Anthocyanins from the Red Flowers of Camellia saluenensis Stapf ex Bean

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Seven anthocyanins, cyanidin 3,5-di-O-β-glucopyranoside, cyanidin 3-O-(6-O-(Z)-p-coumaroyl-β-glucopyranoside)-5-O-β-glucopyranoside, cyanidin 3-O-(6-O-(E)-p-coumaroyl-β-glucopyranoside)-5-O-β-glucopyranoside, cyanidin 3-O-β-glucopyranoside, cyanidin 3-O-(6-O-(Z)-p-coumaroyl-β-glucopyranoside), cyanidin 3-O-(6-O-(E)-p-coumaroyl)-β-glucopyranoside, and cyanidin 3-O-β-galactopyranoside were isolated from the red flowers of Camellia saluenensis. Out of seven anthocyanins, cyanidin 3-O-(6-O-(Z)-p-coumaroyl-β-glucopyranoside)-5-O-β-glucopyranoside was found to be isolated from the red flowers of genus Camellia for the first time. The floral distribution of anthocyanin composition was also determined by HPLC analysis between C. saluenensis and C. pitardii var. pitardii.

Key Words: acylated anthocyanin, Camellia pitardii var. pitardii, Camellia saluenensis, Nuijiang Shan-Cha, Saruwin Tsubaki.

Introduction

Camellia saluenensis Stapf ex Bean (Theaceae) is widely and commonly distributed at 1000–3200 m elevation in central and western Yunnan Province, in southern Sichuan Province, and in western Guizhou Province, China. The species is also known by its Chinese name, Nuijiang Shan-Cha (Feng et al., 1986) and Japanese name, Saruwin Tsubaki. The plant is a shrub, 1–5 m high, young twigs pubescent but glabrous with aging. Leaves are oblong-elliptic to elliptic, 3–3.5 cm by 1–2.3 cm width, apices are obtuse to acute, glabrous beneath but pubescent on midrib. Flowers are small, axillary or subterminal, solitary or paired, diameter ca. 4–5 cm. It often has a striking color variation from white to rose. The blooming period is from December to February (Chang, 1981). This species is resistant to dry and cold conditions, sturdy and regenerates from stumps after deforestation. C. saluenensis crosses easily with other Camellia species, particularly C. japonica, and many of these hybrids are sturdy and distinguished by having greater cold tolerance, faded blooms falling off unaided, and flowers in great profusion (Gao et al., 2005). Thus, C. saluenensis has been considered to be an important species to produce new and very different hybrids, and it is also of great importance to clarify the structure of anthocyanins in flowers of C. saluenensis.

For Camellia genus, Hayashi and Abe (1953) first reported cyanidin 3-O-β-glucoside in C. japonica. Sakata et al. (1986) reported cyanidin 3-galactoside, and Saito et al. (1987) reported cyanidin 3-O-(6-O-(E)-p-coumaroyl)-β-glucoside in C. japonica subsp. rusticana. Recently, Terahara et al. (2001) identified the existence of delphinidin glycosides, delphinidin 3-O-β-glucoside, delphinidin 3-O-β-galactoside, and delphinidin 3-O-(6-O-(E)-p-coumaroyl)-β-galactoside in the leaves of Benibana-cha (C. sinensis). Sakata (2004) reported the existence of two known pigments, cyanidin 3,5-di-O-β-glucopyranoside and cyanidin 3-O-(6-O-(E)-p-coumaroyl-β-glucopyranoside)-5-O-β-glucopyranoside in C. saluenensis, but the existence of these two pigments has not yet been strictly confirmed in this species. Despite these efforts, the anthocyanin composition is still unclear and to discuss the chemical taxonomy of C. saluenensis, we need to know the precise anthocyanin composition in flowers of the genus Camellia.

