Antioxidative Constituents in Camu-camu Fruit Juice Residue

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Camu-camu (Myrciaria dubia, Myrtaceae) is a tropical fruit, and is known to be rich in vitamin C. The fruit is mainly used in juice or vinegar production, and large amounts of byproduct, composed of seed and peel, is generated as agricultural and industrial waste. Our studies on camu-camu seed and peel revealed that the seed contains large amounts of polyphenols (400 mg/g). Further studies on the seed resulted in the isolation of vescalagin and castalagin, C-glycosidic ellagitannins with a hexahydroxydiphenoyl group and a nonahydroxyterphenoyl group attached to the open chain d-glucose, as the main polyphenolic constituents. The structures of these tannins were characterized by their $^1$H- and $^{13}$C-NMR spectra and mass spectrum. The two C-glycosidic ellagitannins represent about 15% of the polyphenolics in the seed of camu-camu fruit, and are likely responsible for the anti-oxidative activity of the seed extract’s anti-oxidative activity.

Keywords: camu-camu, Myrciaria dubia, seed, polyphenol, vescalagin, castalagin, NMR

Introduction

Tropical fruits have been attracting attention from the food industry because of their unique appearance, flavor and nutritional value. Camu-camu (Myrciaria dubia, Myrtaceae) is a tropical fruit, and has recently been used for processed food in advanced countries. Camu-camu is a bushy tree native to the Amazon rainforest, mainly in Peru (Rodrigues et al., 2004). Its fruit is known to be rich in vitamin C, with higher levels reported than those of acerola fruit (Bradfield and Roca, 1964). Camu-camu fruit has a characteristically mild flavor, but its high acid content induces a bitter taste. Therefore, it is mainly consumed as a juice or used as an ingredient in prepared foods (Villachica, 1997; Franco and Jantzetti, 2005). During juice production, byproduct, composed of peel and seed, is generated as agricultural and industrial waste on a large scale. Therefore, utilization of this residue is recommended from both a commercial and ecological point of view.

In a previous paper (Myoda et al., 2010), we reported that the seed extract of camu-camu fruit contains a large amount of polyphenols (400 mg/g), approximately four and ten times that of the residues of acerola and passion fruit juice production, respectively (Oliveira et al., 2009). We also reported that the lipophilic fraction of the seed extract showed antimicrobial activity against Staphylococcus aureus at 1.0 mg/mL. On the other hand, the hydrophilic fraction exhibited strong 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (IC$_{50}$ = 32.2 μg/mL), and the polyphenol content of the fraction was shown to be responsible for the activity. In this paper, we report the polyphenolic constituents in the seed extract of camu-camu fruit and their contributions to the anti-oxidative activity.

Materials and Methods

Materials and chemicals

Dried powder of seeds obtained from camu-camu juice production was obtained from Empresa Agroindustrial del Peru S.A. (Peru). These samples were used after drying at room temperature. Gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and other chemicals were purchased from Kanto Chemicals (Tokyo, Japan). Purified water was obtained using an Elix EV3 system (Millipore, Japan).

$^1$H- and $^{13}$C-NMR spectra were measured with an Agilent MR-400 NMR spectrometer in acetone-$d_6$ or D$_2$O. Chemical shifts were determined by using acetone-$d_6$ (δ$_H$: 2.04 ppm, δ$_C$: 29.8 ppm) as the internal reference. Mass spectra were measured with a JEOL JMS-700 spectrometer in positive FAB mode. Optical rotations were measured on a JASCO P-2100 polarimeter. UV-Vis spectra were measured with a Shimadzu UV-1700 spectrophotometer. Gradient HPLC chromatograms were obtained with a JASCO LC-2000 Plus.

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HPLC system equipped with a MD-2010 Plus photodiode array detector and an Atlantis T3 column (3 μm, 4.6 mm i.d. × 150 mm, Waters, Milford, MA, USA), and the solvent system of acetonitrile - water - formic acid was used. Preparative HPLC was performed with a Shimadzu LC-8A pump, a Hitachi L-4200 detector with a prep cell (2 mm) and an Inertsil ODS-3 column (20 mm i.d. × 250 mm, GL Sciences, Tokyo, Japan). The mobile phase was composed of 10% acetonitrile containing 0.1% of acetic acid. Sephadex LH-20 (GE Healthcare, Sweden) was used for column chromatography.

