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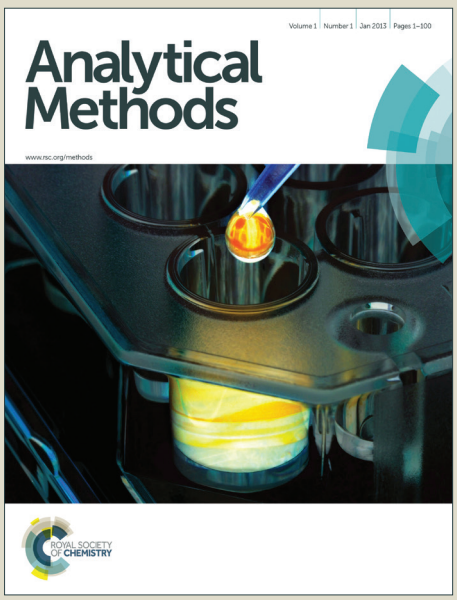

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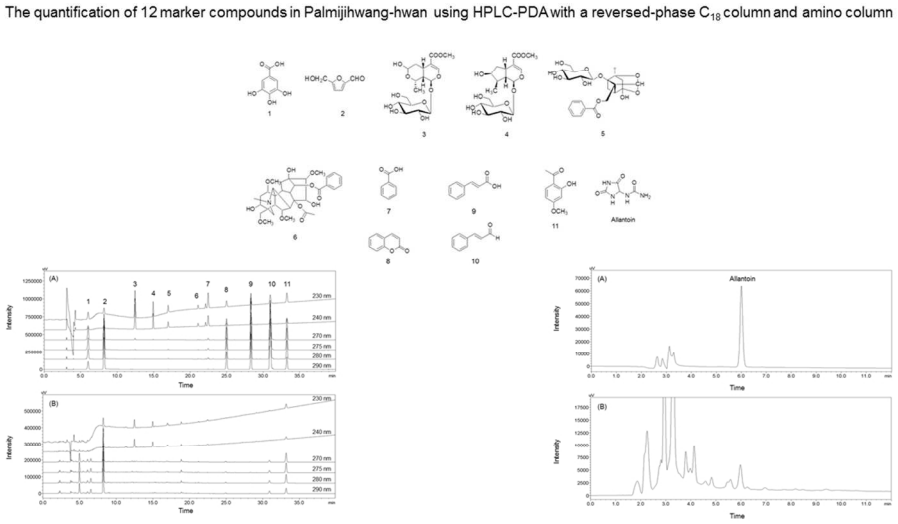

The quantification of 12 marker compounds in Palmijihwang-hwan using HPLC-PDA with a reversed-phase C18 column and amino column
338x190mm (96 x 96 DPI)

Development of a quantitative analysis method for the 12 marker compounds in Palmijihwang-hwan, a herbal formula, using a reversed-phase C_{18} column and amino column by HPLC

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Abstract

The aim of this study was to determine quantitatively the 12 marker compounds in Palmijihwang-hwan (PJH) using high performance liquid chromatography-photodiode array detector. Separation of marker compounds was performed on a reversed-phase C_{18} column for gallic acid, 5-hydroxymethylfurfural (5-HMF), morroniside, loganin, paeoniflorin, mesaconitine, benzoic acid, coumarin, cinnamic acid, cinnamaldehyde, and paeonol, and an amino (NH2) column for allantoin. The correlation coefficient of marker compounds was ≥ 0.9993, which means good linearity. The limit of detection and limit of quantification values were in the ranges 0.01–0.25 μg/mL and 0.02–0.78 μg/mL, respectively. The within-day precision was 0.06–2.95% and the between-day precision was 0.10–4.44% over five consecutive days. The recovery of marker compounds ranged from 97.20 to 106.36%, with RSD values < 3.5%. The repeatability was < 2.1% of RSD value. The quantification results indicated that the quantities of the 12 marker compounds differed between a water extract and commercial granules of PJH. Cinnamaldehyde and paeonol were particularly difficult to determine in commercial granules because of its low LOQ. Evaluation of the Pearson coefficient and principal component analysis showed clear discrimination between a PJH water extract and commercial granules of PJH. The analytical method established was precise, accurate, and reproducible for evaluating the quality of PJH.

Keywords Palmijihwang-hwan, Marker compounds, Quantitative analysis, Method development

1 Introduction

Herbal formulas of traditional medicines usually comprise multiple herbs in a single formula, with various composition ratios. They are generally prepared by boiling with water to produce extract called a 'decoction'. Numerous chemical constituents can be extracted from a single herbal medicine and they interact with each other during the process of decocting a mixture of herbal medicines, which makes quality control of a herbal formula complicated. Moreover, quantitative analysis using single or several chemical compounds is challenging because of its inability to reflect all the characteristics of a herbal formula. Therefore, multiple active compounds are necessary for the quality evaluation of a herbal formula composed of multiple herbs (multi-herb formula).

