Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Simultaneous Determination of two Diterpenoids, Continentalic Acid and Kaurenoic Acid, in the Water extract of *Aralia continentalis* and their Wound-Healing Activity

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Submitted: 10-Aug-2020 Revised: 10-Sep-2020 Accepted: 01-Dec-2020 Published: 16-Feb-2021

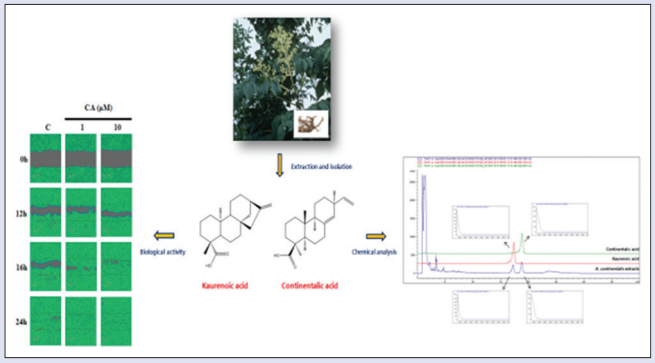
ABSTRACT

Background: The roots of *Aralia continentalis* have been used in traditional Korean medicine to treat pain, lumbago, lameness, and rheumatism. **Objective:** Simultaneous determination of two diterpenoids; continentalic acid (CA) and kaurenoic acid, in the water extract of *A. continentalis* using the high-performance liquid chromatography (HPLC) was established for quality control, as well as evaluation of their wound-healing activity. **Materials and Methods:** The separation was conducted on YMC hydrosphere C_{18} column with isocratic elution of 65% acetonitrile. Furthermore, the isolated diterpenoids were screened for their wound-healing activity using keratinocytes (HaCaT cells). **Results:** The calibration curves were linear over the established range with $R^2 > 0.9999$. The intraday and interday RSDs for each compound were 0.13%–0.89% and 0.14%–0.73%, respectively. The limits of detection and limits of quantification for the two tested diterpenoids were 0.0912, 0.0018 μg/μL, and 0.2764, 0.0056 μg/μL, respectively. In addition, the CA-treated groups showed accelerated wound closure compared to the control group. **Conclusion:** The HPLC method could be accomplished to the quality control and stable experiment for the preparations consisted of two diterpenoids. Compared to the control group, the CA-treated group showed that wound closure was accelerated, indicating its potential in promoting migration of skin cells which is the most important step of wound closure.

Key words: *Aralia continentalis,* continentalic acid, kaurenoic acid, simultaneous determination, wound healing

SUMMARY

- Fast and reliable analytical method for the determination of major diterpenoids of *Aralia continentalis* was developed using high-performance liquid chromatography–ultraviolet techniques
- This method could be accomplished to the quality control and stable experiment for the preparations consisted of two diterpenoids
- Continentalic acid in the water extract of *A. continentalis* showed a potent wound-healing activity.



Abbreviations Used: CA: Continentalic acid; KA: Kaurenoic acid; HPLC: High-performance liquid chromatography; UV: Ultraviolet spectroscopy; ICH: International Conference on Harmonization; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; PS: Penicillin–streptomycin; DMSO: Dimethyl sulfoxide; LOD: Limits of detection; LOQ: Limits of quantification.

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DOI: 10.4103/pm.pm_344_20

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INTRODUCTION

The roots of *Aralia continentalis* (Araliaceae) have been used in traditional Korean medicine to treat pain, lumbago, lameness, and rheumatism.[1,2] In addition, the extract has been reported to possess various pharmacological properties such as antimicrobial, anticancer, and anti-inflammatory activities.[3-9] In previous phytochemical investigations, saponins, flavonoids, essential oils, and diterpenoids were isolated from the roots of this plant.[10,11] Particularly, diterpenoids are the most feature compounds of *A. continentalis*. The major diterpenoids, continentalic acid (CA) and kaurenoic acid (KA), are an *ent*-pimarane and *ent*-kaurane-type diterpenes, respectively. Some of these diterpenoids have been reported to facilitate wound-healing activity.[12-15]

Wound healing is a complex process which includes inflammation, angiogenesis, tissue remodeling, and re-epithelialization with the aim to restore tissue integrity.[16-18] Re-epithelialization involves the

