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The Development and Validation of Simultaneous Multi-Component Quantitative Analysis via HPLC–PDA Detection of 12 Secondary Metabolites Isolated from Drynariae Rhizoma

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Abstract: Drynariae Rhizoma (DR) is a functional food and traditional medicine that has been widely used for bone and joint disorders for thousands of years. In this study, 14 compounds were isolated from DR, and their structures were identified using UPLC/QTOF–MS, UPLC–ESI/LTQ–Orbitrap–HRMS, and 2D NMR and compared with those obtained in previous studies. An HPLC–PDA multi-component simultaneous quantitative determination method was developed for 12 of the 14 DR-derived compounds, excluding compounds with a content <1.5 mg. The developed HPLC method was validated based on linearity ($r^2 \geq 0.999$), limit of detection (0.01–0.65 μg/mL), limit of quantification (0.04–1.97 μg/mL), intra-day precision and accuracy ranges (0.06–2.85% and 95.03–104.75%, respectively), and inter-day precision and accuracy ranges (0.24–2.83% and 95.75–105.75%, respectively). The developed analysis method improved the resolution of compounds **4** and **5**. In addition, this is the first quantitative analysis of compounds **7**, **8**, and **11** and the first simultaneous quantitative analysis of 12 compounds, including compounds **4**, **7**, **8**, **10**, **11**, and **14**. This study developed a rapid, accurate, and economical HPLC method for performing the simultaneous quantitative analysis of 12 secondary metabolites isolated from DR.

Keywords: Drynariae Rhizoma; *Drynaria fortunei* J. Smith; isolation; open column chromatography; HPLC–PDA; multi-component simultaneous quantitative analysis

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1. Introduction

Drynariae Rhizoma (DR) belongs to the Polypodiaceae family, and only *Drynaria fortunei* J. Smith (*D. fortunei*) is recognized by Chinese and Korean pharmacopeias [1,2]. DR is a herb that has widely been used in folk medicine for thousands of years [3]. Research has demonstrated that DR is rich in phenols, such as naringin, neoeriocitrin, and 5,7-dihydroxychromone-7-O-neohesperidoside, and contains phenylpropanoids, such as caffeic acid 4-O-β-d-glucoside and coumaric acid 4-O-β-d-glucoside [4]. Naringin, the main component of DR, possesses antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective properties [5]. Neoeriocitrin has been studied for its osteoblast proliferative, anti-diabetic, and Alzheimer's disease activity [6–8]. The phenolic compound 5,7-dihydroxychromone-7-O-neohesperidoside has been examined for its osteoblast proliferative activity [9]. Coumaric acid 4-O-β-d-glucoside has been suggested as exerting potential preventive and therapeutic effects against inflammatory diseases [10,11]. Caffeic acid 4-O-β-d-glucoside demonstrates notable effects on axonal elongation in cultured cortical neurons of mice subjected to Aβ25–35-induced axonal atrophy [6]. In addition, compounds isolated from DR have been reported as possessing beneficial biological activity, and the separation and quantitative analysis of these key components are considerably useful in evaluating the efficacy and quality of DR. In addition, DR continuously encounters

issues of adulteration and counterfeiting by other species, and research into systematic quality control and evaluation is urgently required [12,13].

High-performance liquid chromatography (HPLC) has been used in the quality control of natural products [14]. It is a chromatographic technique that can be used to analyze plant secondary metabolites, which are the main bioactive substances in natural products, and is used in phytochemistry and analytical chemistry to effectively separate, identify, quantify, and purify unique components within a mixture of compounds [15]. In 2012, Haitao Liu et al. conducted a comprehensive quality evaluation of DR by combining HPLC multi-component determination and fingerprint analysis to analyze the compounds naringin, neoeriocitrin, caffeic acid-4-O-β-D-glucoside, and 5,7-dihydroxychromone-7-O-rutinoside [16]. In 2018, Huang et al. used HPLC to analyze the naringin, neoeriocitrin, protocatechuic acid, and caffeic acid-4-O-β-D-glucoside contents of DR in a study on the angiogenic properties of DR [17]. Yang et al. (2015), in an investigation of potential treatment options for Alzheimer's disease, isolated naringin, neoeriocitrin, 5,7-dihydroxychromone-7-O-neohesperidoside, caffeic acid-4-O-β-D-glucoside, and protocatechuic acid from DR and performed HPLC qualitative analysis [6]. In a metabolite profiling study of DR, Dong et al. (2023) performed an HPLC-based quantitative analysis of seven phenolic compounds, including naringin, neoeriocitrin, 5,7-dihydroxychromone-7-O-neohesperidoside, caffeic acid 4-O-β-d-glucoside, and coumaric acid 4-O-β-d-glucoside, which are major secondary metabolites of *D. fortunei* [4]. To date, previous HPLC quantitative analyses of DR have only simultaneously analyzed five or fewer compounds and have not secured reliability through validation; moreover, some multi-component simultaneous analyses have produced incomplete chromatograms.

As natural products comprise different classes of compounds with complex synergistic interactions, quality control criteria need to be established and standardized through the profiling of the various key bioactive compounds to ensure the safety and quality of natural products [18,19]. In particular, DR is a natural product that has issues with impurities other than *D. fortunei*, and preparing quality control standards based on integrated and diverse compounds is necessary.

In this study, we isolated the main bioactive substances from validated DR. Extracted crude drugs and fractions were selected by measuring total polyphenol content, total flavonoid content, and antioxidant activity using a bioassay guide method [20]. Among the selected fractions, 14 compounds were isolated via open column chromatography using octadecyl silica gel, Sephadex LH-20 resin, and MCI CHP 20P gel. The isolated compounds were structurally identified using mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses and compared with those obtained in previous studies. An HPLC multi-component simultaneous quantitative analysis method was developed for 12 of the 14 separated and identified compounds, excluding compounds with an isolated content <1.5 mg, and validated based on linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, repeatability, and recovery. The developed HPLC method was used to analyze the content of 12 compounds in 15 DR samples in circulation. The HPLC multi-component simultaneous quantitative analysis method for 12 major DR-derived compounds developed in this study is an economical, rapid, and reliable evaluation method. It is anticipated to contribute to various fields, such as the formulation of DR quality control standards as well as quality evaluation.