Palmijihwang-hwan (PJH; Hachimijiogan in Japanese; Ba wei di huang wan in Chinese), a traditional herbal formula, consists of eight herbs, including *Rehmannia glutinosa* Libosch. ex Steudel, *Dioscorea batatas* Decne., *Cornus officinalis* Sieb. et Zucc., *Paeonia suffruticosa* Andrews, *Poria cocos* F.A. Wolf, *Alisma orientale* Juzep., *Cinnamomum cassia* Presl, *Aconitum carmichaeli* Debx. PJH, which is based on Yukmijihwang-hwan (Rokumijiogan in Japanese; Liu wei di huang wan in Chinese), has been applied to improve renal dysfunction and contractile response; to ameliorate spermiotoxicity, diabetes and diabetic nephropathy; and to regulate calcium metabolism in aged-animal models.[1–6] The therapeutic effect of PJH is thought to be exerted through the combination and interaction of multiple components from the above eight compositional herbs.

The chemical compounds contained in the compositional herbs in PJH are known to possess bioactivity: 5-hydroxymethylfurfural (5-HMF) from *Rehmannia glutinosa*; morroniside and loganin from *Cornus officinalis*; gallic acid, paeoniflorin, benzoic acid and paeonol from *Paeonia suffruticosa*; coumarin, cinnamic acid and cinnamaldehyde from *Cinnamomum cassia*); mesaconitine from *Aconitum carmichaeli*; allantoin from *Dioscorea batatas*.[7–15] Therefore, we

considered these compounds to be suitable as markers for the quality control of PJH preparations. Quality assessment of herbal formulas has been carried out by the simultaneous determination of multiple compounds using HPLC with a diode-array detector (DAD). This method is simple, rapid, and precise. [16–18]

In the present study, we quantitatively analyzed the 12 marker compounds in PJH preparations by a validated method using HPLC with a photodiode array detector (PDA). The quantities of the marker compounds present in various commercial PJH granules were compared. One of these compounds, allantoin, showed poor separation on a C_{18} column because of the chemical structure of the nitrogen atom, but it was isolated later on an amino (NH_2) column

2 Experimental

2.1 Chemicals and reagents

HPLC-grade methanol, acetonitrile, and water were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Gallic acid, 5-HMF, benzoic acid, coumarin, and cinnamic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Morroniside, loganin, paeoniflorin, cinnamaldehyde, mesaconitine, and paeonol were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Allantoin was purchased from Acros Organics (New Jersey, USA). The purity of all these reagents was ≥ 98%. The chemical structures of the standard compounds are shown in Figure 1.

Compositional herbal medicines were purchased from the herbal medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Korea) (see Table 1). A voucher specimen (2013-KE35-1–8) has been deposited in the Herbal Medicine Formulation Research Group of the Korea Institute of Oriental Medicine.

2.2 Sample Preparation of PJH Water Extract and Commercial Preparation

Herbal medicines consisting of PJH were mixed and extracted with a 10-fold volume of distilled water (w/v) at 100 ℃ for 2 h under pressure (1 kgf/cm^2) using an electric extractor (COSMOS-660, Kyungseo Machine Co., Incheon, Korea). The extracted decoction was filtered through a standard sieve (no. 270, 53 μm; Chunggyesangongsa, Seoul, Korea) and then lyophilized to create a powdered PJH water extract (PJHWE).

Forty milligrams of PJHWE and 600 mg of commercial PJH granules were accurately weighed and dissolved in 20 mL distilled water. The solutions were filtered through a 0.2 μm syringe filter (SmartPor®, Woongki Science, Seoul, Korea) before injection into the HPLC system.

2.3 Preparation of Standard Solutions of Marker Compounds.

The stock solutions were prepared by dissolving accurately weighed standard compounds in methanol at concentrations of 1000 μg/mL. Working solutions were produced by diluting the stock solutions containing standard compounds. Diluted working solutions were used to construct calibration curves.

2.4 Apparatus

The HPLC-PDA system comprised a Shimadzu LC-20A (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery unit (LC-20AT), autosampler (SIL-20AC), column oven (CTO-20A), degasser (DGU-20A$_3$), and PDA (SPD-M20A). The acquired data were processed using LabSolutions software (Ver. 5.3; Shimadzu, Japan). Separation of compounds except allantoin was performed on a Gemini C_{18} column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) maintained at 40 ℃. The mobile phase consisted of water (A) and acetonitrile (B), both containing 1% acetic acid. Gradient elution of the mobile phase was applied: 5–60% (B) over 0–40 min, 60–70% (B) over 40–45 min, held for 5 min, and then re-equilibriated to 5% until the end of the analysis. The flow rate was 1.0 mL/min and the injection volume was set to

10 μL. The detection wavelengths were optimized according to the maximum absorption wavelengths of the standard compounds.

The separation of allantoin was carried out on a NH_2 column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) using isocratic elution with a 4:6 ratio of water (A) : acetonitrile (B). Other analytical conditions were as described above. The peaks of marker compounds were integrated using 'Valley to Valley' function of software.

2.5 Method Validation

The within-day (intra-day, $n = 5$) and between-day (inter-day, $n = 5$) precisions were determined by analyzing sample extracts added by low, medium, and high concentrations of marker compounds and the values were represented as the RSD [(standard deviation / mean) × 100]. The accuracy of the method used was measured by means of a recovery test. This was performed by adding three known amounts of marker compounds (low, medium, and high) to the samples, followed by extraction using the methods described above. The recovery was calculated as follows: recovery (%) = ((detected concentration – initial concentration) / spiked concentration) × 100. The repeatability was measured by calculating the absolute peak area of each marker compound in PJHWE solution by repetitive analysis ($n = 6$) and the value was also represented as RSD.