Cite this article as: Yoo G, Lee TH, Lee JH, Kang KB, Yang H, Park YK, *et al.* Simultaneous determination of two diterpenoids, continentalic acid and kaurenoic acid, in the water extract of *Aralia continentalis* and their wound-healing activity. Phcog Mag 2020;16:745-9.

proliferation of keratinocytes and migration to cover the wounded surface.[19] Plant-derived materials can play an effective and positive role in the treatment of damaged cells during wound healing and the previous reports stated that plants have traditionally been used to promote wound healing.[20]

To recover bioactive compounds from the natural product, extraction is widely used which constitutes the first key step.[21] Commonly used extraction solvents are hexane, chloroform, acetonitrile, ethyl acetate, and alcohol. These organic solvents can be employed for the extraction of both polar and non-polar organic compounds such as alkaloids, terpenes, phenolics, steroids, flavone, and fatty acids.[22] However, organic solvents have been strictly regulated in the food and dietary supplement industry due to their toxicity and controversy as an environmental issue. Recently, simultaneous determination of major components of *A. continentalis* using ethanol extracts has been developed, but there is no research conducted using water extracts.[23]

In this study, we performed a simultaneous analysis of two diterpenoids, CA and KA, obtained from the water extract of *A. continentalis* using high-performance liquid chromatography–ultraviolet spectroscopy (HPLC–UV) technique. In addition, these two diterpenoids were then screened for their wound-healing activity using keratinocytes (HaCaT cells).

MATERIALS AND METHODS

Plant materials

The roots of *A. continentalis* were collected at Imsil-gun, Jeollabuk-do province, South Korea, in July 2016 and authenticated by Dr. Jonghee Park, a professor emeritus of College of Pharmacy, Pusan National University. A voucher specimen (YIPS-AC-160203) was deposited at the Herbarium of College of Pharmacy, Yonsei University, Incheon, Korea.

Chemicals and materials

Standard compounds, KA and CA, were purchased from ChemFaces (Wuhan, China). The chemical structures of marker compounds, KA and CA, are shown in Figure 1 and their purities were >99.0% according to the HPLC analysis. LC analysis grade water and acetonitrile were purchased from J.T. Baker (Chemical Co., New Jersey, USA).

High-performance liquid chromatography apparatus and conditions

The chromatographic analysis was conducted using an Agilent Technologies 1200 series HPLC system (Santa Clara, CA, USA) consisting of 1260 quaternary pump, 1260 Infinity auto-sampler, and 1260 Infinity DAD detector. Data were collected and processed by LC solution software (version 04.03, ChemStation). Separation of KA and CA was carried out on the YMC hydrosphere C_{18} analytical column (5 μm, 4.6 mm i.d. ×250 mm). The mobile phase for chromatographic separation of the two diterpenoids was distilled water and acetonitrile with isocratic elution (i.e., 65% acetonitrile). The flow rate was 1.4 mL/min, the column temperature was maintained at 30°C, and the detection wavelength of quantification was set at 205 nm. The injection volume was 10 μL.

Preparation of sample solutions and standard solutions

Roots of *A. continentalis* (3 g) were extracted using distilled water (30 mL) by ultrasonication-assisted extraction. The extracted solution was evaporated at 45°C using a rotary evaporator under a vacuum. To obtain the dry extract, *A. continentalis* extracts were conducted by freeze drying. The powder was accurately weighted and dissolved in methanol at a concentration of 10 mg/mL. Standard compounds, KA and CA, were accurately weighed and dissolved in methanol at a concentration of 1 mg/mL. Each standard solution was subjected to serial dilution with methanol. Two standard solutions and *A. continentalis* extracts were filtered through 0.2 μm membrane filters before injection into HPLC.