**Extraction and fractionation** The dried seed samples (400 g) were defatted with n-hexane, and then extracted with 50% aqueous acetone (v/v) three times at room temperature. The combined extracts were concentrated under reduced pressure at 40°C to dryness, and crude extracts (43.9 g) were obtained. The crude extracts (5.0 g) were dissolved in 50% methanol (MeOH, 500 mL) and applied to a Sephadex LH-20 column (5.0 mm i.d. × 300 mm). The column was eluted with a solvent system of H2O - MeOH - acetone, and 8 fractions were obtained: 50% MeOH Fr. (1.60 g), 60% MeOH Fr. (1.30 g), 70% MeOH Fr. (403 mg), 80% MeOH Fr. (268 mg), 90% MeOH Fr. (113 mg), 100% MeOH Fr. (127 mg), 50% acetone Fr. (1.30 g) and 100% acetone Fr. (232 mg) were obtained.

The 70% and 80% MeOH Fr. were dissolved in 10% acetonitrile, and the soluble portions were subjected to preparative HPLC; compound 1 (vescalagin) and 2 (castalagin) were obtained. The crude extracts (5.0 g) were dissolved in 50% aqueous acetone (v/v) three times at room temperature. After addition of the reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. After addition of 1 N NaOH (500 μL), absorbance at 750 nm was measured. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of sample. The assays were carried out in triplicate.

**Compound 1 (vescalagin)** A light brown amorphous powder. [α]D = −80° (c = 0.14, MeOH), HR-MS; m/z = 935.0792 [M+H]+ (calcd. for C41H27O26, 935.0780). 1H-NMR (400 MHz, acetone-d6+D2O): δ 4.82 (1H, d, J = 2.1 Hz, H-1), 5.19 (1H, dd, J = 1.3, 2.1 Hz, H-2), 4.47 (1H, dd, J = 1.3, 7.0 Hz, H-3), 5.10 (1H, t, J = 7.0 Hz, H-4), 5.54 (1H, dd, J = 2.4, 7.0 Hz, H-5), 4.94 (1H, dd, J = 2.4, 12.4 Hz, H-6), 4.00 (1H, d, J = 12.4 Hz, H-6’), 6.55 (1H, s), 6.70 (1H, s), 6.71 (1H, s). 13C-NMR (100 MHz, acetone-d6+D2O): δ 66.7 (C-1), 73.9 (C-2), 66.1 (C-3), 69.0 (C-4), 71.0 (C-5), 65.2 (C-6), 107.3, 108.5, 112.5, 114.1, 114.7, 115.7, 115.8, 121.6, 124.6, 124.7, 126.1, 127.1, 134.7, 135.7, 136.2, 136.5, 137.8, 143.4, 143.9, 144.2, 144.3, 144.5, 144.9, 145.0, 145.2, 146.4, 164.9, 165.5, 166.6, 167.2, 169.2, 175.1.

**Compound 2 (castalagin)** A light brown amorphous powder. [α]D = −136° (c = 0.05, MeOH), HR-MS; m/z = 935.0792 [M+H]+ (calcd. for C41H27O26, 935.0780). 1H-NMR (400 MHz, acetone-d6+D2O): δ 4.82 (1H, d, J = 2.6, 12.5 Hz, H-1), 4.95 (1H, dd, J = 1.1, 4.6 Hz, H-2), 4.91 (1H, dd, J = 1.1, 7.0 Hz, H-3), 5.11 (1H, t, J = 7.0 Hz, H-4), 5.50 (1H, dd, J = 2.6, 7.0 Hz, H-5), 4.97 (1H, dd, J = 2.6, 12.5 Hz, H-6), 3.99 (1H, d, J = 12.4 Hz, H-6’), 6.57 (1H, s), 6.71 (1H, s).
means ± standard error of triplicate assays. The data were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test for comparison of crude extract and each fraction, with p value < 0.01 indicating significance.