Now we are focusing on the isolation and structure determination of seven anthocyanins from the crude acetic-methanolic extract of the flowers of C. saluenensis by using open column chromatography (CC), and their structures were determined on the basis of spectroscopic analyses, i.e., 1H-nuclear magnetic resonance (1H-NMR), 13C-NMR, field-gradient heteronuclear multiple quantum correlation (FG-HMQC), heteronuclear multiple bond correlation (HMBC), electro spray ionization mass (ESI-MS), and ultraviolet and visible (UV-vis). Out of seven anthocyanins, cyanidin 3-O-(6-O-(Z)-p-coumaroyl-β-
glucopyranoside)-5-O-β-glucopyranoside was found for the first time in the *Camellia* genus. In addition, the determination of anthocyanin distribution on the red flowers of *C. saluenensis* and *C. pitardii* var. *pitardii* was also carried out by HPLC analysis. The result indicated that these two species had similar anthocyanin composition, excluding cyanidin 3-O-(6-O-((Z)-p-coumaroyl))-β-glucopyranoside.

**Materials and Methods**

*General procedures*

$^1$H- and $^{13}$C-NMR spectra were recorded with a NMR spectrometer (JNM-ECA600, JEOL, Japan) and chemical shifts are expressed on a $\delta$ (ppm) scale with tetramethylsilane (TMS) as an internal standard (Tables 1 and 2). Solvents for NMR measurements were used with a combination of methanol-$d_4$ and trifluoroacetic acid-$d$ in a ratio of 9:1. To check the purity of each anthocyanin, an HPLC system (JASCO GULLIVER SERIES, Japan) was used at 525 nm with a TSK gel ODS-80Ts QA column (4.5 mm i.d. × 150 mm, Tosoh, Japan) at 40°C. The flow rate was 0.8 mL·min$^{-1}$ with a linear flow gradient elution for 35 min and solution B (1.5% H$_3$PO$_4$-20% HCOOH-15% CH$_3$CN-10% tetrahydrofuran (THF) in H$_2$O, v/v) increased linearly from 18% to 70% in solvent A (1.5% H$_3$PO$_4$ in H$_2$O, v/v). It should be noted that anthocyanins were dramatically and clearly separated when including 10% THF in solution B. TLC was performed on precoated Kiesel gel 60 F$_{254}$ (Merck) with a benzene-ethyl formate-formic acid-water system as described in the former report (Hashimoto et al., 2002). UV-vis spectra were recorded on MPS-2000 spectrophotometer (Shimadzu Co., Ltd, Japan) in 0.01% HCl-MeOH. ESI-MS was measured on MAT900XL (Finnigan Inc., Japan) in 10% AcOH-MeOH without matrix in a positive mode.

*Extraction and isolation*

Fresh petals of the wild *Camellia saluenensis* were collected continuously in Dali and Fuyuan Counties, Yunnan Province, China, as well as from the Experimental Farm, Kagoshima University, in January 2002–2006. The petals were dipped into boiling water (for about 4–6 s) to kill polyphenol enzymes, and were dried at room temperature (Sakata, 1988). The dried petals (ca. 3.5 kg) of *C. saluenensis* were immersed in 50% AcOH-MeOH overnight (ca. 48 h) and the extracted solution was filtered. The extraction was repeated twice. The extracts contained seven anthocyanins and their retention time on the HPLC chromatogram was as follows: pigment 1, 10.2 (min); pigment 7, 12.3; pigment 4, 13.5; pigment 2, 22.1; pigment 3, 26.8; pigment 5, 28.6; and pigment 6, 32.1 (Fig. 1). The crude extract was subjected to MCI gel CHP 20P CC with 5% AcOH-MeOH overnight (ca. 48 h) and the extracted solution was filtered. The extraction was repeated twice. The crude extract was subjected to Sephadex LH-20 CC and ODS-gel CC repeatedly, to furnish seven pigments: 1, 17 mg; 2, 17 mg; 3, 32 mg; 4, 27 mg; 5, 11 mg; 6, 26 mg; 7, 12 mg.

