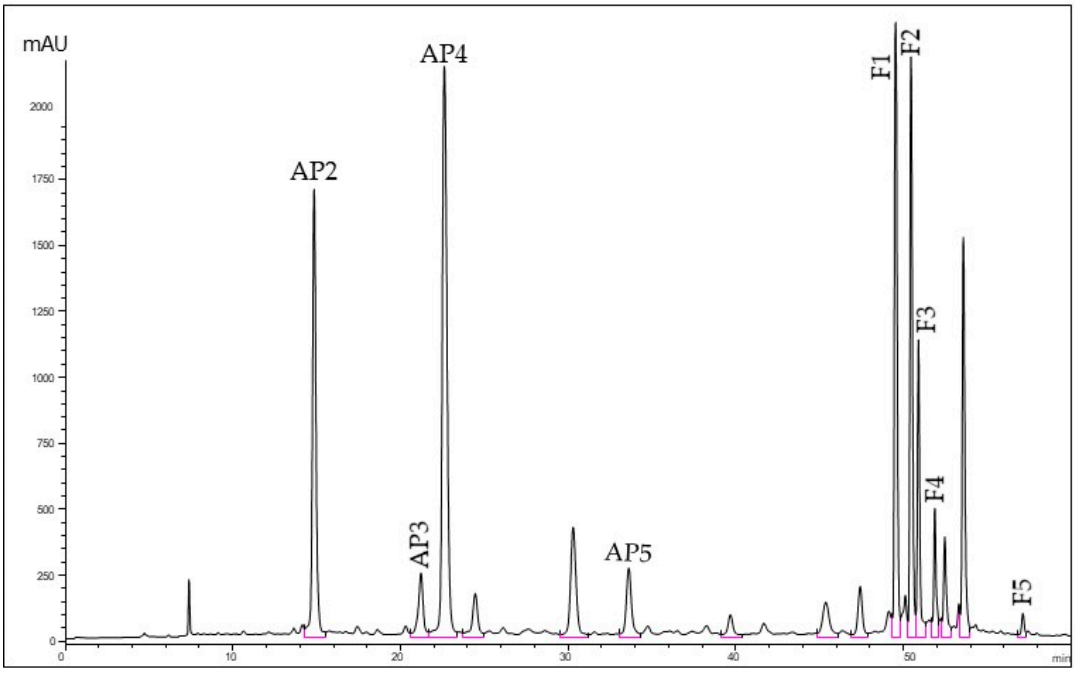


Figure 4. HPLC-DAD (λ = 325 nm) of polyphenols obtained from *C. procera* leaf extracts. Profile recorded from RO extract using a semipreparative HPLC instrument (Model 1200 series, Agilent, Les Ulis, France). Abbreviations: AP1-AP5—phenolic acids, F1-F5—flavonols.