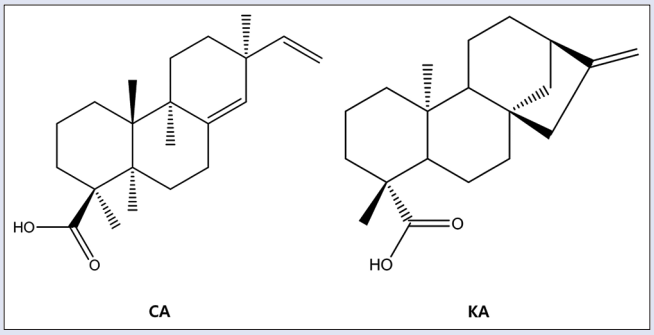

Figure 1: The chemical structures of two major compounds from *Aralia continentalis*

Validation of the high-performance liquid chromatography method

Method validation was performed according to the International Conference on Harmonization guidelines by determination of the precision, recovery, and linearity test.[24]

Cell culture

HaCaT cell lines were obtained from the Korean Cell Line Bank (KCLB, Korea). HaCaT cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium with 1% penicillin–streptomycin and 10% fetal bovine serum. Cultures were incubated in a humidified atmosphere at 37°C with 5% CO_2.[18]

Treatment with two diterpenoids

HaCaT cells were seeded into 6-well plates (1.3×10^5 cells/well). Once cultures were 80% confluent, they were rinsed twice with phosphate-buffered saline (PBS, GIBCO, Grand Island, NY, USA). For treatment with two diterpenoids, an appropriate volume of media was used to dilute the stock solution prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA) to 1 or 10 mM.[18]

Cell migration assay

Cells seeded in 96-well plates were treated with media only for 1 h before scratching. The monolayer of cultured cells was subjected to scratch wounds with a WoundMaker tool (Sartorius, Göttingen, Germany) and the media was removed by suction. Cells were then washed twice with PBS and incubated for 24 h in either the presence or absence of diterpenoids in serum-free media.[18] IncuCyte ZOOM® System (Essen Bioscience, MI, USA) was used to inspect cultures every 4 h.

RESULTS AND DISCUSSION

Optimization of high-performance liquid chromatography conditions

HPLC conditions including column temperature, column type, and mobile phases were evaluated to accomplish the simultaneous

separation of the two major components. For the optimized separation of two diterpenoids, columns including YMC hydrosphere analytical C_{18} (5 μm, 4.6 mm i. d. ×250 mm), Thermo Scientific Hypersil GOLD C_{18} (5 μm, 4.6 mm i.d. ×250 mm), Waters XBridge C_{18} (5 μm, 4.6 mm i.d. ×250 mm), Phenomenex Luna C_{18} (5 μm, 4.6 mm i.d. ×250 mm), and Phenomenex Kinetex C_{18} (5 μm, 4.6 mm i.d. ×250 mm) were examined at column temperatures of 30°C, 40°C, and 45°C, with a range of mobile phases composed of acids such as phosphoric acid, formic acid, and acetic acid and organic solvents such as methanol and acetonitrile. As a result, the most efficient separations were selected using YMC hydrosphere C_{18} analytical column (5 μm, 4.6 mm i.d. ×250 mm) with isocratic elution of distilled water and acetonitrile at 30°C. The optimum detection wavelength was set at 205 nm and the peak of each compound was confirmed by comparing both the retention times in the HPLC chromatogram and the UV spectrum of each marker compound. The retention times of the two diterpenoids, KA and CA, under the optimized conditions were 17.22 and 18.81 min, respectively. Representative HPLC chromatograms of the *A. continentalis* water extracts and standard solutions are shown in Figure 2.

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

The linearity of the method was evaluated from the correlation coefficient (R^2) of the calibration curves of each compound. We found that the two diterpenoids showed good linearity with $R^2 > 0.9999$ in five different concentration ranges. The limit of detections and limit of quantifications for the two tested diterpenoids were 0.0912, 0.0018 μg/μL and 0.2764, 0.0056 μg/μL, respectively [Table 1].

Recovery and precision

The intraday and interday variations for two diterpenoids were assessed by analyzing the *A. continentalis* extract. The intraday and interday RSDs for each compound were 0.13%–0.89% and 0.14%–0.73%, respectively, and these findings are summarized in Table 2. Recovery of the two diterpenoids was in the range of 99.13%–100.65% at the three different concentrations and the RSD values were <1.341%. The recovery data are summarized in Table 3. These results suggested that the established method has satisfactory recovery, reproducible , and precision.

