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Characterization of Unusual Proanthocyanidins in Leaves of Bayberry (*Myrica rubra* Sieb. et Zucc.)

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ABSTRACT: Extractable and unextractable proanthocyanidins (EPAs and UEPAs) from leaves of bayberry were characterized. Both EPAs and UEPAs were analyzed by acid catalysis in the presence of excess phloroglucinol. The main cleavage product, epigallocatechin-3-*O*-gallate-(4β→2)-phloroglucinol, was successfully identified. The EPAs were of the prodelphinidin type. In fact, epigallocatechin-3-*O*-gallate (EGCG) and traces of epigallocatechin (EGC) were detected as the extension units, but only EGCG was present in the terminal units. All of the compounds exhibited a 2,3-*cis* configuration, and >98% of them were galloylated. The mean degree of polymerization (mDP) of bayberry leaf EPAs was 6.5, and the most abundant EPAs were the polymers, with mDP values of 9.5−26.7. The UEPAs were highly polymerized prodelphinidins consisting of EGCG and traces of EGC. In addition, EGCG, three EPA dimers, and two trimers were identified. The EPAs and UEPAs consisted mostly of EGCG, which is unusual in the plant kingdom.

KEYWORDS: Bayberry, leaves, proanthocyanidins, prodelphinidins, epigallocatechin-3-*O*-gallate (EGCG)

INTRODUCTION

Proanthocyanidins (PAs), a class of natural phenolic compounds found abundantly in the plant kingdom, can be divided into different types depending on the substitution pattern of their monomeric flavan-3-ol units. The most widely distributed PAs in foods are procyanidins, prodelphinidins, and propelargonidins, which consist of (epi)catechin, (epi)gallocatechin, and (epi)-afzelechin units, respectively. These units are most frequently linked via C4−C8 or C4−C6 linkages (B-type PAs). Occasionally an additional C2−O−C7 or C2−O−C5 linkage may exist, leading to doubly bonded A-type PAs. The length of the PA chain is known as the degree of polymerization (DP), and it varies between the different types of PAs.[1] The flavan-3-ol subunits can also be esterified or glycosylated at the C-3 position or C-5 position.[2,3] For instance, the most common esters are 3-*O*-gallates formed with gallic acid and flavan-3-ol subunits. PAs usually occur in complex mixtures in plants, making the study of their chemical and biological properties difficult. Sephadex LH-20, C18 Sep-Pak cartridges, and polyamide cartridges are widely used methods for the fractionation and purification of PAs. Acid catalysis in the presence of excess nucleophiles, mass spectrometry (MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC) are common techniques used for the characterization of PAs. There is abundant literature on the characterization of PAs in the aqueous−organic extracts of plant material, that is, extractable PAs (EPAs), assuming that all or most of the PAs are extracted by aqueous−organic solvents. However, a considerable fraction of PAs remains in the residues of extraction as unextractable proanthocyanidins (UEPAs) and needs to be further analyzed.[4]

Proanthocyanidins are known for their notable pharmacological effects, which include antioxidative,[5] antimicrobial,[6] antiviral,[7] and antitumor [8] properties, and confer cardioprotective activities.[9] The presence of galloyl groups and the number and position of hydroxyl groups are said to enhance their antioxidative activity.[10] Therefore, uncovering additional sources of PAs and analyzing their composition qualitatively and quantitatively pose attractive challenges.

PAs have been found in abundance in a wide variety of foods, for example, peanuts, grapes, apple, tea, cocoa, barley, red wine, and beer.[10,11] Bayberry (*Myrica rubra* Sieb. et Zucc.) is another source of PAs, but few studies on its composition have been described thus far. Bayberry is a tree that belongs to the family Myricaceae.[12] Bayberry has been cultivated mainly in southern China for more than 2000 years.[13] Bayberry fruits, with their exquisite taste, flavor, and attractive red color, are popular among local people. Bayberry trees flush two or three times a year. Foliar growth is luxuriant, and leaves remain green throughout the year. Bayberry trees are pruned every year more than once. Generally, the leaves are discarded and remain underutilized. Extracts of bayberry leaves are reported to have antioxidative,[14] antimicrobial,[15] and antiviral properties.[16] Zou and Li[17] isolated some flavonoids such as myricetin, myricetin glycoside, quercetin, and quercetin glycoside from bayberry leaves using reversed phase HPLC (RP-HPLC). However, little information is available about PAs in bayberry leaves. Li et al.[18] reported that leaves of an unknown bayberry cultivar are rich in condensed tannins and that (epi)gallocatechin-*O*-gallate is invariably the basic unit; however, more accurate and detailed information about the composition of EPAs and UEPAs, as well as the structure of flavan-3-ol monomers and oligomers from bayberry leaves is needed.

The purpose of the present work is to further investigate the structural features of bayberry leaf PAs by using fractionation on a Sephadex LH-20 column, acidic degradation in the presence of excess phloroglucinol, HPLC coupled to photodiode array or UV

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detection and electrospray ionization MS (HPLC-DAD-UV/ESI-MS), and NMR analysis.

MATERIALS AND METHODS

Reagents. (−)-Epigallocatechin-3-*O*-gallate (EGCG), (+)-catechin, and phloroglucinol were obtained in the form of commercial samples from Sigma. Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences AB (Sweden). Methanol and acetonitrile for HPLC analysis were of HPLC grade. All other reagents and solvents used were of analytical grade, unless stated otherwise.