2. Materials and Methods

2.1. Reagents and Instruments

An analytical grade solvent acquired from Samchun Pure Chemical (Pyeongtaek, Gyeonggi, Republic of Korea), was used in the extraction, fractionation, and open column chromatography processes. Octadecyl silica gel (ODS), Sephadex LH-20 resin (SLH-20), and MCI CHP 20P gel (MCI) obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) were used for open column chromatography. The solvents used in NMR analysis, namely, chloroform-d (CDCl3-d), dimethyl sulfoxide-d6 (DMSO-d6), and methanol-d4 (CD3OD-

d4), were purchased from Sigma-Aldrich Co., USA. HPLC solvents, namely, acetonitrile and distilled water, were obtained from J. T. Baker® (Phillipsburg, PA, USA). Ammonium chloride, sodium nitrite, gallic acid, and catechin were purchased from Sigma-Aldrich Co., USA. Other chemicals and solvents used were of analytical grade.

2.2. Plant Materials

The DR samples included nine samples purchased from Gyeongdong Market, Seoul, Republic of Korea, and six samples collected in China. The collected samples were confirmed via genetic analysis in collaboration with the Ministry of Food and Drug Safety (Osong, Cheongju, Chungcheongbuk, Republic of Korea), and certified by Professor Wan Kyunn Whang (Department of Pharmacy, Chung-Ang University, Dongjak, Seoul, Republic of Korea). Plants identified as *Drynaria fortunei* J. Smith through the verification process were stored as specimens in the Laboratory of Pharmaceutical Resources and Plant Sciences, College of Pharmacy, Chung-Ang University, Dongjak, Seoul, Republic of Korea. Each sample's classification is provided in Table 1.

Table 1. List of Drynariae Rhizoma samples.

Sample	Species	Native	Note
S1	*Drynaria fortunei*	Hubei, China	Collected
S2	*Drynaria fortunei*	Guizhou, China	Collected
S3, S4, S5, S6	*Drynaria fortunei*	Hunan, China	Collected
S7, S8, S9, S10	*Drynaria mollis*	Myanmar	Market-purchased
S11	*Drynaria mollis*	China	Market-purchased
S12	*Drynaria mollis*	China	Market-purchased
S13, S14, S15	*Davallia mariesii*	Republic of Korea	Market-purchased

2.3. Material Evaluation

Of the 15 DR samples, 6 samples identified as *D. fortunei* via genetic analysis were extracted using 80% methanol (MeOH), powdered, and dissolved in 10% DMSO at an appropriate concentration for use as material evaluation samples. Additionally, during material evaluation, the DR sample with the highest total polyphenol content (TPC) and total flavonoid content (TFC) and excellent antioxidant activity was partitioned into dichloromethane (DCM), ethyl acetate (EtOAc), n-butanol (BuOH), and water fractions. The four fractions were powdered, dissolved in 10% DMSO at an appropriate concentration, and used as material evaluation samples.

2.3.1. Determination of TPC and TFC

TPC and TFC were analyzed using a slight modification of the method provided by Ku et al. [21]. TPC assay conditions were as follows: the sample or gallic acid standard solution (20 µL) was added to each well of a 96-well plate. Standard solution final concentrations ranged from 5 to 100 µg/mL. Thereafter, 100 µL of 0.2 N Folin–Ciocalteu reagent was added to each well. After 3 min, 7.5% sodium carbonate solution (8 µL) was added to the mixture, followed by incubation at room temperature for 1 h. The absorbance of the resulting mixture was measured at 750 nm using a spectrophotometer (BioTek Epoch 2, Winooski, VT, USA). The results are expressed in milligrams of gallic acid equivalent (GAE) per gram. Experiments were conducted in triplicate.

TFC assay conditions were as follows: the sample or catechin standard solution (20 µL) was added to each well of a 96-well plate. Standard solution final concentrations ranged from 3 to 50 µg/mL. Subsequently, 40 µL of distilled water and 6 µL of 5% (w/v) sodium nitrite were added to each well. After 5 min, 10% (w/v) aluminum chloride (12 µL) was added. After 6 min, 40 µL of 1 M sodium hydroxide was added to the mixture, followed by 42 µL of distilled water. The absorbance of the resulting mixture was measured at 151 nm using a spectrophotometer (BioTek Epoch 2, Winooski, VT, USA). The results are expressed

in milligrams of (+)–catechin equivalent (CE) per gram. Experiments were performed in triplicate. The TPC and TFC content results of the samples are shown in Table 2.

Table 2. Material evaluation results.

Sample	Total Content		Antioxidant Activity Assay		
	TPC (mg GAE [1]/g)	TFC (mg CE [2]/g)	ABTS	DPPH IC50 (μg/mL)	FRAP
GA [3]	-	-	1.74 ± 0.01	2.75 ± 0.02	0.34 ± 0.00
1	161.94 ± 2.20	545.89 ± 5.67	432.50 ± 8.46	253.30 ± 3.66	-
2	160.36 ± 1.38	38.21 ± 0.17	224.85 ± 7.94	310.18 ± 2.71	162.41 ± 2.30
3	213.19 ± 1.25	52.17 ± 0.13	151.58 ± 4.53	287.26 ± 7.14	114.34 ± 1.67
4	222.95 ± 0.75	54.75 ± 0.00	108.46 ± 4.75	156.13 ± 4.33	94.21 ± 2.28
5	193.49 ± 2.20	46.96 ± 0.39	151.65 ± 3.18	282.52 ± 5.10	133.54 ± 2.54
6	233.42 ± 0.89	57.51 ± 0.04	99.50 ± 2.20	154.77 ± 2.98	88.03 ± 2.60
DCM [4]	22.95 ± 0.38	0.90 ± 0.09	1816.61 ± 35.17	3158.47 ± 82.69	940.84 ± 7.27
EtOAc [5]	483.31 ± 3.31	100.30 ± 0.41	38.62 ± 1.27	79.98 ± 2.41	37.93 ± 1.54
BuOH [6]	411.09 ± 0.07	119.38 ± 0.48	81.77 ± 2.38	117.44 ± 1.99	69.78 ± 0.93
Water [7]	44.06 ± 0.82	3.32 ± 0.18	293.50 ± 7.78	582.39 ± 7.24	435.28 ± 8.16

[1] GAE, gallic acid equivalent; [2] CE, catechin equivalent; [3] GA, gallic acid; [4] DCM., dichloromethane fraction of Sample 6; [5] EtOAc, ethyl acetate fraction of Sample 6; [6] BuOH, n-butanol fraction of Sample 6; [7] Water, water fraction of Sample 6.

2.3.2. Antioxidant Activity Assay

Antioxidant activity was evaluated using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging, and ferric reducing/antioxidant power (FRAP) assays.