2.6 Evaluation of Pearson coefficient and principle component analysis

Evaluation of the Pearson coefficient and principle component analysis (PCA) were performed based on the rows (PJH samples) and columns (the amounts of 12 marker compounds) using open-source software R (ver. 3.0.2).

3 Results and discussion

3.1 Optimization of chromatographic conditions

The HPLC chromatography conditions considered for the analysis of PJH were the column, the mobile phase, and the UV wavelength of PDA. Because a C_{18} column is most commonly used in the analysis of chemical components in herbal medicines, we also employed C_{18} column to detect 11 compounds in PJH simultaneously. However, allantoin (a diureide of glyoxylic acid), which is a highly polar compound, was poorly separated on a C_{18} column because it is not retained by a reversed phase C_{18} column.[19] Therefore, we elected to use an amino phase (NH_2) instead of a reversed C_{18} phase. The mobile phase consisted of water (A) and acetonitrile (B), both containing 1% acetic acid, because of acidity of some marker compounds, and gradient elution was applied after testing for various A:B ratios. The gradient elution conditions were as follows: 5–60% (B) over 0–40 min, 60–70% (B) over 40–45 min, held for 5 min. The UV wavelength was tested in the UV spectrum from 190 nm to 400 nm to determine the optimal absorption for each marker compound: allantoin at 210 nm; paeoniflorin and benzoic acid at 230 nm; morroniside, loganin, and mesaconitine at 240 nm; gallic acid at 270 nm; coumarin, cinnamic acid, and paeonol at 275 nm; 5-HMF at 280 nm; and cinnamaldehyde at 290 nm. The 11 marker compounds that were analysed on the C18 column, and the allantoin that was analysed on the NH2 column, were reasonably separated on a chromatogram of PJHWE, without overlapping or interception of adjacent peaks (Figs 2 and 3). The system suitability was evaluated via capacity factor, theoretical plate number, resolution, and symmetry of marker compounds. The capacity factor and theoretical plate number ranged from 1.81 to 14.42, and from 14986 to 229886, respectively. The resolutions of marker compounds were > 1.3, which also demonstrated that the compound peaks were not disturbed by adjacent peaks in the quantification of their amounts. The symmetry factor of the compounds peaks were in the range from 0.9 to 1.4, indicating that severe peak fronting or tailing was not found (Table 2).

3.2 Linear regression, limit of detection (LOD), and limit of quantification (LOQ)

Stock solutions were diluted to six levels of concentration to produce calibration curves of marker compounds. The correlation coefficient (r^2) of compounds ranged from 0.9993 to 1.0000, showing good linearity. The values of LODs and LOQs, with signal-to-noise ratios at 3 and 10, respectively, were 0.01–0.25 μg/mL for LODs and 0.02–0.78 μg/mL for LOQs (Table 3).

3.3 Precision, recovery, and repeatability

The precisions of the 12 marker compounds were represented as RSD values, calculated as the percentage of standard deviation divided by the mean values. The intra-day and inter-day precisions ranged from 0.06% to 2.95% and from 0.10% to 4.44%, respectively (Table 4). The recoveries of the 12 marker compounds were in the range 97.20–106.36%, with RSD values < 3.5%; and the repeatability, which was represented as RSD values, ranged from 0.57 to 2.01% (Table 5). These results indicate that the established analytical method was precise, accurate and reproducible for the analysis of the 12 marker compounds in PJHWE.

3.4 Quantification of twelve marker compounds in PJHWE and commercial granules

The validated method was then successfully applied to quantifying marker compounds in PJHWE and commercial granules. The 11 marker compounds, which were apparent as peaks in PJHWE, were not all detected in the commercial PJH granules. Cinnamaldehyde was not apparent in all commercial granules. Paeonol was detected in PJH01 but only slightly in PJH02; however, it was unapparent in PJH03 (Fig. 4A). Allantoin, separated on a NH_2 column, was detected in all samples containing PJHWE and the commercial granules in 6 min (Fig. 4B).

There was noticeable variation in the amounts of the 12 marker compounds in the PJH samples, including PJHWE and commercial granules. In PJHWE, 5-HMF was present in the highest amount, followed by morroniside and allantoin, while cinnamic acid was present in the lowest amount, followed by mesaconitine and benzoic acid. On the other hand, in three

commercial granules, gallic acid and paeoniflorin were contained in higher amounts, whereas cinnamic acid, mesaconitine, and coumarin were present in lower amounts. As the amount of cinnamaldehyde was below the LOQ, it was not possible to quantify the content in all commercial PJH granules. Paeonol, in a higher amount in PJHWE, could be quantified in PJH01 and PJH02 but not in PJH03 because of its low LOQ level (Table 6). This is because cinnamaldehyde and paeonol, the main compound in *Cinnamomum cassia* and *Paeonia suffruticosa*, are volatile and are vaporized at high temperature. Hence, the manufacturing process may contribute to the absence of those two compounds as well as differences in the amounts of marker compounds in commercial PJH granules.[20,21]

The Pearson coefficient represented as a boxplot showed correlation between PJH samples given as correlation coefficient. The median value of the coefficient of PJHWE was quite low compared with those of three commercial granules, which means that PJHWE was not closely correlated with the commercial samples (Fig. 5). The biplot from PCA also supported the results of the Pearson coefficient–there were a clear classification between PJH samples. The principle component (PC) 1, which had the greatest influence on classification, clearly divided the PJH samples into two groups, PJHWE and commercial granules. This means PJHWE is not closely correlated with the commercial granules as closer PC scores mean the closer relationships.[22] The 11 compounds, except for gallic acid which headed for PJH03, contributed the separation of PJHWE from the commercial granules (Fig. 6).