Plant Material. Leaves of 'Biqi', the major cultivar used in the industry, were hand-harvested randomly in July 6, 2009 in Cixi, Zhejiang province, southeastern China. The leaves were washed, dried under vacuum at 40 °C for 12 h, ground finely, and stored at −20 °C until required.

Determination of Total Phenolics and EPAs. The finely ground powder (1 g each) was extracted with 10 mL of 70% aqueous acetone containing 0.1% ascorbic acid for 15 min at room temperature. For the determination of the total phenolic content, extracts were prepared in the absence of ascorbic acid. The process was repeated three times. After 10 min of centrifugation at 2000 rpm, the supernatant was rotary-evaporated under vacuum at 40 °C to remove the acetone and the aqueous phase was diluted to 1000 mL with methanol. All samples were prepared and processed in triplicate.

Total phenolics were estimated using the modified Folin−Ciocalteu method.[19] Briefly, 1 mL of prepared sample solution was added to a 25 mL volumetric flask filled with 9 mL of distilled water. A blank sample was prepared using distilled−deionized water. Folin−Ciocalteu phenol reagent (0.5 mL) was added to the mixture, and the mixture was shaken vigorously for 5 min; 5 mL of Na_2CO_3 solution was added to the mixture; the resulting mixture was immediately diluted to 25 mL with distilled water and mixed thoroughly, and then it was allowed to stand for 60 min before its absorbance at 750 nm was measured. The absorbance was compared with that of the prepared blank. Total polyphenol contents were expressed as milligrams per gram of gallic acid equivalent.

The contents of EPAs were determined according to the modified vanillin assay.[20] One milliliter of the prepared sample solution was mixed first with 2.5 mL of 1% (w/v) vanillin in methanol and then with 2.5 mL of 20% (v/v) H_2SO_4 in methanol to undergo vanillin reaction. A blank sample was prepared using methanol. The vanillin reaction was carried out in a 30 °C water bath for 15 min. The absorbance was measured at 500 nm versus the prepared blank. EPAs contents were expressed as milligrams per gram of (+)-catechin equivalent.

Extraction of PAs. EPAs were extracted from the leaves of bayberry as described by Karonen et al.[21] and Taylor et al.[22] with some modification. The finely ground powder (100 g) was extracted with 70% aqueous acetone (1000 mL) containing 0.1% ascorbic acid. The extraction was performed three times. The acetone extracts were pooled and rotary-evaporated under vacuum at 40 °C to remove acetone. The aqueous phase (∼800 mL) was recovered; washed first with hexane (3 × 800 mL) and then with dichloromethane (3 × 800 mL) to remove pigments, lipids, and other nonpolar material; the organic solvents were evaporated under vacuum. Finally, the aqueous phase was lyophilized to dryness to obtain an extract (∼27 g).

The residue (∼69 g) after the above extraction procedure was collected using water and lyophilized to perform acid degradation for analyzing UEPAs using HPLC.

Purification and Fractionation of EPAs by a Sephadex LH-20 Column. To purify the EPAs, the 2 g sample extracts obtained by the method described above were dissolved in a methanol/water solution (1:1, v/v) and loaded onto a column (300 × 15 mm) of Sephadex LH-20 equilibrated with a methanol/water solution (1:1, v/v) The column was first eluted with 3 volumes of a methanol/water solution (1:1 v/v) and then with an acetone/water solution (7:3 v/v) to recover the EPAs[23] (Table 1). This fraction (180 mL) containing the EPAs was named total EPAs, and it was reduced to an aqueous phase by vacuum evaporation before it was lyophilized to dryness.

Table 1. Procedures of EPAs[a] Fractionation on Sephadex LH-20 Column

	volume of pooled fraction (mL)	acetone (vol %)	methanol (vol %)	water (vol %)
preparation of total EPAs				
−	180	0	50	50
total EPAs	180	70	0	30
fractionation of bayberry leaf EPAs				
−	180	0	50	50
fraction 1	180	0	90	10
fraction 2	180	50	0	50
fraction 3	180	70	0	30

[a] EPAs, extractable proanthocyanidins.

To fractionate the EPAs, the extracts weighing 2 g were also dissolved in the methanol/water solution (1:1, v/v) and applied onto a column identical to the one mentioned above. The column was developed with the sequence of solvent mixtures shown in Table 1. The fractions (collected in lots of 180 mL) were labeled fractions 1, 2, and 3, respectively, rotary-evaporated under vacuum to remove the organic solvents, and lyophilized to dryness. All of the fractions thus collected were subjected to acid catalysis.

Acid Catalysis of PAs in the Presence of Phloroglucinol. The PAs (as samples weighing 5 mg each) were dissolved in 1 mL of freshly prepared methanol solution containing 0.2 N HCl, 50 g/L phloroglucinol, and 10 g/L ascorbic acid. The solution was maintained at 60 °C for 1 h for the reaction to proceed, which was then stopped by adding aqueous 200 mM sodium acetate in equal volumes. Fraction 1 underwent acid catalysis in the presence of excess phloroglucinol at 50 °C for 15 min.[24,25] Phloroglucinol adducts were then filtered though a 0.45 μm membrane and analyzed by HPLC-DAD and HPLC-UV- ESIMS immediately.

