Simultaneous determination three phytosterol compounds, campesterol, stigmasterol and daucosterol in *Artemisia apiacea* by high performance liquid chromatography-diode array ultraviolet/visible detector

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Submitted: 23-04-2014 Revised: 29-04-2014 Published: 12-03-2015

INTRODUCTION

For 1,000s of years, herbal product is used for prevention and treatment of various diseases in many countries. These herbal medicines have lower toxicity with high compliance and as single components, these exhibit therapeutic effects for multiple diseases.¹ Therefore, herbal products have gained increasing popularity and have become a popular form of healthcare.²³

*Artemisia* species are genus of the family *Compositae* consisting of more than 350 species. *Artemisia apiacea* is widely distributed at wasteland and river beaches of Korea, China and Japan. *A. apiacea* traditionally used for treatment of dermatomycosis, jaundice, eczema, decubitus and alopecia.⁴⁵ The recent studies about the isolated compounds from *A. apiacea* show the presence of campesterol, stigmasterol, β-sitosterol, daucosterol, artemisterol, 7-methoxycoumarin, 7,8-dimethoxycoumarin, daphnetin, 7-hydroxy-8-methoxycoumarin, artemisinin, artemisitin, scopoletin, protocatechualdehyde, and volatile constituents, including apicin, α-pinene and *Artemisia* ketone.⁶⁻¹⁴ Recent studies about *Artemisia* species showed various biological activities including antimarial, antiviral, antitumor, antipyretic, antihemorrhagic, antioxidant, antihepatitis and anticomplementary activities.¹⁵⁻¹⁶ Biological activity of *A. apiacea* was reported that it has hair-growth activity.¹⁷ *A. apiacea* was found to possess the antioxidant activity and protective property in CCl₄-intoxicated rats.¹⁸ Furthermore, *A. apiacea* showed antinflammation activity via nuclear factor-κB inactivation.¹⁹
The phytosterol derived from vegetable oils or wood pulp has various bioactivities. Phytosterols, including stigmasterol, campesterol and daucosterol were detected in *Artemisia apiacea*. Stigmasterol has antiostearthritic, neutralization of viper and cobra venom, thyroid hormone and glucose regulatory activities. In recent study, it also exhibited cognitive ameliorative effects against scopolamine-induced memory impairments in mice. Campesterol have antiangiogenic activity. Daucosterol exhibits immunoregulatory activity and promotion activity for the proliferation of neural stem cells.

The natural products contained various chemical compounds such as terpenoid, flavonoid, alkaloid, saponin and phenol etc. Chemical composition of compounds was varied depending on several factors, such as plant origins, geographic area, harvest time and even storage method. This variability can result in significant differences in pharmacological activity. Therefore, the establishing reliable and accurate analytical quality control method for natural products is necessary for evaluation of safety and efficacy. In many approaches, high performance liquid chromatography (HPLC) is a simple and popular method for the analysis of natural products. Due to its easy operation, side suitability and high accuracy, HPLC method extensively applied to analysis of natural product over the past decades.

In this study, a simple and reliable HPLC-diode array ultraviolet/visible detector (UV/VIS) (DAD) and liquid chromatography–mass spectrometry (LC-MS) method has been established for simultaneous determination of three phytosterol compounds, campesterol, stigmasterol and daucosterol in *Artemisia apiacea* [Figure 1].

**MATERIALS AND METHODS**

**Plant materials**  
*Artemisia apiacea* samples were purchased from Kyung-Dong Market in Seoul (Korea) and were authenticated by Dr. Young Bae Seo, a professor of the College of Oriental Medicine, Daejeon University (Korea). A voucher specimen (no. CJ064M) was deposited at the Kangwon National University in Chuncheon (Korea).

**Reagents**  
Campesterol, stigmasterol and daucosterol used for standard compounds were isolated from *Artemisia apiacea* by silica gel column chromatography. Structures of isolated three compounds were determined by spectroscopic methods, including nuclear magnetic resonance spectrum and compared with spectroscopic data of the literatures.