4 Conclusions

A validated HPLC-PDA analytical method was established for the simultaneous determination of 11 marker compounds in PJH, using a C_{18} column, and for separation of allantoin, using a NH_2 column, and was successfully applied to quantification in PJH samples. The quantities of the 12 marker compounds differed among PJH samples. Their correlation was evaluated by the

Pearson coefficient and PCA. The developed method was precise, accurate, and reliable. It could therefore be applied to quality assessment of herbal formulas.

Conflict of interest

No conflict of interest exists.

Acknowledgements

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Table 1 Composition of herbal medicines in Palmijihwang-hang (PJH)

Herbal medicine	Region of origin	Amount (g)
Rehmannia glutinosa Libosch. ex Steudel	Euiseong, Gyeongbuk, Korea	30.00
Dioscorea batatas Decne.	Andong, Gyeongbuk, Korea	15.00
Cornus officinalis Sieb. et Zucc.	Gurye, Jeonnam, Korea	15.00
Paeonia suffruticosa Andrews	Jecheon, Chungbuk, Korea	11.25
Poria cocos F.A. Wolf	Pyeongchang, Gangwon, Korea	11.25
Alisma orientale Juzep	Namyangju, Gyeonggi, Korea	11.25
Cinnamomum cassia Presl	Vietnam	3.75
Aconitum carmichaeli Debx.	China	3.75
Sum	-	101.25

Table 2 System suitability: Capacity factor, resolution, and symmetry

Compound	Capacity factor (k)	Theoretical plate (N)	Resolution (Rs)	Symmetry factor (As)
Gallic acid	1.83	18245	2.52	1.02
5-HMF	2.82	32807	9.35	1.05
Morroniside	4.84	90679	2.08	1.05
Loganin	6.12	134948	1.55	0.99
Paeoniflorin	6.95	135728	1.69	1.35
Mesaconitine	8.35	191001	1.96	1.03
Benzoic acid	8.72	90626	3.47	0.96
Coumarin	10.59	134659	2.36	0.91
Cinnamic acid	12.15	229886	9.93	1.12
Cinnamaldehyde	13.36	183211	9.93	1.02
Paeonol	14.42	238460	8.15	1.00
Allantoin*	2.31	14985	1.33	1.11

* Allantoin was analysed on a NH_2 column.

Table 3 Linear equation, correlation coefficients (r^2), LOD, and LOQ for the marker compounds in PJH

Compound	Detection wavelength (nm)	Linear equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	270	y = 25005x – 4345.1	0.9998	0.63 – 20.00	0.17	0.52
5-HMF	280	y = 76404.25x + 46886.09	0.9996	0.78 – 100.00	0.02	0.05
Morroniside	240	y = 19045.28x + 8257.94	0.9999	0.78 – 100.00	0.02	0.08
Loganin	240	y = 16074.06x + 2227.70	1.0000	0.39 – 50.00	0.02	0.08
Paeoniflorin	230	y = 9577.1x - 964.6	0.9993	0.56 – 50.00	00.12	0.39
Mesaconitine	240	y = 8825.41x + 3678.38	1.0000	0.78 – 100.00	0.03	0.09
Benzoic acid	230	y = 23211x + 692.25	1.0000	0.63 – 20.00	0.19	0.57
Coumarin	275	y = 22649.93x + 1003.82	1.0000	0.08 – 10.00	0.03	0.09
Cinnamic acid	275	y = 46667.35x + 4952.83	0.9998	0.08 – 10.00	0.01	0.03
Cinnamaldehyde	290	y = 105207x + 64706.4	0.9995	0.16 – 200.00	0.01	0.02
Paeonol	275	y = 56170x + 34750.67	0.9998	0.39 – 50.00	0.01	0.03
Allantoin*	210	y = 5767.5x – 1826	0.9999	2.50 – 80.00	0.25	0.78

LOD, limit of detection; LOQ, limit of quantification

y, peak area (mAU); x, concentration of compound (μg/mL).

* Allantoin was analysed on a NH_2 column.