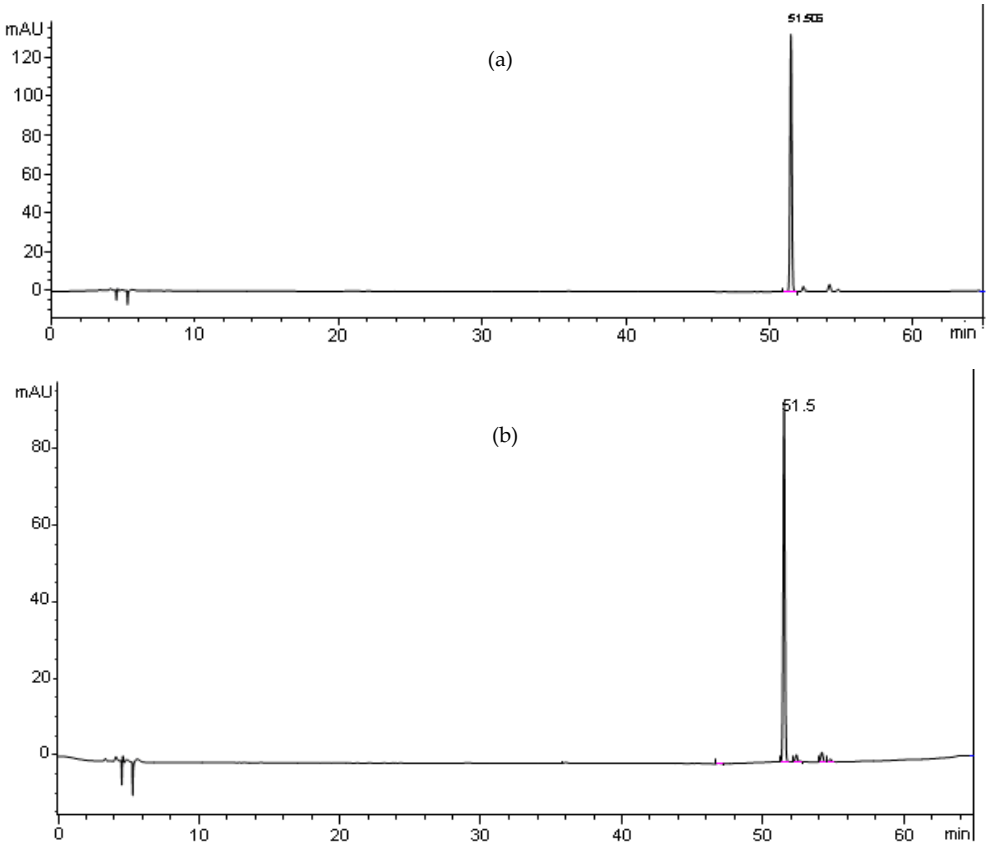


Figure 5. Compared chromatography of quercetin 3-*O*-rutinoside (standard (**a**) and purified sample (**b**)). Profiles recorded using an analytical HPLC instrument (Model 1100 series, Agilent, Les Ulis, France).

According to those data, peak F1 (RT = 46.5 min) had a molecular ion at *m*/*z* 611 in full MS, and in MS^2 fragment, ions at *m*/*z* 465 and 303 (aglycone of quercetin) corresponding, respectively, to losses of 146 u (one molecule of rhamnose) and then 162 u (one molecule of hexose: glucose or galactose). We observed that this compound had a maximal absorption at 356 nm with a UV–vis spectrum similar to a quercetin compound. F1 was suggested to be a glycoside of quercetin. The sugar moieties (rhamnose and hexose) may be linked together or not. We confirmed this result by matching the chromatogram of the standard of quercetin 3-*O*-rutinoside (rutin) with that of the corresponding purified compound (Figure 5).

For peak F2 (RT = 47.2 min), the UV–vis spectrum (356 nm) and characteristics of fragmentation were the same as those obtained with peak F1 in MS^2. Compound F2 lost a fragment ion *m*/*z* 162 (one molecule of hexose: glucose or galactose) in full scan from a molecular ion *m*/*z* 465.

MS^2 data of peak F3 revealed exactly the same fragmentation and UV–vis characteristics with F2 but a different retention time (RT = 47.7 min). This means that F3 is a quercetin glycoside as well, but these two compounds (F2 and F3) differ by their sugar moieties, which have the same molecular weight but not the same nature. Because of the elution order reported in literature, such a result led us to suggest for F2 and F3 the structure of quercetin galactoside and quercetin glucoside, respectively [19].

MS data of peak F4 (RT = 49.0 min) showed in full scan a molecular ion at *m*/*z* 595 and in MS^2 fragments at *m*/*z* 449 (loss of 146 amu: rhamnose) and 287 (loss of 162 amu: glucose or galactose), with a λ_{max} = 348 nm. F4 was suggested to be a kaempferol linked to one molecule of rhamnose and one molecule of glucose or galactose: kaempferol rhamnosyl-hexoside [20]. According to the work of Kumar et al., who described this compound on the basis of the same data, in *Phyllanthus* species extracts, F4 was suggested to be kaempferol rutinoside [21].

In the case of peak F5 (RT = 58.6 min, λ_{max} = 368 nm), the fragmentation provided a molecular ion at *m*/*z* 303, described and confirmed as an aglycone of quercetin on the basis of comparison with the commercial standard.

