Characterization of UV-Sensitive Marker Constituents of Polygala Root for TLC: Applications in Quality Control of Single Crude Drug Extract Preparations

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Polygala Root (the root of Polygala tenuifolia Willd. [Japanese name “Onji”], a well-known crude drug, traditionally used as an expectorant and sedative, has been attracting increased interest in recent years owing to its newly found pharmacological effect related to neuroprotection. However, there is no specific method for identifying and estimating the quality of this crude drug in the Japanese Pharmacopoeia, 17th edition. Therefore, in order to develop a TLC-based simple and convenient identification method using characteristic chemical marker(s) for the drug and its extract products, UV-sensitive constituents of Polygala Root were first investigated. A total of 23 aromatic compounds were isolated and characterized. Two new compounds, namely, polygalaonjisides A (1) and B (2), were characterized as syringic acid 4-O-(2’-O-β-D-apiosylo)-β-D-glucoside and 2-O-(β-D-glucosyl)-3’-O-benzoylsucrose, respectively. Based on these physicochemical results, a TLC method focusing on three marker spots with RF value of approximately 0.4–0.5 due to tenuifolides A and B and 3,6-di-O-sinapoylsucrose was proposed as a simple and convenient test to identify Polygala Root or its single-extract products on the market. The data presented in this paper could be useful in stipulating a confirmation test to identify Polygala Root.

Key words Polygala Root; Polygala tenuifolia; TLC; aromatic compound; polygalaonjiside A; polygalaonjiside B

Polygala Root (Japanese name: “Onji”) is described as the root or the root bark of Polygala tenuifolia Willd. (Polygalaceae) in the 17th edition of the Japanese Pharmacopoeia (JP17).1) It is an important crude drug commonly incorporated in Kampo prescriptions such as Kamikihito, Ninjinyoeito, and Kamiuntanto, which are used as an anti-neurosis agent, tonic, and sedative agent, respectively. Polygala Root has also been reported as the main constituents of Polygala Root in addition to abundant saponins. Polygala Root is listed as Polygala Radix in the Chinese Pharmacopoeia, and the standard values of three of its major components—tenuifolin, 3,6-di-O-sinapoylsucrose, and polygalaxanthone III—have also been stipulated based on HPLC quantification.38)

The aims of this study were to characterize UV-sensitive constituents of Polygala Root and establish a simple, convenient, and reliable TLC-based quality control test applicable to Polygala Root or its extract. Here, we report on the isolation and characterization of water-soluble aromatic constituents, including two newly found compounds from Polygala Root, and proposal a TLC-based simple identification test focusing on the marker compounds.

Results and Discussion

Characterization of UV-Sensitive Components of Polygala Root

A homogenate of Polygala Root in methanol (MeOH) was concentrated and defatted by extraction with n-hexane. The residual water extract was subjected to suc-
cessive chromatographic profiles on Diaion HP-20, YMC GEL ODS-AQ, and Chromatex ODS with aqueous MeOH and preparative TLC to afford 23 compounds including two new glycosides designated as polygalaonjisides A (1) and B (2). The known compounds were identified as 4-hydroxybenzoic acid (3),\(^{1}\) monomericophenol A (4),\(^{20}\) 4-hydroxy-3-methoxybenzoic acid (vanillic acid) (5),\(^{19}\) hesmeylanumoid (6),\(^{21}\) sibirioside A3 (7),\(^{22}\) 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) (8),\(^{23}\) sibiricose A5 (9),\(^{24}\) sibiricose A6 (10),\(^{25}\) trans-ferulic acid (11),\(^{22}\) sibiricose A1 (12),\(^{22}\) glomeratose A (13),\(^{24}\) sibiricoxanthone B (14),\(^{11}\) tenuifoliside B (15),\(^{11}\) polygalaxanthone XI (16),\(^{11}\) polygalaxanthone III (17),\(^{11}\) 3,6'-di-O-sinapoylsucrose (18),\(^{11}\) tenuifoliside A (19),\(^{11}\) 1'-cinnamoyl-3'-benzoyl-(2-O-β-glucosyl)-sucrose (20),\(^{11}\) 6'-4''-(4''''-trimethoxycinnamoyl)sucrose (21),\(^{11}\) 1,3,7-trihydroxysucrose (22),\(^{25}\) and tenuifoliol A (23)\(^{15}\) (Fig. 1). The known compounds, 3, 6, 8, and 11, as well as the novel compounds 1 and 2, were obtained from Polygala Root in this study.