Extraction for HPLC analysis was as described in a previous report (Hashimoto et al., 2002). Briefly, anthocyanins in dried flowers (ca. 1 g) were extracted with 5 mL of a modified acetic acid methanol-solution (MeOH-H$_2$O-HCOOH-CF$_3$COOH, 70:27:2:1, v/v/v%,), which gives good separation. For HPLC analysis, the petals of *C. saluenensis* and *C. pitardii* var. *pitardii* were collected in Dali and Chuxiong Cities, respectively, Yunnan Province, China, in February, 2002. It should be noted that *C. saluenensis*, and *C. pitardii* var. *pitardii* were authenticated and certified by Prof. Li-Fang Xia of Kunming Institute of Botany at the Chinese Academy of Sciences, China, and thus the two wild species presented in this paper were confirmed to be typical phenotypes.

*Acid hydrolysis*

Each anthocyanin (ca. 2 mg) was dissolved in 2N HCl (ca. 2 mL) and the mixture was heated at 95°C for 2 h. The acid hydrolyzed mixture was directly compared by TLC with authentic sugars (glucose, galactose, mannose, rhamnose, xylose, fructose, fucose, and arabinose). The following solvent systems for TLC were used for distinguishing sugars; n-BuOH-AcOH-H$_2$O (2 : 1 : 1), and CH$_3$Cl-MeOH-H$_2$O (7 : 3 : 0.5), and sugars were detected by spraying with 5% H$_2$SO$_4$, followed by heating. Acid hydrolysis of pigments 1–6 gave cyanidin and glucose, and 7 gave cyanidin and galactose.

*ESI-MS and UV-vis data*

**Pigment 1**

ESI-MS $m/z$ 611.39 [$M^+$] (Calcd for C$_{37}$H$_{31}$O$_{16}$ 611.43). UV-vis $\lambda_{max}$ 313 (2.82), 452 (2.64), 570 (3.44). $E_{acryl}/E_{vis, max}$ = $E_{331}/E_{526}$ = 0.103; $E_{440}/E_{vis, max}$ = 0.125.

**Fig. 1.** Structure of anthocyanins isolated from the red flowers of *Camellia saluenensis*. 20 CC and ODS-gel CC repeatedly, to furnish seven pigments: 1, 17 mg; 2, 17 mg; 3, 32 mg; 4, 27 mg; 5, 11 mg; 6, 26 mg; 7, 12 mg.

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**Fig. 1.** Structure of anthocyanins isolated from the red flowers of *Camellia saluenensis*.
Pigment 2
ESI-MS $m/z$ 757.44 [M]$^+$ (Calcd for C$_{36}$H$_{37}$O$_{18}$ 757.55). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 281 (4.25), 294 (4.22), 530 (4.34); AlCl$_3$-MeOH: 281 (4.19), 312 (4.21), 421 (3.68), 571 (4.37). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{294/530} = 0.603$; $E_{440/530}^\text{vis, max} = 0.137$.

Pigment 3
ESI-MS $m/z$ 757.44 [M]$^+$ (Calcd for C$_{36}$H$_{37}$O$_{18}$ 757.55). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 284 (4.58), 314 (4.46), 530 (4.59); AlCl$_3$-MeOH: 285 (4.51), 313 (4.54), 570 (4.67). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{314/530} = 0.638$; $E_{440/530}^\text{vis, max} = 0.119$.

Pigment 4
ESI-MS $m/z$ 449.21 [M]$^+$ (Calcd for C$_{21}$H$_{20}$O$_{11}$ 449.31). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 283 (4.08), 332 (3.64), 530 (4.19); AlCl$_3$-MeOH: 288 (3.99), 313 (3.94), 571 (4.28). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{332/530} = 0.287$; $E_{440/530}^\text{vis, max} = 0.221$.

Pigment 5
ESI-MS $m/z$ 595.19 [M]$^+$ (Calcd for C$_{30}$H$_{27}$O$_{13}$ 595.44). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 284 (4.33), 311 (4.17), 375sh (3.58), 530 (4.35); AlCl$_3$-MeOH: 289 (4.26), 311 (4.27), 411 (3.70), 573 (4.44). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{311/530} = 0.669$; $E_{440/530}^\text{vis, max} = 0.225$.

