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Development and Validation of a High-Performance Liquid Chromatography Method for Quality Assessment of Oriental Medicine, Dokhwalgisaeng-Tang

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Abstract: Dokhwalgisaeng-tang (DHGST) is an herbal medicine formula that is frequently used in the treatment of arthritis in Korea and consists of 16 medicinal herbs. In this study, a simultaneous analysis method for quality assessment of DHGST by universal and widely used high-performance liquid chromatography was developed and validated. Twenty-four marker components were separated on a reverse-phase SunFire C18 column (4.6 × 250 mm, particle size; 5 μm) maintained at 40 °C using a gradient elution of two mobile phase systems (0.1% aqueous formic acid and 0.1% formic acid in acetonitrile). The developed method was validated via linearity, limit of detection, limit of quantification, recovery, and precision. Using the developed method, 24 marker components in DHGST were founded at 0.23–14.68 mg/g, and this method will be used as basic data for the quality assessment of DHGST or other herbal medicine prescriptions.

Keywords: high–performance liquid chromatography; quality assessment; Dokhwalgisaeng-tang

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1. Introduction

Herbal medicine prescriptions are generally composed of two or more herbal medicines and can be prepared in various formulations such as tang (decoction), pills, granules, and powders. Dokhwalgisaeng-tang (DHGST; alternatively, Duhuojisheng-tang in Chinese) is a typical herbal formula used in Korean medicine for the treatment of rheumatoid arthritis and neuritis [1]. Since DHGST was first recorded in the Bei Ji Qian Jin Yao Fang (备急千金要方) written by Sun Simiao in the Tang Dynasty in China, it has also been included in Donguibogam (東醫寶鑑) written by Heo Jun in the Joseon Dynasty, a well-respected Korean oriental medicine book [1,2]. According to the Donguibogam, DHGST is composed of 16 medicinal herbs (*Aralia continentalis* Kitag., *Angelica gigas* Nakai, *Paeonia lactiflora* Pall., *Taxillus chinensis* (D.C.) Danser, *Rehmannia glutinosa* (Gaertn.) D.C., *Cnidium officinale* Mak., *Panax ginseng* C.A. Mey., *Poria cocos* Wolf, *Achyranthes bidentata* Blume, *Eucommia ulmoides* Oliv., *Gentiana straminea* Maxim., *Asarum heterotropoides* F. Schmidt, *Saposhnikovia divaricate* (Turcz.) Schischk., *Cinnamomum cassia* (L.) J. Presl, *Glycyrrhiza uralensis* Fisch., and *Zingiber officinale* Roscoe) and has been used to treat muscle cramps, bone pain, lower-back pain, and knee pain caused by liver–kidney Yin deficiency [1].

Many systematic reviews and meta-analyses have been reported for the use of DHGST in the treatment of rheumatoid arthritis, lumbar disk herniation, postmenopausal osteoporosis, and knee osteoarthritis [3–7], and research has been published by Liu et al. [8] on the effect of DHGST on stromal-cell-derived factor-1-induced inflammation and extracellular matrix degradation in human nucleus pulposus cells. It has also been reported that DHGST regulates autophagy and the P38/MAPK signaling pathway to prevent compression-induced matrix degradation and cell apoptosis in a rat model [9].

Each raw herbal medicine that makes up DHGST contains a variety of phytochemicals: namely, diterpenoids (kaurenoic acid and continentalic acid) from *A. continentalis*, coumarins (nodakenin, decursin, and decursinol angelate) from *A. gigas*, phenols (gallic

acid and benzoic acid) and monoterpenoids (albiflorin and paeoniflorin) from *P. lactiflora*, cardiac glycosides (neritaloside and odoroside H) from *T. chinensis*, miscellaneous (5-hydroxymethylfurfural) from *R. alutinosa*, phenylpropanoids (ferulic acid) and miscellaneous (senkyunolide A and (*Z*)-ligustilide) from *C. officinale*, triterpenoids (ginsenoside Rb1 and ginsenoside Rg1) from *P. ginseng*, triterpenoids (pachymic acid and polyporenic acid C) from *P. cocos*, steroids (ecdysterone) from *A. bidentata*, iridoids (geniposide and geniposidic acid) and lignans (pinoresinol diglucoside) from *E. ulmoides*, iridoids (gentiopicroside and loganic acid) from *G. straminea*, phenylpropanoids (methyleugenol and safrole) from *A. heterotropoides*, chromones (*prim-O*-glucosylcimifugin and 5-*O*-methylvisammioside) from S. divaricate, phenylpropanoids (cinnamic acid and cinnamaldehyde) from *C. cassia*, flavonoids (liquiritin and liquiritin apioside) and triterpenodis (glycyrrhizin) from *G. uralensis*, and phenols (6-gingerol and 6-shogaol) from *Z. officinale* [10–25].