ABTS analysis of the samples was performed using a protocol published by Re et al. [22], with minor modifications. Briefly, 7.4 mM ABTS ammonium was dissolved in potassium phosphate buffer (pH 7.4) and treated with 2.5 mM potassium persulfate. The mixture was subsequently left at room temperature for 15–24 h until it turned dark blue. Before use, the ABTS+ solution was diluted with methanol, and the absorbance at 732 nm was 1.0–1.2 units, as measured using a spectrophotometer. Thereafter, 10 μL of the sample or gallic acid standard solution was added to the ABTS+ radical solution (190 μL) in a 96-well plate. After 5 min of incubation, absorbance was measured at 732 nm using a spectrophotometer (BioTek Epoch 2, Winooski, VT, USA).

The DPPH assay was performed on the samples according to the protocol described by Hatano et al. [23], with minor modifications. Briefly, 12 μL of the sample or gallic acid standard solution was added to each well of a 96-well plate. Subsequently, 188 μL of 0.2 mM DPPH EtOH solution was added to each well. The mixture was shaken for 10 s and incubated for 25 min at room temperature. The absorbance of the resulting mixture was measured at 517 nm using a spectrophotometer (BioTek Epoch 2, Winooski, VT, USA).

The FRAP assay was performed on the samples according to the instructions published by Benzie et al. [24], with minor modifications. The FRAP reagent was prepared by (1) adding glacial acetic acid to 0.3 M sodium acetate to produce a buffer of pH 3.6, (2) dissolving 2,4,6-tripyridyl triazine in 40 mM hydrogen chloride to produce a 10 mM solution, and (3) adding 20 mM iron (III) chloride hexahydrate. The solvents prepared in this manner were mixed in a 10:1:1 (*v*:*v*:*v*) ratio. For FRAP assay measurements, 10 μL of the sample or gallic acid standard solution was added to each well of a 96-well plate, followed by 290 μL of FRAP reagent. The mixture was shaken for 10 s and incubated for 8 min at room temperature. The absorbance of the resulting mixture was measured at 594 nm using a spectrophotometer (BioTek Epoch 2, Winooski, VT, USA).

The ABTS, DPPH, and FRAP assays were conducted in triplicate. The content of each sample is expressed in terms of the microliter concentration required to inhibit radical formation by 50% (IC50), calculated from the inhibition curve. Inhibitory activity was calculated using the following formula: inhibition rate (%) = AC − AS/AC × 100, where

AS and AC are the absorbance values of the sample and negative control, respectively. Data are expressed as the mean ± standard deviation (SD). The antioxidant assay results of the samples are shown in Table 2.

2.4. Extraction and Fractionation

2.4.1. Extraction

In the material evaluation, Sample 6 (700 g), which had the highest TPC and TFC and exhibited excellent antioxidant activity, was dried with powder and extracted using 80% MeOH (10 L × 6) at room temperature. The extract was filtered, concentrated to dryness in vacuo (103.03 g), suspended in water, and partitioned into dichloromethane (DCM), ethyl acetate (EtOAc), butanol (BuOH), and water fractions. As a result, DCM (21.72 g), EtOAc (5.86 g), BuOH (11.92 g), and water (31.13 g) fractions were derived. Among these four fractions, the EtOAc and BuOH fractions had the highest TPC and TFC and displayed excellent antioxidant capacity according to the material evaluation. Therefore, open column chromatography (CC) of the EtOAc and BuOH fractions was repeated.

2.4.2. Isolation of Compounds from the EtOAc Fraction

The EtOAc fraction (5.00 g) was isolated using a SLH-20 column with an elution gradient range of 5–30% EtOH, yielding two sub-fractions (EtOAc 1 and EtOAc 2). Sub-fraction EtOAc 1 underwent SLH-20 CC with 5–10% EtOH, resulting in three sub-fractions (EtOAc 1-1, 1-2, and 1-3). The EtOAc 1-1 sub-fraction was subjected to SLH-20 CC with 5% EtOH, undergoing two repetitions to isolate compound **1** (1.3 mg). Additionally, sub-fraction EtOAc 1-2 underwent SLH-20 CC twice to obtain compound **2** (3.2 mg). Subjection of sub-fraction EtOAc 1-3 to SLH-20 CC with 7% EtOH three times resulted in the isolation of compound **3** (2.1 mg). Similarly, sub-fraction EtOAc 2 was subjected to SLH-20 CC with 7% EtOH three times to obtain compound **6** (1.1 mg).

2.4.3. Isolation of Compounds from the BuOH Fraction

The BuOH fraction (10.00 g) underwent ODS CC with 5–45% MeOH, yielding six sub-fractions (BuOH 1 to BuOH 6). Sub-fraction BuOH 1 underwent SLH-20 CC with 5–10% EtOH, resulting in three sub-fractions (BuOH 1-1, 1-2, and 1-3). Sub-fraction BuOH 1-1 was applied to a SLH-20 column with 7% EtOH to isolate compound **4** (6.5 mg). Additionally, BuOH 1-2 underwent SLH-20 CC with 7% EtOH and MCI CC with 10% MeOH to obtain compound **5** (11.4 mg). BuOH 1-3 was subjected to SLH-20 CC with 10% EtOH and MCI CC with 15% MeOH to obtain compound **7** (5.6 mg). BuOH 2 underwent SLH-20 CC with 15% EtOH three times and MCI CC with 15% MeOH to obtain compound **8** (5.1 mg). BuOH 3 was subjected to SLH-20 CC with 15% EtOH and MCI CC with 15% MeOH to obtain compound **9** (4.2 mg). BuOH 4 was applied to a SLH-20 column with 20% EtOH and MCI CC with 20% MeOH to obtain compound **10** (8.2 mg). BuOH 5 underwent SLH-20 CC with 25% EtOH three times, resulting in sub-fractions BuOH 5-1 and BuOH 5-2. BuOH 5-1 underwent SLH-20 CC with 30% EtOH and MCI CC with 40% EtOH to yield compound **11** (4.5 mg). BuOH 5-2 was applied to SLH-20 and MCI columns with 40% EtOH to yield compound **12** (18.7 mg). BuOH 6 was applied to a SLH-20 column with 40% EtOH, producing sub-fractions BuOH 6-1 and BuOH 6-2. BuOH 6-1 underwent SLH-20 CC with 40% EtOH to obtain compound **13** (24.7 mg). BuOH 6-2 was applied to SLH-20 and MCI columns with 45% EtOH to yield compound **14** (1.8 mg).

2.5. Compound Identification

2.5.1. NMR Spectroscopy

The recorded ^{1}H- and ^{13}C-NMR spectra, utilizing a JEOL spectrometer (JEOL, Tokyo, Japan), were obtained at 600 and 150 MHz, respectively. Chemical shifts are reported on the δ scale in parts per million (ppm), and coupling constants (J) are expressed in Hertz (Hz). Each isolated compounds **1**–**14** was weighed (1–7 mg) and dissolved in 700 μL of each solvent. DMSO-d6, CD3OD-d4, and CDCl3-d were used to dissolve samples for NMR analysis.

