

Simultaneous determination of nine marker compounds in the traditional Korean medicine, Dangguisu-san by high-performance liquid chromatography

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ABSTRACT

Background: Dangguisu-san (DGSS) has been widely used to treat ecchymosis, blood stagnation and pain resulting from physical shock in Korea. **Objective:** A high-performance liquid chromatography–photodiode array detection (HPLC–PDA) method for simultaneous analysis of nine components, albiflorin (1), paeoniflorin (2), liquiritin (3), nodakenin (4), coumarin (5), liquiritigenin (6), cinnamic acid (7), cinnamaldehyde (8), and glycyrrhizin (9) in DGSS extract has been developed for the first time. **Materials and Methods:** The analytical column for separation of the nine constituents used a Gemini C₁₈ column kept at 40°C by the gradient elution with 1.0% (v/v) acetic acid in water and 1.0% (v/v) acetic acid in acetonitrile as mobile phase. The flow rate was 1.0 mL/min and the injection volume was 10 µL. **Results:** Calibration curves of all compounds showed good linearity ($r^2 \geq 0.9999$) within the test ranges. The limits of detection and quantification for all analytes were 0.01–0.27 µg/mL and 0.04–0.89 µg/mL, respectively. All recoveries of the nine marker compounds were 96.62–102.47% with relative standard deviations (RSD) < 1.72%. The RSDs of intra-day and inter-day precision were < 1.32% and 1.61%, respectively. The amounts of the nine marker components ranged from 0.10 mg/g to 13.71 mg/g. **Conclusion:** The developed and validated HPLC–PDA method may help for the quality control of DGSS.

Key words: Dangguisu-san, high-performance liquid chromatography–photodiode array detection, simultaneous determination, traditional Korean medicine

INTRODUCTION

In general, traditional Korean medicines include many herbs, which also contain various components. Thus, they have been widely used to prevent and treat many diseases associated with multiple targets.^[1,2] Dangguisu-san (DGSS), also known as Dangguisu-san in Chinese and Tokishusan in Japan, is a well-known traditional Korean herbal medicine prescription, consisting of nine commonly used herbal medicines, Radix Angelicae Gigantis, Radix Paeoniae Rubra, Radix Linderae, Rhizoma Cyperi, Lignum Sappan, Flos Carthami, Semen Persicae, Cinnamon Bark, and Radix et Rhizoma Glycyrrhizae.^[3] It has been widely used to treat ecchymosis, blood stagnation, and pain resulting from physical shock in Korea.^[3,4] The

pharmacological effects of DGSS have been investigated *in vitro* and *in vivo* for anti-inflammatory,^[5] bone healing,^[6] ecchymoma,^[7] and anti-thrombotic^[8] effects. Effects on allergic purpura^[9] and tension headache^[10] have been reported in case reports. Recently, nitric oxide-dependent effects of DGSS on cerebral ischemic injury have been reported.^[4] A number of methods have been reported for the simultaneous determination of bioactive compounds in many herbal formulas. However, no simultaneous determination of bioactive compounds for the quality control of DGSS has been reported. Therefore, we conducted qualitative and quantitative analyses to improve the quality control of DGSS. In the present study, we improved the quality control of DGSS through the simultaneous determination of nine bioactive compounds, albiflorin (1) and paeoniflorin (2) from Radix Paeoniae Rubra, liquiritin (3), liquiritigenin (6), and glycyrrhizin (9) from Radix et Rhizoma Glycyrrhizae, nodakenin (4) from Radix Angelicae Gigantis, and coumarin (5), cinnamic acid (7), and cinnamaldehyde (8) from Cinnamon Bark using a rapid

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and precise high-performance liquid chromatography–photodiode array detection (HPLC–PDA) method.

MATERIALS AND METHODS

Chemicals and materials

Reference standard, compounds 5 and 7 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and compounds 1, 2, 3, 8, and 9 were purchased from Wako Chemicals (Osaka, Japan). Compounds 4 and 6 were obtained from NPC Bio Technology (Yeongi, Korea) and Chengdu Biopurify Phytochemicals (Chengdu, China), respectively. The chemical structures of marker compounds 1–9 are shown in Figure 1 and their purities were >98.0% according to their HPLC analysis. HPLC-grade methanol, acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA) and analytical reagent-grade, glacial acetic acid was purchased from Merck (Darmstadt, Germany).

