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Discrimination of *Phellodendron amurense* and *P. chinense* Based on DNA Analysis and the Simultaneous Analysis of Alkaloids

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Phellodendri Cortex is the bark of the stems of *Phellodendron amurense* Ruprecht or *P. chinense* Schneider (Rutaceae), which is orginated from periderm. The internal transcribed spacer sequences of 20 originated plants and identified samples were analyzed. The result showed that the 99% of the base sequences of *P. amurense* were identical to that of *P. chinense*, but the differentiation of *P. amurense* and *P. chinense* was difficult. In addition, the ribulose-1, 5-bisphospate carboxylase large subunit (*rbcL*) intergenic spacer sequences of specific parts produced the same result. However, when the analysis was carried out by using the RAPD (randomly amplification polymorphism DNA) analysis method, which utilizes 48 randomly primers, it allowed us to confirm the polymorphism of *P. amurense* and *P. chinense* in 12 primers. A high-performance liquid chromatographic (HPLC) method was developed and validated for the simultaneous quantitation of berberine, palmatine and jatrorrhizine in a traditional herbal drug, Phellodendri Cortex. The HPLC method was applied successfully to the quantification of three constituents in the extract of twenty Phellodendri Cortex. The results indicated that the established HPLC and RAPD methods are suitable for the quantitative analysis and the quality control multi-simultaneous discrimination in Phellodendri Cortex.

Key words: Phellodendri Cortex, Simultaneous determination, RAPD (randomly amplification polymorphism DNA)

INTRODUCTION

Phellodendri Cortex is the dried bark of two botanical species, *Phellodendron amurense* Ruprecht and *P. chinense* Schneid (both Family Rutaceae). Phellodendri Cortex, 'Hwangbaek' in Korean and 'Huangbai' in Chinese, is widely employed in the practices of traditional oriental medicine. The dry bark was considered to be one of the 50 fundamental herbs in Chinese herbalism. This bark has found application in the Chinese traditional medicine for various diseases like cirrhosis, bacillary dysentery, pneumonia, tuberculosis and liver cirrhosis. Phellodendri Cortex show quite variable quality, because of the two Phellodendron species comprised the source of the medicinal herb on the market. It has been widely used as an important natural source of berberine (Ida et al., 1993). Alkaloid components are active ingredients that exhibit a wide spectrum of biological and pharmacological activites, including the following effects: antimicrobial (Yu et al., 2005), anti-inflammation (Lee et al., 1989), anti-gastric ulcer (Uchiyama et al., 1989; Lee et al., 2005), anticancer (Kumar et al., 2007), and antihypersensitivety (Mori et al., 1994, 1995).

In Korea, despite the two different species of Phello-

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dendri Cortex are currently being used as a same medicinal plant, in which the contents of active secondary metabolites, such as berberine, palmatine and Jatorrhizine, in two species differ. Thus, chemical and genetical methods to distinguish these two different species are required. The discrimination of Phellodendri Cortex from its adulterants is currently limited to the methods of morphology and chemical fingerprinting. So far, many studies have examined the constituents and efficacy of Phellodendri Cortex, but molecular genetic reports have been insufficient. The major chemical constituents of the bark of Phellodendri Cortex are alkaloids, e.g. berberine, palmatine, magnoflorine, phellodendrine, jatrorrhizine and candicin; phytosterols, e.g. campesterol, β-sitosterol, 7-dehydrostigmasterol and their fatty acid esters; as well as limonoidal triterpenes, e.g. limonin, obakunone and flavone glucosides have also been isolated from the bark and the leaves (Ikuta et al., 1998). HPLC methods have been developed for the determination of berberine from *P. amurense* Rupr. (Huang et al., 2002; Lu and Yang, 2004; Tan et al., 2004). Yang et al. (2010) had determined the content of the two subclasses, including limonin and alkaloids in Phellodendri Cortex. It has been known that Phellodendri Cortex is very complicated with respect to its sources on the market, thus, a systematic comparative study on the commercial articles is important.

In addition, the ribulose-1, 5-bisphospate carboxylase large subunit (*rbcL*) intergenic spacer sequences of specific parts produced the same result. However, when the analysis was carried out by using the RAPD (random amplification polymorphism DNA) analysis method, which uses 48 random primers, it allowed us to confirm the polymorphism of *P. amurense* and *P. chinense* in 12 primers. With the development of molecular biology and the innovation of related technology, the identification of plants and medicinal substances on the DNA molecular level has become a feasible and a widely used method. A comparison of nucleotide sequences, forming the internal transcribed spacer (ITS) region and ribulose-1, 5-bisphospate carboxylase large subunit (*rbcL*) region have been reported to be highly useful for the study of species discrimination (Polans et al., 1986; Jensen and Straus, 1993; Graham and Olmstead, 2000). Also, RAPD analysis was conducted for the detection of genetic polymorphisms, with a single short oligonucleotide primer. Among the many molecular methods that are currently available for genetic studies, it appears particularly suitable for analysis of any species (Williams et al., 1990; Castiglioni and de Campos Bicudo, 2005; Cesniene et al., 2010).

For this study, we performed experiments to discrimination of Phellodendri Cortex by ITS-PCR, *rbcL*-PCR and RAPD analysis. Further, we collected twenty samples from different herbs in Korea and China, assayed the contents of three alkaloids by HPLC/UV and identified and categorized them.