3.2.3. Phenolic Acid Identification

The chromatographic profile (Figure 6) showed many peaks at 280 nm. Among them, it was possible to distinguish peaks AP1–AP5 as phenolic acids. From the composition comparison with standards (peak AP1) and reported data (peaks AP2–AP5), we were able to describe the molecular structures of dissolved phenolic acids.

The MS data (Table 4) of peak AP1 (RT = 15.4 min) showed a deprotonated molecular ion at *m*/*z* 153. In MS^2, the loss of *m*/*z* 44 (corresponding to a CO_2 unit) led to a fragment at *m*/*z* 109. The UV–vis spectrum also showed absorption maxima at 217, 260, and 295 nm and was identical to that of protocatechuic acid. A comparison with standard data showed that this compound (AP1) can be confirmed to be protocatechuic acid.

For peak AP2 (RT = 18.6 min), according to the MS^2 data shown in Table 4, a molecular ion was observed at *m*/*z* 353. In MS^2, the loss of *m*/*z* 162 (hexose moieties), 12, and 44 (CO_2 unit) gave fragments 191 (quinic acid), 179 (caffeic acid), and 135, respectively. The UV–vis spectrum showed a maximum absorption at 308 nm. We suggest that compound AP2 was a caffeoylquinic acid derivative. These observations have already been reported by Parejo et al. [22], who described this compound as 3-caffeoylquinic acid in Fennel (*Foeniculum vulgare*).

However, MS^2 data (Table 4) of peak AP3 (RT = 23.6 min) showed a molecular ion at *m*/*z* 353 but fragments at *m*/*z* 191 corresponding to quinic acid (loss of hexose moieties, 162 amu) and 163 (loss of CO, 28 amu), and the UV–vis spectrum showed maximum absorption at 313 nm. This compound was suggested to be a caffeoylquinic acid derivative as well. The fragmentation described above was also identical to that reported in the works of Clifford et al. and Rakesh et al. [23,24]. AP3 was then identified as 5-caffeoylquinic acid.