Polygalaonjiside A (1) was isolated as a light brown amorphous powder. Its molecular formula was determined to be C\(_{25}\)H\(_{36}\)O\(_{17}\) based on its HR-ESI-MS data ([m/z] 631.1832 [M+Na]+; Calcd for C\(_{25}\)H\(_{36}\)O\(_{17}\)+Na: 631.1845) and \(^{13}\)C-NMR spectrum (25 \(^{13}\)C signals). The UV absorption bands at 231 and 279sh nm suggested that it is an aromatic compound. The \(^{1}H\)-NMR spectrum of 2 exhibited signals characteristics of mono-substituted benzene [δ 8.10 (2H, dd, J = 1.0, 8.0Hz), δ 7.50 (2H, t, J = 8.0Hz), and δ 7.61 (1H, tt, J = 1.0, 1.0, 8.0, 8.0Hz)] and three sets of sugar proton signals. The \(^{13}\)C-NMR spectrum showed six aromatic carbon signals and one ester carbonyl carbon signal [δ 131.1, 131.0 (2C), 129.7 (2C), 134.5, 167.3] assignable to a benzoyl unit, which was confirmed by acid hydrolysis followed by HPLC showing the production of benzoic acid. The sugar proton and carbon signals in the NMR spectra were assigned by \(^{1}H–^{1}H\) COSY, HSQC, and HMBC experiments as shown in Table 1. These spectral data implied close similarity to those of 2-O-β-glucosylsucrose.\(^{16,30,31}\) The HMBC spectrum showed long range correlations among H-1' (δ 5.65)/C-2' (δ 105.1), H-1'' (δ 4.41)/C-2 (δ 82.1) and H-3' (δ 5.66)/C-7'' (δ 167.3) (Fig. 2). Based on these spectral data, polygalaonjiside B (2) was established to be 2-O-(β-D-glucosyl)-3'/O-benzoylsucrose.

**TLC-Based Evaluation of Polygala Root**

Examination of various TLC conditions to detect the UV-sensitive constituents of Polygala Root revealed the following conditions to be suitable to test the quality of this drug. As for the developing solvent on silica gel TLC, a mixture of ethyl acetate (EtOAc), MeOH and water (6:1:1) was found to provide well-separated spots that included three clear spots (I–III). These three spots clearly distinguished Polygala Root from a closely related crude drug, Senega (root of Polygala senega), which shows a quite different high performance (HP) TLC pattern under the same conditions as shown in Fig. 3. Furthermore, HPTLC comparison of nine samples (Table 2) of Polygala Root available on the Japanese market indicated that these three spots were characteristic of Polygala Root, and suggested to be useful as markers to estimate of their identity and quality (Fig. 4).

The compounds corresponding to the three marker spots obtained from TLC analysis were identified as (spot I) tenuifoliside A (19), (spot II) tenuifoliside B (15), and (spot III) 3,6'-di-O-sinapoylsucrose (18), by comparing the respective spots of the isolated compounds (Fig. 3). Tenuifolisides A (19) and B (15) were first isolated from Polygala Root,\(^{14}\) whereas 3,6'-di-O-sinapoylsucrose (18) is a previously known characteristic ingredient of this drug.\(^{32}\) These three compounds have also been reported as marker components in the multi-component analysis of Polygala Root by ultra-performance liquid chromatography (UPLC) and LC/MS analysis,\(^{32,33}\) further supporting the validity that they are characteristic components of Polygala Root. These compounds have been reported to exhibit neuroprotective, cognitive improving, and cerebroprotective effects.\(^{34–37}\) 3,6'-Di-O-sinapoylsucrose (18) has also been reported to synergistically interact with 19.\(^{38}\) In addition, sinapic acid and 3,4.5-trimethoxycinnamic acid, which are aglycones of these compounds, have been reported to possess cerebroprotective and cognition-improving effects,\(^{39,41}\) implying these compounds to be prodrugs of the active aglycones. The single crude drug extract product of Polygala Root has been prescribed for mitigating the problem.
Fig. 1. Structures of Compounds 1–23
Optical rotations were measured using a JASCO P-1020 digital polarimeter (JASCO Corporation, Tokyo, Japan). The UV spectra were recorded using a Shimadzu UVmini-1240 (Shimadzu Corporation, Kyoto, Japan) and a JASCO V-530 (JASCO Corporation). ESI and HR-ESI-MS spectra were recorded using a microOTOF-Q (Bruker Daltonics, Billerica, MA, U.S.A.) mass spectrometer with acetonitrile as the reagent, but the present method, which uses only UV irradiation, required to be as simple, inexpensive, and safe as possible. The TLC method does not require expensive equipment such as HPLC or LC/MS, and the drugs can be evaluated easily and accurately. Thus, the TLC method has been adopted as the confirmation test for many crude drugs in JP; however, no TLC method for Polygala Root has been reported thus far. The main components of the Polygala Root are known to be saponins, as described above. Detection of saponins using TLC must be carried out by heating after spraying the acid reagent, but the present method, which uses only UV irradiative detection, enables elimination of these steps. Therefore, the TLC analytical method proposed for the first time in this study is applicable for the identification and quality analysis of both the crude drug of Polygala Root and its extracts.