MATERIALS AND METHODS

Plant material

A total of 20 Phellodendri Cortex samples were collected for this study, including 4 Phellodendri Cortex samples (k-1 ~ k-4) from Korea, 8 Phellodendri Cortex samples (c-1 ~ c-8) from China, 3 authentic *P. chinense* samples (s-4, s-7 and s-8) from the Sichuan market in China and 5 authentic *P. amurense* samples (s-1 from Bonghwa-gun, Korea; s-2 from Yeongcheon, Korea; s-3 and s-5 from Jilin, China; s-6 from Liaoning, China) collected from Korea and China (Table I). A voucher specimen (k-1 ~ k-4, c-1 ~ c-8 and s-1 ~ s-8) has been deposited at the College of Pharmacy, at Yeungnam University, located in Gyeongsan, Korea.

Reagents

All the standards, berberine, palmatine and jatrorrhizine were provided by Prof. S. H. Lee, Yeungnam University, Korea. Their purities were above 97%, as determined by HPLC and LC MS/MS analysis, and the standard compound structures were shown in Fig. 1. Internal standard, *n*-propylparaben was purchased from Aldrich (purity 99.0%). HPLC grade reagents, acetonitrile and methanol were purchased from Burdick & Jackson. 1-Octanesulfonic acid sodium salt was obtained from Wako, potassium dihydrogen phosphate was obtained from Sinyo Pure Chemicals Co. Ltd. Hydrochloric acid (HCl) was analytical-reagent grade, obtained from Matsunoen Chemical Ltd.

Instrumentation

The Shimadzu HPLC system consisted of a LC-20AD pump, SPD-20A UV/VIS detector, SIL-20A auto-sampler injector, DGU-20A_5 solvent degasser and CTO-20A column oven, Ultra-sonicator was Branson 3210, Shaking incubator was J-SIL-R (JISICO).

Preparation of HPLC samples

To determine the content of three marker compounds of Phellodendri Cortex samples, dried Phellodendri Cortex was powdered and through 50 mesh, and 100 mg of the powder were accurately weighed and added 50 mL 70% methanol:10% HCl (100:1), accurately measured weight and ultrasonicated for 30 min at room temperature. The solution was cooled, which was weighed again, and then made up the loss in weight with the above solvent. The solution was filtered through

Table I. Twenty Phellodendri Cortex samples

Code	Sample	Source	Obtained
k-1	Phellodendri Cortex	Yeongcheon, Korea	purchased
k-2	Phellodendri Cortex	Gangwon-do, Korea	purchased
k-3	Phellodendri Cortex	Gyeongju, Korea	purchased
k-4	Phellodendri Cortex	Yeongcheon, Korea	purchased
c-1	Phellodendri Cortex	Jilin, China	purchased
c-2	Phellodendri Cortex	Jilin, China	purchased
c-3	Phellodendri Cortex	Jilin, China	purchased
c-4	Phellodendri Cortex	Heilongjiang, China	purchased
c-5	Phellodendri Cortex	Heilongjiang, China	purchased
c-6	Phellodendri Cortex	Heilongjiang, China	purchased
c-7	Phellodendri Cortex	Liaoning, China	purchased
c-8	Phellodendri Cortex	Shandong, China	purchased
s-1	*Phellodendron amurense* Ruprecht	Bonghwa-gun, Korea	collected
s-2	*P. amurense* Ruprecht	Yeongcheon, Korea	collected
s-3	*P. amurense* Ruprecht	Jilin, China	collected
s-4	*P. chinense* Schneid	Sichuan, China	collected
s-5	*P. amurense* Ruprecht	Jilin, China	collected
s-6	*P. amurense* Ruprecht	Liaoning, China	collected
s-7	*P. chinense* Schneid	Sichuan, China	collected
s-8	*P. chinense* Schneid	Sichuan, China	collected

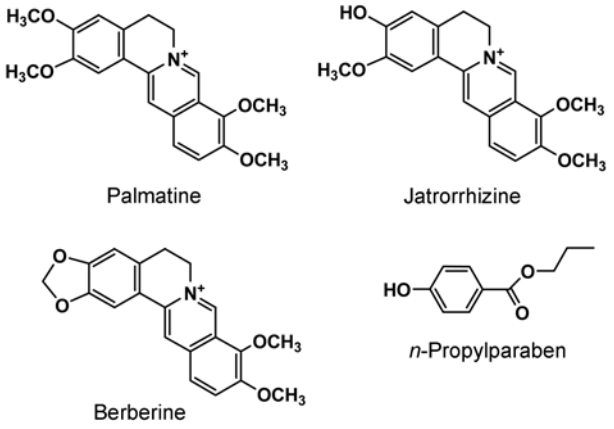

Fig. 1. Chemical structures of standards.

a 0.45 μm membrane filter and the filtrate was used as the test solution.