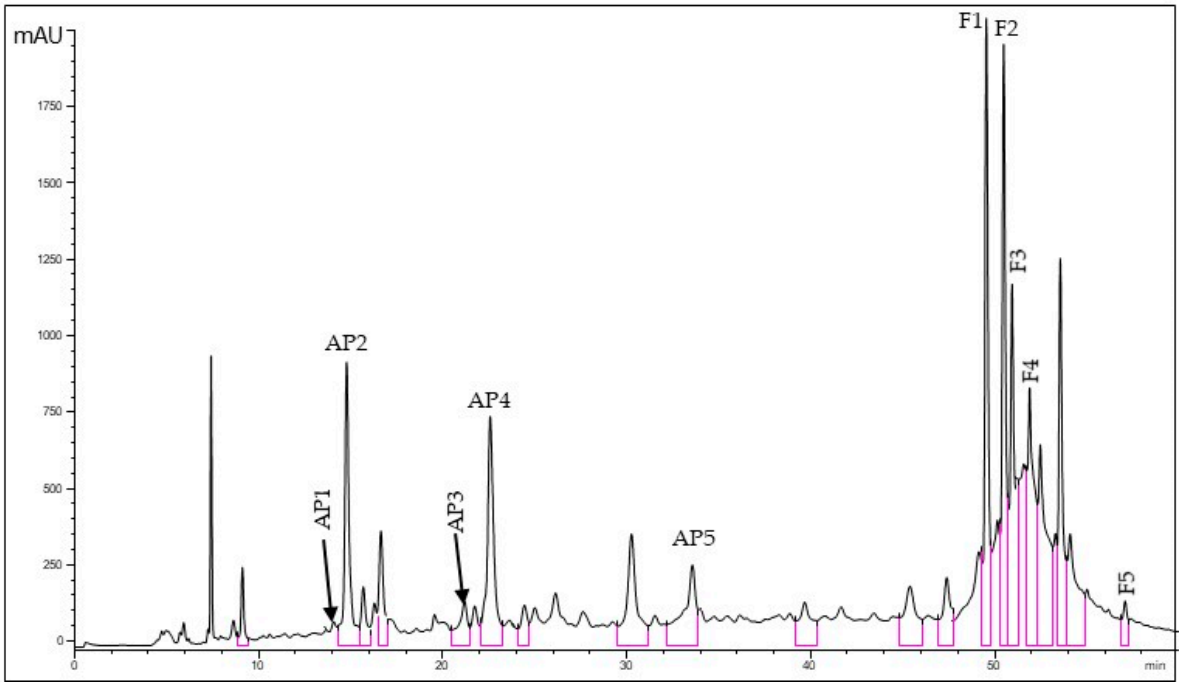


Figure 6. HPLC-DAD (λ = 280 nm) of polyphenols obtained from *C. procera* leaf extracts. Profile recorded from RO extract using a semipreparative HPLC instrument (Model 1200 series, Agilent, Les Ulis, France).

Table 4. LC-ESI-MS of phenolic acids extracted from *C. procera* leaves.

Peak n°	RT (min)	λ_{max} (nm)	$[M - H]^-$ *m/z*	$[M - X]^+$ *m/z*	Identified Phenolic Acids	%
AP1	15.4	217, 260, and 295	153	109 [M-44]	protocatechuic acid	3
AP2	18.6	308	353	191 [M-162] 179 [M-12] 135 [M-44]	3-caffeoylquinic acid	20
AP3	23.6	313	353	191 [M-162] 163 [M-28]	5-caffeoylquinic acid	a
AP4	25.4	327	353	191 [M-162] 179 [M-12] 135 [M-44]	4-caffeoylquinic acid	50
AP5	33.5	313	337	191 [M-146] 163 [M-28]	coumaroylquinic acid	a

a: less than 2%.

MS^2 data of peak AP4 (RT = 25.4 min) showed the following characteristics of fragmentation: molecular ion at *m/z* 353 in full MS and fragments at *m/z* 191 (quinic acid), 179 (caffeic acid), 173 (quinic acid dehydrated derivative), and 135 in MS^2 and maximum absorption (λ_{max} = 327 nm). According to reported data [22,23], this compound (AP4) was identified as 4-caffeoylquinic acid. These authors reported that isomers of chlorogenic acid eluted preferentially in the following order: 3-caffeoylquinic, 5-caffeoylquinic, and 4-caffeoylquinic acids. When comparing fragment *m/z* 191 intensity on both 3- and 4-isomer spectra, it was found that the *m/z* intensity was greater for 3-caffeoylquinic than for 4-caffeoylquinic, as shown in Figure 7.

For peak AP5 (RT = 33.5 min), a molecular ion was observed at *m/z* 337 (Table 4) in full MS and fragments 191 corresponding to quinic acid (loss of *m/z* 146: rhamnose) and 163 corresponding to coumaric acid (loss of *m/z* 28: CO) in MS^2. This compound had UV–vis spectrum 313 nm and was found

to be coumaroylquinic acid [23]. Protocatechuic (3%), 3-caffeoylquinic (20%), and 5-caffeoylquinic acids (50%) were found as major compounds of phenolic acids in *C. procera* leaves.