Identification and Quantification of PA Cleavage Products. Because authentic standards of the extension subunit−phloroglucinol adducts were not available, we prepared them from bayberry leaves as described above and from grape skins as follows. Grape skin (200 g) was extracted by using the extraction method for bayberry leaf EPAs with acetone/water (2:1, v/v). The extract powder obtained was purified by a Sephadex LH-20 column. The column (300 × 15 mm) was equilibrated with a methanol/water solution (1:1, v/v). Next, the extract powder was dissolved in this mobile phase and applied to the column. The column was then rinsed with 3 column volumes of methanol/water solution (1:1, v/v) and methanol/water solution (9:1, v/v). The PAs were then eluted with 3 column volumes of acetone/water solution (7:3, v/v). Then, the eluent was concentrated under vacuum to remove the organic solvents and lyophilized to dryness. The grape skin PAs obtained (as samples weighing 5 mg each) were then degraded in the presence of phloroglucinol using the method described above.

The extension subunit−phloroglucinol adducts were purified by semipreparative RP-HPLC on an XTerra Prep RP18 OBD column (300 × 19 mm, 10 μm) (Waters) with a guard column containing the same material. The HPLC was run under the linear gradient condition with a flow rate of 15 mL/min at room temperature. The mobile phase consisted of 0.1% v/v aqueous formic acid (solvent A) and methanol

(solvent B). The elution program was as follows: 15% B (0−9 min), 15−25% B (9−10 min), 25% B (10−22 min), 25−100% B (22−23 min), and 100% B (23−30 min). The isolated compounds were concentrated by rotary evaporation and lyophilized to a dry powder.

The extension subunit−phloroglucinol adducts were identified by chromatographic comparisons with standards prepared, UV absorption spectra, MS, and NMR. ^{1}H and ^{13}C NMR spectra were recorded in D_2O with a Bruker AvIII500 at 500 and 150 MHz, respectively. The hydrolyzed terminal subunits were identified by comparison with the authentic standard, UV absorption spectra, and MS.

The extension subunit−phloroglucinol adducts of samples were quantified by injecting a solution with known concentrations of the standards prepared above. The concentration of the hydrolyzed terminal subunits was determined from standard curves prepared from commercial standards. The mean degree of polymerization (mDP) of EPAs, galloylation, and qualitative data were on a molar basis.

Isolation of Individual Flavan-3-ol Monomers and Oligomeric EPAs. Individual flavan-3-ol monomers and EPA oligomers were isolated according to a method published earlier.[26] The extracts (as a sample weighing 1 g) were dissolved in 20 mL of water. The solution was centrifuged at 2000 rpm for 5 min, and the supernatant was recovered. Crystallized NaCl was then added to reach the saturation point to precipitate the polymerized EPAs. The preparation was filtered, and the filtrate was extracted with ethyl acetate (3 × 20 mL). The ethyl acetate phase (60 mL) was washed with 10 mL of water and concentrated under vacuum to ∼2 mL, and chloroform (10 mL) was added to precipitate phenolics. The suspension was filtered; the filtrate was discarded, and the residue was redissolved in methanol for analysis by HPLC-DAD and HPLC-UV-ESIMS.

HPLC-DAD and HPLC-UV-ESIMS Analyses. Individual flavan-3-ol monomers, oligomeric EPAs, and cleavage products were analyzed by HPLC-DAD and HPLC-UV-ESIMS. HPLC-DAD was performed on a Waters platform, composed of a Waters 2695 HPLC and a Waters 2998 photodiode array detector. UV−vis absorption spectra were recorded online during HPLC analysis. Spectral measurements were made at 200−400 nm. HPLC-UV-ESIMS analysis was done by electrospray ionization in the negative mode on ESQUIRE3000 PIUS (Bruker). Samples were separated on a Zorbax SB-C18 (Agilent, USA) column (250 × 4.6 mm, 5 μm) protected by a guard column of the same material and detected at 280 nm.

For analysis of individual flavan-3-ol monomers and oligomeric EPAs, HPLC was carried out under the following conditions: 0.1% formic acid (solvent A) and acetonitrile (solvent B) were used as mobile phase at a flow rate of 0.7 mL/min at 30 °C, and the linear gradient was isocratic 10% B (5 min), 10−30% B (5−35 min), and 30−90% B (35−40 min).

For analysis of cleavage products, the mobile phase was a linear gradient of 0.1% formic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min at 35 °C, and the elution program comprised 5% B (0−10 min), 5−20% B (10−20 min), 20−40% B (20−30 min), and 40−90% B (35−37 min).

RESULTS AND DISCUSSION

Contents of Total Phenolics and EPAs. The contents of total phenolics and EPAs in bayberry leaves, on a dry weight basis, were 194.0 and 117.5 mg/g, respectively. Compared to some other leaves, the contents of total phenolics in bayberry leaves were lower than those of *Nelumbo nucifera* leaves, which contained 358−487 mg/g dry plant material,[27] and *Chromolaena odorata* leaves, which contained 242 mg/g dry plant material,[28] but higher than those of *Calpurnia aurea* leaves, which contained 9.62 mg/g dry plant material,[29] and *Stevia rebaudiana* Bert. leaves, which contained 61.50 mg/g dry plant material.[30] Besides, the contents of EPAs in leaves of *N. nucifera*, *Calliandra*, and *C. aurea* are 124−179 mg/g,[27] 74.7−106 mg/g,[31] and 4.37 mg/g[29] dry plant material, respectively. The value of EPA content in this study falls within the range of the leaves mentioned above. These observations suggest that bayberry leaves are a considerably useful source of phenolics and EPAs. Because many phenolics and EPAs are well-known antioxidants, the studied bayberry leaves are useful material with potential antioxidative activities.