HPLC/UV condition

The HPLC analysis was performed using the Shimadzu HPLC system with LC-20AD pumps SPD-20A UV detector and SIL-20A autosampler. The HPLC column was used Inertsil® ODS-3 column (250 × 4.6 mm, 5 μm, GL Sciences), which was used with the guard column of C18 cartridge (4.0 × 3.0 mm). A mixture of acteonitrile (30 mM potassium dihydrogen phosphate) and water (20 mM 1-octanesulfonic acid) (40:60, v/v %) was used as the mobile phase. The mobile phase was filtered under a vacuum through a 0.45 μm membrane filter and had been degassed prior to use. The column temperature was maintained at 40°C. The analysis was carried out at a flow rate of 1.0 mL/min, with the detection wavelength set to 254 nm, and the total run time was 20 min. The injection volume was 10 μL.

Analytical method validation

Linearity, limits of detection and limits of quantification

Stock solution of berberine (500 μg/mL), palmatine (500 μg/mL) and jatrorrhizine (100 μg/mL) were prepared in methanol and kept below at 4°C. Standard solutions were prepared by serial dilution of the stock solutions to working ranges of these substances with mobile phase. An internal standard (*n*-propylparaben, 1,000 μg/mL) was prepared with methanol to the concentration. Calibration graphs were prepared from the peak area-ratio of analytes to I.S. in the concentration range, berberine 0.50-200.00 μg/mL, palmatine 0.50-100.00 μg/mL and jatrorrhizine 0.10-20.00 μg/mL, respectively. Limit of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were determined from signal-to-noise (S/N) of 3 and 10, respectively.

Recovery, precision and accuracy

For the preparation of the crude extract, the powders of the dried drugs were sieved though a 50 mesh. Pul-

verized drug specimen was weighed accurately, 100 mg into 50 mL volume metric flask and different amounts of berberine (100 μg/mL; 1.1, 2.2, 4.4 mL), palmatine (100 μg/mL; 0.95, 1.9, 4.75 mL), and jatrorrhizine (100 μg/mL; 0.16, 0.4, 0.8 mL) were added into the samples, respectively. Each 1.0 mL aliquots of the I.S. solution (1,000 μg/mL) was spiked with the spiked samples, then the aqueous of 70% methanol:10% HCl (100:1) were added to mark the 50 mL volume metric flask. The sample mixture was extracted for 30 min in an ultrasonic bath at room temperature. After extraction, the sample mixture was filtered through 0.45 μm membrane filter and the 10 μL aliquots of the filtrate were injected into HPLC. The average recovery was represented by the formula: R (%) = [(amount from the sample spiked standard − amount from the sample)/ amount from the spiked standard] × 100.

Genomic DNA and polymerase chain reaction

Genomic DNA was prepared from dry bark tissue and was extracted using the CTAB method (Doyle and Doyle, 1987). The extracted genomic DNA was amplified by PCR, which used each of the ITS (ITS1; 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4; 5'-TCCTCCGCTTATTGATATGC-3') primer, *rbcL* (*rbcL* 1F; 5'-ATGTCACCACAAACAGAAAC-3' and *rbcL* 1352R; 5'-CAGCAACTAGTTCAGGRCTCC-3') primer and universal Operon primers. The Total volume of 30 μL reaction were mixed with a pair of primers (10 pmole/μL of each), 10 × PCR buffer, 1.5 μM $MgCl_2$, 2 μL dNTP mixtures, 0.1 μL Taq DNA polymerase (Takara), and distilled water to a final volume. The PCR program was 35 cycles of 30 sec at 94°C, 40 sec at 55°C (ITS and *rbcL*) or 37°C (Operon), 1 min at 72°C.

Cloning of DNA fragments and sequence analysis

The amplified DNA fragments were electrophoresed on a 1.5% agarose gel. The bands of the expected size were separated from the gel and the DNA fragments were purified using the gel elution kit (Promega). These fragments were then subcloned into the pGEM T-easy vector (Promega). The sequenced nucleotides were analyzed by Clustal W (Thompson et al., 1994) and the percentage of homology was compared by Blast in NCBI (National center for biotechnology information).

RESULTS

Optimization of chromatographic condition

The HPLC conditions were selected by the requirement for obtaining the chromatograms with a better resolution of the adjacent peaks, within a short retention time. For the optimization of chromatographic condition, the effect of the composition of mobile phases on the separation was examined. Among them, an isocratic mobile phase consisting of acetonitrile (30 mM potassium dihydrogen phosphate), and water (20 mM 1-octanesulfonic acid sodium salt) (40:60) resulted in a good resolution, as well as satisfactory peak symmetry and shape. All compounds could be resolved with the baseline separation at 254 nm with the maximum absorption. The typical chromatograms of samples and standard mixture are shown in Fig. 2, from which, one can observe that all target compounds and internal standard are completely separate within 20 min. The chromatographic peaks of the analytes in sample the solution were identified by comparing their retention time with those of the reference standards, and were further confirmed by spiking the samples with the reference compounds.