2.5.2. Mass Spectrometry

Confirmation of the molecular weights of compounds from the 80% MeOH extraction of Sample 6 was achieved using ultra-high performance liquid chromatography–electrospray ionization/quadrupole time-of-flight mass spectrometry (UPLC–ESI/QTOF–MS) (Waters, Milford, MA, USA). The Sample 6 extract was dissolved in 80% MeOH. The column (Acquity BEH C18, 2.1 × 100 mm, 1.7 μm (Waters, Milford, MA, USA)) and sample temperatures were set at 30 and 15 °C, respectively. The mobile phase comprised solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient conditions included the following intervals: 0–5 min, 5–30% B; 5–8 min, 30–60% B; 8–10 min, 60% B; and 10–10.5 min, 60–5% B. The flow rate was maintained at 0.3 mL/min, with a sample injection volume of 5.0 μL. The optimized analysis conditions were as follows: spray capillary voltage of 2.5 kV; source and desolvation temperatures of 120 and 500 °C, respectively; cone and desolvation gas flow rates of 0 and 700 L/h, respectively; acquisition range of m/z 50–1200; and collision energy ramping range of 20–40 V.

The molecular weights of compounds **1**–**14** isolated from Sample 6 were further validated using high-performance liquid chromatography–electrospray ionization/linear ion trap quadrupole–Orbitrap-high-resolution mass spectrometry (UPLC–ESI/LTQ–Orbitrap-HRMS) (Thermo, Darmstadt, Germany). All samples were dissolved in 80% MeOH distilled water. The column (Agilent ZORBAX SB C18, 2.1 × 50 mm, 1.8 μm (Agilent Technologies, Santa Clara, CA, USA)) and sample temperatures were set at 30 and 15 °C, respectively. The mobile phase comprised solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient conditions were as follows: 0–18 min, 5–50% B, and 18–23 min, 50–100% B. The flow rate was maintained at 0.3 mL/min, with sample injection volumes of 5.0 and 2.0 μL for the standard and extract solutions, respectively. Optimal analysis conditions included a spray capillary voltage of 3.0 kV, S-lens RF level of 50.0 V, capillary temperature of 360 °C, heater temperature of 300 °C, sheath gas flow rate of 45 L/h, auxiliary gas flow rate of 10 L/h, full MS resolution of 35,000 (full width at half maximum of m/z 200), full MS automatic gain control target of 3e6, and full MS maximum IT of 200 ms.

2.6. HPLC Analysis

2.6.1. Preparation of Standard Solution

Among the 14 compounds isolated from Sample 6, compounds **1** and **6**, each having a mass <1.5 mg, were considered to have insufficient content in DR and were excluded from the main compounds. Therefore, compounds **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, and **14** were used as standard solutions. For HPLC analysis, standard solutions were prepared by diluting each of the 12 selected compounds in 80% methanol. The dilutions were strategically adjusted to achieve a concentration range of 3.9–83.3 μg/mL, categorized into five specific concentrations. Subsequently, the solutions were refined through a 0.45-μm polyvinylidene fluoride (PVDF) membrane filter. All working solutions were then stored at 4 °C until use.

2.6.2. Preparation of Sample Solution

Each of the 15 samples from the six species of *Drynaria fortunei* J. Smith, six species of *Drynaria mollis* Bedd, and three species of *Davallia mariesii* T. Moore ex Baker was ground with a grinder, and 5 g of sample powder was subsequently extracted in an ultrasonic bath for 90 min by adding 50.0 mL of 80% methanol. Thereafter, all extracted samples were concentrated under reduced pressure and dissolved in 80% MeOH to achieve a final concentration of 5500 μg/mL before usage. All analyzed solutions were filtered using a 0.45-μm PVDF syringe filter. For the analysis, 10 μL of each sample solution was injected into the HPLC system.

2.6.3. HPLC Analysis Method Development

For the simultaneous quantification of 12 compounds, screening was conducted under ultraviolet (UV) radiation (210–400 nm) using HPLC coupled with a photodiode array detector (HPLC–PDA). The HPLC–PDA analysis utilized Empower Pro 2.0 software, and compounds were determined using the following equipment: a Waters 996 photodiode array detector, Waters 2695 system pump, Waters 717 plus auto-sampler, and column oven (Waters, Milford, MA, USA). The separation column employed was a Develosil RPAQUEOUS, 4.6 × 250 mm, 5 μm (Nomura Chemical Co., Ltd., Seto, Japan), maintained at 30 °C. The mobile phase comprised 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 1.0 mL/min. Linear gradient elution was employed based on the following conditions: 0–30 min, 85% A; 30–40 min, 70% A; and 40–50 min, 50% A, for column equilibration. All eluents underwent filtration using a 0.45-μm PVDF syringe filter. The injection volume was set to 10 μL, and 280 nm was selected as the optimal wavelength for UV detection.

2.6.4. Validation of HPLC Analysis

Validation of the developed HPLC method encompassed various evaluation criteria, including linearity, LOD, LOQ, intra- and inter-day accuracy and precision, repeatability, and recovery. All procedures were executed in triplicate.

Specificity

Each compound was distinguished from the others in the standard and sample solutions. In particular, compounds **4** and **5** were hydroxycinnamic acids and exhibited similar chemical structures and molecular weights. In previous studies, HPLC peak aggregation and overlapping of these two compounds were confirmed by HPLC–PDA analysis, and in this study, specificity was increased by improving HPLC peak aggregation and overlap using an ODS C30 column (Develosil RPAQUEOUS, 4.6 × 250 mm, 5 μm (Nomura Chemical Co., Ltd., Seto, Japan)) [6,16]. For verification, the ODS C30 column was used in this study for comparison with the ODS C18 column (CAPCELL PAK C18 MG II, 4.6 × 250 mm, 5 μm (OSAKA SODA, Osaka, Japan)) used in previous studies. Other conditions remained the same. In addition, the compound was identified by analyzing the *D. fortunei* 80% MeOH extract via UPLC–ESI/QTOF–MS.

Linearity

Five concentrations of solutions for compounds **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, and **14** in 80% MeOH were prepared. Calibration curves were then constructed for these five standards using concentration (X, μg/mL), peak area (Y), and the triplicated mean value (M) ± SD. Subsequently, the contents of the analytical solutions were determined from the calibration curves.