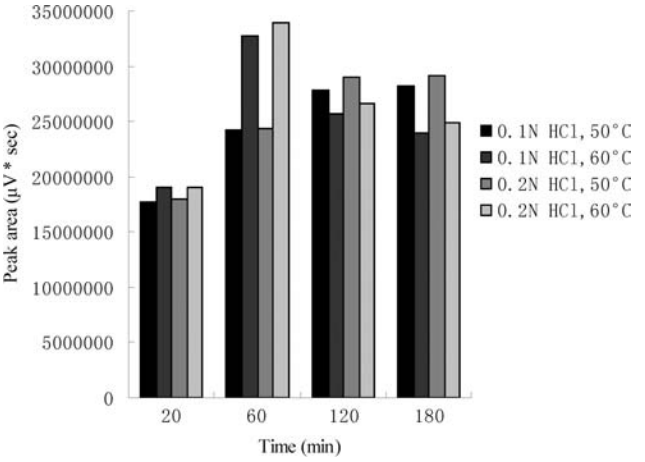

Figure 1. Effect of reaction temperature, time, and concentration of HCl on HPLC chromatograms of main reaction product (EGCG-ph) of fraction 2 and 3 mixture.

Acid Catalysis of PAs and Identification of the Cleavage Products. The EPAs were fractionated by column chromatography with Sephadex LH-20 into the three following fractions: 0.38 g of fraction 1 with a methanol/water solution (9:1, v/v), 0.59 g of fraction 2 with an acetone/water solution (1:1, v/v), and 0.12 g of fraction 3 with an acetone/water solution (7:3, v/v). The chromatographic procedures described above yielded 1.06 g of total EPAs.

To search for the optimal reaction conditions, a mixture of fractions 2 and 3 was submitted to acid degradation under different reaction temperatures and for various durations on the basis of the method described by Michodjehoun-Mestres et al.[25] The results in Figure 1 show a higher yield of cleavage product at higher reaction temperatures and over longer durations. The yield then remains roughly constant or decreased. The highest yield of the main cleavage product was recorded when acid catalysis was carried out using 0.2 N HCl, thus allowing the reaction to proceed at 60 °C over 1 h.

The cleavage products were analyzed by HPLC-DAD and HPLC-UV-ESIMS immediately after acid catalysis. Two main products (peaks 4 and 5) showed up in the chromatograms (Figure 2), one of which (peak 5) was identified as EGCG by comparison with authentic standard and showed the following properties: UV spectra parameter with λ_{max} at 273.5 (shoulder from 290 to 325 nm), and a pseudomolecular ion $[M - H]^-$ at m/z 457.1.

Peak 4, which showed a UV spectrogram very similar to that of EGCG, had a pseudomolecular ion $[M - H]^-$ at m/z 581.1 and a duple molecular ion $[2M - H]^-$ at m/z 1163.3, indicating that it may have been derived from EGCG and was likely to be either EGCG-ph or gallocatechin-*O*-gallate-ph (GCG-ph). The ^{13}C NMR chemical shifts of peak 4 (Table 2) were very similar to those of the following products: epicatechin-3-*O*-gallate-(4β→2)-ph (ECG-ph),[24] gallocatechin-(4α→2)-ph (GC-ph), epigallocatechin-(4β→2)-ph (EGC-ph),[32,33] and EGCG. The C-2 signal of peak 4 was observed at δ 74.4, which is characteristic of a flavan