Pigment 6
ESI-MS $m/z$ 595.14 [M]$^+$ (Calcd for C$_{30}$H$_{27}$O$_{13}$ 595.44). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 284 (4.51), 314 (4.39), 530 (4.52); AlCl$_3$-MeOH: 289 (4.45), 313 (4.47), 570 (4.60). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{313/530} = 0.795$; $E_{440/530}^\text{vis, max} = 0.218$.

Pigment 7
ESI-MS $m/z$ 449.23 [M]$^+$ (Calcd for C$_{21}$H$_{20}$O$_{11}$ 449.31). UV-vis $\lambda_{\text{max}}$ 0.01% HCl-MeOH (nm) (log $\varepsilon$): 282 (4.54), 331 (3.97), 529 (4.74); AlCl$_3$-MeOH: 272 (4.43), 312 (4.24), 571 (4.85). $E_{\text{acryl/MeOH}}^\text{vis, max} = E_{331/529} = 0.171$; $E_{440/530}^\text{vis, max} = 0.212$.

Results and discussion
HPLC analysis revealed that the composition of anthocyanins in C. saluenensis flower had seven major peaks, including minor peaks. Among them, the seven major anthocyanin pigments (1–7) were isolated as reddish amorphous powders of acetic acid salts (Fig. 1). In order to determine the detailed chemical structures of

Table 1. $^1$H-NMR data for anthocyanins 1–7 (6 ppm) & $J$ (Hz), in acidified CD$_3$OD).

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<thead>
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<th>Pigments</th>
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<td>(Z)-p-coumaroyl</td>
<td>(E)-p-coumaroyl</td>
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<td>7.32 d (15.8)</td>
<td>6.58 (d, 12.8)</td>
<td>7.42 (d, 15.8)</td>
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<td>$\beta$</td>
<td>5.76 (d, 13.0)</td>
<td>6.22 d (15.8)</td>
<td>5.73 d (12.8)</td>
<td>6.21 (d, 15.8)</td>
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<td>$\gamma$</td>
<td>5.69 (d, 13.0)</td>
<td>6.20 d (15.8)</td>
<td>5.69 (d, 12.8)</td>
<td>6.21 (d, 15.8)</td>
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$s$ = singlet, $d$ = doublet, $dd$ = doublet, $t$ = triplet, $br$ = broad.
these anthocyanins, 1H-NMR (Table 1), 13C-NMR (Table 2), ESI-MS, and acid hydrolysis were performed. In NMR measurements, long-range correlation spectra such as the 2D FG-HMQC spectrum and HMBC spectrum were measured to investigate linkages among the aglycone, sugar and acyl units (data not shown). The structures of the seven isolated anthocyanins are as follows: cyanidin 3,5-di-O-β-glucopyranoside (1), cyanidin 3-O-(6-O-(Z)-p-coumaroyl)-β-glucopyranoside)-5-O-β-glucopyranoside (3), cyanidin 3-O-β-glucopyranoside (4), cyanidin 3-O-(6-O-(Z)-p-coumaroyl)-β-glucopyranoside (5), cyanidin 3-O-(6-O-(E)-p-coumaroyl)-β-glucopyranoside (6), and cyanidin 3-O-β-galactopyranoside (7) (Fig. 1).

Pigment 1 was directly compared with the data of cyanidin 3,5-di-O-β-glucopyranoside isolated from the red petals of rose. Pigment 3 was found to be shisonin, which was first isolated from Perilla leaves (Kuroda et al., 1935), and was also reported by Takeda et al. (1964). Pigment 2 was also from Perilla leaves and reported by Yoshida et al. (1990). Pigments 4 and 6 were first reported by Saito et al. (1987). Pigment 5 was identified as cyanidin 3-O-(6-O-(Z)-p-coumaroyl)-β-glucopyranoside by Boido et al. (2006), but the spectral data were not presented briefly. Pigment 7 was from direct comparison with the authentic sample (Sakata and Arisumi, 1986), and was also isolated by Terahara et al. (2000). From the genus Camellia, pigment 2 was identified for the first time, and the detailed chemical structure is presented herein in red flowers of C. saluenensis.