Methods for the quality control of DHGST based on high-performance liquid chromatography (HPLC) have been published by Chen et al. [26] and Wang et al. [27]; however, the analysis time in the former method was very long (500 min), and only four components (ferulic acid, osthole, gentiopicroside, and paeoniflorin) were detected. The method developed by Wang et al. [27] was based on only six components (chlorogenic acid, gentiopicrin, paeoniflorin, ferulic acid, glycyrrhizin, and osthole). Moreover, these studies focused on method efficacy rather than component analysis; thus, only a selection of component herbs (*P. lactiflora*, *C. officinale*, and *G. uralensis*) was examined, and no assay verification was performed. The development and validation of a simultaneous analysis method based on HPLC consistent quality evaluation of DHGST were therefore required and are described herein.

In this study, a simultaneous analysis method for the quality assessment of DHGST was developed and validated using standard HPLC equipment. The assay was used to monitor 24 marker components: gallic acid (**1**), 5-hydroxymethylfurfural (**2**), geniposidic acid (**3**), loganic acid (**4**), chlorogenic acid (**5**), gentiopicroside (**6**), pinoresinol diglucoside (**7**), albiflorin (**8**), *prim-O*-glucosylcimifugin (**9**), paeoniflorin (**10**), liquiritin apioside (**11**), liquiritin (**12**), ferulic acid (**13**), nodakenin (**14**), 5-*O*-methylvisammioside (**15**), benzoic acid (**16**), coumarin (**17**), cinnamic acid (**18**), cinnamaldehyde (**19**), glycyrrhizin (**20**), methyleugenol (**21**), safrole (**22**), decursin (**23**), and decursinol angelate (**24**).

2. Materials and Methods

2.1. Plant Materials

The 16 raw herbal medicines used in this experiment are listed in Table S1; the plant names were confirmed on the website "The Plant List" (http://www.theplantlist.org/, accessed on 9 August 2021). These materials were purchased from Kwangmyungdag Medicinal Herbs (Ulsan, Korea). The origins of the raw herbal medicines were morphologically confirmed by Dr. Goya Choi, Korea Institute of Oriental Medicine (KIOM, Naju, Korea) according to guidelines and previous study protocols [28,29], and each material (2018–KE74–1 to 2018–KE74–16) was kept in KIOM.

2.2. Chemicals and Reagents

Compounds **1**–**24** (Figure S1) were purchased from commercial manufacturers: compounds **1**, **2**, **16**, **17**, **21**, and **22** from Merck KGaA (Darmstadt, Germany); compounds **3**, **8**, **13**, and **18**–**20** from Fujifilm Wako Pure Chemical Co. (Osaka, Japan); compounds **4**, **7**, and **9** from ChemNorm Biotech Co. (Wuhan, China); compound **5** from Acros Organics (Pittsburgh, PA, USA); compounds **6**, **12**, **15**, and **24** from Chengdu Biopurify (Chengdu, China); compounds **10**, **11**, and **23** from Shanghai Sunny Biotech Co. (Shanghai, China); and compound **14** from ChemFaces (Wuhan, China). The purity of the reference compounds was confirmed to be ≥98.0% by HPLC analysis.

Methanol (Cat No. 9093-88, ≥99.9%), acetonitrile (Cat No. 9017-88, ≥99.0%), distilled water (Cat No. 4218-88), and formic acid (Cat No. 100264, 98–100%) were HPLC-grade

solvents or reagents and were purchased from J.T.Baker (Phillipsburg, NJ, USA) or Merck KGaA (Darmstadt, Germany).

2.3. DHGST Sample Preparation

DHGST powder extract was prepared according to a previously developed protocol [29]; the 16 herbal medicines were mixed in the weight ratio (w/w) shown in Table S1, then 50 L of distilled water was added, and the mixture was extracted at 100 °C for 2 h using an electric extractor. The extract solution was freeze-dried with an LP110R freeze-dryer (IlShinBioBase, Dongducheon, Korea) to obtain 1113.6 g (yield 22.3%) of a powder sample. The prepared DHGST sample was stored at −20 °C.

2.4. HPLC Simultaneous Quantification of the 24 Marker Compounds

Simultaneous quantification of the selected 24 marker analytes in the DHGST sample was conducted using a modification of a protocol developed in a previous study [29]. A Prominence LC-20A series (Shimadzu, Kyoto, Japan) HPLC instrument coupled with a photodiode array (PDA) detector capable of scanning the 190–800 nm region was used. The system was controlled and operated with LabSolution software (Ver. 5.53, SP3,Shimadzu, Kyoto, Japan). Full details of the analysis conditions are provided in Table S2.

