high-performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector.

MATERIALS AND METHODS

Plant material

The roots of *A. lappa* Decne used in this study was purchased from HMAX (Jecheon, Korea) in July 2009. The botanical origin of this sample was confirmed taxonomically by Prof. Je-Hyun Lee, Dongguk University, Gyeongju, Republic of Korea. A voucher specimen (2009-KIOM62) has been deposited at the K-herb Research Center Korea Institute of Oriental Medicine.

Chemicals and materials

Reference compounds 1–3 were purchased from ChemFaces (Wuhan, China). The purities of the three sesquiterpene lactones were >98.0% by HPLC analysis.

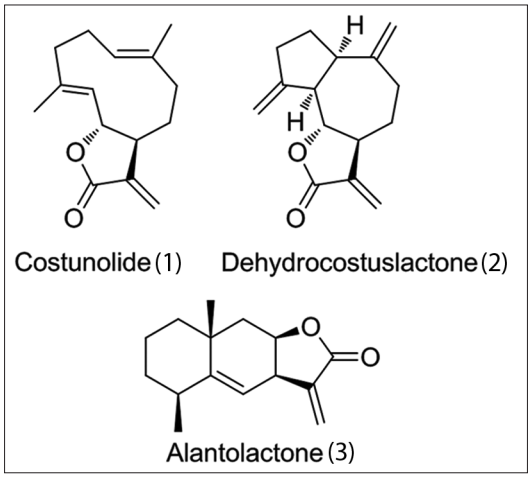

Figure 1: Chemical structures of the three biomarker compounds in *Aucklandia lappa*

Table 1: System suitability of marker compounds

Analyte	Capacity factor (*K*□)	Relative retention (*α*)	Resolution (*R*s)	Theoretical plate number (*n*)	TF
1	2.88	1.78	5.34	1392	1.20
2	5.12	1.14	1.56	3340	1.12
3	5.82	1.14	1.56	3319	1.12

TF: Tailing factor

HPLC-grade solvents, methanol, acetonitrile, and water were obtained from JT Baker (Phillipsburg, NJ, USA). Glacial acetic acid (analytical grade) was purchased from Merck KGaA (Darmstadt, Germany).

Preparations of 70% methanol extract and sample solution

Dried sample powder of *A. lappa* (100 g) was extracted 3 times with 70% methanol (1 L) by heating to reflux for 90 min. The extracted solution was filtered through filter paper, evaporated at 40°C using a Büchi R-210 rotary evaporator (Flawil, Switzerland) under vacuum to dryness and freeze-dried. The yield of the freeze-dried 70% methanol extract obtained was 28.57% (28.57 g). For the HPLC analysis of the compounds 1–3, the 70% methanol extract (20 mg) was dissolved in 10 mL of 70% methanol and extracted by sonication for 30 min. The solution was filtered through a 0.2 μm membrane filter (Woongki Science, Seoul, Korea) before injection into the HPLC instrument.

Apparatus and conditions

The simultaneous determination was performed with a Shimadzu Prominence LC-20A series HPLC (Shimadzu Co., Kyoto, Japan) consisting of a solvent delivery unit (LC-20AT), on-line degasser (DGU-20A$_3$), column oven (CTO-20A), auto sample injector (SIL-20AC), and PDA detector (SPD-M20A). Data were collected and processed by LCsolution software (Version 1.24, Shimadzu, Kyoto, Japan). Separation of compounds 1–3 was carried out on a reversed-phase SunFire™ C$_{18}$ analytical column (Waters, Milford, MA, USA; 150 mm × 4.6 mm and 5 μm particle size). The mobile phase for chromatographic separation of the three analytes was distilled water (A) and acetonitrile (B) with isocratic elution (i.e. 40% A and 60% B). The flow rate was 1.0 mL/min, the column temperature was maintained at 35°C, and the detection wavelength of quantification was set at 225 nm. The injection volume was 10 μL.