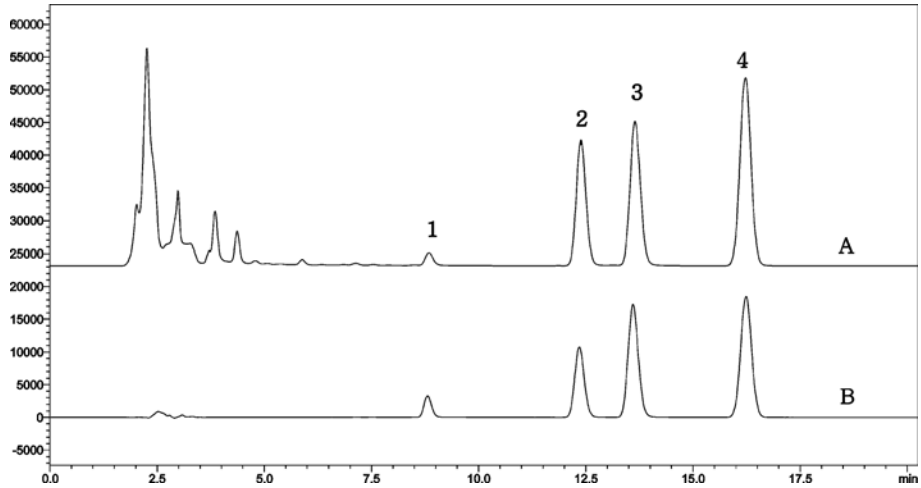

Fig. 2. Chromatographies of Phellodendri Cortex extract (**A**) and standard mixture (**B**): Jatrorrhizine (1), Palmatine (2), Berberine (3) and *n*-Propylparaben (4).

Table II. Calibration curve data, linear ranges, LOD and LOQ

Compounds	Linear range (μg/mL)	Slope	Intercept	Correlation coefficient (r^2)	LOD[a] (ng/mL)	LOQ[b] (ng/mL)
Jatrorrhizine	0.10 ~ 20.00	0.0299	-0.0045	0.9997	115.4	384.6
Palmatine	0.50 ~ 100.00	0.0319	-0.0170	0.9998	166.7	555.6
Berberine	0.50 ~ 200.00	0.0336	-0.0169	0.9999	176.5	588.2

[a]LOD = 3×S/N; [b]LOQ = 10×S/N

Validation

Linearity, LOD and LOQ

The linearity of the peak area ratio with respect to the concentration was examined under the optimal HPLC/UV conditions, and is described as a regression equation. Each correlation coefficient (r^2) was > 0.999, as determined by least square analysis, suggesting good linearity between the peak areas and the compound concentrations, over a wide concentration range (berberine, 0.50-200.00 μg/mL, palmatine, 0.50-100.00 μg/mL and jatrorrhizine, 0.10-20.00 μg/mL) (Table II). The ranges of LOD and LOQ were 115.4-176.5 ng/mL and 384.0-588.2 ng/mL, respectively (Table II).

Specificity and selectivity

Specificity refers to the ability of a method to accurately determine the analyte level in the presence of all other components in the sample matrix. As shown in Fig. 2, all analytes were well separated from the backgrounds.

Recovery

The extraction recovery test was performed by extracting a known amount of the three compounds from the Phellodendri Cortex powder samples. A known amount of each standard compound at three different levels was mixed with the sample powder and extracted, as described in the experimental section. The recovery (%) of each standard ranged from 95.7 to 107.8%, and the RSD was less than 1.8% (Table III).

Table III. Recovery of marker compounds by standard addition method (n = 3)

Compounds	Fortified Conc. (μg/mL)	Observed Conc. (μg/mL)	Mean recovery (%)	Recovery RSD (%)
Jatorrhizine	0.00	1.58 ± 0.00	-	-
	0.80	2.44 ± 0.00	107.50	0.00
	1.60	3.25 ± 0.00	104.44	0.10
	3.20	5.03 ± 0.00	107.81	0.08
Palmatine	0.00	16.81 ± 0.07	-	-
	3.80	20.80 ± 0.01	104.97	1.75
	7.60	24.42 ± 0.03	100.13	0.58
	19.00	35.14 ± 0.03	96.60	0.47
Berberine	0.00	24.30 ± 0.04	-	-
	4.40	29.01 ± 0.01	107.21	1.72
	8.80	33.08 ± 0.13	99.82	1.06
	17.60	41.14 ± 0.04	95.70	0.13

Precision and accuracy

Precision and accuracy were determined by multiple analysis (n = 3) of quality control samples prepared at lower, medium and higher concentration, which spans the calibration range. Intra-assay precision and accuracy were determined from the variability of replicate an-

Table IV. Precision and accuracy of analytical results

Compounds	Spiked Conc. (μg/mL)	Inter-day (n = 3)				Inter-day (n = 3)			
		Observed Conc. (μg/mL)	SD	Precision RSD (%)	Accuracy (%)	Observed Conc. (μg/mL)	SD	Precision RSD (%)	Accuracy (%)
Jatorrhizine	0.80	0.81	0.00	0.32	101.16	0.82	0.00	0.46	102.23
	1.60	1.57	0.00	0.10	97.83	1.56	0.02	1.10	97.47
	3.20	3.22	0.00	0.09	100.54	3.22	0.01	0.22	100.63
Palmatine	3.80	4.00	0.01	0.19	105.33	4.06	0.01	0.28	106.84
	7.60	7.71	0.03	0.45	101.46	7.56	0.01	0.14	99.48
	19.00	18.90	0.03	0.17	99.48	18.51	0.02	0.10	97.41
Berberine	4.40	4.73	0.07	1.44	107.49	4.67	0.02	0.45	106.24
	8.80	8.97	0.14	1.58	101.90	8.77	0.12	1.33	99.65
	17.60	17.40	0.04	0.25	98.85	17.42	0.06	0.35	98.98

alyses of quality control samples analyzed within the same analytical run. The remaining quality control samples had the intra-assay precision below 2% and accuracy between 97.8% and 107.5%. Inter-assay precision and accuracy were evaluated from the variability of triplicate analyses of quality control samples analyzed on single analytical run and extended for consecutive three days. The remaining quality control samples had the inter-assay precision lower than 2% and accuracy between 97.5% and 106.8%. The above data reflects that the developed method is highly reproducible and precision and accuracy data are presented in Table IV.