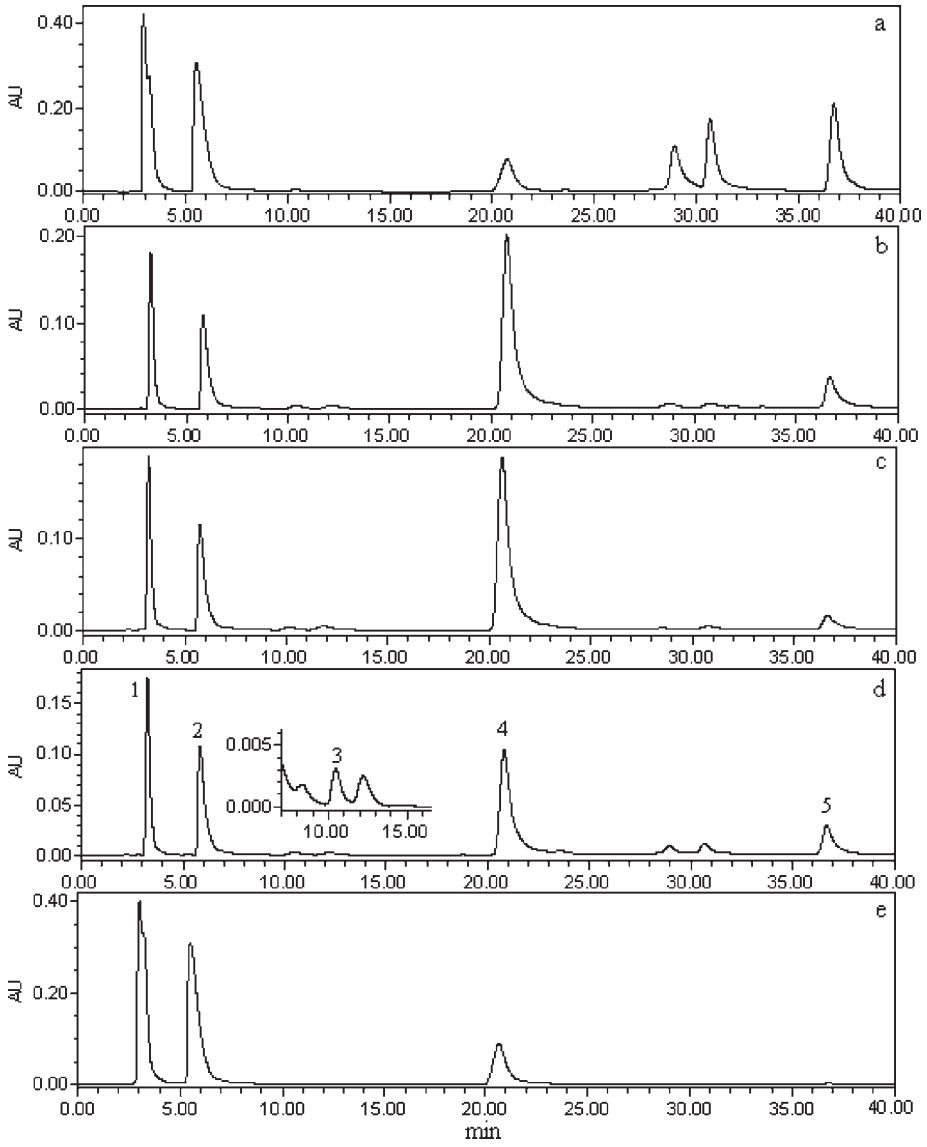

Figure 2. HPLC chromatograms (detected at 280 nm) of reaction products of fractions collected from Sephadex LH-20 column and UEPAs with the presence of excess phloroglucinol: (a) fraction 1; (b) fraction 2; (c) fraction 3; (d) total EPAs; (e) UEPAs. Peaks: 1, ascorbic acid; 2, phloroglucinol; 3, EGC-ph; 4, EGCG-ph; 5, EGCG.

with a 2, 3-*cis* configuration compared to those of ECG-ph (δ 75.1), EGC-ph (δ 76.9), and GC-ph (δ 84.2). Compared to ECG-ph (δ 74.7) and EGCG (δ 72.5), the C-3 signal of peak 4 observed at δ 72.7 indicated that the flavan was esterified at the C-3 position. The carbon signal at δ 166.2 is characteristic of C═O, confirming that the flavan was esterified. The carbon signals of the B-ring were two sets of carbon signals at δ 105.8 and 143.9, which were very similar to those of EGCG, suggesting that the B-ring was symmetrical. This was confirmed by the observation of only one singlet at δ 6.48 (B-2 and B-6 protons) in the ^{1}H NMR spectrum (Table 2). It was reported that the B-2 and B-6 protons of GC and EGC appeared at δ 6.50 and 6.60, respectively.[32] Eventually, peak 4 was identified as epigallocatechin-3-*O*-gallate-(4β→2)-ph (EGCG-ph). The structure of peak 4 is shown in Figure 3.

In addition to the two main cleavage products (EGCG and EGCG-ph), a minor peak (peak 3, Figure 2) was also observed in all samples to elute shortly after phloroglucinol and showed the following properties: $UV_{\lambda max}$ at 269.9 nm (no shoulder from 290 to 325 nm) and a pseudomolecular ion $[M - H]^-$ at m/z 429.1. By comparison with the authentic standard prepared from grape skin earlier, the small peak was identified as EGC-ph.

Furthermore, we failed to find A-type dimers and their phloroglucinol adducts. Another two unknown peaks were detected in the chromatogram of the cleavage products of total EPAs and fraction 1 (Figure 2a,d). They were also detected as peaks 2 and 3 in the chromatogram corresponding to the flavan-3-ol monomeric and oligomeric fraction (Figure 4). Consequently, the peaks were thought to be artifacts resulting from the incomplete purification of proanthocyanidins.

Structural Composition of PAs. The HPLC-DAD-UV/ESI-MS chromatograms of the cleavage products yielded information useful in elucidating the polymeric structure of PAs. The chromatogram, monitored at 280 nm (Figure 2), showed that the terminal unit was released as EGCG, and the extension units were released as EGCG and EGC phloroglucinol adducts. Therefore,

Table 2. ^{1}H and ^{13}C NMR Chemical Shift Values of Peak 4 (Reaction Product) in Figure 2 Recorded in D_2O

carbon no.	chemical shift of carbon (δ)	chemical shift of proton (δ)
C═O	166.2	−
A-5	155.2	−
A-6	95.0	6.07, d (J = 2.33 Hz)
A-7	154.9	−
A-8	93.9	6.19, d (J = 1.99 Hz)
4α	101.9	−
8α	154.8	−
B-1	129.4	−
B-2	105.8	6.48, s
B-3	143.9	−
B-4	131.4	−
B-5	143.9	−
B-6	105.8	6.48, s
C-2	74.4	5.41, s
C-3	72.7	5.33, s
C-4	31.6	4.55, s
D-1	119.8	−
D-2	109.3	6.95, s
D-3	144.5	−
D-4	137.6	−
D-5	144.5	−
D-6	109.3	6.95, s
E-1	105.9	−
E-2	155.2	−
E-3	95.0	6.02, bs
E-4	155.4	−
E-5	95.0	6.02, bs
E-6	155.2	−

EPAs in bayberry leaves were essentially of the prodelphinidin type; all of the compounds exhibited a 2,3-*cis* configuration.