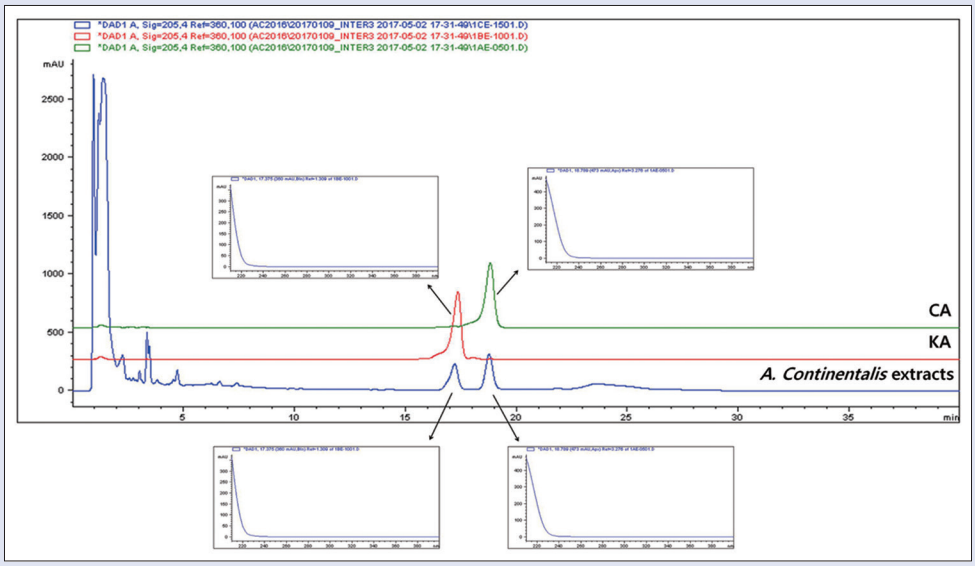

Figure 2: The high-performance liquid chromatography chromatogram of standard solution and *Aralia continentalis* extracts

Table 1: Analytical results of linearity, limit of detection, and limit of quantification

Compound	Regression equation	R^2	Linear range[a] (μg/μL)	LOD (μg/μL)	LOQ (μg/μL)
CA	y=860.52548x+151.9875	0.9999	0.0625-1.0000	0.0018	0.0056
KA	y=846.6829x+122.4875	0.9999	0.0625-1.0000	0.0912	0.2764

[a]y: Peak area (mAU) of compounds; x: Concentration of compounds. LOD: Limit of detection; LOQ: Limit of quantification; CA: Continentalic acid; KA: Kaurenoic acid

Table 2: Analytical results of intraday and interday tests

Compound	Concentration (μg/μL)	Intraday			Interday		
		Mean (μg/μL)	RSD (%)	Accuracy (%)	Mean (μg/μL)	RSD (%)	Accuracy (%)
CA	10	9.96	0.13	100.41	9.97	0.31	100.27
	5	5.02	0.26	99.43	4.99	0.57	100.04
	2.5	2.52	0.82	99.17	2.58	0.45	96.68
KA	10	10.02	0.69	99.79	9.97	0.34	100.33
	5	5.01	0.57	99.64	5.00	0.73	99.90
	2.5	2.49	0.89	100.11	2.57	0.14	97.34

RSD: Relative standard deviation; CA: Continentalic acid; KA: Kaurenoic acid

Quantitative analysis

The proposed HPLC-UV analytical method was successfully applied for the simultaneous quantification of two diterpenoids in *A. continentalis*. The peaks of each compound in *A. continental* were identified by comparison of the retention time and UV spectra with the standard compounds. The contents of two diterpenoids, KA and CA, were 0.21 mg/g and 0.32 mg/g, respectively.

Effects of the diterpenoids on skin cells migration

To test whether the isolated diterpenoids have a positive effect on wound repair, we used scratch wound model in HaCaT cells. Cells grown in the presence of CA exhibited faster growth rates and improved morphology than cells grown without CA. Treatment with 10 mM CA resulted in faster growth and migration rates compared with 5 mM [Figure 3a and b]. These results were supported by the cell viability assay which indicated that CA treatment increased the viability of HaCaT cells in a dose-dependent manner. However, the KA-treated group did not have wound closure and showed inhibition of cell migration compared to the control group [Figure 3c and d].