LOD and LOQ

Using regression equations based on the calibration curves, the concentrations of the 12 standard compounds were calculated. Additionally, LOD and LOQ values were determined at signal-to-noise ratios of 3 and 10, respectively.

Intra- and Inter-Day Accuracy and Precision

The precision and accuracy of the analyses were evaluated using 12 standard compound solutions. Inter- and intra-day precision and accuracy measurements were performed three times a day and repeated over 3 days.

Repeatability

The 12 standard solutions were analyzed with six repetitions under the same conditions to confirm the repeatability of the HPLC method and reproducibility of retention time and peak area.

Recovery

The recovery rate was confirmed by spiking the Sample 6 80% MeOH extraction solution with 12 standard solutions at a concentration of 8–31 μg/mL or 10–42 μg/mL.

2.6.5. Content Analysis

The content of each of the 15 samples (six *Drynaria fortunei* J. Smith species, six *Drynaria mollis* Bedd species, and three *Davallia mariesii* T. Moore ex Baker species) was analyzed using HPLC multi-component simultaneous quantitative analysis of the 12 developed compounds.

3. Results and Discussion

3.1. Structural Identification of Isolated Compounds **1–14**

In this study, chromatographic separation of EtOAc and BuOH fractions from Sample 6 of the *Drynaria fortunei* J. Smith species was performed. The isolated compounds were identified as gallic acid (1) [25], 5-hydroxymethylfurfural (2) [26], protocatechuic acid (3) [27], coumaric acid 4-O-β-D-glucopyranoside (4) [28–30], caffeic acid 4-O-β-D-glucopyranoside (5) [31–35], 4-hydroxybenzoic acid (6) [26], lavandoside (7) [29,36], trans-p-sinapoyl-β-D-glucopyranoside (8) [33,36], 5,7-dihydroxychromone-7-O-neohesperidoside (9) [9,31], kaempferol 3-O-rhamnoside 7-O-glucoside (10) [31], kaempferol 3-O-glucopyranoside-7-O-arabinofuranoside (11) [31,37], neoeriocitrin (12) [7], naringin (13) [38], and hesperidin (14) [38] through NMR and MS analyses and comparison with previous research results. The chemical structure of the isolated compound is presented in Figure 1, and the corresponding NMR and MS data are provided in Table S1.

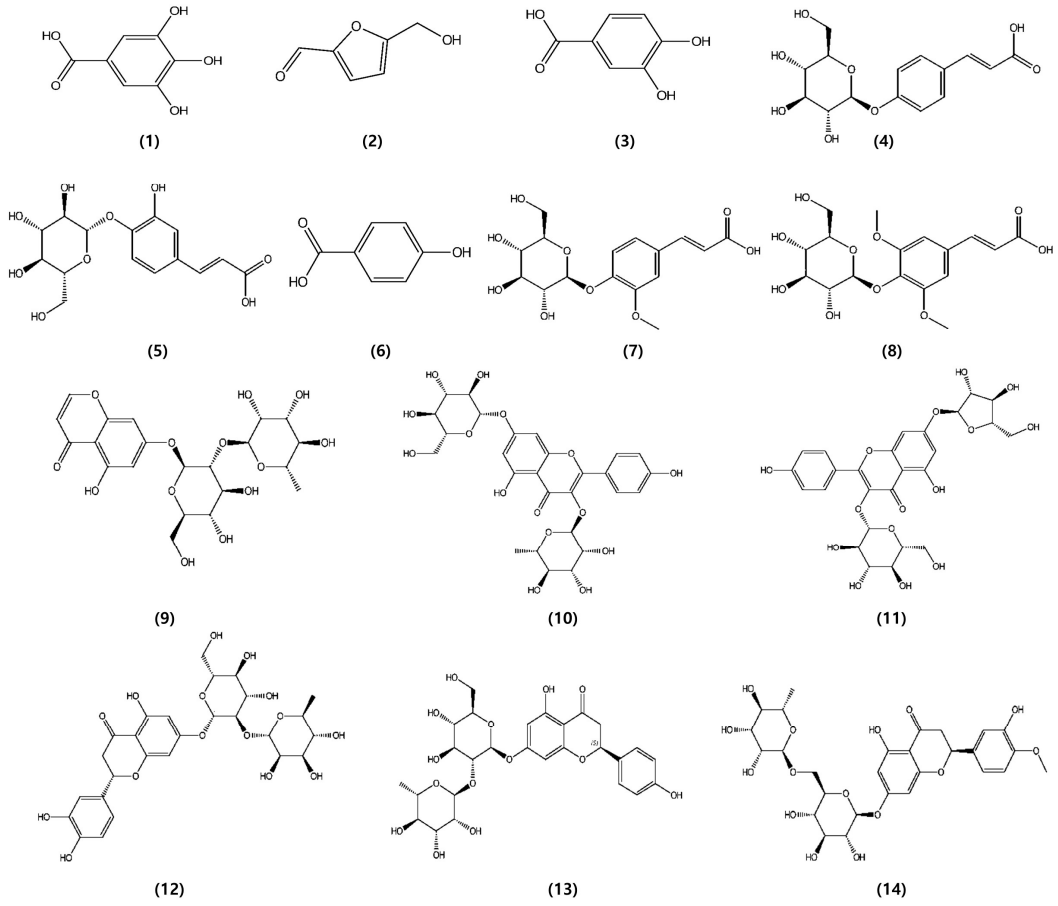

Figure 1. Chemical structures of 14 compounds isolated from *Drynaria fortunei* J. Smith.

3.2. Validation of HPLC Analysis

3.2.1. Specificity

On comparing the standard mixture of 12 compounds with the 80% MeOH extracts from *D. fortunei*, *D. mollis*, and *D. mariesii*, the extracts appeared to be well separated without interference (Figure 2). In addition, the analysis of these 12 compounds was compared between the ODS C18 column used in previous studies and the ODS C30 column used in this study. In the ODS C18 column, the peaks overlapped at a retention time of 15.93 min; however, in the ODS C30 column, the retention times were 19.32 and 20.19 min (Figure 3). UPLC–ESI/QTOF–MS analysis revealed peak aggregation between 2.43 and 2.51 min of the UPLC–UV retention time. The MS spectrum retention times of the aggregated portion were 2.36 and 2.43 min, and the $[M-H]^-$ values were 341.0848 and 325.0909, respectively. Therefore, the HPLC peak compounds separated using the ODS C30 column were confirmed to be coumaric acid 4-O-β-D-glucopyranoside and caffeic acid 4-O-β-D-glucopyranoside. Figure 4A shows the HPLC chromatogram of the standard mixture of 12 compounds analyzed using the ODS C18 column, while Figure 4B shows the 80% MeOH extracts of Sample 6′s UPLC–QTOF–MS chromatogram. Table 3 shows the results of the UPLC–QTOF–MS analysis of the aggregated peaks (compounds **4** and **5**).