Calibration curves, limits of detection, and quantification

To prepare the stock solutions, reference compounds 1–3 were accurately weighed and dissolved in methanol to a concentration of 1.0 mg/mL; the samples were stored below 4°C. The calibration curves for 1–3 were calculated by plotting the peak areas (*y*) versus the

Table 2: Regression equation, linear range, correlation coefficient, LOD, and LOQ for marker compounds

Analyte	Linear range (μg/mL)	Regression equation[a]	Correlation coefficient (r^2)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
1	1.56-200.00	y=19517.29x+25885.26	0.9999	0.06	0.21
2	2.34-300.00	y=12298.18x+3968.28	0.9999	0.11	0.37
3	0.23-30.00	y=10505.61x+331.17	1.0000	0.13	0.42

[a]y: Peak area (mAU) of compounds; x: Concentration (μg/mL) of compounds; [b]LOD: 3×signal-to-noise ratio; [c]LOQ: 10×signal-to-noise ratio; LOD: Limits of detection; LOQ: Limits of quantification

concentrations (x, μg/mL) using the standard solutions. The tested concentration ranges for calibration curves were 1.56–200.00, 2.34–300.00, and 0.23–30.00 μg/mL for compounds 1, 2, and 3, respectively. The limits of detection (LOD) and limits of quantification (LOQ) for the three sesquiterpene lactones were calculated at signal-to-noise ratios of 3 and 10, respectively.

Precision, reproducibility, and accuracy

Intra-day and inter-day variations, which were used to evaluate the precision of the established HPLC method, were determined using the standard addition method for samples spiked with low, medium, and high concentration levels of analyte. The relative standard deviation (RSD) was used as a measurement of precision. To confirm the reproducibility of the proposed method, we measured six replications using the mixed standard stock solutions. The RSD values of peak areas and retention times of the three compounds were evaluated to establish the reproducibility of the method, and recovery tests were performed to evaluate the accuracy of the method. The test for recovery was carried out by adding known amounts of low, medium, and high concentration levels of the three reference compounds to 20 mg of *A. lappa* extract. This test was evaluated using the calibration curves determined for compounds 1–3.

Table 3: Recoveries for the assay of the three investigated compounds

Analyte	Spiked amount (μg/mL)	Detected amount (μg/mL)	Recovery (%)[a]	SD	RSD (%)
1	4.00	41.12	100.91	0.95	0.95
	15.00	51.85	98.41	0.18	0.18
	30.00	67.98	103.00	0.56	0.54
2	8.00	68.00	97.27	0.99	1.02
	30.00	89.58	97.88	0.68	0.69
	60.00	118.90	97.80	0.20	0.20
3	1.00	1.01	99.19	0.90	0.91
	2.00	2.04	100.98	0.59	0.58
	4.00	4.04	100.48	0.91	0.91

[a]Recovery (%): Detected amount/spiked amount ×100; SD: Standard deviation; RSD: Relative standard deviation

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

High-performance liquid chromatography conditions including column type, column temperature, and mobile phases were assessed to accomplish the simultaneous separation of the three analytes 1–3. For the optimized separation of the three components, columns including Phenomenex Gemini C_{18} (250 mm × 4.6 mm, 5 μm), Waters SunFire C_{18} (250 mm × 4.6 mm, 5 μm), Waters SunFire C_{18} (150 mm × 4.6 mm, 5 μm), and OptimaPak C_{18} (250 mm × 4.6 mm, 5 μm), various column temperatures (30, 35, and 40°C), and a range of mobile phases composed of acids such as acetic, formic, and phosphoric acid, and organic solvents methanol and acetonitrile, were examined. As a result, optimized simultaneous separation of the three analytes with respect to baseline, resolution, and peak shape could be achieved using a Waters SunFire™ C_{18} column (150 mm × 4.6 mm, 5 μm) with isocratic elution of distilled water (A) and acetonitrile (B) at 35°C. The optimized conditions enabled the three compounds to be eluted within 15 min with a resolution of better than 1.56. Detection wavelength for quantification of the three analytes was set at 225 nm, and each compound in the HPLC chromatogram was identified by comparing the retention times and ultraviolet spectra with those of standards and by co-injection with an authentic sample. The retention times of compounds 1–3 under the optimized conditions were 7.81, 8.70, and 10.22 min, respectively. Representative HPLC chromatograms of standard solutions and the *A. lappa* extracts are shown in Figure 2.

System suitability

The system suitability, that is., capacity factor (K), relative retention (α), resolution (Rs), theoretical plate number (N) and tailing factor (TF) was measured in order to evaluate

Table 4: Precision and repeatability of the analysis

Analyte	Spiked concentration (μg/mL)	Intra-day (n=5)			Inter-day (n=5)			Repeatability (RSD (%), n=6)	
		Detected concentration (μg/mL)	SD	RSD (%)	Detected concentration (μg/mL)	SD	RSD (%)	Retention time	Peak area
1	4.00	4.09	0.02	0.50	4.07	0.03	0.72	0.08	0.28
	15.00	14.52	0.06	0.39	14.57	0.11	0.72		
	30.00	30.23	0.03	0.09	30.20	0.05	0.16		
2	8.00	7.97	0.08	0.97	8.11	0.05	0.63	0.09	0.21
	30.00	30.03	0.15	0.49	29.80	0.12	0.42		
	60.00	59.99	0.06	0.11	60.08	0.06	0.09		
3	1.00	0.99	0.01	0.69	1.00	0.00	0.45	0.09	0.19
	2.00	2.01	0.01	0.67	1.97	0.02	1.06		
	4.00	4.00	0.01	0.15	4.01	0.01	0.25		