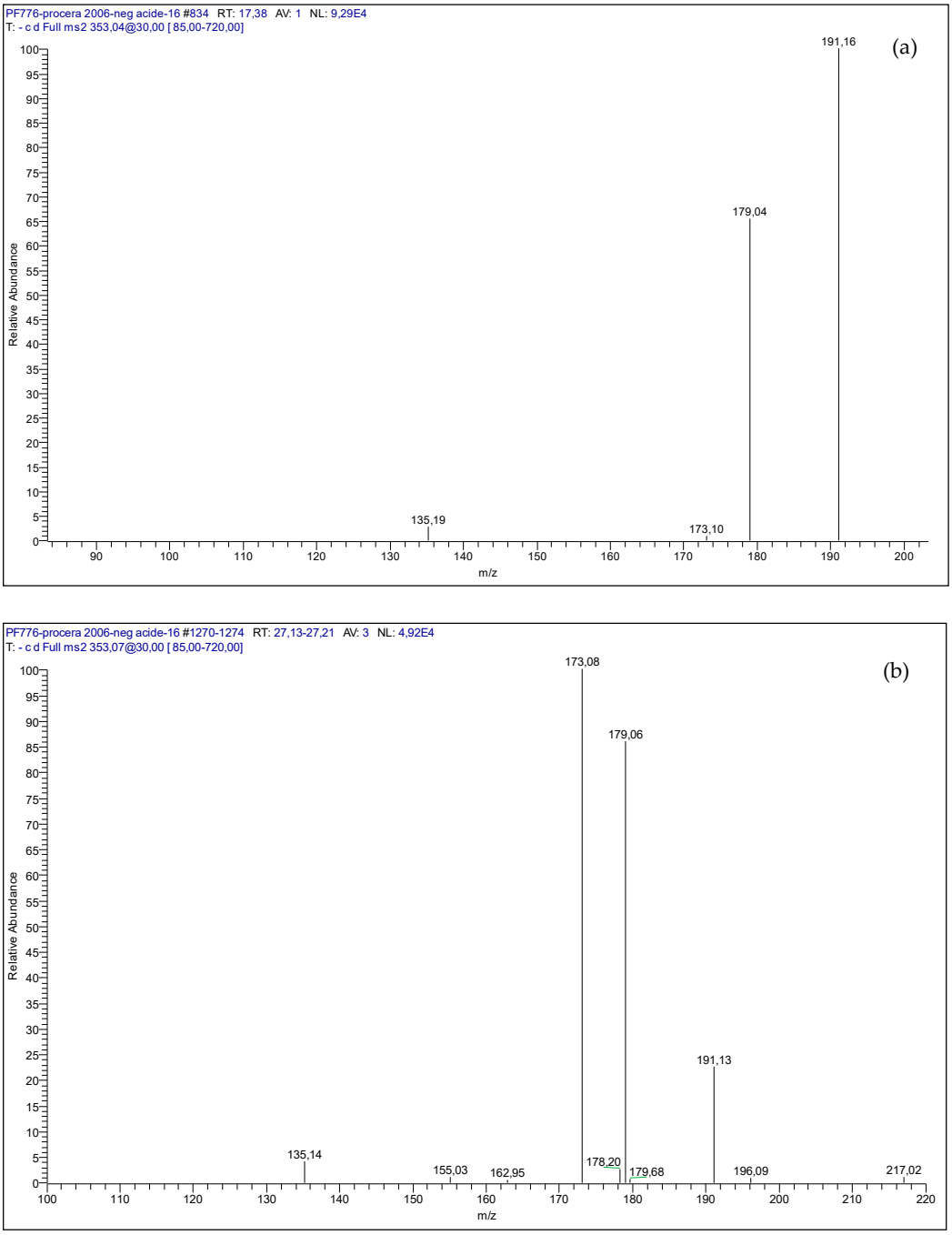


Figure 7. MS^2 of peaks of 3-caffoylquinic and 4-caffeoylquinic acids. (**a**): MS of Peak AP2 (3-caffeoylquinic acid); (**b**): MS of Peak AP4 (4- caffeoylquinic acid).

3.3. Effect of Extraction Process Steps on Polyphenol Content

During the entire process (CrE, CFM, and RO), the contents of the three families of polyphenols (anthocyanins, flavonols, and phenolic acids) previously identified and the concentration factor (CF) for each family were determined (Table 5).

Table 5. Polyphenol family contents of *C. procera* leaves at each step of the process (CrE, CFM, and RO).

Process Coproducts	Anthocyanins (μmol L^{-1} CE)	Flavonols (μmol L^{-1} QE)	Phenolic Acids (μmol L^{-1} GAE)
CrE	2.0 ± 0.4 [a]	230 ± 6 [a]	367 ± 4 [a]
CFM	1.6 ± 0.3 [a]	226 ± 6 [a]	361 ± 2 [a]
RO	28.4 ± 0.3 [b]	1587 ± 3 [b]	3650 ± 10 [b]
CF	18	7	10

In each column, the averages not followed by the same lowercase letter [a,b] are statistically different at $p < 0.05$. Abbreviations: CrE—crude extract; CFM—cross-flow microfiltration; CF—concentration factor; CE—cyanidin equivalents; QE—quercetin equivalents.