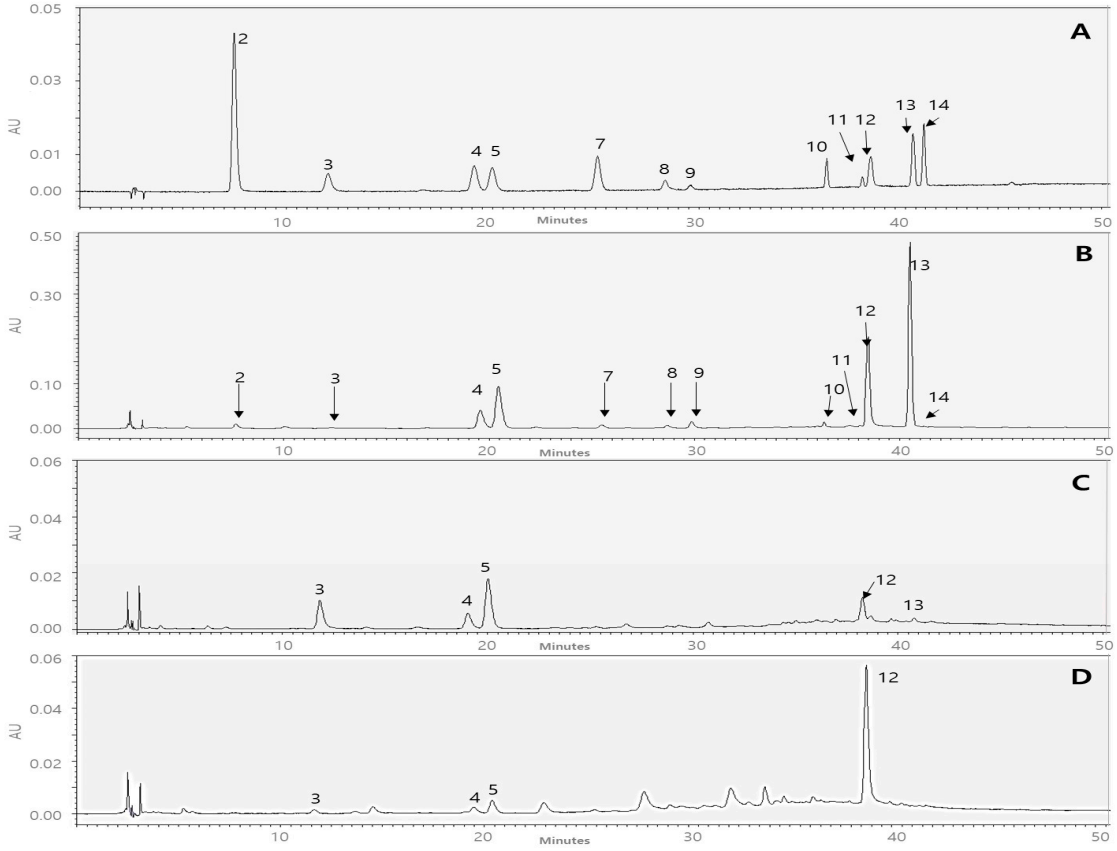

Figure 2. HPLC chromatograms of the (**A**) standard mixture, (**B**) *D. fortunei* extract, (**C**) *D. mollis* extract, and (**D**) *D. mariesii* extract. 2: compound **2**, 3: compound **3**, 4: compound **4**, 5: compound **5**, 7: compound **7**, 8: compound **8**, 9: compound **9**, 10: compound **10**, 11: compound **11**, 12: compound **12**, 13: compound **13**, 14: compound **14**.

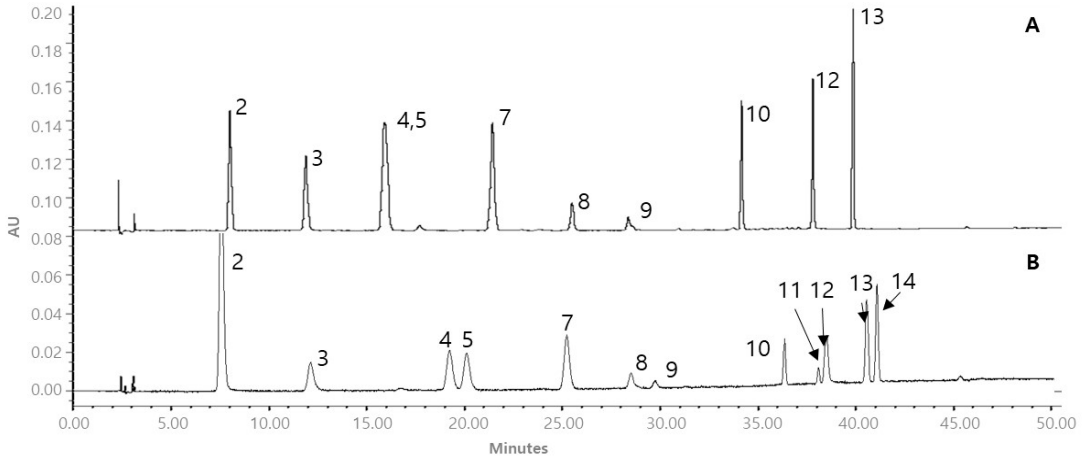

Figure 3. HPLC chromatograms comparing the analysis results of the standard mixture of 12 compounds between the (**A**) ODS C18 Column and (**B**) ODS C30 Column. 2: compound **2**, 3: compound **3**, 4: compound **4**, 5: compound **5**, 7: compound **7**, 8: compound **8**, 9: compound **9**, 10: compound **10**, 11: compound **11**, 12: compound **12**, 13: compound **13**, 14: compound **14**.