The mDP and details of the structural composition of bayberry leaf EPAs were therefore ascertained, and the results are presented in Table 3. The mDP of total EPAs was 6.5, and that of the three fractions (fractions 1−3) ranged from 1.6 to 26.7. Table 3 also shows that EGCG (>97% of total extension units) was the main extension unit of bayberry leaf EPAs, whereas EGC was present only in trace amounts (<3% of all extension units). EGCG was also the only terminal unit of bayberry leaf EPAs; galloylated units accounted for about 98% of all units.

EPAs containing EGC were not detected in an unknown cultivar of bayberry leaves by Li et al.[18] using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). This might be because the amount of EPAs containing EGC was so small, compared to EPAs containing only EGCG, that they could not be easily detected under the experimental conditions used by Li et al.[18] In our work, we used acid catalysis of EPAs in the presence of phloroglucinol; this method resulted in the total separation of EGC-ph and EGCG-ph by HPLC, thus yielding more informative spectra, and allowed us to find EGC as a monomeric flavan-3-ol unit of bayberry leaf EPAs. In addition, our data indicated that all of the monomeric flavan-3-ol units conformed to the 2,3-*cis* configuration, which Li et al.[18] did not determine because MALDI-TOF/MS cannot distinguish between epimers.

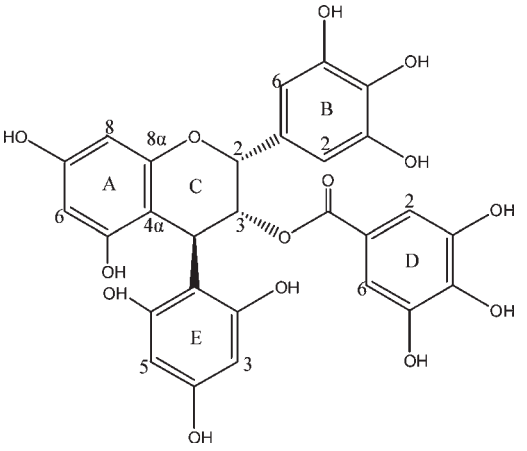

Figure 3. Structure of peak 4 (reaction product) in Figure 2.

Our data indicated that bayberry leaf EPAs contained polymers with a DP of >26. However, Li et al.[18] estimated the DP of bayberry leaf tannins at 2−7, an estimate clearly different from our results. This disparity in the findings was mainly due to the different analytical methods we used. A previous study[22] reported that MALDI-TOF/MS is limited in the detection of large polymers, and acid catalysis can supply more accurate results to characterize EPAs because the latter correlated well with gel permeation chromatography data. In our work, the fractionation steps used before acid catalysis separated larger polymers from other bayberry leaf EPAs, resulting in the detection of polymers with an mDP of 26.7.

The results of acid catalysis of UEPAs from bayberry leaves in the presence of excess phloroglucinol are shown in Figure 2 and Table 3. The cleavage products of UEPAs were identified by comparison with the authentic standards prepared earlier. Our HPLC chromatographic results showed that acid catalysis of UEPAs from bayberry leaves in the presence of excess phloroglucinol resulted almost exclusively in EGCG-ph with only traces of EGC-ph and EGCG; this confirmed that UEPAs in the residue of bayberry leaves were mostly polymers of EGCG. Although UEPAs are strong complexes with insoluble polymeric plant materials (e.g., cell wall polysaccharides) and cannot be extracted by aqueous−organic solvents,[4] they may exert antioxidant activity via a surface reaction in the small intestine and reach the colon in association with vegetable cell walls. There they are fermented by bacterial microflora, yielding different metabolites that may counteract the effects of dietary pro-oxidants.[34] The generation of an antioxidant environment in the colon may have important effects on gastrointestinal health, including a chemopreventive effect for colorectal cancer.

Compared to other plant PAs, the structural profile of bayberry leaf PAs is unusual. The PAs are entirely of the prodelphinidin type. To our knowledge, procyanidins, consisting of (epi)catechin, exist most widely in plants (e.g., grape seed, broomcorn, pine bark, and peanut). Prodelphinidins and propelargonidins mostly coexist with procyanidins (e.g., barley and grape skin). Plants containing only prodelphinidins are seldom reported. Furthermore, the composition of bayberry leaf PAs is simple; EGCG is the exclusive subunit of bayberry leaf PAs, whereas most other PAs contain more than one kind of subunit. The PAs of pine bark are mainly of the procyanidin type, with catechin and epicatechin as their subunits.[35] Subunits of birch leaf PAs include catechin, epicatechin, and (epi)gallocatechin.[21]

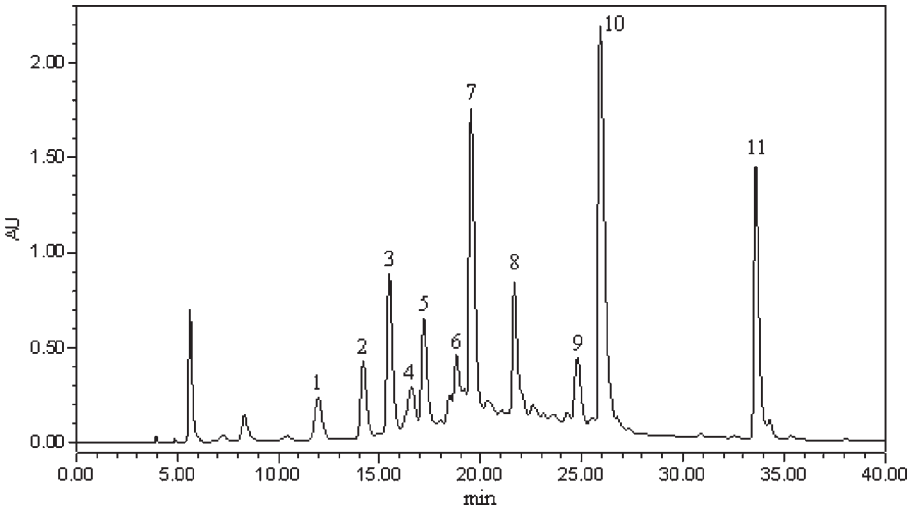

Figure 4. HPLC chromatogram (detected at 280 nm) of individual flavan-3-ol monomers and oligomeric proanthocyanidins of bayberry leaves. Peak numbers refer to Table 4.