The data analyzed by HPLC-DAD of RO samples showed high contents of phenolic acids (3650 ± 10 μmol L^{-1} GAE), followed by flavonols (1587 ± 1 μmol L^{-1} QE). Few anthocyanins were quantified (28.4 ± 0.3 μmol L^{-1} CE). In addition, when reducing the volume (volume reduction factor, VRF) 23 times, it was possible to concentrate more anthocyanins than phenolic acids and flavonols (CF 18 times for anthocyanins while 10 and 7 times for phenolic acids and flavonols, respectively). This indicates that only anthocyanins were better accumulated under the effect of transmembrane pressure during the reverse osmosis operation.

In Table 6, the amounts of concentrated extracts of polyphenols of *C. procera* leaves, in terms of anthocyanins, flavonols, and phenolic acids, are reported. For anthocyanins (28.4 ± 0.3 μmol L^{-1} CE), a high content of cyanidin-3-*O*-rutinoside was found (25.5 ± 0.2 μmol L^{-1} CE) compared with cyanidin-3-*O*-glucoside (2.9 ± 0.2 μmol L^{-1} CE). Among the five flavonols identified (1587 ± 3 μmol L^{-1} QE), quercetin-3-*O*-rutinoside and quercetin-3-*O*-galactoside were the most important compounds (613 ± 1 and 519 ± 1μmol·L^{-1} QE, respectively).

Table 6. Polyphenolic compounds in the final RO extract of *C. procera* leaves.

Types of Polyphenols	Compounds	Content (μmol L^{-1})	%
Anthocyanins (CE)	cyanidin-3-*O*-glucoside	2.9 ± 0.2	10.2
	cyanidin-3-*O*-rutinoside	25.5 ± 0.2	89.8
	Total 1	28.4 ± 0.3	100
Flavonols (DE)	quercetin-3-*O*-rutinoside	613 ± 1	38.6
	quercetin-3-*O*-galactoside	519 ± 1	32.7
	quercetin-3-*O*-glucoside	257 ± 1	16.2
	kaempferol rhamnosyl-hexoside	198	12.5
	Quercetin	-*	-*
	Total 2	1587 ± 3	100
Phenolic acids (GAE)	protocatechuic acid	756 ± 3	20.7
	caffeoylquinic acid	2310 ± 1	63.3
	coumaroylquinic acid	583 ± 5	16.0
	Total 3	3650 ± 10	100

-*: less detectable.

Phenolic acids (3650 ± 10 μmol L^{-1} GAE) such as caffeoylquinic acids were the major compounds (2310 ± 1 μmol L^{-1} GAE). This led us to conclude that leaves are rich sources of chlorogenic acids. The polyphenol composition of *C. procera* leaves has significant similarity to that of *D. regia*, except the chlorogenic acid composition. To our knowledge, this is the first time that the polyphenol composition has been described in *C. procera* leaf aqueous extracts: two glycosides of cyanidin, four quercetin derivatives, one kaempferol glycoside, and three kinds of phenolic acids (protocatechuic, caffeoylquinic, and coumaroylquinic). Figure 8 shows the chemical structures of most of the characterized compounds.