A sample solution for simultaneous determination of the 24 marker analytes in the DHGST sample was prepared at a concentration of 10.0 mg/mL using 70% methanol, followed by ultrasonic extraction for 60 min. A standard solution of each reference standard compound was prepared at a concentration of 1.0 mg/mL using methanol and then stored in a refrigerator. All of the prepared solutions were filtered through a 0.2-µm membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) before injection into the HPLC.

2.5. System Suitability Test of the Analytical Method

System suitability tests were conducted to evaluate the retention factor (k'), relative retention (α), resolution (Rs), number of theoretical plates (N), and tailing factor (Tf) to ensure the adequate performance of the chromatography system for the developed method.

2.6. Method Validation of the Developed HPLC Analytical Assay

Validation of the analytical method developed in this study was performed with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision as described in the previous studies [29,30].

3. Results and Discussion

3.1. Selection of Marker Components for Quality Assessment of DHGST

As shown in Figure S2, we analyzed and compared the major components of each raw herbal medicine to select the marker compounds. All herbal medicines and components were scanned from 190 to 400 nm with a PDA detector during HPLC analysis using a mobile phase system of distilled-water–acetonitrile, with both phases containing 0.1% formic acid. Each marker component was confirmed by comparing its retention time and UV spectrum with those of the corresponding reference standard.

As shown in Figure S3, 40 main components in DHGST were analyzed using HPLC–PDA to enable the selection of the marker components. As a result, 24 components were identified as suitable markers in the DHGST sample, and these analytes were used as marker compounds for quality control of the DHGST sample in the subsequent studies.

3.2. Establishment of Optimal HPLC–PDA Conditions

HPLC–PDA analytical conditions for efficient separation of the 24 selected markers were determined using a range of reverse-phase C18 columns (I. D. 4.6 mm × length 250 mm, particle size 5 µm), Waters SunFire (Milford, MA, USA), Thermo Scientific Hypersil GOLD (Waltham, MA, USA), Phenomenex Gemini (Torrance, CA, USA), and Shiseido Capcell Pak UG80 (Tokyo, Japan), acids (0.1% formic acid, 0.1% trifluoroacetic acid, and

1.0% acetic acid), and column oven temperatures (30, 40, and 50 °C). As a result of comparative analysis, as shown in Table S2, a SunFire C18 reverse-phase column, 0.1% formic acid, and a column oven temperature of 40 °C were established as the optimal analysis conditions. Compounds **1**–**24** were eluted within 60 min and detected at 6.21, 8.70, 9.43, 12.26, 14.79, 16.63, 17.42, 17.86, 18.75, 18.96, 20.90, 21.30, 22.04, 22.52, 23.36, 25.64, 28.45, 32.79, 36.05, 42.76, 47.93, 53.20, 55.39, and 55.83 min, respectively (Figure 1).