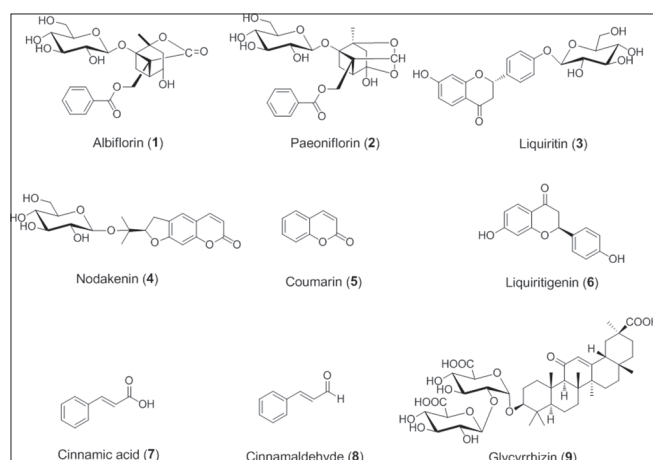


Figure 1: Chemical structures of the nine marker compounds in Dangguisu-san

Each sample of DGSS was composed of nine herbal components purchased from the Korean herbal market, Kwangmyungdang (Ulsan, Korea). The origin of these herbal components was confirmed taxonomically by Prof. Je Hyun Lee, Dongguk University, Gyeongju, Korea. Voucher specimens (2012–KE33-1 through KE33-9) have been deposited at the Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine.

Apparatus and conditions

The chromatographic analysis was conducted using a Shimadzu Prominence LC-20A series system (Shimadzu, Kyoto, Japan) consisting of a solvent delivery unit (LC-20AT), online degasser (DGU-20A₃), column oven (CTO-20A), auto sample injector (SIL-20AC) and PDA detector (SPD-M20A). Data were acquired and processed using LC solution software (version 1.24, Shimadzu, Kyoto, Japan). Compounds 1–9 were separated on a 250 mm × 4.6 mm Phenomenex Gemini C₁₈ column with 5 μm particles (Torrance, CA, USA). The gradient elution of two mobile phase systems with 1.0% (v/v) acetic acid in water (A) and 1.0% (v/v) acetic acid in acetonitrile (B) was as follows: 10–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min and 100–10% B for 45–50 min, with a re-equilibrium time of 10 min. The flow rate was kept constant at 1.0 mL/min, column temperature was maintained at 40°C, and the injection volume was 10 μL. The PDA detector wavelength ranged from 190 to 400 nm and was monitored at 230, 254, 275, and 335 nm.

A Waters (Milford, MA, USA) triple quadruple mass spectrometer (MS) equipped with an electrospray ionization source was used. The MS conditions were as follows: Capillary voltage 3.3 kV, extractor voltage