Results and Discussion

The IC50 values of DPPH radical scavenging activity of the crude extract and 8 fractions obtained by Sephadex LH-20 column chromatography are shown in Table 1. The IC50 value of the crude extract was 29.2 ± 0.2 µg/mL, and the activity was about one third of that for gallic acid. Among the fractions, the 80% MeOH Fr. showed the highest activity (19.4 ± 0.0 µg/mL) and the 50% MeOH Fr. showed the lowest (155.5 ± 0.2 µg/mL). The fractions obtained after elution with 70% MeOH exhibited significantly higher (p < 0.01) activity than the crude extract. Therefore, many anti-oxidative compounds are likely contained in the seed of camu-camu fruit. The polyphenol contents are shown in Table 1; the 80% MeOH Fr. showed the highest polyphenol content (740 ± 7.4 mg/g). The polyphenol content in the 70% MeOH Fr. exceeded 500 mg/g, significantly higher than the crude extract, and the polyphenol content was closely correlated with DPPH radical scavenging activity.

The HPLC chromatograms of the crude extract, 70% MeOH Fr., 80% MeOH Fr. and 90% MeOH Fr. showed a number of peaks were detected at 280 nm (Fig. 1). In the chromatogram of the crude extract, gallic acid and ellagic acid were identified by comparing their retention times with those of authentic samples. The chromatogram of the most active fraction (80% MeOH Fr.) showed a primary single peak of compound 2, and that of the 70% MeOH Fr. showed two main peaks of compounds 1 and 2. Compound 2 was observed in the chromatogram of 90% MeOH Fr. As no other large peaks were observed in the chromatograms of the other fractions, the constituents in 70% MeOH Fr. and 80% MeOH Fr. were determined to be the main polyphenols of the seed extract of camu-camu fruit. Therefore, these two fractions were further fractionated by preparative HPLC, and compounds 1 and 2 were obtained.

In the 1H-NMR spectrum of compound 1, three aromatic protons were observed, along with 7 protons assignable to hexose. Methylation with diazomethane and dimethyl sulfate/K2CO3 indicated that compound 1 had 15 phenolic hydroxyl groups. These results indicated the presence of hexahydroxydiphenoyl (HHDP) and nonahydroxyterphenoyl (NHTP) groups. The 1H- and 13C-NMR spectra showed no signal assignable to esterified anomeric proton and carbon; accordingly, the presence of C-glycoside linkage was implied. Therefore, the structure of compound 1 was presumed to be a C-glycosidic ellagitannin with a HHDP group and a NHTP group, which esterized with an open chain hexose with C-glycoside linkage at anomeric position. Compound 2 showed closely related 1H- and 13C-MNR data with those of compound 1, and was presumed to be structurally related to compound 1. In a comparison of their NMR and other spectral data with the literature (Waltter and Johannes, 1969; Nonaka et al., 1987, 1990; Chang and Shen, 2012), compounds 1 and 2 were characterized as vescalagin and castalagin, respectively, C-glycosidic ellagitannins with HHDP and NHTP groups attached to the open chain d-glucose at C-2-C-6 position by ester linkage, and C-1 position of glucose is attached directly to NHTP by C-glycosidic linkage.

Chirinos et al. (2010) isolated catechins, ellagic acid derivatives, anthocyanins, flavonols and flavanones from a mixture of juice and peel of camu-camu fruit. We also identified gallic acid and ellagic acid in the seed extract, and ellagic acid is likely derived from vescalagin or castalagin and