Robustness

The robustness was determined in order to evaluate the reliability of the established HPLC methods. All of the parameters were maintained so there would not be any interference with the other peaks for the Phellodendri Cortex extract. The experimental conditions, such as the column temperature and column species were purposely altered. The theoretical plate (*N*), capacity factor (*k'*), separation factor (α) and resolution (*Rs*) were evaluated. To evaluate the suitability of three different columns, Inertsil® ODS-3, SunFire™ C-18 and Luna C-18, were compared with regards to the four analytical factors (theoretical plate plate (*N*), capacity factor (*k'*), separation factor (α) and resolution (*Rs*)) on the column temperature of 25°C. When using Inertsil® ODS-3 column, the values of resolution and theoretical plate were higher than that of the others (Table V). Therefore we selected Inertsil® ODS-3 column to analyze the samples throughout this work. Six different column temperatures, 25, 30, 35, 40, 45 and 50°C, were compared with regards to the four analytical factors, using Inertsil® ODS-3 column. When the column temperature was 40°C, the values of resolution and theoretical plate were satisfied than the other column temperatures (Table VI). Therefore we selected the column temperature as 40°C.

Stability

The stability of standards in methanol under two different states of storage, room temperature and cold-storage at 4°C were tested at 0, 1, 2, 5, 10, 15 and 30 days. During this period, peak areas of standards were measured by comparison to the 1st day response. The resulting data indicate that all marker analytes showed a long-term stability with RSD ≤ 1.8%, as shown in Table VII.

Sample analysis

The developed HPLC/UV method was then applied to the simultaneous determination of the three compounds, berberine, palmatine and jatrorrhizine, in Phellodendri Cortex. Twenty Phellodendri Cortex samples were obtained from Korea and China. The developed analytical method was subsequently applied to the simultaneous determination of the three components in Phellodendri Cortex extract. The quantity of each compound present in samples was determined and the results are summarized in Table VIII. Each sample

Table V. Efficiency (%) of marker compounds in different column

Column	Surface area (m^2/g)	Pore size (Å)	Analytes (Mean ± S.D.)		
			Jatrorrhizine	Palmatine	Berberine
Theoretical plate (N)					
Inertsil® ODS-3	450	100	3313 ± 8.86	3450 ± 0.00	4268 ± 7.55
SunFire™ C18	349	90	2954 ± 10.04	2040 ± 26.13	2401 ± 0.00
Luna C18	400	100	2500 ± 0.00	2601 ± 0.00	3077 ± 6.40
Capacity factor (k')					
Inertsil® ODS-3	450	100	1.95 ± 0.00	3.06 ± 0.00	3.43 ± 0.00
SunFire™ C18	349	90	1.65 ± 0.00	2.51 ± 0.00	2.82 ± 0.00
Luna C18	400	100	1.78 ± 0.03	2.78 ± 0.04	3.12 ± 0.05
Separation factor (α)					
Inertsil® ODS-3	450	100	1.57 ± 0.00	1.13 ± 0.00	1.13 ± 0.00
SunFire™ C18	349	90	1.53 ± 0.00	1.12 ± 0.00	1.12 ± 0.00
Luna C18	400	100	1.56 ± 0.00	1.12 ± 0.00	1.12 ± 0.00
Resolution (Rs)					
Inertsil® ODS-3	450	100	8.61 ± 0.00	2.68	2.68 ± 0.00
SunFire™ C18	349	90	6.31 ± 0.02	1.79 ± 0.00	1.79 ± 0.00
Luna C18	400	100	7.16 ± 0.11	2.14 ± 0.04	2.14 ± 0.04

All columns are those of analytical, 4.6 × 250 mm, 5 μm; Inertsil® ODS-3 (G.L. Science Inc.); SunFire™ C18 (Waters); Luna C18 (Phenomenex).