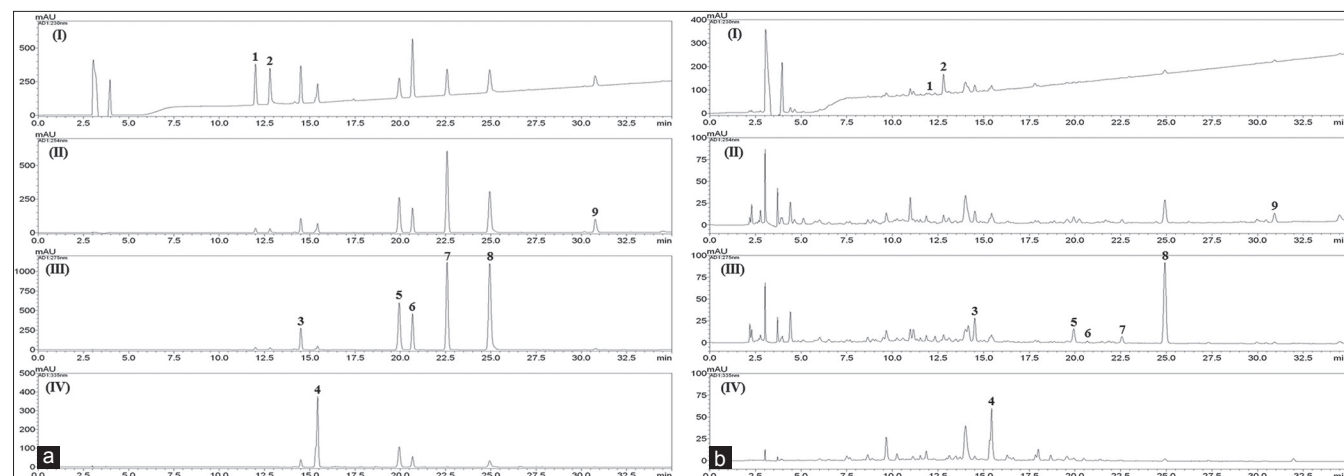


Figure 2: High-performance liquid chromatography of a standard mixtures (a) and Dangguisu-san extracts (b) at 230 nm (I), 254 nm (II), 275 nm (III), and 335 nm (IV). Albiflorin (1); paeoniflorin (2); liquiritin (3); nodakenin (4); coumarin (5); liquiritigenin (6); cinnamic acid (7); cinnamaldehyde (8); and glycyrrhizin (9)

Table 1: Composition of DGSS

Herbal medicine	Scientific name	Supplier	Location of origin	Amount (g)
Radix Angelicae Gigantis	<i>Angelica gigas</i>	Kwangmyungdang	Bonghwa, Korea	5.625
Radix Paeoniae Rubra	<i>Paeonia obovata</i>	Kwangmyungdang	Uiseong, Korea	3.75
Radix Linderae	<i>Lindera strichnifolia</i>	Kwangmyungdang	China	3.75
Rhizoma Cyperi	<i>Cyperus rotundus</i>	Kwangmyungdang	Yeongcheon, Korea	3.75
Lignum Sappan	<i>Caesalpinia sappan</i>	Kwangmyungdang	Indonesia	3.75
Flos Carthami	<i>Carthamus tinctorius</i>	Kwangmyungdang	China	3.0
Semen Persicae	<i>Prunus persica</i>	Kwangmyungdang	South Africa	2.625
Cinnamon Bark	<i>Cinnamomum cassia</i>	Kwangmyungdang	Vietnam	2.25
Radix et Rhizoma Glycyrrhizae	<i>Glycyrrhiza uralensis</i>	Kwangmyungdang	China	1.875
Total amount				30.375