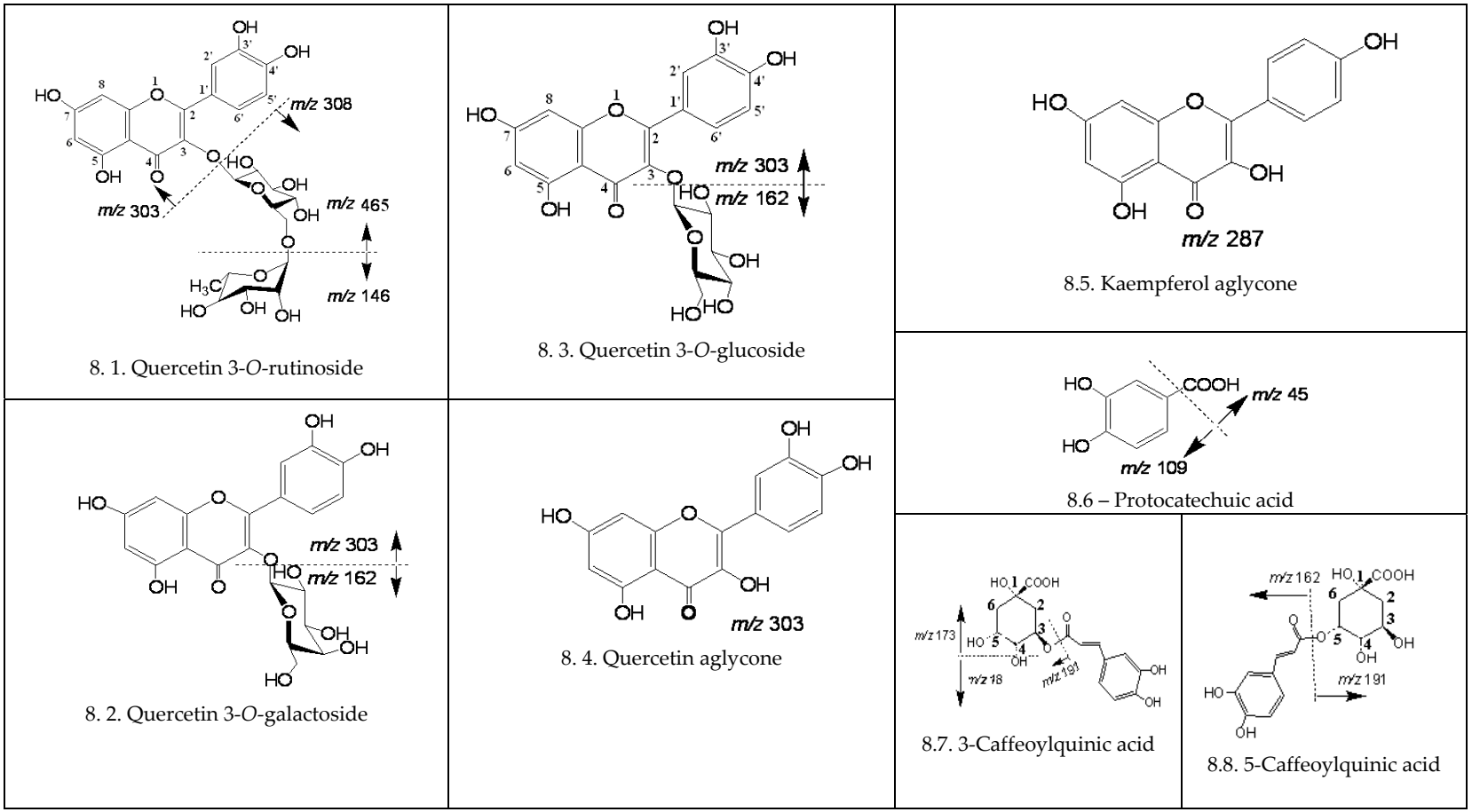


Figure 8. Structures of some flavonols (8.1–8.5) and phenolic acids (8.6–8.8) described in *C. procera* leaf extracts.

4. Conclusions

This study revealed that the aqueous extracts obtained from leaves of *C. procera* are a potential source of phenolic compounds. Their reddish coloring is due to the presence of anthocyanins. Other phenolic compounds, such are flavonols and phenolic acids, were also identified. In total, 12 phenolic compounds (2 anthocyanins, 5 flavonols, and 5 phenolic acids) were characterized for the first time in the studied leaf extracts, suggesting the great importance of this part of the plant. The multistep process including UAE, CFM, and RO that was used to perform the extractions allowed us to produce high-added-value concentrates. Many of the beneficial properties (antioxidant, anti-inflammatory, antibacterial, antiobesity, and anticarcinogenic activities) reported about the leaf extracts could be justified by the presence of such bioactive and functional compounds.

Author Contributions: This work was carried out in collaboration among all authors. F.A.A. designed the study, wrote the protocol, and wrote the first draft of the manuscript. E.N.K. contributed to collecting the biological material, the statistical treatment of the data, and the redaction. K.Y.K. provided important contributions by collecting the biological material, optimizing the extraction process, and document formatting according to the *Beverages* template. E.M. performed LC-MS analyses. A.A.A., P.R.L., Y.F.L., and E.M.G. were the scientific coordinators of this work. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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