Table VI. Effects of column temperature on system suitability

Temperature (°C)		Analytes (Mean ± S.D.)		
		Jatrorrhizine	Palmatine	Berberine
Theoretical plate (N)	25	2122 ± 10.65	3216 ± 0.00	3505 ± 0.00
	30	2408 ± 11.34	3697 ± 18.07	3927 ± 28.91
	35	2285 ± 25.26	3494 ± 31.53	3559 ± 19.88
	40	3762 ± 0.00	3243 ± 11.70	3193 ± 13.82
	45	2256 ± 0.00	3287 ± 11.70	3047 ± 13.82
	50	2162 ± 0.00	3906 ± 11.70	2788 ± 13.82
Capacity factor (k')	25	2.35 ± 0.00	3.61 ± 0.00	4.37 ± 0.01
	30	2.36 ± 0.01	3.70 ± 0.00	4.68 ± 0.02
	35	2.23 ± 0.01	3.50 ± 0.01	4.05 ± 0.01
	40	2.16 ± 0.06	3.35 ± 0.01	3.82 ± 0.05
	45	1.99 ± 0.01	3.02 ± 0.02	3.30 ± 0.00
	50	1.88 ± 0.00	2.87 ± 0.00	3.09 ± 0.00
Separation factor (α)	25	1.54 ± 0.00	1.21 ± 0.00	1.21 ± 0.00
	30	1.56 ± 0.00	1.18 ± 0.00	1.18 ± 0.00
	35	1.57 ± 0.00	1.16 ± 0.00	1.16 ± 0.00
	40	1.58 ± 0.00	1.13 ± 0.00	1.13 ± 0.00
	45	1.52 ± 0.00	1.10 ± 0.00	1.10 ± 0.00
	50	1.52 ± 0.00	1.08 ± 0.00	1.08 ± 0.00
Resolution (Rs)	25	7.59 ± 0.05	3.98 ± 0.07	3.98 ± 0.07
	30	8.45 ± 0.02	3.85 ± 0.03	3.85 ± 0.03
	35	8.16 ± 0.01	3.13 ± 0.02	3.13 ± 0.02
	40	8.90 ± 0.01	2.50 ± 0.00	2.50 ± 0.00
	45	5.90 ± 0.06	1.51 ± 0.01	1.51 ± 0.01
	50	5.99 ± 0.00	1.20 ± 0.00	1.20 ± 0.00

was analyzed in triplicate to ensure the reproducibility of the quantitative result. The results indicated that, berberine (0.50-8.75%) and palmatine (0.04-0.95%) were found to be the most abundant components in Phellodendri Cortex, whereas, jatrorrhizine (0.02-0.10%) was a minor ingredient of Phellodendri Cortex. On the other hand, contents of berberine in *Phellodendron chinense* samples (s-4, s-7 and s-8) were about three times more than those in *P. amurense*, whereas, the contents of palmatine in *P. chinense* were found to be less than a half of those in *P. amurense*.

Analysis of ITS region and *rbcL* region

To obtain the sequence information of each 20 Phellodendri Cortex samples, isolated genomic DNA was used, as a template to amplify ITS and *rbcL* region using PCR. The amplification band size for ITS and *rbcL* was 503 bp and 1,280 bp, respectively. The result showed that the 99.9% and 100% separately of the nucleotide sequences of *P. amurense* were identical to that of *P. chinense*, but the differentiation of *P. amurense* and *P. chinense* was difficult. Also, the base sequences of ITS analyzed showed 99.9% homology with the NCBI database (*P. amurense*; DQ225837, *P. chinense*; DQ225840) reported any of ITS nucleotide regions, and in the NCBI database not reported any of *rbcL* nucleotide regions from Phellodendri Cortex (Fig. 3).

RAPD (randomly amplification polymorphism DNA) analysis

We tested RAPD analysis, using 48 universal primers (Table IX), because ITS and *rbcL* gene analysis were limited on the discrimination of Phellodendri Cortex. As a result of RAPD, 12 primers (OPAA11, HK1, OPAM18, OPAE01, OPAC05, OPAB05, OPAB06, OPAB-10, OPAB12, OPAM08, OPAB03) showed genetic diversity between *P. amurense* and *P. chinense* (Fig. 4). The DNA fragment pattern (arrow) could be efficiently discriminated of *P. amurense* and *P. chinense* in 12 primers.

DISCUSSION

A rapid and optimized chromatographic method with UV detection was designed for the quality control of Phellodendri Cortex, well-known oriental folk medicine. Validation data indicates that the developed analytical methods are suitable to measure the concentration of three compounds of Phellodendri Cortex. Our results confirm that berberine can serve as the species-specific marker compounds to distinguish authentic *Phellodendron amurense* and *P. chinense*. Therefore the results demonstrated that the developed method could

Table VII. Stability of analytes in MeOH

Compounds	Temperature (°C)	Day							Mean (n = 3)	SD	RSD (%)
		0	1	2	5	10	15	30			
Jatorrhizine	R.T[a]	100.0	102.2	100.8	100.8	100.0	103.1	102.5	101.3	1.25	1.23
	4°C	100.0	99.0	100.3	100.8	100.0	103.2	102.6	100.8	1.51	1.50
Palmatine	R.T	100.0	102.0	100.6	100.6	100.6	103.0	101.9	101.2	1.07	1.06
	4°C	100.0	99.9	99.5	101.2	101.0	103.6	102.3	101.1	1.47	1.45
Berberine	R.T	100.0	99.7	99.8	100.6	101.0	103.0	101.8	100.8	1.21	1.20
	4°C	100.0	99.7	99.5	101.7	102.2	104.0	102.9	101.5	1.82	1.79

[a]Room temperature

Table VIII. Analytical results (w.t.%) of the marker compounds in Phellodendri Cortex