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH radical scavenging activity (IC50, µg/mL)</th>
<th>Polyphenol content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>29.2 ± 0.2</td>
<td>404.6 ± 0.8</td>
</tr>
<tr>
<td>50% MeOH Fr.</td>
<td>155.5 ± 0.2 **</td>
<td>122.5 ± 1.8”</td>
</tr>
<tr>
<td>60% MeOH Fr.</td>
<td>47.0 ± 0.6 **</td>
<td>349.6 ± 6.9”</td>
</tr>
<tr>
<td>70% MeOH Fr.</td>
<td>27.0 ± 0.4 **</td>
<td>554.8 ± 8.2”</td>
</tr>
<tr>
<td>80% MeOH Fr.</td>
<td>19.4 ± 0.0 **</td>
<td>740.0 ± 7.6”</td>
</tr>
<tr>
<td>90% MeOH Fr.</td>
<td>20.4 ± 0.3 **</td>
<td>578.2 ± 9.4”</td>
</tr>
<tr>
<td>100% MeOH Fr.</td>
<td>24.3 ± 0.1 **</td>
<td>552.4 ± 3.1”</td>
</tr>
<tr>
<td>50% acetone Fr.</td>
<td>23.3 ± 0.1 **</td>
<td>559.5 ± 4.3”</td>
</tr>
<tr>
<td>100% acetone Fr.</td>
<td>24.1 ± 0.2 **</td>
<td>623.5 ± 5.8”</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>10.1 ± 0.2</td>
<td>–</td>
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</table>

n = 3, mean ± SEM. **: p < 0.01, crude extract vs. each fraction.
Fig. 1. HPLC chromatograms of the crude seed extract of camu-camu fruit and fractions obtained by Sephadex LH-20 column chromatography.

Detection: PDA detector (280 nm)
Gradient condition: 5% acetonitrile to 100% acetonitrile containing 0.2% HCOOH
A: crude extract, B: 70% MeOH Fr., C: 80% MeOH Fr., D: 90% MeOH Fr.
other related tannins in the extract. Our results demonstrated that the seed of camu-camu contains high molecular weight C-glycosidic ellagitannins, and further studies may reveal the presence of more tannins related to vescalagin and castalagin.

The contents of vescalagin and castalagin in the crude seed extract were analyzed by HPLC, and were determined as 1.97% and 4.19%, respectively; the sum of these contents was about 15% of the total polyphenol content. From the yield of crude extract, the contents of vescalagin and castalagin in the seed were calculated as 0.22% and 0.46%, respectively (Table 2). In the chromatograms, gallic acid and ellagic acid were identified along with other tannins and related compounds. Characterization of these compounds is currently ongoing.

As shown in Table 3, the IC50 values of DPPH radical scavenging activity of vescalagin and castalagin were 20.89 ± 0.18 μg/mL and 19.44 ± 0.40 μg/mL, respectively, and these values were almost the same as that of the 80% MeOH Fr., and about two times of that of gallic acid. The polyphenol content in the seed extract of camu-camu was determined as 400 mg/g EAG; the sum of vescalagin and castalagin was 61.56 mg/g. Therefore, these two compounds are the likely main polyphenolic constituents in the seed of camu-camu fruit.

Vescalagin and castalagin have been isolated from several plants belonging to Fagaceae, Combretaceae, Lythraceae and Myrtaceae (Quideau et al., 2004). Camu-camu belongs to the family Myrtaceae; however, this is the first report of the presence of vescalagin and castalagin in camu-camu. In addition to its anti-oxidative activities, C-glycosidic ellagitannins has been reported to have anti-microbial (Taguri et al., 2004) and anti-herpes (Quideau et al., 2004) activities. Therefore, the seed of camu-camu fruit is a potential functional resource for the food industry.

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References


Fig. 2. Structures of vescalagin and castalagin.

Table 2. Contents of vescalagin and castalagin in the crude extract and dried seed of camu-camu.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crude extract (mg/g)</th>
<th>Dried seed (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vescalagin</td>
<td>19.65 (1.97%)</td>
<td>2.16 (0.22%)</td>
</tr>
<tr>
<td>Castalagin</td>
<td>41.91 (4.19%)</td>
<td>4.60 (0.46%)</td>
</tr>
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</table>

Table 3. DPPH radical scavenging activities of vescalagin and castalagin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH radical scavenging activity (IC50; µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% MeOH Fr.</td>
<td>20.5 ± 0.4</td>
</tr>
<tr>
<td>Vescalagin</td>
<td>20.9 ± 0.2</td>
</tr>
<tr>
<td>Castalagin</td>
<td>19.4 ± 0.4</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>10.1 ± 0.2</td>
</tr>
</tbody>
</table>

n = 3, mean ± SEM.