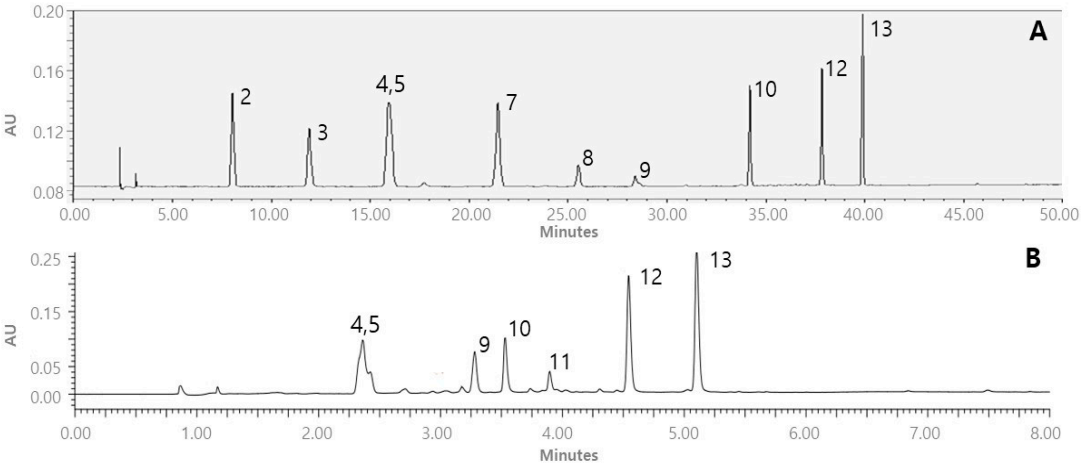

Figure 4. Chromatograms of the standard mixture of 12 compounds via (**A**) HPLC (using the ODS C18 column) and the 80% MeOH extract of Sample 6 via (**B**) UPLC/QTOF–MS. 2: compound **2**, 3: compound **3**, 4: compound **4**, 5: compound **5**, 7: compound **7**, 8: compound **8**, 9: compound **9**, 10: compound **10**, 11: compound **11**, 12: compound **12**, 13: compound **13**, 14: compound **14**.

Table 3. Qualitative analysis of compounds 4 and 5 via UPLC/QTOF–MS.

	Compound 4	**Compound 5**
Retention time (UV)	2.51 min	2.43 min
Retention time (MS)	2.43 min	2.36 min
Predicted Formula [M]	$C_{15}H_{18}O_8$	$C_{15}H_{18}O_9$
Monoisotopic Mwt *	326.2986	342.0951
$[M - H]^-$	325.0909	341.0848
[M + H COOH − H]−	371.0959	-
[2M − H]−	-	683.1767
Identification of compound	Coumaric acid 4-O-β-D-glucopyranoside	Caffeic acid 4-O-β-D-glucopyranoside.

* Mwt: monoisotopic molecular weight computed using PubChem 2.1.

3.2.2. Linearity, LOD, and LOQ

The linearity of the 12 compounds was measured and evaluated at five concentrations (range: 3.9–83.3 μg/mL). In all calibration curves, the correlation coefficients (r^2) of the

12 compounds exceeded 0.999. The LOD values of compounds **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, and **14** ranged from 0.01 to 0.65 μg/mL, while their LOQ values ranged from 0.04 to 1.97 μg/mL (Table 4).

Table 4. Calibration curves and linear range of the 12 compounds.

Compound No	Rt [1] (min)	Regression Equation	r^2 *	Linear Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
2	7.56	y = 74,736x + 52,127	0.999	3.9–62.5	0.65	1.97
3	12.13	y = 10,100x − 6844.1	0.999	5.2–83.3	0.46	1.39
4	19.32	y = 14,172x + 4127.5	0.999	5.2–83.3	0.45	1.37
5	20.19	y = 13,076x + 4101.6	1	5.2–83.3	0.22	0.65
7	25.28	y = 17,140x + 2496.6	0.999	5.2–83.3	0.03	0.08
8	28.54	y = 4482.1x − 4792.3	0.999	5.2–83.3	0.21	0.64
9	29.75	y = 1577.8x + 2011.2	0.999	5.2–83.3	0.01	0.04
10	36.29	y = 6002.6x + 3961	1	5.2–83.3	0.62	1.88
11	38.00	y = 1991.6x + 1564.7	1	5.2–83.3	0.57	1.71
12	38.45	y = 10,522x − 2012.8	0.999	5.2–83.3	0.20	0.61
13	40.47	y = 14,009x + 10,548	1	5.2–83.3	0.02	0.06
14	41.00	y = 9620.6x + 48,604	0.999	5.2–83.3	0.18	0.55

[1] Rt: retention time; * r^2: the correlation coefficient of the equation.

3.2.3. Intra- and Inter-Day Precision and Accuracy

To evaluate the precision and accuracy of the assay, concentration triplicates (15–80 μg/mL) of each of the 12 standard compounds were assayed for inter- and intra-day accuracy (%) and precision (coefficient of variation) within one or three consecutive days. The intra-day precision range for the 12 standard compounds was 0.06–2.85%, while the inter-day precision range was 0.24–2.83%. Intra-day accuracy ranged from 95.03 to 104.75%, while inter-day accuracy ranged from 95.75 to 105.75% (Table S2).

3.2.4. Repeatability

Regarding repeatability, the 12 compounds' standard solutions were repeatedly analyzed six times under the same conditions to confirm the repeatability of the HPLC method and reproducibility of retention time and peak area. The relative standard deviation (RSD) of the retention times ranged from 0.03% to 0.33%, while the peak areas ranged from 0.68% to 2.23% (Table S3).

3.2.5. Recovery

The concentrations of 11 compounds (1–11; 5, 25,20 and 50 μg/mL) were spiked in the Sample 6 extract solution. The recovery rate of the method was in the range of 97.94–103.96%. The method was deemed accurate with respect to the results. The results are shown in Table S4.

3.3. Simultaneous Quantitative HPLC Analysis of 12 Marker Components of D. fortunei, D. mollis, and D. mariesii

The 12 marker components of the 15 samples were simultaneously quantified using the developed and validated HPLC method. The compound content data for each sample are presented in Table 5, and the samples' chromatograms are displayed in Figure S1.

Table 5. The contents (μg/mL) of 12 compounds in the *D. fortunei*, *D. mollis*, and *D. mariesii* samples.