High performance liquid chromatography-grade acetonitrile (ACN) and water were purchased from J. T. Baker (USA). Trifluoroacetic acid (TFA) was purchased from DAE JUNG (Korea). Methanol and dimethyl sulfoxide (DMSO) was purchased from DAE JUNG (Korea).

**Preparation of standard and sample solutions**  
Standard stock solution of campesterol (500 µg/mL), stigmasterol (620 µg/mL) and daucosterol (640 µg/mL) were prepared in 2% DMSO in MeOH, respectively and stored below 4ºC. The working standard solutions were prepared by appropriate dilution of stock solutions with MeOH. These diluted working solutions were used for establishment of calibration curves.

The herb of *Artemisia apiacea* sample was extracted by ultrasonication in 80% MeOH. The solvent was removed by vacuum evaporator and the residue was freeze-dried. The dried sample was dissolved in 5 mL 2% DMSO in MeOH. All sample solutions were filtered through a 0.45 µm membrane filter before HPLC analysis.

**High performance liquid chromatography-diode array ultraviolet/visible detector analysis condition**  
The HPLC equipment used was Dionex system (Dionex, Germany) composed of a pump (LPG 3X00), an auto sampler (ACC-3000), a column oven (TCC-3000SD) and DAD-3000(RS). System control and data analyses were

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**Figure 1**: Chemical structure of three standard compounds of *Artemisia apiacea*.
The standard stock solution containing three marker compounds was diluted to a series of appropriate concentrations with MeOH for the construction of calibration curves. Each diluted standard solutions were analyzed in triplicate. The calibration curves were constructed by plotting the peak areas versus the concentrations of analytes and obtained regression equations. The correlation of coefficient ($R^2$) was used as measure of linearity. The limit of detection and limits of quantification (LOQ) values were determined at signal-to-noise (S/N) ratios of 3 and 10 times, respectively. The precision of developed method was estimated by inter- and intra-day variations. The relative standard deviation (RSD) (%) was considered as a measure of precision. Accuracy of the method was evaluated using a spike recovery test. The accurate amounts of mixed standard solution were added to A. apiacea sample, and then analyzed three different concentrations in triplicate, respectively. The spike recoveries were calculated by the equation:

\[ \text{Spike recovery (\%)} = \frac{\text{amount found} - \text{original amount}}{\text{amount spiked}} \times 100 \text{ (\%)} \]

**Quantification of Artemisia apiacea samples**

Twelve A. apiacea samples (A1–A12) were separated by established method for quality control and each sample was analyzed in three times. A1–A6 samples were collected from Korea and A7–A12 samples were collected from China. The content of three standard compounds in A. apiacea samples was calculated from calibration curves of standard compounds.

**RESULTS AND DISCUSSION**

Pharmacological effects of A. apiacea have been attributed to the bioactivity compounds. Stigmasterol, campesterol and daucosterol were important phytosterols of A. apiacea and considered to be responsible for therapeutic effect, such as antiosteoarthritic, cognitive ameliorative effect, antiangiogenic activity and immunoregulatory activity.

Quality control of herbal medicine could identify and quantitate variation of compounds by cultivation environment. Quantitative analysis method of A. apiacea has not yet reported. Thus, efficient analysis method of A. apiacea need for quality control. We applied HPLC coupled to DAD technique to establish analysis method and simultaneously determined three compounds, stigmasterol, campesterol and daucosterol.

**Optimization of high performance liquid chromatography-diode array ultraviolet/visible detector condition**

To development of optimal analytic condition, different HPLC parameters were tested including column type, mobile phase, elution system and detection wavelength. The analytical conditions were optimized considering with resolution, baseline and elution time. In mobile phase, TFA (0.1% in water) was added to obtain the inhibition of peak tailing and improvement in peak shape. Due to differentiation in highest detection wavelength of each standard compounds, the detection wavelength was optimized at 205 nm (daucosterol) and 254 nm (campesterol and stigmasterol) [Figure 2]. Injection volume was 20 µL. All peaks of each compound were separated successfully within 65 min. HPLC chromatogram of the three standards is shown in Figure 3a. The identification of the each compound’s peaks was performed by comparing the retention time and UV spectrum. The retention time of campesterol, stigmasterol and daucosterol were 30.61, 57.62 and 60.12 min, respectively.
Identification of standard compounds
Liquid chromatography-electrospray ionization-mass spectrometry was used to identify peaks of campesterol, stigmasterol and daucosterol obtained by HPLC-DAD analysis. MS spectra of campesterol, stigmasterol and daucosterol in positive ion mode were shown in Figure 4. In MS spectra, the fragments of three compounds exhibited at m/s 424 [M + Na] + for campesterol, m/z 413 [M + H] + for stigmasterol and m/z 608 [M + Na + 9H] + for daucosterol.