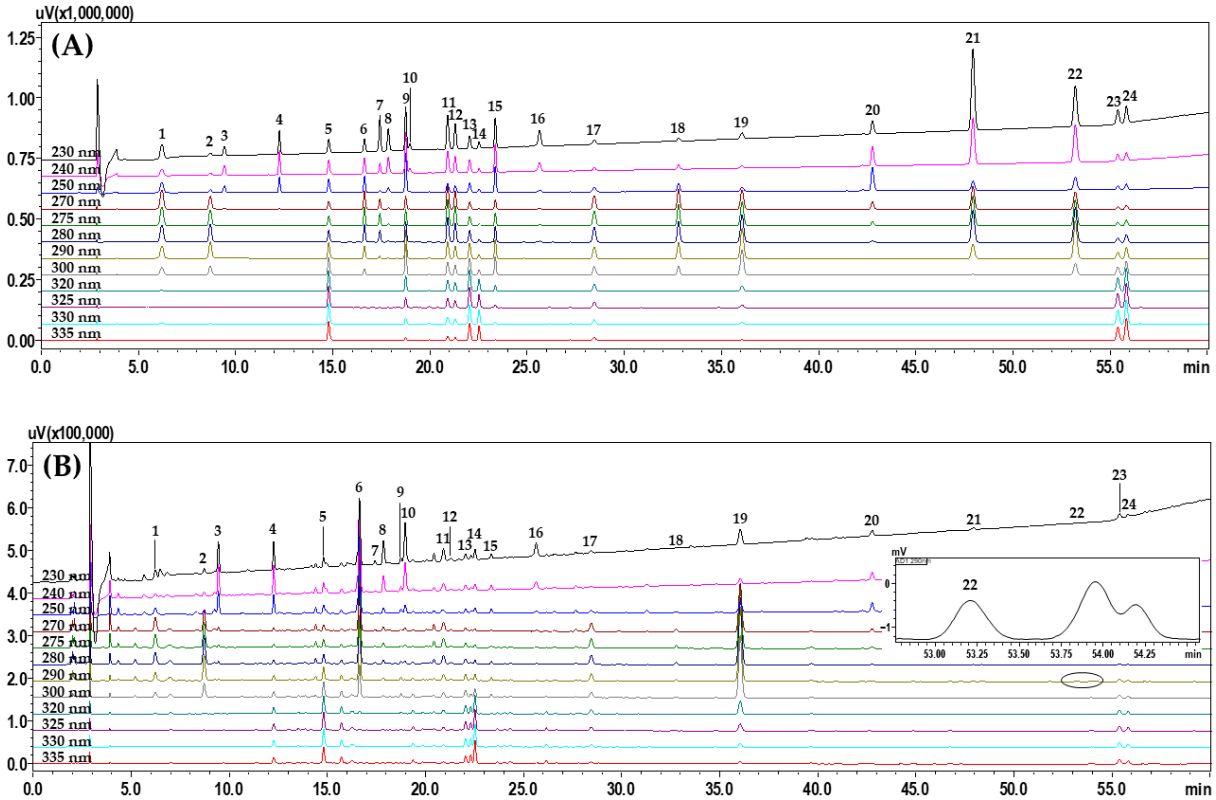

Figure 1. Representative HPLC chromatograms of (**A**) standard solution and (**B**) 70% methanol extract of DHGST sample. Peaks correspond to gallic acid (**1**), 5-hydroxymethylfurfural (**2**), geniposidic acid (**3**), loganic acid (**4**), chlorogenic acid (**5**), gentiopicroside (**6**), pinoresinol diglucoside (**7**), albiflorin (**8**), *prim-O*-glucosylcimifugin (**9**), paeoniflorin (**10**), liquiritin apioside (**11**), liquiritin (**12**), ferulic acid (**13**), nodakenin (**14**), 5-*O*-methylvisammioside (**15**), benzoic acid (**16**), coumarin (**17**), cinnamic acid (**18**), cinnamaldehyde (**19**), glycyrrhizin (**20**), methyleugenol (**21**), safrole (**22**), decursin (**23**), and decursinol angelate (**24**).

3.3. System Suitability and Method Validation of the Developed HPLC Analytical Method

For efficient simultaneous analysis of markers in the DHGST sample, the suitability of the HPLC instrument was confirmed with respect to k'(1.15–18.38), α (1.01–1.75), N (16865–2158966), Rs (1.27–18.17), and Tf (0.92–1.20) (Table S3). The regression equations for all the calibration curves showed excellent linearity, with a coefficient of determination (r^2) of 0.9999 to 1.0000 over the tested concentration range. The LOD and LOQ were calculated to be 0.004–0.061 μg/mL and 0.012–0.184 μg/mL, respectively (Table 1). Recovery was tested by adding three levels (low, medium, and high) of each standard solution to the DHGST sample, with results in the range of 95.47–102.81% (Table 2). The repeatability of the assay with respect to retention time and peak area was measured using a DHGST sample, and the RSD (%) was found to be ≤0.44% and ≤1.78%, respectively (Tables S4 and S5). Good RSD (%) values for intraday and interday precisions of ≤0.79% and ≤1.42%, respectively, were also recorded (Table 3). Having validated the method as described, it was concluded that the current HPLC simultaneous analysis method is suitable for the quality assessment of DHGST samples.

Table 1. Linear range, regression equation, coefficient of determination (r^2), limit of detection (LOD), and limit of quantification (LOQ) of 24 marker analytes for quantification by HPLC (n = 3).