DGSS: Dangguisu-san

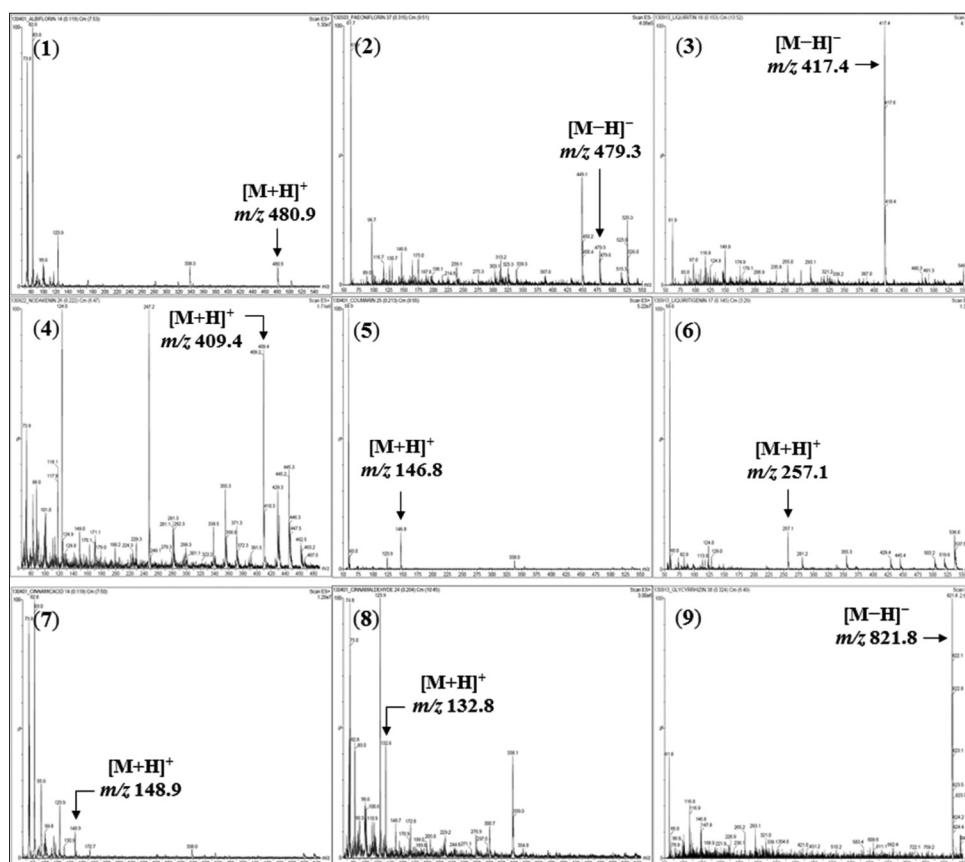


Figure 3: Mass spectra of standard compounds 1–9. Albiflorin (1); paeoniflorin (2); liquiritin (3); nodakenin (4); coumarin (5); liquiritigenin (6); cinnamic acid (7); cinnamaldehyde (8); and glycyrrhizin (9)

3.0 V, radio frequency lens voltage 0.3 V, source temperature 120°C, desolvation temperature 300°C, desolvation gas 600 L/h, cone gas 50 L/h and collision gas 0.14 mL/min. Data were processed using Waters MassLynx Software (version 4.1, Milfore, MA, USA).

Preparation of standard solutions

Reference compounds 1–9 were accurately weighed and dissolved in methanol at a concentration of 1.0 mg/mL. Each standard stock solution was kept at

4°C and used after serial dilution with methanol before HPLC analysis.

Preparation of sample solutions

The nine crude dried herbal components, Radix Angelicae Gigantis, Radix Paeoniae Rubra, Radix Linderae, Rhizoma Cyperi, Lignum Sappan, Flos Carthami, Semen Persicae, Cinnamon Bark, and Radix et Rhizoma Glycyrrhizae were mixed as indicated in Table 1 (5.0 kg; 130.375 g × 164.6) and extracted in a 10-fold mass of water at 100°C for

2 h. After filtration of the extract, the resulting solution was lyophilized by freeze-drying (845.4 g). The yield of DGSS extract was 16.9%. For simultaneous analysis of the soluble components by HPLC, 40 mg of the lyophilized DGSS extract was dissolved in 20 mL of 70% methanol. The solution was filtered through a 0.2 μm syringe filter (Woongki Science, Seoul, Korea) before HPLC analysis.

Calibration curves, limits of detection, and quantification

For the calibration curves, each concentration was measured in triplicate. The calibration curves of compounds 1–9 were calculated by plotting the peak areas (y) versus the corresponding concentrations (x , $\mu\text{g/mL}$) using standard solutions. The tested concentration ranges were as follows: Compounds 1, 8, and 9, 0.31–40.00 $\mu\text{g/mL}$, compound 2, 0.78–100.00 $\mu\text{g/mL}$, compounds 3 and 5, 0.16–20.00 $\mu\text{g/mL}$, compound 4, 0.39–50.00 $\mu\text{g/mL}$, and compounds 6 and 7, 0.08–10.00 $\mu\text{g/mL}$, respectively. The standard solutions of reference compound 1–9 were diluted with methanol to determine limits of detection (LOD) and limits of quantification (LOQ) values. The LOD and LOQ data under the present chromatographic conditions were determined at signal-to-noise ratios of 3 and 10, respectively.