Analytes	Content (%)								
	Jatrorrhizine			Palmatine			Berberine		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
k-1	0.08	0.000	0.071	0.92	0.000	0.026	1.32	0.001	0.038
k-2	0.09	0.000	0.050	0.77	0.001	0.091	1.83	0.002	0.102
k-3	0.10	0.000	0.039	0.85	0.000	0.022	2.16	0.000	0.018
k-4	0.09	0.000	0.071	0.76	0.001	0.088	1.85	0.001	0.045
c-1	0.10	0.000	0.071	0.95	0.000	0.020	2.05	0.000	0.014
c-2	0.07	0.000	0.245	0.57	0.001	0.164	1.31	0.000	0.027
c-3	0.05	0.000	0.039	0.51	0.000	0.029	1.00	0.001	0.048
c-4	0.06	0.000	0.062	0.59	0.001	0.119	1.20	0.002	0.130
c-5	0.07	0.000	0.066	0.69	0.000	0.031	1.22	0.000	0.030
c-6	0.03	0.000	0.126	0.42	0.001	0.273	0.50	0.002	0.345
c-7	0.04	0.000	0.039	0.48	0.001	0.203	0.76	0.001	0.175
c-8	0.06	0.000	0.083	0.51	0.490	0.001	1.21	0.001	0.094
s-1	0.07	0.000	0.482	0.88	0.000	0.034	1.26	0.001	0.060
s-2	0.08	0.000	0.269	0.94	0.001	0.122	2.23	0.003	0.113
s-3	0.04	0.001	1.313	0.59	0.000	0.044	1.03	0.001	0.078
s-4	0.07	0.000	0.495	0.16	0.000	0.252	5.15	0.001	0.015
s-5	0.03	0.000	0.831	0.35	0.000	0.016	0.70	0.001	0.119
s-6	0.08	0.000	0.255	0.48	0.000	0.081	1.09	0.000	0.031
s-7	0.02	0.000	0.560	0.04	0.000	0.081	6.03	0.002	0.025
s-8	0.02	0.000	0.442	0.04	0.001	0.084	8.75	0.001	0.015

Table IX. List of universal primers used in this study

No.	Primer name	Sequence (5'-3')	No.	Primer name	Sequence (5'-3')
1	HK 1	TGGTCGCTGA	25	OPAB-11	GTGCGCAATG
2	HK 2	AAGCTCCCCG	26	OPAB-12	CCTGTACCGA
3	HK 3	CCAGACCCTG	27	OPAB-15	CCTCCTTCTC
4	OPB-11	GTAGACCCGT	28	OPAB-16	CCCGGATGGT
5	OPB-16	TTTGCCCGGA	29	OPAC-05	GTTAGTGCGG
6	OPB-18	CCACAGCAGT	30	OPAD-05	ACCGCATGGG
7	OPM-06	CTGGGCAACT	31	OPAD-13	GGTTCCTCTG
8	OPO-01	GGCACGTAAG	32	OPAD-16	AACGGGCGTC
9	OPO-10	TCAGAGCGCC	33	OPAD-17	GGCAAACCCT
10	OPO-12	CAGTGCTGTG	34	OPAE-01	TGAGGGCCGT
11	OPAA-11	ACCCGACCTG	35	OPAE-03	CATAGAGCGG
12	OPAA-13	GAGCGTCGCT	36	OPAG-10	ACTGCCCGAC
13	OPAA-17	GAGCCCGACT	37	OPAG-12	CTCCCAGGGT
14	OPAB-01	CCGTCGGTAG	38	OPAG-16	CCTGCGACAG
15	OPAB-02	GGAAACCCCT	39	OPAJ-01	ACGGGTCAGA
16	OPAB-03	TGGCGCACAC	40	OPAK-01	TCTGCTACGG
17	OPAB-04	GGCACGCGTT	41	OPAK-02	CCATCGGAGG
18	OPAB-05	CCCGAAGCGA	42	OPAK-05	GATGGCAGTC
19	OPAB-06	GTGGCTTGGA	43	OPAK-12	AGTGTAGCCC
20	OPAB-07	GTAAACCGCC	44	OPAL-11	GTCACGTCCT
21	OPAB-08	GTTACGGACC	45	OPAL-12	CCCAGGCTAC
22	OPAB-09	GGGCGACTAC	46	OPAM-08	ACCACGAGTG
23	OPAB-10	TTCCCTCCCA	47	OPAM-18	ACGGGACTCT
24	OPAB-13	CCTACCGTGG	48	OPAM-16	TGGCGGTTTG