Analyte	Quantification Wavelength (nm)	Linear Range (μg/mL)	Regression Equation [a] $y = ax + b$	r^2	LOD [b] (μg/mL)	LOQ [c] (μg/mL)
1	270	0.31–20.00	$y = 50{,}316.71x - 21.01$	1.0000	0.013	0.040
2	280	0.31–20.00	$y = 95{,}555.03x + 935.74$	1.0000	0.013	0.039
3	240	0.47–30.00	$y = 15{,}452.83x + 1286.82$	1.0000	0.061	0.184
4	230	0.31–20.00	$y = 13{,}392.15x + 165.98$	1.0000	0.012	0.037
5	325	0.31–20.00	$y = 34{,}622.74x + 153.82$	1.0000	0.054	0.164
6	275	0.47–30.00	$y = 9752.57x + 486.57$	1.0000	0.005	0.017
7	275	0.31–20.00	$y = 6007.63x + 176.53$	1.0000	0.009	0.028
8	230	0.31–20.00	$y = 12{,}743.33x - 1091.63$	0.9999	0.057	0.173
9	300	0.31–20.00	$y = 24{,}286.05x + 859.92$	1.0000	0.025	0.074
10	230	0.31–20.00	$y = 15{,}281.10x - 925.73$	1.0000	0.051	0.155
11	275	0.31–20.00	$y = 15{,}640.67x + 491.83$	1.0000	0.005	0.015
12	275	0.31–20.00	$y = 28{,}890.59x + 763.74$	1.0000	0.011	0.032
13	320	0.31–20.00	$y = 76{,}887.30x + 2445.47$	1.0000	0.006	0.018
14	335	0.31–20.00	$y = 39{,}517.99x + 1130.43$	1.0000	0.008	0.025
15	290	0.31–20.00	$y = 24{,}810.62x + 623.99$	1.0000	0.031	0.095
16	230	0.31–20.00	$y = 66{,}259.50x + 1750.85$	1.0000	0.004	0.012
17	275	0.31–20.00	$y = 65{,}542.40x + 1891.13$	1.0000	0.005	0.014
18	275	0.31–20.00	$y = 108{,}376.64x + 3458.00$	1.0000	0.013	0.039
19	290	0.31–20.00	$y = 158{,}240.87x + 3787.46$	1.0000	0.005	0.016
20	250	0.31–20.00	$y = 8285.09x + 3641.95$	1.0000	0.058	0.176
21	280	0.31–20.00	$y = 14{,}330.97x + 305.25$	1.0000	0.006	0.019
22	290	0.31–20.00	$y = 22{,}822.38x + 385.08$	1.0000	0.023	0.070
23	330	0.31–20.00	$y = 50{,}159.85x + 796.04$	1.0000	0.016	0.049
24	330	0.31–20.00	$y = 33{,}400.18x + 668.55$	1.0000	0.013	0.038

[a] y and x indicate the peak area and concentration of each analyte, respectively; LOD [b] = $3.3 \times \sigma/S$ and LOQ [c] = $10 \times \sigma/S$ (where σ and S are the standard deviation of the y-intercept and the slope of the calibration curve, respectively).

Table 2. Recovery (%) of 24 marker analytes in the developed analysis method (n = 5).

Analyte	Spiked Conc. (μg/mL)	Measured Conc. (μg/mL)	Recovery (%) [a]	SD	RSD (%) [b]
	2.00	2.00	100.04	0.77	0.77
1	4.00	4.08	101.92	0.98	0.96
	8.00	7.94	99.19	1.12	1.13
	2.00	1.97	98.46	1.25	1.27
2	4.00	3.99	99.87	0.44	0.44
	8.00	7.78	97.27	0.51	0.52
	1.00	1.01	101.30	1.16	1.14
3	2.00	2.03	101.44	1.84	1.82
	4.00	4.07	101.82	1.55	1.52
	1.00	1.02	101.83	2.05	2.02
4	2.00	1.97	98.55	0.55	0.56
	4.00	4.02	100.41	0.92	0.92
	2.00	2.03	101.39	1.20	1.19
5	5.00	5.03	100.54	0.53	0.53
	10.00	9.96	99.59	0.93	0.93
	3.00	2.98	99.37	1.32	1.33
6	7.50	7.30	97.38	1.47	1.50
	15.00	14.48	96.52	2.34	2.43
	2.00	1.94	97.25	1.31	1.34
7	5.00	4.92	98.45	0.82	0.84
	10.00	10.08	100.78	1.49	1.47
	1.00	0.97	97.42	1.63	1.67
8	2.00	1.97	98.73	1.72	1.74
	4.00	3.86	96.49	1.16	1.20