### Experimental

**General** Optical rotations were measured using a JASCO P-1020 digital polarimeter (JASCO Corporation, Tokyo, Japan). The UV spectra were recorded using a Shimadzu UVmini-1240 (Shimadzu Corporation, Kyoto, Japan) and a JASCO V-530 (JASCO Corporation). ESI and HR-ESI-MS spectra were recorded using a microOTOF-Q (Bruker Daltonics, Billerica, MA, U.S.A.) mass spectrometer with acetonitrile as the solvent. The NMR spectra were recorded using a Bruker AVANCE500 instrument (Bruker BioSpin, Billerica, MA, U.S.A.; 500 and 126MHz for \(^1\)H and \(^{13}\)C, respectively) and chemical shifts were expressed relative to those of the solvent.

<table>
<thead>
<tr>
<th>Position</th>
<th>(\delta_C)</th>
<th>(\delta_H) ((J) in Hz)</th>
<th>Position</th>
<th>(\delta_C)</th>
<th>(\delta_H) ((J) in Hz)</th>
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<tbody>
<tr>
<td>1</td>
<td>129.7</td>
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<td>1</td>
<td>93.3</td>
<td>5.65 (d, (J=3.5))</td>
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<td>2</td>
<td>108.6</td>
<td>7.32 (s)</td>
<td>2</td>
<td>82.1</td>
<td>3.47 (dd, (J=3.5, 10.0))</td>
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<td>3</td>
<td>154.0</td>
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<td>3</td>
<td>73.7</td>
<td>3.75 (t, (J=10.0))</td>
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<tr>
<td>4</td>
<td>139.1</td>
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<td>4</td>
<td>70.9</td>
<td>3.44 (t, (J=10.0))</td>
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<tr>
<td>5</td>
<td>154.0</td>
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<td>5</td>
<td>74.4</td>
<td>3.93 (ddd, (J=2.5, 4.5, 7.0))</td>
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<td>6</td>
<td>108.6</td>
<td>7.32 (s)</td>
<td>6</td>
<td>62.3</td>
<td>3.77–3.85((^a))</td>
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<td>7</td>
<td>171.0</td>
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OCH\(_3\)-3,5 57.0 3.86 (6H, s) 1 66.3 3.58 (d, \(J=11.5\)) 1 66.3 3.58 (d, \(J=11.5\))

a, b Overlapped signals.

Fig. 2. Key HMBC Correlations of Compounds 1 and 2
[MeOH-\textit{d}_4 (\delta_H 3.30; \delta_C 49.0) and dimethyl sulfoxide (DMSO)\textit{d}_6 (\delta_H 2.50; \delta_C 39.5)] on a tetramethylsilane scale in parts per million (ppm). The standard pulse sequences programmed for the instrument (AVANCE 500) were used for 2D measurement (COSY, HSQC, and HMBC). J_{CH} was set at 10 Hz for HMBC analysis.