Table 4 Intra- and inter-day precision of the marker compounds in PJH

Compound	Spiked conc. (μg/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
		Detected conc. (μg/mL)	RSD (%)	Accuracy (%)	Detected conc. (μg/mL)	RSD (%)	Accuracy (%)
Gallic acid	1.00	0.95	1.75	94.50	0.95	1.86	94.56
	2.00	2.07	1.53	103.61	2.06	2.08	102.83
	4.00	3.98	0.41	99.44	3.99	0.47	99.63

5-HMF	4.00	3.91	0.81	97.66	3.88	0.71	97.02
	10.00	10.07	1.28	100.68	9.98	0.32	99.78
	20.00	19.98	0.32	99.92	20.03	0.06	100.17
Morroniside	4.00	3.98	1.04	99.61	3.96	1.60	98.89
	10.00	10.02	0.89	100.15	10.10	1.38	101.01
	20.00	20.00	0.21	99.98	19.96	0.29	99.79
Loganin	2.00	2.00	0.72	100.23	1.98	1.18	98.82
	5.00	4.97	0.99	99.45	5.15	2.08	103.09
	10.00	10.01	0.24	100.13	9.93	0.53	99.27
Paeoniflorin	2.00	1.95	2.01	97.57	1.94	1.46	96.83
	4.00	3.97	1.79	99.17	4.05	3.05	101.24
	8.00	8.03	0.33	100.36	7.99	0.74	99.89
Mesaconitine	4.00	3.97	0.60	99.18	3.96	0.84	98.99
	10.00	9.90	0.56	99.05	10.07	2.00	100.65
	20.00	20.05	0.15	100.27	19.98	0.51	99.88
Benzoic acid	0.50	0.51	2.95	101.14	0.50	2.78	100.24
	1.00	1.02	2.60	102.36	1.01	4.44	101.40
	2.00	1.99	0.53	99.34	1.99	1.13	99.63
Coumarin	1.00	0.99	0.37	99.00	1.00	0.94	100.32
	2.00	2.00	0.27	99.84	1.98	1.11	99.19
	4.00	4.00	0.06	100.10	4.01	0.31	100.18
Cinnamic acid	1.00	1.00	0.31	100.48	0.98	0.49	98.23
	2.00	2.03	0.22	101.39	2.05	0.81	102.55
	4.00	3.99	0.06	99.64	3.98	0.10	99.46
Cinnamaldehyde	1.00	0.99	1.34	98.71	0.99	0.38	98.73
	2.00	1.99	1.25	99.58	1.99	0.64	99.35
	4.00	4.01	0.29	100.21	4.01	0.18	100.24
Paeonol	2.00	1.99	0.45	99.39	1.95	0.32	97.54
	5.00	5.05	0.49	100.93	5.14	0.67	102.76
	10.00	9.98	0.11	99.79	9.94	0.17	99.41
Allantoin	5.00	5.01	1.33	100.19	5.03	1.29	100.69
	10.00	9.68	1.24	96.80	9.68	1.24	96.80
	20.00	20.16	0.23	100.79	20.15	0.24	100.76

Conc., concentration; SD, standard deviation; RSD, relative standard deviation (%) = (standard deviation/mean) × 100.

Table 5 Recovery and repeatability of the marker compounds in PJH (n = 5)

Compound	Initial conc. (μg/mL)	Spiked conc. (μg/mL)	Detected conc. (μg/mL)	Recovery (%)	RSD (%)	Repeatability (n = 6, %)
Gallic acid	4.55	1.00	5.54	99.38	1.71	1.09
		2.00	6.67	106.36	2.99	
		4.00	8.73	104.60	2.07	
5-HMF	24.14	4.00	28.06	97.98	1.41	0.99
		10.00	34.31	101.39	1.28	
		20.00	44.37	101.12	1.45	
Morroniside	19.39	4.00	23.37	99.70	0.86	0.88
		10.00	29.42	100.33	1.14	
		20.00	39.42	100.18	0.72	
Loganin	12.40	2.00	14.42	100.86	0.88	0.57
		5.00	17.42	100.38	0.79	
		10.00	22.52	101.18	0.81	
Paeoniflorin	7.89	2.00	9.86	98.17	3.30	1.88

		4.00	11.90	100.13	2.88	
		8.00	16.07	102.19	1.34	
Mesaconitine	2.34	4.00	6.31	99.28	0.81	1.67
		10.00	12.36	100.27	0.18	
		20.00	22.72	101.90	0.88	
Benzoic acid	1.78	0.50	2.28	100.73	3.10	1.87
		1.00	2.80	102.28	2.72	
		2.00	3.72	97.20	2.14	
Coumarin	2.29	1.00	3.28	98.74	0.34	0.38
		2.00	4.29	100.05	0.27	
		4.00	6.31	100.54	0.34	
Cinnamic acid	0.24	1.00	1.25	100.54	0.33	2.01
		2.00	2.26	100.86	0.26	
		4.00	4.19	98.78	0.40	
Cinnamaldehyde	3.69	1.00	4.69	99.31	1.36	1.14
		2.00	5.71	100.98	1.08	
		4.00	7.78	102.02	1.37	
Paeonol	17.42	2.00	19.42	100.16	0.54	0.95
		5.00	22.49	101.29	0.35	
		10.00	27.42	100.02	0.39	
Allantoin	16.32	5.00	21.41	101.90	1.84	1.15
		10.00	26.53	102.11	1.24	
		20.00	37.49	105.84	0.97	

Conc., concentration; RSD, relative standard deviation (%) = (standard deviation/mean) × 100.