For the Camellia genus, Hayashi and Abe (1953) first reported cyanidin 3-O-β-glucoside (4) in C. japonica. Sakata et al. (1981) reported the constitution of anthocyanins in cultivars of Camellia japonica, C. japonica subsp. rusticana, C. sasanqua, C. hiemalis, C. vernalis and C. wabisuke by TLC analysis where cyanidin 3-glucoside (spot 1) and two major pigments dominated the diversity of these species. The major spot 1 was detected as a mixture of cyanidin 3-glucoside (4) and cyanidin 3-galactoside (7) in C. japonica subsp. rusticana (Sakata et al., 1986), but Saito et al. (1987) succeeded in isolating cyanidin 3-galactoside (7) and cyanidin 3-O(6-O-(E)-p-coumaroyl)-β-glucoside (6). The anthocyanins of wild Camellia species, i.e., C. japonica ssp. rusticana, C. japonica ssp. hozanensis, C. japonica f. macrocarpa were also reported and the presence of 3-glucoside (4) and 3-galactoside (7) was briefly described (Sakata et al., 1987). The constitution of anthocyanins was also discussed in C. reticulata, C. saluenensis, C. pitardii, C. hongkongensis, C. rosaeflora and C. maliflora by two-dimensional TLC analysis; however, the detailed chemical structures of these pigments have not been reported yet but the existence of delphinidin derivatives in C. hongkongensis have been determined (Sakata and Arisumi, 1985). The existence of cyanidin 3-glucoside (4), 3-galactoside (7) and 3-p-coumaroylglucoside was discussed in flowers of C. semiserrata and C. chekiangoleosa together with the other twelve unknown pigments (Sakata and Arisumi, 1992). The 3,5-diglucoside-series and 3-diglucoside-series of cyanidin were also presumed to be present in flowers of C. saluenensis and C. reticulata, respectively.

Recently, Terahara et al. (2001) identified the existence of delphinidin glycosides, delphinidin 3-O-β-glucoside, delphinidin 3-O-β-galactoside and delphinidin 3-O(6-O-(E)-p-coumaroyl)-β-galactoside in the leaves of Benibana-cha.

Table 2. 13C-NMR data for anthocyanins 1–7 (δ ppm), in acidified CD3OD.

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</table>

(ZC): (Z)-p-coumaroyl; (E)C: (E)-p-coumaroyl.
(C. sinensis). Sakata (2004) also issued the existence of the two known pigments, cyanidin 3,5-di-O-β-glucopyranoside (1) and cyanidin 3-O-(6-O-(E)-p-coumaroyl-β-glucopyranosido)-5-O-β-glucopyranoside (3) in C. saluenensis, but the existence of the two pigments has not yet been strictly confirmed in this species. In order to discuss the chemical taxonomy of C. saluenensis, we obtained precise information about the anthocyanin composition in the flowers of C. saluenensis and C. pitardii var. pitardii based on HPLC analysis.

The content of pigments 1–7 and their quantities were compared in HPLC chromatograms between the flowers of C. saluenensis and C. pitardii var. pitardii (Table 3). The quantities of pigments 1, 7, and 3 (retention time order) of C. saluenensis and C. pitardii var. pitardii were 137 and 166, 33 and 58, and 346 and 337 (nmol·g⁻¹ dried petal), respectively, which were quite similar. However, the quantity of pigment 4 of C. pitardii var. pitardii were 1204 (nmol·g⁻¹ dried petal), which was over four times that of C. saluenensis (268 nmol·g⁻¹ dried petal), and pigment 5 was not found in the flowers of C. pitardii var. pitardii (Table 3).

We believe that our findings could be the basis for future classification of Camellia species and could be utilized for the analysis of many Camellia species and cultivars based on floral anthocyanin composition.

### Acknowledgements

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### Literature Cited


<table>
<thead>
<tr>
<th>Species</th>
<th>Value (nmol·g⁻¹ dried petal)</th>
<th>Anthocyanins</th>
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<td>C. saluenensis</td>
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<tr>
<td>C. pitardii var. pitardii</td>
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