CONCLUSION

We established a simple, accurate, and reliable HPLC-UV method for the quantitative analysis of two major diterpenoids in water extracts of *A. continentalis*. Validation of the method showed high linearity, repeatability, precision, and recovery. Moreover, this method has been successfully applied to the simultaneous analysis of two major components for the quality control of *A. continentalis*. Compared to the previous literature, the concentrations of the two diterpenoids were relatively lower in the water extract than in the ethanol extract as expected since these two compounds are more soluble in non-polar organic solvents.[23] These results are similar to previous studies which claim that extraction solvents have a significant effect on the recovery of chemical components of plant materials.[15-30] These differences in chemical components can be explained by the dielectric constant and polarity of the solvent used.[31] The established method might be valuable and efficient for the quality control of *A. continentalis* and related botanical preparations which have been generally extracted by water. From the wound-healing activity, we found that CA-treated groups

Table 3: Recovery test of continentalic acid and kaurenoic acid in the water extract of *Aralia continentalis*

Compound	Spiked amount (μg/μL)	Measured amount (μg/μL)	Recovery[a] (*n*=3, %)	RSD (%)
CA	4.33	4.36	100.60	1.27
	4.00	4.02	100.47	0.92
	3.67	3.69	100.51	1.34
KA	4.04	4.01	99.30	0.53
	3.56	3.58	100.65	0.66
	3.08	3.05	99.13	0.14

[a]Recovery (%) = Measured amount/Spiked amount ×100. RSD: Relative standard deviation; CA: Continentalic acid; KA: Kaurenoic acid

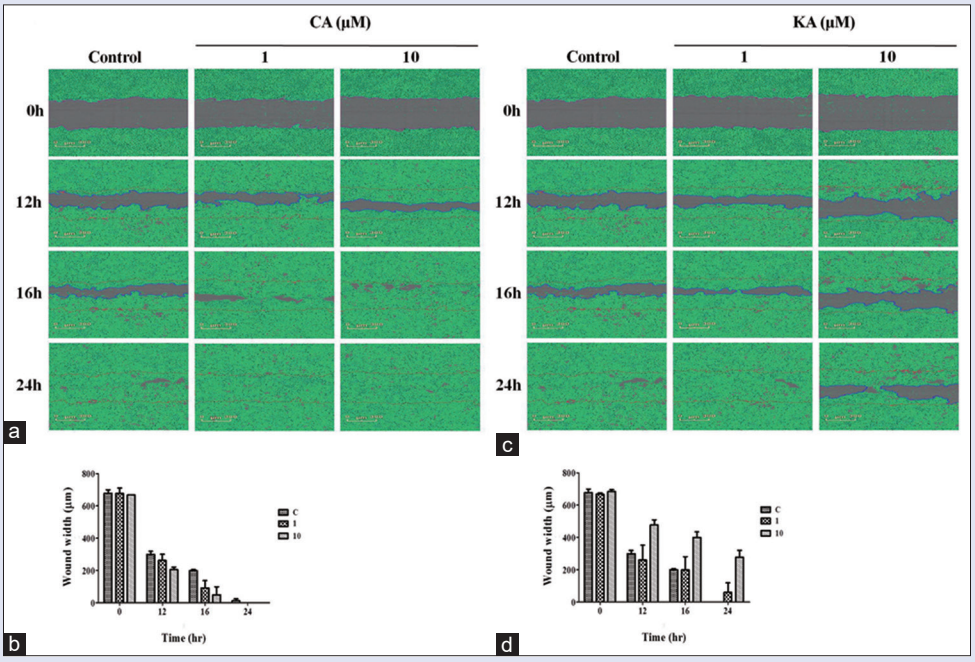

Figure 3: The effects of continentalic acid and kaurenoic acid on scratch wound healing and proliferation in HaCaT cells. (a) HaCaT cells grown with different concentrations of continentalic acid after cell scratching showed that 10 μM continentalic acid induced better migration compared to 1 μM continentalic acid. (b) Graph showing the average wound size in control and continentalic acid-treated HaCaT cells over a period of 24 h. (c) The kaurenoic acid-treated group showed inhibition of cell migration compared to the control group. (d) Graph showing average wound size in control and kaurenoic acid-treated HaCaT cells over a period of 24 h

showed accelerated wound closure compared to the controls, indicating its potential in promoting migration of skin cells which is the most important step of wound closure.

Financial support and sponsorship

This research was supported by a grant (16182MFDS227) from the Ministry of Food and Drug Safety and by the Main Research Program E0164400-05 of the Korea Food Research Institute funded by the Ministry of Science.

Conflicts of interest

There are no conflicts of interest.

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