Table 3. Acid Catalysis Results of Collected Fractions from Sephadex LH-20 Column and UEPAs [b] in the Presence of Excess Phloroglucinol

sample	sample (g)	EGC[c]/ EGCG[d] of extensional unit/EGCG[d] of terminal unit (%)[e]	mDP[f]	galloylation (%)[e]
total EPAs[a]	1.06	2:83:15	6.5	98.0
fraction 1	0.38	1:37:62	1.6	99.0
fraction 2	0.59	2:88:10	9.5	98.4
fraction 3	0.12	1:95:4	26.7	98.7
UEPAs[b]	−	EGCG[d]	−	99.9

[a] EPAs, extractable proanthocyanidins. [b] UEPAs, unextractable proanthocyanidins. [c] EGC, epigallocatechin. [d] EGCG, epigallocatechin-3-*O*-gallate, EGCG-ph with traces of EGC-ph and EGCG. [e] (%), Mol %, % = [EGCG]/([EGCG] + [EGC]) × 100. [f] mDP, mean degree of polymerization, mDP = ([extension units] + [terminal units])/[terminal units].

Subunit composition is one factor leading to the high complexity of PAs. The complexity of PAs makes it difficult to determine both their chemical and biological properties. Bayberry leaf PAs with a simpler composition might be used as standard compounds for the qualitative and quantitative analysis of PAs, as well as for the elucidation of their biological activity in the future. Besides, almost all of the constitutive units of bayberry leaf PAs were 3-*O*-galloylated; this is a rare scenario. The galloylation of cashew apple tannins was reported as 20−44%.[25] The presence of galloyl groups was reported to potentially enhance the antioxidative activity of PAs.[10] Therefore, bayberry leaf PAs might have greater potential as antioxidants.

Identification of Individual Flavan-3-ol Monomers and Oligomeric EPAs. Individual flavan-3-ol monomers and oligomeric EPAs were prepared and then characterized with HPLP-DAD and HPLP-UV-ESIMS (Figure 4). One flavan-3-ol monomer, three EPA dimers, and two trimers were isolated. The composition of each compound, which was determined by their spectral characteristics, pseudomolecular ions ($[M - H]^-$), and product ions, is given in Table 4. The dimers and trimers were all of B-type. Whereas one dimer and one trimer (peaks 1 and 4 in Figure 4) contained EGCG and EGC, others consisted of only EGCG.

To identify the structure of peak 1, a hypothetical structure based on the product ions detected by ESIMS (Figure 5) was drawn with EGC as the extension unit and EGCG as the terminal unit. Earlier studies have reported three characteristic fragmentation routes for EPAs: retro-Diels−Alder fission (RDA), quinone methide fission (QM), and heterocyclic ring fission (HRF).[36] RDA was found to be the most important fragmentation route for elucidating the structure of dimers and to lead to the loss of ring B in the extension unit.[36] Peak 1, eluting at 11.97 min, showed a pseudomolecular ion $[M - H]^-$ at m/z 761.3 with fragment ions at m/z 591.2 and 423.1, which correspond to further loss of *O*-galloyl[37] and the RDA product ion (m/z 168),[38] respectively. On the basis of these results, we concluded that EGC was the extension unit and EGCG was the terminal unit. Product ions derived from fragmentation can be used only for determining the sequence of monomeric units, not for distinguishing between epimers. However, acid catalysis of PAs in the presence of excess phloroglucinol produced only EGC-ph and EGCG-ph in the present experiment. Therefore, peak 1 was identified as EGC−EGCG.

Peak 4, eluting at 16.58 min, showed a pseudomolecular ion $[M - H]^-$ at m/z 1217.4 with two fragment ions at m/z 1065.3 and 913.4, which confirmed the loss of one galloyl residue ($[M - H - 152]^-$) and two galloyl residues ($[M - H - 152 - 152]^-$),[37] respectively. Because there were no more fragment ions, especially the most important fragment ions to characterize trimeric EPAs produced by the cleavage of interflavanoid linkages,[37] the structure of this peak could not be determined with certainty. Therefore, peak 4 was provisionally identified as a

Table 4. Identification of Individual Flavan-3-ol Monomers and Oligomeric Proanthocyanidins of Bayberry Leaves by Using Their Spectral Characteristics, Negative Ions in HPLC-DAD/HPLC-UV-ESIMS, and Respective Standards

peak	HPLC t_R (min)	λ_{max}[a] (nm)	mol wt	HPLC-ESIMS, $[M-H]^-$, m/z	tentative identification
1	11.97	272.3	762	761.3, 591.2, 423.1	EGC[b]−EGCG[c]
2	14.47	271.1	468	935.0, 467.4	unknown
3	15.74				
8	22.15				
4	16.58	272.3	1218	1217.4, 1065.3, 913.4	2EGCG[c] + EGC[b]
5	17.19	273.5	914	913.4, 743.2, 573.2	2EGCG[c]
9	24.81				
6	18.81	273.5	1370	1369.4, 1217.4, 1049.2	3EGCG[c]
7	19.53	273.5	458	915.1, 457.1, 304.8	EGCG[c]
10	26.21	260, 350	−	−	flavones
11	34.85				

[a] λ_{max}, wavelength of maximum absorption in UV. [b] EGC, epigallocatechin; [c] EGCG, epigallocatechin-3-*O*-gallate.