Table 2: System suitability of the nine marker compounds

Compounds	Capacity factor (k')	Separation factor (α)	Number of theoretical plates (n)	Resolution (R_s)
1	2.97	1.09	58,887	3.69
2	3.23	1.09	44,807	3.69
3	3.89	1.08	38,847	2.49
4	4.21	1.08	18,198	2.49
5	5.73	1.05	31,950	2.09
6	5.99	1.05	73,730	2.09
7	6.62	1.10	45,197	5.07
8	7.43	1.12	36,594	5.12
9	9.22	1.24	101,155	11.69

Precision and recovery

Intra- and inter-day variations, which were used to determine the precision of the new HPLC method, were determined using a standard addition method with the samples spiked with low, middle, and high concentration levels of standard compounds. The relative standard deviation (RSD) was used as a measure of precision. To confirm the repeatability, six replicates were analyzed using the standard stock solutions. The RSDs of peak areas and retention times for each compound were used to evaluate the repeatability of the method. A recovery test was used to evaluate the accuracy of the method. The recovery test was conducted by adding known low, middle, and high concentration levels of standard solutions to 40 mg of DGSS extract. This test was assessed using the calibration curves of compounds 1–9.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

High-performance liquid chromatography conditions such as column types, column temperatures, and mobile phases were optimized to accomplish the simultaneous separation of the nine analytes including the four terpenoids, compounds 1, 2, and 9, two flavonoids, compounds 3 and 6, two coumarins, compounds 4 and 5, and phenolic acids, compounds 7 and 8. To accomplish the efficient separation of the nine components, we evaluated columns such as the Phenomenex Gemini C_{18} , Waters SunFire C_{18} , and OptimaPak C_{18} columns, column temperatures of 30, 35, and 40°C, and various mobile phases including acetic acid, formic acid, and phosphoric acid and organic solvents including methanol and acetonitrile. The most efficient separations were selected using a Phenomenex Gemini C_{18} column (250 mm \times 4.6 mm, 5 μm) with a gradient of acetonitrile in 1.0% (v/v) acetic acid at 40°C after comparing the baselines, resolution and peak shapes of compounds

Table 3: Regression equations, linearity, LODs, LOQs and detected ions of the nine marker compounds

Compounds	Linear range ($\mu\text{g/mL}$)	Regression equation ^a	Correlation coefficient	LOD ^b ($\mu\text{g/mL}$)	LOQ ^c ($\mu\text{g/mL}$)	Detected ion (m/z)
1	0.31–40.00	$y=9216.04x+358.35$	1.0000	0.21	0.69	480.9
2	0.78–100.00	$y=8438.37x-1069.11$	1.0000	0.27	0.89	479.3
3	0.16–20.00	$y=17273.80x+570.67$	1.0000	0.05	0.16	417.4
4	0.39–50.00	$y=27944.62x+1859.01$	1.0000	0.04	0.13	409.4
5	0.16–20.00	$y=46607.58x+915.18$	1.0000	0.03	0.09	146.8
6	0.08–10.00	$y=35854.18x+190.26$	1.0000	0.03	0.09	257.1
7	0.08–10.00	$y=91081.26x+1182.04$	1.0000	0.01	0.04	148.9
8	0.31–40.00	$y=113032.51x+8583.45$	1.0000	0.01	0.04	132.8
9	0.31–40.00	$y=7156.34x+1051.03$	0.9999	0.07	0.22	821.8

^a y : Peak area (mAU) of compounds; x : Concentration ($\mu\text{g/mL}$) of compounds; ^bLOD=3 \times signal-to-noise ratio, ^cLOQ=10 \times signal-to-noise ratio. LOD: Limits of detection; LOQ: Limits of quantification