Table 6 Quantification of 12 marker compounds in PJH samples

Compound	Content (mg/g)			
	PJHWE	PJH01	PJH02	PJH03
Gallic acid	0.901 ± 0.002	1.008 ± 0.010	0.504 ± 0.008	1.805 ± 0.015
5-HMF	5.037 ± 0.007	0.207 ± 0.001	0.438 ± 0.001	0.395 ± 0.003
Morroniside	3.867 ± 0.058	0.380 ± 0.010	0.231 ± 0.007	0.444 ± 0.004
Loganin	2.378 ± 0.005	0.230 ± 0.013	0.135 ± 0.003	0.342 ± 0.009
Paeoniflorin	1.588 ± 0.020	0.865 ± 0.025	0.657 ± 0.008	0.550 ± 0.003
Mesaconitine	0.381 ± 0.003	0.052 ± 0.000	0.020 ± 0.001	0.062 ± 0.001
Benzoic acid	0.360 ± 0.008	0.260 ± 0.002	0.169 ± 0.005	0.254 ± 0.003
Coumarin	0.476 ± 0.004	0.027 ± 0.000	0.058 ± 0.001	0.052 ± 0.000
Cinnamic acid	0.048 ± 0.001	0.016 ± 0.001	0.016 ± 0.001	0.031 ± 0.001
Cinnamaldehyde	0.699 ± 0.005	< LOQ	< LOQ	< LOQ
Paeonol	3.096 ± 0.006	0.268 ± 0.001	0.004 ± 0.000	< LOQ
Allantoin	3.279 ± 0.004	0.228 ± 0.007	0.163 ± 0.002	0.157 ± 0.001

PJHWE, PJH water extract; PJH01–03, commercial granules supplied by Korean manufacturers.
< LOQ, below the limit of quantification.

Figure legends

Fig. 1 Chemical structures of 12 standard compounds in Palmijihwang-hwan (PJH).

1, Gallic acid; 2 5-hydroxymethylfurfural; 3, morroniside; 4, loganin; 5, paeoniflorin; 6, mesaconitine; 7, benzoic acid; 8, coumarin; 9, cinnamic acid; 10, cinnamaldehyde; 11, paeonol.

Fig. 2 Chromatograms of 11 marker compounds (A) and PJHWE (B) at the optimal detection wavelength. 1, Gallic acid; 2 5-hydroxymethylfurfural; 3, morroniside; 4, loganin; 5, paeoniflorin; 6, mesaconitine; 7, benzoic acid; 8, coumarin; 9, cinnamic acid; 10, cinnamaldehyde; 11, paeonol.

Fig. 3 Chromatograms of allantoin (A) and PJH water extract (B) at 210 nm.

Fig. 4 Representative chromatograms of 11 marker compounds (A) and allantoin (B) in PJH samples at 254 nm. 1, Gallic acid; 2 5-hydroxymethylfurfural; 3, morroniside; 4, loganin; 5, paeoniflorin; 6, mesaconitine; 7, benzoic acid; 8, coumarin; 9, cinnamic acid; 10, cinnamaldehyde; 11, paeonol.

PJHWE: PJH water extract, PJH01–PJH03: commercial PJH granules.

Fig. 5 Pearson coefficient of PJH samples. PJHWE: PJH water extract, PJH01–PJH03: commercial PJH granules.

Fig. 6 Biplot of principal components (PC1 vs. PC2) on the variables (amounts of marker compounds) with the objectives (PJH samples).

The effects of marker compounds on the distribution of PJH are represented by arrows. PC1 and PC2 contributed to 87% and 11% of total variance, respectively.

PJHWE: PJH water extract, PJH01–PJH03: commercial PJH granules.