Linearity, limits of detection and limits of quantification
Calibration curves were plotted for each standard compounds and relative regression coefficients (R²) were calculated to validate their linearity. The calibration data of the three standard compounds showed good linearity (R² > 0.9994) in a relatively wide concentration range. The limits of detection and LOQ values of all standard compounds were in the range 0.55–7.07 μg/mL and 1.67–21.44 μg/mL, respectively [Table 1]. These results indicate that established HPLC-DAD method has good sensitivity.

Precision and accuracy
The precision of developed method was evaluated by repetitive intra- and inter-day test. Mixed standard solutions of three different concentrations were prepared and analyzed by developed HPLC method. The intra-day test was determined by analyzing each mixed solution five times within 1-day. For the inter-day test, the same mixed solutions were analyzed five times within each three successive days. The result of detected amount of each compound was calculated using the corresponding calibration curve. The Precision was expressed by the RSD values. As a result, the RSD values of the intra- and inter-day test were found to be within the ranges 0.41–2.85% and 0.91–2.93%, respectively. Accuracy of intra- and inter-day assay was ranged 96.60–109.57% and 97.24–107.24%, respectively. The results of the intra- and inter-day tests are shown in Table 2.

To assess the accuracy of the method, the recovery test of three standard compounds was performed. The recovery of the selected standard compounds ranged from 90.16% to 104.91%, and their RSD values were < 2.59% [Table 3]. These results showed that the established method has

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**Table 1: The regression data, LOD and LOQs of three compounds in Artemisia apiacea**

| Compounds  | Linear range (μg/mL) | Regression equation | R²   | LOD  (μg/mL) | LOQ  (μg/mL) |
|------------|----------------------|---------------------|------|--------------|--------------|
| Campesterol | 20.84-500.00         | y=0.0054 x-0.0431  | 0.9998 | 0.55         | 1.67         |
| Stigmasterol | 25.84-620.00        | y=0.0008 x+0.0009a | 0.9999 | 2.18         | 6.61         |
| Daucosterol  | 26.68-640.00        | y=0.0511 x-0.2023  | 0.9994 | 7.07         | 21.44        |

*a: Peak area; x: Amount (μg). LOD: Limits of detection; LOQ: Limits of quantification*
Table 2: Intra- and inter-day precision of three compounds in *Artemisia apiacea*

| Compounds   | Concentration (μg/mL) | Intra-day (n=5) | Inter-day (n=5) |
|-------------|-----------------------|-----------------|-----------------|
|             | Mean±SD (μg/mL)       | RSD (%)         | Accuracy (%)    | Mean±SD (μg/mL) | RSD (%) | Accuracy (%) |
| Campesterol | 166.67                | 160.55±0.66     | 0.41            | 96.33           | 163.86±1.50  | 0.91     | 98.32         |
|             | 83.34                 | 83.40±0.40      | 0.48            | 109.57          | 83.79±1.18   | 1.41     | 100.54        |
|             | 41.67                 | 40.25±0.37      | 0.93            | 109.57          | 41.00±0.40   | 0.98     | 108.38        |
| Stigmasterol| 206.67                | 226.45±2.51     | 1.11            | 107.24          | 221.63±5.45  | 2.46     | 105.40        |
|             | 103.34                | 112.00±2.48     | 2.22            | 108.38          | 108.93±1.83  | 1.68     | 105.01        |
|             | 51.67                 | 53.13±1.31      | 2.46            | 102.82          | 54.26±1.59   | 2.93     | 101.38        |
| Daucosterol | 213.33                | 208.68±5.24     | 2.51            | 97.82           | 207.44±1.97  | 0.95     | 97.24         |
|             | 106.67                | 106.26±1.71     | 1.61            | 99.62           | 105.62±1.36  | 1.28     | 99.02         |
|             | 53.36                 | 55.36±1.58      | 2.85            | 103.76          | 54.10±1.47   | 2.72     | 101.38        |

*RSD: Standard deviation; RSD: Relative standard deviations
a suitable precision and accuracy for the simultaneous determination of *A. apiacea*.