Table 2. *Cont.*

Analyte	Spiked Conc. (μg/mL)	Measured Conc. (μg/mL)	Recovery (%) [a]	SD	RSD (%) [b]
	1.00	1.00	100.20	2.80	2.79
9	2.00	2.00	99.77	0.81	0.81
	4.00	4.05	101.34	0.96	0.95
	1.00	0.98	98.30	1.58	1.61
10	2.00	2.02	101.24	2.36	2.34
	4.00	3.99	99.78	0.80	0.80
	1.00	0.99	99.17	0.73	0.74
11	2.00	1.93	96.42	0.52	0.54
	4.00	4.03	100.75	1.24	1.23
	1.00	1.00	99.75	0.78	0.78
12	2.00	2.02	100.75	1.00	0.99
	4.00	4.05	101.22	0.88	0.87
	1.00	1.03	102.81	0.75	0.73
13	2.00	1.96	97.79	0.50	0.51
	4.00	3.90	97.55	0.84	0.86
	2.00	2.02	100.97	1.17	1.16
14	5.00	5.05	100.95	1.46	1.44
	10.00	10.15	101.53	0.82	0.81
	1.00	0.99	99.32	2.67	2.69
15	2.00	1.99	99.27	1.24	1.25
	4.00	4.01	100.20	1.05	1.05
	1.00	0.96	95.92	0.79	0.83
16	2.00	1.92	96.04	0.96	0.99
	4.00	3.86	96.57	1.31	1.35
	1.00	0.96	96.49	0.81	0.84
17	2.00	1.92	96.03	0.54	0.56
	4.00	3.98	99.43	0.36	0.36
	1.00	0.96	96.28	0.34	0.36
18	2.00	1.91	95.68	0.71	0.75
	4.00	3.84	95.90	0.24	0.25
	1.00	1.02	101.96	0.10	0.10
19	2.00	1.92	96.01	0.45	0.47
	4.00	3.90	97.55	0.46	0.47
	1.00	1.02	101.91	0.78	0.77
20	2.00	2.00	100.05	0.66	0.66
	4.00	4.01	100.35	1.47	1.47
	1.00	0.96	95.68	0.49	0.51
21	2.00	1.97	98.62	0.33	0.34
	4.00	3.95	98.70	0.25	0.26
	1.00	0.96	95.77	1.01	1.05
22	2.00	1.91	95.47	0.54	0.56
	4.00	3.87	96.83	0.41	0.42
	1.00	0.98	98.10	1.93	1.97
23	2.00	1.97	98.32	0.32	0.32
	4.00	4.09	102.16	0.22	0.21
	1.00	0.98	97.75	0.95	0.97
24	2.00	1.95	97.69	0.41	0.42
	4.00	4.02	100.52	0.29	0.29

Recovery (%) [a] = measured concentration/spiked concentration × 100%; RSD (%) [b] = standard deviation (SD)/mean × 100%.

Table 3. Precision and accuracy of the developed analysis method using 24 markers.

Analyte	Conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)		
		Measured Conc. (μg/mL)	Precision (RSD, %) [a]	Accuracy (%)	Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)
1	5.00	4.88	0.46	97.64	4.87	0.53	97.38
	10.00	9.72	0.44	97.23	9.68	0.48	96.83
	20.00	19.50	0.19	97.51	19.49	0.32	97.44
2	5.00	4.97	0.47	99.42	4.96	0.48	99.24
	10.00	9.85	0.73	98.52	9.79	1.24	97.90
	20.00	19.79	0.39	98.93	19.65	0.57	98.27
3	7.50	7.52	0.14	100.29	7.58	1.19	101.05
	15.00	14.97	0.25	99.81	15.07	0.99	100.43
	30.00	29.96	0.79	99.88	30.04	0.80	100.14
4	5.00	5.03	0.20	100.65	5.05	0.81	101.03
	10.00	9.96	0.25	99.64	9.99	0.58	99.93
	20.00	19.83	0.18	99.17	19.98	0.84	99.92
5	7.50	7.26	0.70	96.78	7.19	0.87	95.17
	15.00	14.46	0.73	96.38	14.30	0.95	95.32
	30.00	29.01	0.53	96.71	28.80	0.62	96.00
6	7.50	7.48	0.18	99.78	7.55	1.20	100.04
	15.00	14.86	0.20	99.08	14.96	0.83	99.75
	30.00	29.63	0.19	98.78	29.88	0.90	99.61
7	5.00	4.99	0.35	99.75	5.03	1.16	100.55
	10.00	9.90	0.23	98.97	9.96	0.87	99.60
	20.00	19.75	0.10	98.76	19.89	0.77	99.45
8	5.00	4.86	0.37	97.13	4.90	1.10	98.00
	10.00	10.00	0.32	99.98	9.86	1.53	98.55
	20.00	19.71	0.40	98.56	19.89	0.93	99.43
9	5.00	5.00	0.32	100.01	5.04	1.20	100.75
	10.00	9.93	0.27	99.31	10.00	0.86	99.98
	20.00	19.80	0.22	99.01	19.95	0.84	99.77
10	5.00	4.97	0.85	99.50	5.02	1.09	100.32
	10.00	9.91	0.62	99.05	9.95	0.62	99.50
	20.00	19.83	0.63	99.13	19.95	1.02	99.73
11	5.00	4.98	0.14	99.68	5.02	1.04	100.26
	10.00	9.90	0.22	98.99	9.96	0.75	99.43
	20.00	19.74	0.22	98.69	19.95	0.84	99.38
12	5.00	4.99	0.11	99.81	5.03	1.12	100.50
	10.00	9.91	0.19	99.14	9.97	0.79	99.56
	20.00	19.76	0.17	98.80	19.92	0.86	99.43
13	5.00	4.97	0.14	99.49	5.01	1.14	100.66
	10.00	9.89	0.18	98.90	9.94	0.78	99.78
	20.00	19.73	0.20	98.64	19.88	0.84	99.57
14	5.00	4.98	0.21	99.65	5.03	1.42	100.70
	10.00	9.90	0.22	98.99	9.96	1.09	100.51
	20.00	19.74	0.15	98.68	19.89	0.55	100.93
15	5.00	4.99	0.10	99.83	5.03	1.18	100.70
	10.00	9.92	0.16	99.20	9.98	0.92	99.89
	20.00	19.76	0.20	98.78	19.91	1.00	99.63
16	5.00	4.97	0.28	99.47	5.04	1.17	100.55
	10.00	9.95	0.20	99.50	10.05	0.86	99.67
	20.00	20.13	0.33	100.66	20.19	0.92	99.54
17	5.00	4.99	0.12	99.70	5.04	1.14	100.71
	10.00	9.90	0.18	99.04	9.99	0.87	100.09
	20.00	19.74	0.21	98.72	19.93	0.96	99.79
18	5.00	4.98	0.12	99.63	5.03	1.07	99.22
	10.00	9.90	0.17	98.98	9.97	0.72	100.23
	20.00	19.74	0.18	98.72	19.91	0.56	99.86