Table 4: Recoveries for the assay of the nine analytes in DGSS

Analytes	Original concentration (µg/mL)	Spiked concentration (µg/mL)	Found concentration (µg/mL)	Recovery ^a ±SD (%)	RSD (%)
1	3.79	1.00	4.79	99.70±0.82	0.83
		2.00	5.81	101.31±0.79	0.78
		4.00	7.79	100.44±1.14	1.13
2	26.95	4.00	31.00	99.38±0.89	0.90
		10.00	36.89	99.91±1.08	1.08
		20.00	46.93	99.90±0.93	0.93
3	5.82	1.00	6.82	100.59±0.77	0.77
		2.00	7.82	98.89±0.86	0.87
		4.00	9.84	100.26±1.21	1.20
4	7.87	2.00	9.84	98.18±0.12	0.13
		5.00	12.78	100.45±1.73	1.72
		10.00	18.11	102.47±0.48	0.47
5	1.48	1.00	2.49	100.90±0.58	0.57
		2.00	3.52	100.62±1.35	1.34
		4.00	5.51	102.29±0.66	0.64
6	0.20	1.00	1.21	97.29±0.91	0.94
		2.00	2.15	100.72±0.22	0.22
		4.00	4.23	100.58±0.31	0.31
7	0.33	1.00	1.32	99.07±0.43	0.43
		2.00	2.26	100.48±1.17	1.16
		4.00	4.27	96.62±0.49	0.51
8	3.60	1.00	4.60	98.66±0.69	0.70
		2.00	5.57	98.59±0.15	0.15
		4.00	7.69	102.24±0.41	0.40
9	6.66	1.00	7.65	99.30±0.88	0.88
		2.00	8.65	99.70±0.90	0.90
		4.00	10.67	100.34±0.92	0.92

^aRecovery (%)=(Found concentration-Original concentration)/spiked concentration×100. SD: Standard deviation; RSD: Relative standard deviation; DGSS: Dangguisu-san

Table 5: Repeatability of retention times and peak responses for the nine analytes (n=6)

Compounds	Retention time (min)		Peak response (mAU)	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
1	11.63±0.01	0.06	261741.83±1069.09	0.41
2	12.42±0.01	0.06	411327.67±972.47	0.24
3	14.12±0.01	0.07	903419.33±5651.87	0.63
4	1504±0.01	0.07	1391562.17±8899.38	0.64
5	19.44±0.01	0.05	1245194.67±8831.91	0.71
6	20.21±0.01	0.05	828856.67±5892.65	0.71
7	22.01±0.01	0.05	944776.50±6953.58	0.74
8	24.37±0.01	0.05	1251609.83±8186.21	0.65
9	29.50±0.01	0.04	707579.50±5841.55	0.83

SD: Standard deviation; RSD: Relative standard deviation

1–9 under the various conditions. Quantification was achieved using a PDA detector at 230 nm for compounds 1 and 2, 254 nm for compound 9, 275 nm for compounds 3, 5–8, and 335 nm for compound 4, based on retention time and ultra violet spectra compared with those of the standards. In the optimized method, the nine compounds were resolved within 40 min and showed the suitability of the separation system without obstruction from other

components [Table 2]. Representative chromatograms of standard solutions and the DGSS extracts are shown in Figure 2.

The MS conditions were optimized in full scan mode using the reference compounds [Figure 3]. Compounds 1 and 4–8 were detected in the positive ion mode $[M + H]^+$ at m/z 480.9, m/z 409.4, m/z 146.8, m/z 257.1, m/z 148.9,

Table 6: Precision data for the assay of the nine analytes in DGSS

Compounds	Fortified concentration (µg/mL)	Intraday (n=5)		Interday (n=5)	
		Observed concentration (µg/mL)	Precision ^a (%)	Observed concentration (µg/mL)	Precision (%)
1	1.00	0.99	0.88	1.00	0.63
	2.00	2.02	0.69	2.01	0.33
	4.00	3.99	0.14	4.00	0.12
2	4.00	4.05	0.93	3.99	0.94
	10.00	9.95	1.16	10.12	1.61
	20.00	20.01	0.25	19.94	0.38
3	1.00	1.00	0.79	0.99	0.72
	2.00	2.00	1.32	1.99	0.85
	4.00	4.00	0.34	4.01	0.21
4	2.00	2.00	0.72	1.97	0.71
	5.00	4.87	0.28	4.98	0.88
	10.00	10.07	0.09	10.03	0.24
5	1.00	0.99	0.25	1.00	0.68
	2.00	2.02	0.48	2.01	0.91
	4.00	3.99	0.13	4.00	0.22
6	1.00	1.02	1.20	1.00	0.75
	2.00	1.95	0.76	1.96	1.03
	4.00	4.02	0.14	4.02	0.27
7	1.00	1.01	0.41	1.01	0.48
	2.00	1.97	0.33	1.98	0.42
	4.00	4.01	0.06	4.01	0.10
8	1.00	1.00	1.00	1.01	0.80
	2.00	1.95	0.70	1.99	0.87
	4.00	4.02	0.13	4.00	0.18
9	1.00	1.00	0.14	1.00	0.71
	2.00	1.99	0.29	1.99	0.80
	4.00	4.00	0.08	4.00	0.17