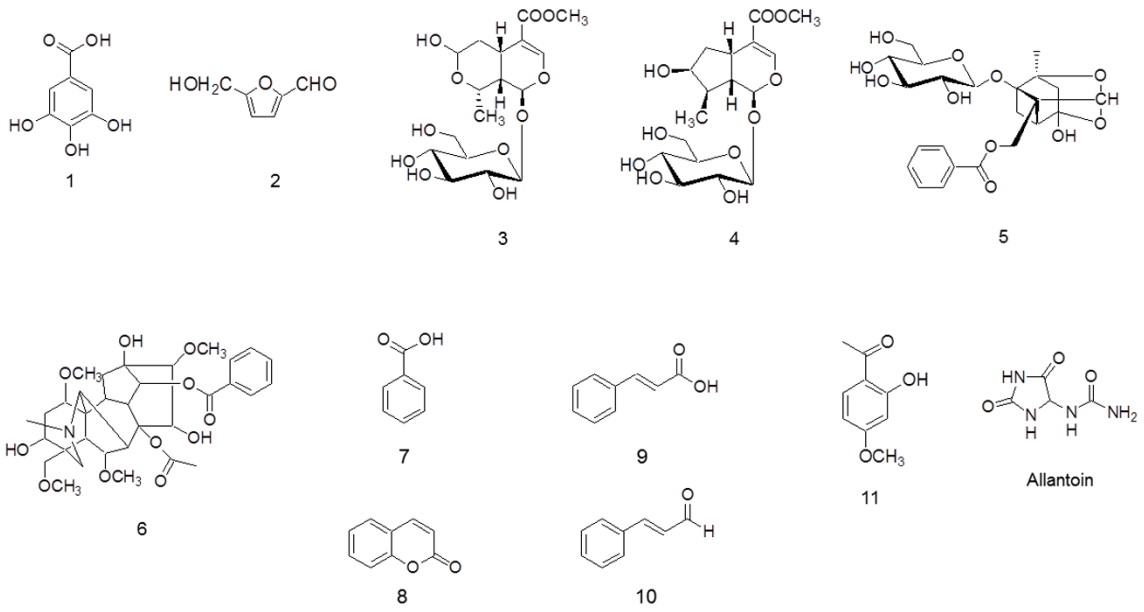


Fig. 1

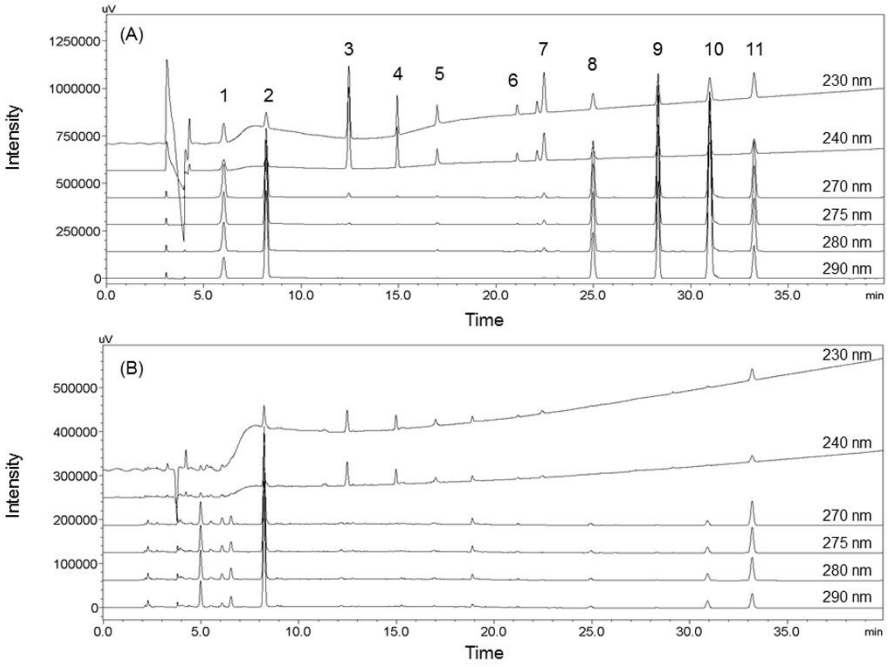


Fig. 2

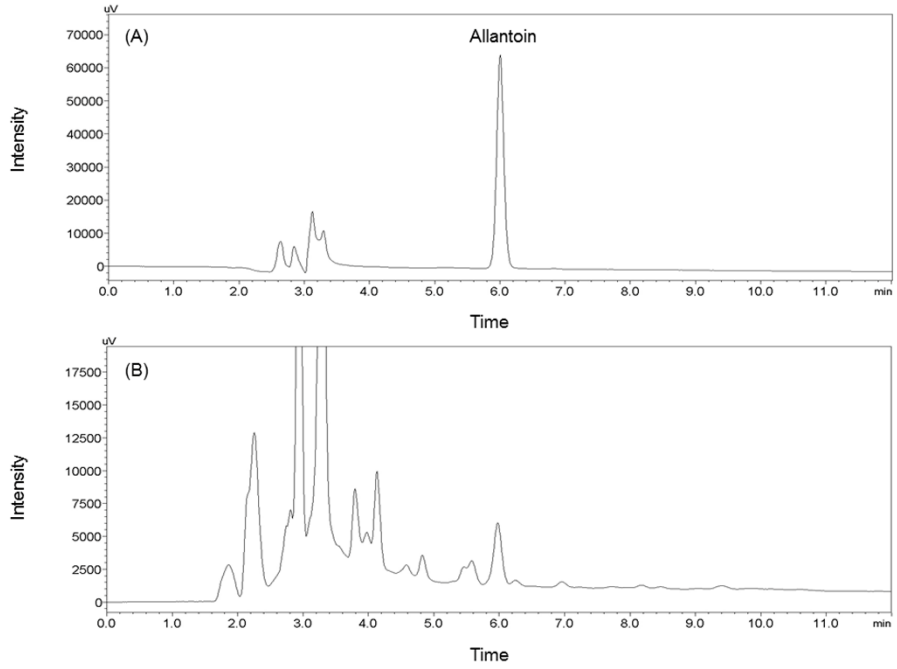
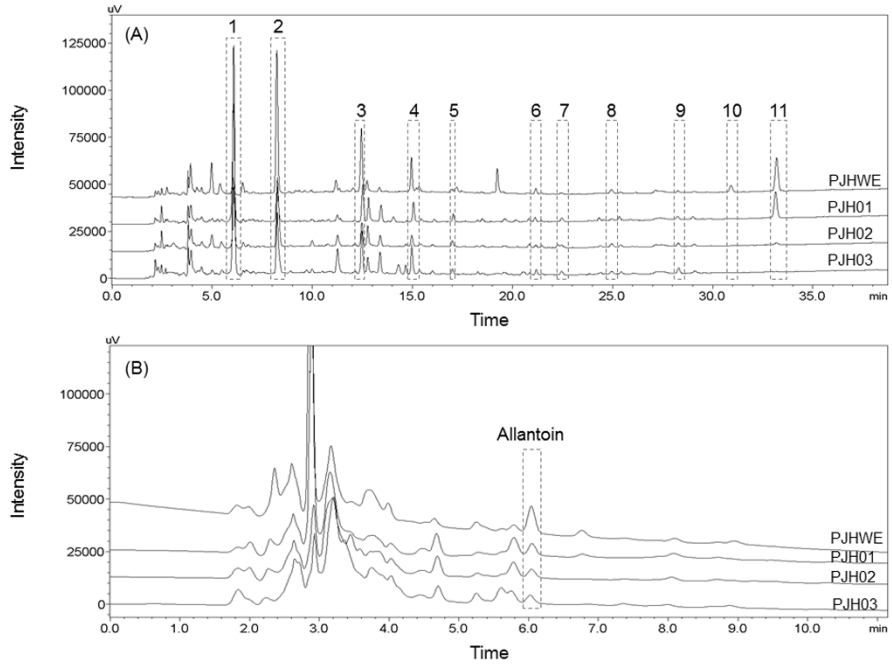