SD: Standard deviation; RSD: Relative standard deviation

the reliability of optimized HPLC method. As shown in Table 1, the capacity factor and relative retention were 2.88–5.82 and 1.14–1.78. The resolutions of the compounds 1–3 were >1.5, which suggested that the peaks of three components were not severely overlapped by adjacent peaks and interference from other components. The number of theoretical plate and TF were 1392–3340 and 1.12–1.20.

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

Each regression equation ($y = ax + b$) was calculated based on the ratio of peak area (y), and concentration (x, μg/mL) of reference compounds 1–3. Standard curves plotted with compounds 1–3 showed high linearity, with $r^2 \geq 0.9999$ in the eight different concentration ranges tested. The LODs and LOQs compounds 1–3 were 0.06–0.13 and 0.21–0.42 μg/mL, respectively. The results are summarized in Table 2.

Precision, reproducibility, and accuracy

As shown in Table 3, the recoveries of the three analytes were 97.27–13.00% at low, medium, and high concentration levels, and the RSD values were <1.02%. The RSD for reproducibility of analysis of compounds 1–3 was 0.19–0.28% for peak area and 0.08–0.09% for retention times [Table 4]. The precisions (RSD) of intra- and inter-day variations for the three analytes were <0.97 and 1.06%, respectively. The results, which are summarized in Table 4, therefore, suggest that the established method was suitable for quantitative analysis of the three investigated compounds.

Quantitative analysis

The established HPLC analytical method was applied for the simultaneous quantification of the compounds 1–3 in *A. lappa*. The amounts of three sesquiterpene lactones, costunolide (1), dehydrocostus lactone (2), and alantolactone (3) were 17.32 mg/g, 28.26 mg/g, and 0.01 mg/g, respectively [Table 5].

CONCLUSION

A convenient, simple, and accurate HPLC-PDA method was established for the simultaneous determination of the three sesquiterpene lactones, costunolide (1), dehydrocostus lactone (2), and alantolactone (3), in *A. lappa*. The developed method has been successfully applied to the quantitative analysis for the purpose of quality control and showed good linearity, precision, and accuracy. The established HPLC-PDA method was demonstrated to be a suitable method for quality control of the components of *A. lappa*.

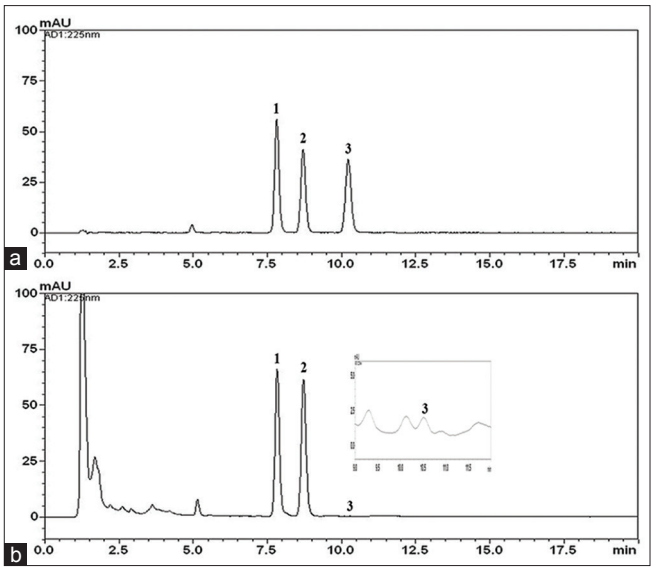

Figure 2: High-performance liquid chromatography chromatogram of standard solution (a) and *Aucklandia lappa* extract (b). Costunolide (1); dehydrocostus lactone (2); and alantolactone (3)

Table 5: The amounts of the three marker compounds in *A. lappa* determined by HPLC analysis

Analyte	Amount (mg/dried g)		
	Mean	SD (×10^{-2})	RSD (%)
1	17.32	2.54	0.15
2	28.26	1.42	0.05
3	0.01	0.03	2.74

HPLC: High-performance liquid chromatography; SD: Standard deviation; RSD: Relative standard deviation; *A. lappa: Aucklandia lappa*

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