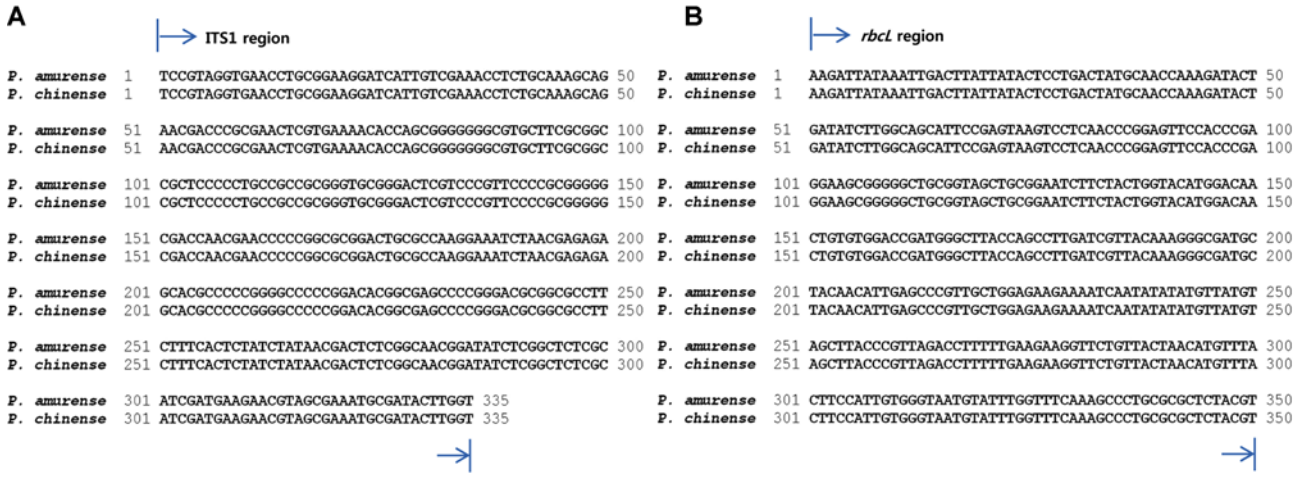

Fig. 3. Aligned sequences of partial ITS (**A**) and *rbcL* (**B**) regions in *P. amurense* and *P. chinense*. Nucleotide sequences were displayed from 5' to 3'.

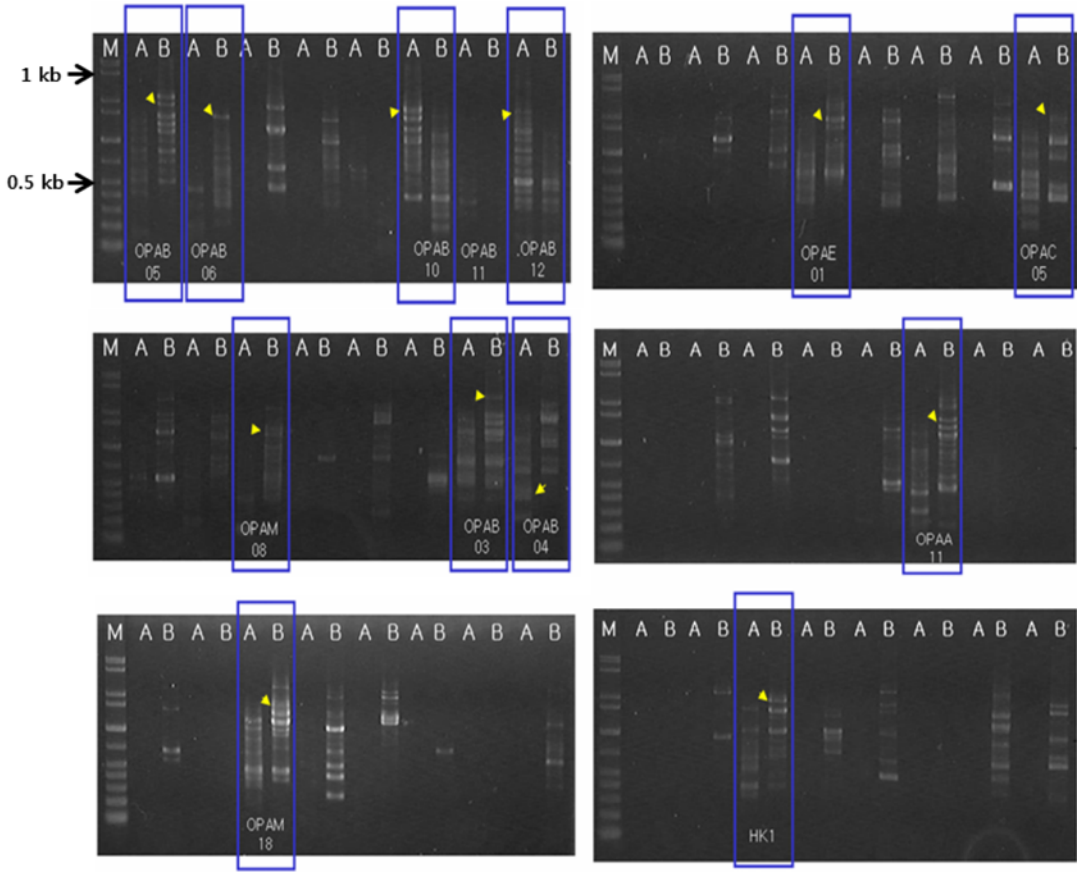

Fig. 4. RAPD polymorphism of the *P. amurense* (A; s-1 sample of Table I) and *P. chinense* (B; s-8 sample of Table I). The arrow showing the different band pattern were observed polymorphism between *P. amurense* and *P. chinense*. Numbers written at the under of box were primer used in this RAPD. M, molecular size marker.

be applied as a reliable and sensitive quality control procedure for Phellodendri Cortex.

Phellodendri Cortex has the difficulty in extracting DNA dried samples, due to large polysaccharide content, which required a long time in its extraction. ITS region was characteristic of biparental inheritance, based on base variation occurring among the species, but the Phellodendri Cortex showed 99.9% homology, among the samples of *P. amurense* and *P. chinense*. In addition, this study about sequencing of ITS and *rbcL*

region was consistent with NCBI database registered in the sequences of a Phellodendri Cortex *trnL-F* region (DQ225993, DQ225989, DQ225991 and DQ225996) However, DNA pattern analysis using universal primer could be discriminated in the two species. Therefore, we provided an effective method for discrimination of *P. amurense* and *P. chinense*. Further studies will be required to verify the result of this study by securing more samples from more diverse regions.

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