Fig 3



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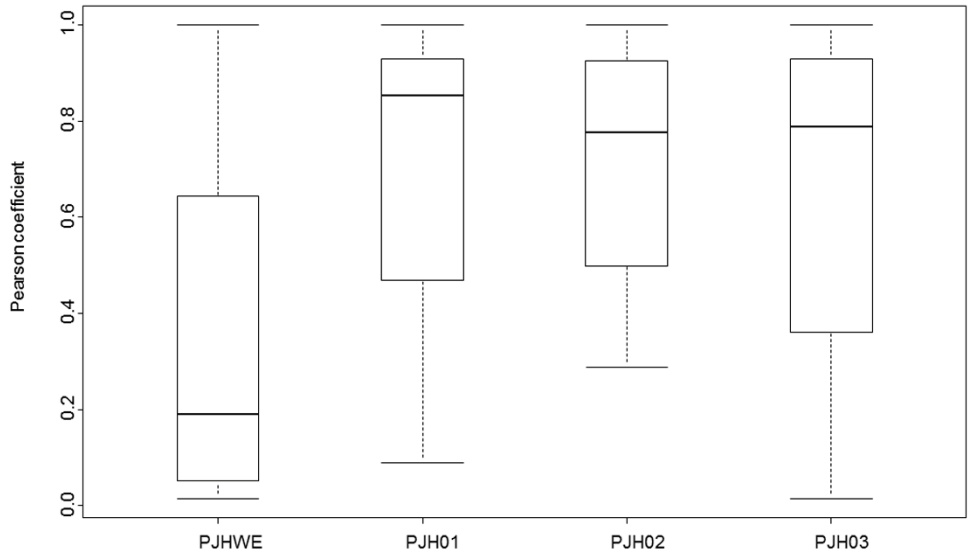

Fig. 5

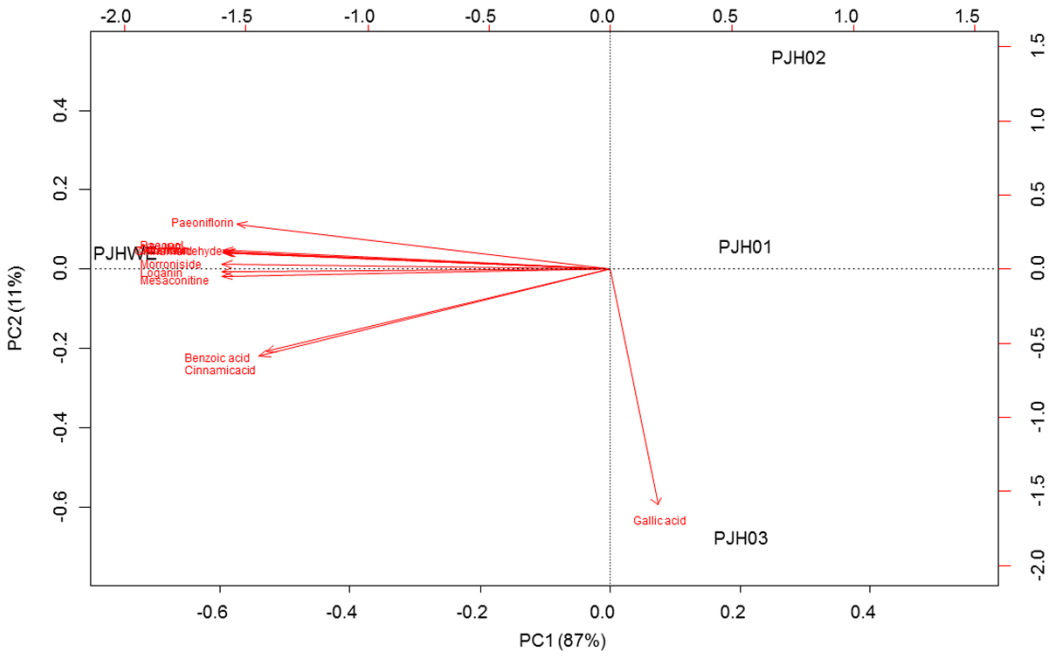

Fig. 6