Table 3. *Cont.*

Analyte	Conc. (μg/mL)	Intraday (n = 5)			Interday (n = 5)		
		Measured Conc. (μg/mL)	Precision (RSD, %) [a]	Accuracy (%)	Measured Conc. (μg/mL)	Precision (RSD, %)	Accuracy (%)
19	5.00	4.99	0.14	99.76	5.04	1.20	100.82
	10.00	9.93	0.13	99.28	10.01	0.94	99.97
	20.00	19.79	0.22	98.94	19.96	0.97	99.74
20	5.00	4.95	0.37	99.09	4.96	0.90	101.19
	10.00	10.04	0.42	100.44	10.02	0.63	100.45
	20.00	19.89	0.24	99.47	19.97	0.72	100.29
21	5.00	4.99	0.29	99.86	5.04	1.11	100.60
	10.00	9.91	0.22	99.14	10.00	0.82	99.64
	20.00	19.78	0.27	98.90	19.95	0.89	99.44
22	5.00	5.03	0.18	100.58	5.06	1.12	100.48
	10.00	10.00	0.10	99.99	10.05	0.80	99.55
	20.00	19.94	0.23	99.72	20.06	0.87	99.42
23	5.00	4.99	0.17	99.75	5.03	1.04	100.26
	10.00	9.90	0.21	99.02	9.96	0.75	99.43
	20.00	19.73	0.18	98.66	19.89	0.84	99.38
24	5.00	4.98	0.19	99.62	5.02	1.12	100.50
	10.00	9.90	0.19	98.96	9.96	0.79	99.56
	20.00	19.73	0.17	98.64	19.88	0.86	99.43

[a] RSD (%) = standard deviation (SD)/mean × 100%.

3.4. Simultaneous Determination of 24 Markers Components for Quality Assessment of DHGST Sample

The established HPLC analysis method was successfully applied to the simultaneous determination of DHGST components for quality assessment. Simultaneous analysis of DHGST using the established assays showed that compounds **1–24** were present in 0.23–14.68 mg/g of freeze-dried sample (Table 4).

Table 4. Content of the 24 marker analytes in the DHGST sample determined using HPLC (n = 3).

Analyte	Content (mg/g Freeze-Dried Sample)						Source [a]
	Batch 1		Batch 2		Batch 3		
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%, $\times 10^{-1}$)	
1	0.70	0.20	0.71	0.28	0.70	0.02	PL, TC
2	0.61	0.30	0.61	0.22	0.61	2.29	RG
3	2.91	0.40	2.99	0.25	2.96	3.61	EU
4	2.73	0.22	2.79	0.25	2.74	0.89	GS
5	0.94	1.22	0.95	0.91	0.95	6.23	EU
6	14.29	0.19	14.68	0.84	14.48	7.08	GS
7	9.81	0.26	9.69	0.44	9.70	5.92	EU
8	3.13	0.28	3.28	1.16	3.28	24.73	PL
9	0.30	1.00	0.30	0.22	0.30	5.05	SD
10	4.68	0.30	4.77	0.28	4.72	2.19	PL
11	1.25	0.51	1.27	0.22	1.25	5.23	GU
12	0.24	1.26	0.23	0.99	0.23	5.14	GU
13	0.23	0.98	0.23	0.09	0.23	1.75	CO
14	0.90	0.23	0.90	0.29	0.90	3.48	AG
15	0.26	0.31	0.26	0.91	0.26	1.66	SD
16	0.52	0.47	0.52	0.28	0.52	0.91	PL
17	0.39	0.57	0.39	0.50	0.39	0.74	CC
18	0.60	0.40	0.60	0.31	0.60	1.80	CC
19	1.36	0.50	1.38	0.48	1.36	4.64	CC
20	2.25	0.44	2.28	1.27	2.26	7.34	GU