Compound No.	Content (μg/mL)					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
2	0.001 ± 0.003	0.000 ± 0.001	ND *	ND *	0.001 ± 0.000	0.032 ± 0.006
3	ND *	ND *	0.171 ± 0.019	ND *	ND *	0.059 ± 0.035

Table 5. *Cont.*

Compound No.	Content (μg/mL)					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
4	1.042 ± 0.001	1.044 ± 0.002	0.329 ± 0.003	0.958 ± 0.001	0.343 ± 0.000	1.082 ± 0.001
5	1.830 ± 0.002	2.398 ± 0.002	0.716 ± 0.000	2.548 ± 0.003	0.984 ± 0.000	2.696 ± 0.003
7	0.050 ± 0.000	0.135 ± 0.000	0.058 ± 0.000	0.098 ± 0.000	0.088 ± 0.000	0.136 ± 0.000
8	0.207 ± 0.000	0.382 ± 0.000	0.226 ± 0.000	0.685 ± 0.015	0.337 ± 0.001	0.455 ± 0.000
9	0.810 ± 0.000	1.660 ± 0.005	1.992 ± 0.000	1.577 ± 0.011	0.669 ± 0.001	2.457 ± 0.000
10	0.175 ± 0.002	0.115 ± 0.009	0.157 ± 0.002	0.290 ± 0.019	0.494 ± 0.000	0.343 ± 0.000
11	0.109 ± 0.003	0.037 ± 0.005	0.247 ± 0.000	0.334 ± 0.000	0.417 ± 0.003	0.361 ± 0.001
12	3.637 ± 0.004	2.364 ± 0.004	4.624 ± 0.003	7.175 ± 0.008	2.770 ± 0.000	4.857 ± 0.003
13	5.488 ± 0.005	4.946 ± 0.010	6.767 ± 0.001	5.827 ± 0.006	8.815 ± 0.002	5.748 ± 0.002
14	ND *	0.016 ± 0.000	0.035 ± 0.000	ND *	ND *	0.032 ± 0.000
Compound No.	**Content (μg/mL)**					
	Sample 7	**Sample 8**	**Sample 9**	**Sample 10**	**Sample 11**	**Sample 12**
3	0.337 ± 0.000	0.141 ± 0.054	0.146 ± 0.017	0.164 ± 0.013	0.286 ± 0.006	0.125 ± 0.016
4	0.142 ± 0.008	0.125 ± 0.001	0.141 ± 0.000	0.103 ± 0.011	0.133 ± 0.000	0.121 ± 0.001
5	0.481 ± 0.028	0.561 ± 0.000	0.515 ± 0.000	0.420 ± 0.029	0.612 ± 0.000	0.442 ± 0.000
12	0.208 ± 0.012	0.466 ± 0.003	0.608 ± 0.001	0.363 ± 0.052	0.304 ± 0.002	0.258 ± 0.001
13	0.007 ± 0.001	0.017 ± 0.000	0.019 ± 0.001	0.094 ± 0.037	0.013 ± 0.000	0.048 ± 0.000
Compound No.	**Content (μg/mL)**					
	Sample 13	**Sample 14**	**Sample 15**			
3	0.039 ± 0.022	0.054 ± 0.024	0.012 ± 0.000			
4	0.072 ± 0.001	0.047 ± 0.000	0.051 ± 0.000			
5	0.086 ± 0.000	0.125 ± 0.000	0.076 ± 0.000			
12	0.560 ± 0.001	1.354 ± 0.001	0.737 ± 0.001			

* ND: non-detection.

Samples 1–6 from the *D. fortunei* species were confirmed to contain compounds **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, and **13**. Samples 7–12 contained compounds **3**, **4**, **5**, **12**, and **13**, while Samples 13–15 contained compounds **3**, **4**, **5**, and **12**. Therefore, compounds **3**, **4**, **5**, and **12** are all contained in *D. fortunei*, *D. mollis*, and *D. mariesii*, all distributed as DR. However, compounds **7**, **8**, **9**, **10**, and **11** are unique compounds found exclusively in the *D. fortunei* species. Additionally, the hydroxycinnamic acid glycoside and flavonoid glycoside contents of the *D. fortunei* species in Samples 1–6 were significantly higher than those in Samples 7–15 (*D. mollis* and *D. mariesii*). In addition, among the currently developed HPLC analysis methods for analyzing *D. fortunei*, the HPLC simultaneous quantitative analysis method of identifying 12 indicator components, including compounds **4**, **7**, **8**, **10**, **11**, and **14**, was developed for the first time, and compounds **7**, **8**, **9**, **10**, and **11** exhibited potential as unique markers for the *D. fortunei* species.

4. Conclusions

In this study, we developed an HPLC-based multi-component simultaneous quantitative analysis method aimed at standardizing and improving the quality control of DR. Fifteen herbs distributed as DR were collected, and samples were classified using genetic analysis. Among the 15 samples, Sample 6, which was certificated via genetic analysis and material evaluation, was extracted using 80% MeOH, and 14 compounds were isolated from the EtOAc and BuOH fractions to identify their structures. The isolated compounds were identified as follows: one furan (compound **2**; 5-hydroxymethylfurfural), three phenolic compounds (compounds **1**, **3**, and **6**; gallic acid, protocatechuic acid, and 4-hydroxybenzoic acid), four hydroxycinnamic acids (compounds **4**, **5, 7**, and **8**; coumaric acid 4-O-β-D-glucopyranoside, caffeic acid 4-O-β-D-glucopyranoside, lavandoside, and trans-p-sinapoyl-β-D-glucopyranoside), one chromone (compound **9**; 5,7-dihydroxychromone-7-O-neohesperidoside), and five flavonoids (compound **10**, **11**, **12**, **13**, and **14**; kaempferol

3-O-rhamnoside 7-O-glucoside, kaempferol 3-O-glucopyranoside-7-O-arabinofuranoside, neoeriocitrin, naringin, and hesperidin) via NMR, MS, and comparison with previous studies' results. In this study, an HPLC multi-component simultaneous quantitative analysis method for examining compounds **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, and **14** using an ODS C30 column (Develosil RPAQUEOUS, 4.6 × 250 mm, 5 µm (Nomura Chemical Co., Ltd., Seto, Japan)) was developed. In addition to its specificity, the developed HPLC analysis method was validated based on its linearity ($r^2 > 0.999$), LOD (0.01–0.65 µg/mL), LOQ (0.04–1.97 µg/mL), repeatability (RSD of retention time: 0.03–0.33%; peak area range: 0.68–2.23%), precision and accuracy (intra-day: 0.06–2.85% and 95.03–104.75%; inter-day: 0.24–2.83% and 95.75–105.75%, respectively), and recovery (recovery rate: 97.940–103.96%). The developed analysis method improved the resolution of compounds **4** and **5**, which exhibited severe peak overlap or peak aggregation in previous studies, and was the first to perform simultaneous quantitative analysis of 12 compounds, including compounds **4**, **7**, **8**, **10**, **11**, and **14**. The developed analytical HPLC method is anticipated to contribute to the standardization and improvement of the quality control of DR and is potentially useful in various industrial fields.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations10120601/s1: Figure S1: HPLC chromatograms of *D. fortunei*, *D. mollis*, and *D. mariesii* samples; Table S1: NMR and mass spectrometry data of 14 compounds isolated from *D. fortunei;* Table S2: intra- and inter-day precision and accuracy of 12 compounds; Table S3: repeatability of retention time and peak area of 12 compounds; Table S4: recovery of 12 compounds for three different spiked concentrations.

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