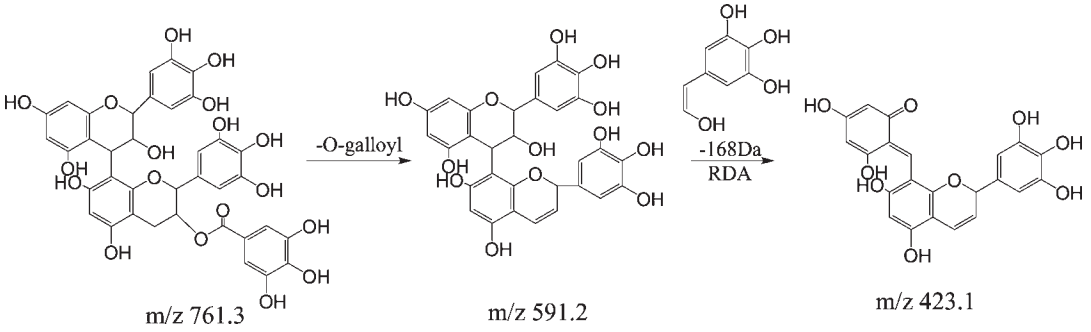

Figure 5. Fragmentation pathway of peak 1 in Figure 4, consistent with a structure composed of EGC (extension unit) and EGCG (terminal unit).

prodelphinidin trimer consisting of two EGCG units and one EGC unit.

Peaks 5 and 9, the other two dimers consisting of two EGCG units, had the same fragment ions: a pseudomolecular ion $[M-H]^-$ at m/z 913.3 with two fragment ions at m/z 743.2 and 573.2, corresponding to further loss of one *O*-galloyl and two *O*-galloyls, respectively. Peak 6 was a trimer containing three EGCG. It had a pseudomolecular ion $[M-H]^-$ at m/z 1369.4 and another two fragment ions at m/z 1217.4, 1049.2 (1217.4−168 Da), which corresponded to the loss of galloyl residue and further loss of RDA product ion (m/z 168). Peak 7 gave a molecular ion $[M-H]^-$ at m/z 457.1, a duple molecular ion $[2M-H]^-$ at 915.1, a fragment ion at m/z 304.8 corresponding to the loss of a galloyl residue, and a fragment ion at m/z 168.8 (the gallic acid fragment), thus indicating that peak 7 was EGCG or GCG. By comparison with the standard, this peak was positively identified as EGCG.

Peaks 2, 3, and 8 (eluting at 14.47, 15.74, and 22.15 min, respectively) showed the same fragment ions at m/z 935.0 and 467.4, indicating that the three peaks had the same molecular weight of 468 and that they might have different stereochemical structures. Furthermore, the three peaks had very similar UV−vis spectrum shapes, with λ_{max} at 271.1 nm and a very broad band shifted to 360 nm. However, the shapes of their UV−vis spectra were different from those of other flavan-3-ol monomers and oligomeric EPAs. Because there was no additional information about the three peaks, their structures could not be confirmed.

Peaks 10 and 11 in Figure 4 were not flavan-3-ol monomers or EPAs but flavones, because the shape of their UV−vis spectra was similar to that of flavones. Other peaks with retention times of 5.62 and 8.32 min were not characterized, because of the unclear mass spectra data we observed under our analytical conditions.

In summary, the present study addressed the characterization of EPAs and UEPAs from leaves of bayberry (*M. rubra* Sieb. et Zucc.). Bayberry leaf EPAs were essentially of the prodelphinidin type, and EGCG was the main flavan-3-ol unit in EPAs. Traces of EGC were also present in the extension units. All of the compounds conformed to the 2,3-*cis* configuration, and >98% of them were galloylated. UEPAs in the residue of bayberry leaves were mostly polymers of EGCG. Such features of bayberry PAs are unusual in the plant kingdom and merit further research regarding their role in promoting health, preventing diseases, and similar practical applications. Besides, one flavan-3-ol monomer, three EPA dimers, and two trimers were identified. The dimers and trimers were all of the B-type. Whereas one dimer and one trimer contained EGCG and EGC, the others consisted of only EGCG.

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ABBREVIATIONS USED

(E)GC, (epi)gallocatechin; (E)GC-ph, (epi)gallocatechin-phloroglucinol; (E)GCG, (epi)gallocatechin-3-*O*-gallate; (E)GCG-ph, (epi)gallocatechin-3-*O*-gallate-phloroglucinol; EPAs, extractable

proanthocyanidins; HRF, heterocyclic ring; HPLC-DAD, high-performance liquid chromatography coupled to photodiode array detection; HPLC-UV-ESIMS, HPLC coupled to UV detection and electrospray ionization mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; mDP, mean degree of polymerization; NMR, nuclear magnetic resonance; QM, quinone methide; RDA, retro-Diels−Alder; RP-HPLC, reversed phase high-performance liquid chromatography; UEPAs, unextractable proanthocyanidins.

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