Column chromatography was carried out over Diaion HP-20(120,573),(870,843), MCI-gel CHP-20P (Mitsubishi Chemical Co., Tokyo, Japan), Chromatorex ODS (Fuji Silysia Chemical Ltd., Aichi, Japan), Sephadex LH-20 (GE Healthcare, Little Chalfont, U.K.), and YMC GEL ODS (YMC Co., Ltd., Kyoto, Japan). Preparative TLC was carried out on TLC Silica gel 60 F\textsubscript{254} glass plates (Merck, Darmstadt, Germany). HPTLC was performed with an CAMAG HPTLC equipment (CAMAG, Muttenz, Switzerland) including a Linomat V applicator (CAMAG) and visualizer documentation system (CAMAG). The samples were spotted on HPTLC Silica gel 60 F\textsubscript{254} glass plates (20×10 cm, Merck) or TLC Silica gel 60 F\textsubscript{254} plates (5×7.5 cm, Merck), and the spots were detected by irradiation at 254 nm. The reversed-phase (RP) HPLC conditions used for monitoring fractions in the column chromatography were as follows. Condition 1: column, YMC-pack ODS AQ-3C2 (5 \textmu m, 150×2.0 mm i.d., YMC Co., Ltd., Kyoto, Japan); mobile phase, solvent A 0.1% formic acid in water, and solvent B MeOH (0–30 min, 0–50% B in A, 30–50 min, 50–60% B in A); column temperature, 40°C; flow rate, 0.25 mL/min; detection wavelength, 200–400 nm. Condition 2: column, YMC-pack ODS AQ-3C2 (5 \textmu m, 150×2.0 mm i.d., YMC Co., Ltd.); mobile phase, 10 mmol/L phosphate buffer–acetonitrile (85:15); column temperature, 40°C; flow-
rate, 0.2 mL/min; detection wavelength, 254 nm. Condition 3: column, YMC-pack ODS AQ-3C2 (5 µm, 150×2.0 mm i.d., YMC Co., Ltd.); mobile phase, 50 mmol/L phosphate buffer–acetonitrile (75:25); column temperature, 35°C; flow-rate, 0.3 mL/min; detection wavelength, 250 nm.

Materials The Polygala Root (Lot. no. 9A60019) used for the phytochemical investigation was purchased from Uchida Wakanyaku Ltd., Tokyo, Japan. Polygala Roots available on the Japanese market were obtained from Japan Kampo Medicines Manufactures Association, Japan Medicinal Plant Federation, and Tokyo Crude Drugs Association (model samples described by the National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN)). Senega (Lot. no. 006105001) was purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). Commercial single crude drug extract products of Polygala Root were purchased from drugstores in Japan (in 2017 and 2018). All other reagents used were of special or analytical grade.

Extraction and Isolation The Polygala Root product (100 g) was homogenized in MeOH (1 L). The homogenate was filtered and concentrated to approximately 200 mL. Next, water (200 mL) was added, and the solution was concentrated to approximately 200 mL. Extraction was performed with 900 mL n-hexane to obtain extracts from the n-hexane (944.6 mg) and H2O layers (ca. 100 mL). The H2O layer was separated using column chromatography over Diaion HP-20 with aqueous MeOH to yield sibiricose A5 (1.9 mg) and B (2.1 mg), additionally the 100% MeOH eluate (2.0 g) was separated using column chromatography over Diaion HP-20 with ethanol and/or MCI-gel CHP-20P with aqueous pyridine (0.2 mL) was then added to each mixture and heated at 60°C for 1 h. o-Tolyl isothiocyanate (1.0 mg) in pyridine (0.2 mL) was then added to each mixture and heated at 60°C for 1 h. The reaction mixtures were directly analyzed using RP-HPLC (Condition 3). The peaks from 1 coincided with those of the derivatives similarly prepared from authentic D-glucose and D-apiose obtained from apiin. The peak from 2 was confirmed to be from D-glucose.

Preparation of Test Solution of Crude Polygala Root for TLC Crude Polygala Root samples were pulverized, and a 1.0 g sample was extracted with MeOH (5.0 mL) via sonication for 3 min. The extract was centrifuged, and the supernatant obtained was used as the test solution. Test solutions for commercial single crude drug Polygala Root extract products were prepared by extracting 100 mg of the product with 1.0 mL of MeOH. For TLC, aliquots (1 or 3 µL) of the test solutions were applied to the TLC or HPTLC plates, developed in a TLC chamber saturated with the mobile phase, which was a 6:1:1 (v/v/v) mixture of EtOAc, MeOH, and H2O. The spots were detected under a UV lamp at 254 nm.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials. Experimental details on 1H- and 13C-NMR spectra of compounds 1 and 2 are provided.

References and Notes