^aPrecision is expressed as RSD (%)=(SD/mean) × 100. SD: Standard deviation; RSD: Relative standard deviation; DGSS: Dangguisu-san

Table 7: The amount of each marker compounds in the DGSS extract (n=3)

Compounds	Amount (mg/g)			Source
	Mean	SD	RSD (%)	
1	1.88	0.02	1.32	Radix Paeoniae Rubra
2	13.71	0.16	1.20	Radix Paeoniae Rubra
3	2.79	0.02	0.77	Radix et Rhizoma Glycyrrhizae
4	4.06	0.00	0.07	Radix Angelicae Gigantis
5	0.73	0.01	0.78	Cinnamon Bark
6	0.10	0.00	1.06	Radix et Rhizoma Glycyrrhizae
7	0.16	0.00	0.90	Cinnamon Bark
8	1.80	0.01	0.69	Cinnamon Bark
9	3.27	0.02	0.61	Radix et Rhizoma Glycyrrhizae

SD: Standard deviation; RSD: Relative standard deviation; DGSS: Dangguisu-san

and m/z 132.8, respectively while compounds 2, 3, and 9 were detected using the negative ion mode $[M - H]^-$ at m/z 479.3, m/z 417.4, and m/z 821.8, respectively [Table 3].

Linearity, range, limits of detection, and limits of quantification

The linearity of the method was evaluated from the correlation coefficient (r^2) of the calibration curves of

each compound. We found that the nine compounds showed good linearity with $r^2 \geq 0.9999$ in eight different concentration ranges. The LODs and LOQs for the tested compounds 1–9 were 0.01–0.27 µg/mL and 0.04–0.89 µg/mL, respectively [Table 3].

Recovery and precision

Recovery of the nine compounds was in the range 96.62–102.47% at the three different concentrations (low, middle, and high), and the RSD values were $\leq 1.72\%$. The recovery data are summarized in Table 4. The RSDs for repeatability of compounds 1–9 were from 0.24% to 0.83% for peak responses and from 0.04% to 0.07% for retention times [Table 5]. Thus, the method showed good repeatability under optimized conditions. The intra- and inter-day variations for compounds 1–9 were assessed by analyzing the DGSS extract. The intra- and inter-day RSDs for each analyte were 0.06–1.32% and 0.10–1.61%, respectively and these findings are summarized in Table 6. These results suggest that the method established has satisfactory recovery, repeatability, and precision.

Quantitative analysis

The newly developed HPLC–PDA method was applied to the simultaneous determination of the nine marker compounds in DGSS. Each DGSS sample was analyzed three times and amounts of the tested compounds 1–9 in the DGSS extracts are shown in Table 7. Among these components, compound 2 and 4, which are marker components of *Radix Paeoniae* and *Radix Angelicae Gigantis*, respectively, were found to be 13.71 mg/g and 4.06 mg/g, respectively, and were the most abundant compounds compared with the others in the DGSS extract.

CONCLUSION

In this work, we have, for the first time to our knowledge, developed a rapid, accurate, and reliable HPLC–PDA method for the quantitative analysis of nine marker components in extracts of the traditional Korean herbal medicine DGSS. Validation of the method showed high linearity, repeatability, intra- and inter-day precision, and recovery. Moreover, this method has been successfully applied to the simultaneous analysis of extract components for the quality control of DGSS. The method may be valuable and efficient for the quality control of DGSS samples and related botanical preparations.

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