**Artemisia apiacea** sample quantitative analysis and cluster analysis

Quantitative analysis of campesterol, stigmasterol and daucosterol in twelve *A. apiacea* samples was performed under the optimized HPLC condition. HPLC-DAD chromatogram of *A. apiacea* sample is shown in Figure 3b. The content (µg/mg) was tabulated in Table 4. Table 4 shows that campesterol was in the range of 16.74–19.53 µg/mg and was highest content among three compounds. The content ranges of stigmasterol and daucosterol were 3.49–4.74 µg/mg and 2.05–2.40 µg/mg, the content of campesterol in A1 was higher than other samples. Stigmasterol and daucosterol was abundant in A6 and A1, respectively. Contents of campesterol, stigmasterol and daucosterol are different between Korea and China.

Hierarchical cluster analysis was performed to confirm homogeneous clusters using IBM SPSS Statistics (IBM, USA) 21. Cluster difference from twelve *A. apiacea* was exhibited by dendrogram [Figure 5]. We found that there are three pair samples (Cluster I, II, III). Cluster I (A2, A4 and A6) was samples collected from Korea. Two of pairs, cluster II (A7, A12, A3 and A8) and III (A9, A10 and A11) were samples collected from China exclude A3 sample. The result showed that contents of compounds in *A. apiacea* samples are different by cultivation environment such as collection region.

**CONCLUSION**

In this study, a reliable and accurate HPLC-DAD and LC-DAD method for the simultaneous determination of three phytosterol compounds (campesterol, stigmasterol and daucosterol) in *A. apiacea* was established. Three compounds, campesterol, stigmasterol and daucosterol confirmed by UV wavelength pattern and MS spectra. The developed method showed good linearity, precision and recovery. This developed method successfully applied to quantitative analysis of campesterol, stigmasterol and daucosterol in twelve *A. apiacea* samples. Thus, this established method can provide improvement quality control of *A. apiacea*.

**ACKNOWLEDGMENTS**

This research was supported by a Basic Science Research Program grant from the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005149).

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**Table 3: Recovery of the 3 compounds in Artemisia apiacea**

| Compounds   | Spiked amount (µg/mL) | Measured amount (µg/mL) | Recovery (%) | RSD (%) |
|-------------|-----------------------|-------------------------|--------------|---------|
| Campesterol | 83.34                 | 80.68±0.25              | 96.81        | 0.31    |
|             | 41.67                 | 41.67±0.53              | 100.01       | 1.27    |
|             | 20.84                 | 21.86±0.57              | 104.91       | 2.59    |
| Stigmasterol| 103.34                | 93.04±0.94              | 90.03        | 1.01    |
|             | 51.67                 | 46.96±0.56              | 90.88        | 1.20    |
|             | 25.84                 | 23.54±0.40              | 91.11        | 1.71    |
| Daucosterol | 106.67                | 110.61±1.89             | 103.69       | 1.71    |
|             | 53.36                 | 48.11±0.48              | 90.16        | 0.99    |
|             | 26.68                 | 25.59±0.49              | 95.90        | 1.93    |

*Recovery (%): (amount found-original amount)/amount spiked x100 (%). RSD: Relative standard deviations*
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Cite this article as: Lee J, Weon JB, Yun BR, Eom MR, Ma CJ. Simultaneous determination three phytosterol compounds, campesterol, stigmasterol and daucosterol in Artemisia apiacea by high performance liquid chromatography-diode array ultraviolet/visible detector. Phcog Mag 2015;11:297-303.

Source of Support: Nil, Conflict of Interest: None declared.