Table 4. *Cont.*

Analyte	Content (mg/g Freeze-Dried Sample)						Source [a]
	Batch 1		Batch 2		Batch 3		
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%, $\times 10^{-1}$)	
21	1.38	0.30	1.39	0.82	1.39	5.41	AH
22	0.50	1.16	0.50	0.63	0.50	9.28	AH
23	0.25	0.20	0.25	0.01	0.25	3.45	AG
24	0.24	0.23	0.25	0.11	0.24	1.48	AG

[a] PL, *P. lactiflora*; TC, *T. chinensis*; RG, *R. glutinosa*; EU, *E. ulmoides*; GS, *G. straminea*; SD, *S. divaricate*; GU, *G. uralensis*; CO, *C. officinale*; AG, *A. gigas*; CC, *C. cassia*; and AH, *A. heterotropoides*.

4. Conclusions

A simultaneous HPLC analysis method for quality assessment of DHGST, a traditional herbal medicine prescription that has been used for arthritis and neuritis for a long time, was developed. The method was validated with respect to linearity, LOD, LOQ, recovery, and precision. As a result of these investigations, components of *G. straminea*, *E. ulmoides*, and *P. lactiflora* in the DHGST sample, such as gentiopicroside (**6**, 14.29–14.68 mg/g), pinoresinol diglucoside (**7**, 9.69–9.81 mg/g), and paeoniflorin (**10**, 4.68–4.77 mg/g), were found in the highest amounts. The developed and validated method is suitable for use in the quality assessment of DHGST or other herbal medicine prescriptions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11177829/s1, Figure S1: Chemical structures of the 24 marker components in DHGST; Figure S2: HPLC chromatogram of the herbal medicine extract and its main components. (A): *A. continentalis*; (B): *A. gigas*; (C), *P. lactiflora*; (D), *T. chinensis*; (E), *R. glutinosa*; (F), *C. officinale*; (G), *P. ginseng*; (H), *P. cocos*; (I), *A. bidentata*; (J), *E. ulmoides*; (K), *G. straminea*; (L), *A. heterotropoides*; (M), *S. divaricate*; (N), *C. cassia*; (O), *G. uralensis*; and (P), *Z. officinale*; Figure S3: HPLC chromatograms of the standard solution (A) and 70% methanol of DHGST sample (B). Gallic acid (**1**), 5-hydroxymethylfurfural (**2**), geniposidic acid (**3**), loganic acid (**4**), cimufugin (**5**), chlorogenic acid (**6**), geniposide (**7**), gentiopicroside (**8**), pinoresinol diglucoside (**9**), albiflorin (**10**), *prim-O*-glucosylcimifugin (**11**), paeoniflorin (**12**), genipin (**13**), liquiritin apioside (**14**), liquiritin (**15**), ecdysterone (**16**), ferulic acid (**17**), nodakenin (**18**), 5-*O*-methylvisammioside (**19**), quercitrin (**20**), benzoic acid (**21**), ginsenoside Rg1 (**22**), coumarin (**23**), cinnamic acid (**24**), benzoylpaeoniflorin (**25**), ginsenoside Rb1 (**26**), cinnamaldehyde (**27**), glycyrrhizin (**28**), 6-gingerol (**29**), aristolochic acid II (**30**), aristolochic acid I (**31**), β-asarone (**32**), methyleugenol (**33**), α-asarone (**34**), safrole (**35**), decursin (**36**), decursinol angelate (**37**), pachymic acid (**38**), continentalic acid (**39**), and kaurenoic acid (**40**); Table S1: Information and composition of DHGST; Table S2: HPLC operating conditions for simultaneous quantification of the 24 marker components in DHGST; Table S3: System suitability for the analysis of the 24 marker compounds with the developed HPLC method; Table S4: Repeatability of retention time of the 24 marker analytes using HPLC (n = 6); Table S5: Repeatability of peak area of the 24 marker analytes by HPLC (n = 6).

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