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# Simultaneous qualitative and quantitative analysis of morroniside and hederacoside D in extract mixture of *Cornus officinalis* and *Stauntonia hexaphylla* leaves to improve benign prostatic hyperplasia by HPLC-UV

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**Abstract:** With the improvement in the standard of living and extension of life expectancy, the incidence of prostate diseases has increased yearly, thus becoming a serious disease affecting the health of men. The extract mixture of *Cornus officinalis* and *Stauntonia hexaphylla* leaves is a developed functional food formula to improve prostate health. This study developed a simultaneous analytical method of bioactive compounds for quantifying the mixture of *Cornus officinalis* and *S. hexaphylla* leaves using high-pressure liquid chromatography-ultraviolet (HPLC–UV). HPLC analytical condition was performed on a Hector $C_{18}$ column with a mobile phase of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) under the following gradient conditions: 0-50 min, 12 %-40 % (B) at a flow rate of 1.0 mL/min. Meanwhile, this method was validated properly and successfully used to quantify the bioactive components of morroniside and hederacoside D in 20 sample batches and assess the quality of different ages and seasons of *S. hexaphyll*a leaves. The result showed that the content of morroniside in the extract mixture of *Cornus officinalis* and *S. hexaphylla* leaves ranged from 1.38-1.62 mg/g, and the hederacoside D ranged from 28.42-32.02 mg/g, suggesting that this novel analytical method will be suitable for the quality control of the extract mixture to improve benign prostatic hyperplasia.

**Key words:** benign prostatic hyperplasia, HPLC-UV, *cornus officinalis*, *stauntonia hexaphylla*, LC-ESI-MS/MS

## 1. Introduction

Benign prostatic hyperplasia (BPH) is a common symptom of the urinary system in old age.[1] Recently, the incidence rate of BPH has increased yearly owing to the increasing trend of global population aging. Unfortuna- tely, no effective drugs have been identified for treating BPH yet. Also, people are paying more attention to using natural herbal extracts to improve prostate health.[2]

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Plant extracts were frequently used for treating BPH globally.[3] The extract mixture of *Cornus officinalis* and *Stauntonia hexaphylla* leaves, called CS extract, is a developed functional food formula to improve prostate health by Chong Kun Dang Healthcare, which could significantly reduce the expression of type 2 5α-reductase and proliferating cell nuclear antigen to attenuate BPH.[4] *Cornus officinalis* is known as "Sansuyu" in Korea, and it has various pharmacological activities such as anti-inflammation, antioxidant, immune regulation, antineoplasia, antidiabetic nephropathy, antihyperglycemia, hepatoprotection, and antisepsis effects.[5] Besides, chemical constituents from *Cornus officinalis,* which have been studied, mainly include iridoids, organic acids, triterpenes, cornus tannins, and carbohydrates.[5,6] Particularly, the representative component morronisides were considered the active compounds in treating prostate disease, which could be a marker compound to control the quality of this developed functional food.[7,8]

*S. hexaphylla* is widely distributed as thickets in lowlands and foothills of warmer regions of Korea, Japan, and China. The leaves of *S. hexaphylla* are used as a diuretic in China and Korea. The reported chemical constituents of *S. hexaphylla* include triterpenoids, glucosides, flavonoids, phenylpropanoids, phenolic glucosides, and chlorogenic acid analogs.[9] Among these ingredients, triterpenoid saponins were admitted to be the bioactive component responsible for treating BPH. The most representative saponin component, hederacoside D can enhance prostate disease by inhibiting 5α-reductase and α-adrenergic receptor blocks.[10] Therefore, hederacoside D was a promising marker compound to assess the quality of *S. hexaphylla* leaves.

However, the effectiveness of HPLC analytical methods to evaluate CS extract quality has not yet been reported. Previously, some developed HPLC analysis methods were established to detect the active components in *Cornus officinalis*, but these methods were unsuitable for evaluating CS extract quality because another extract of *S. hexaphylla* leaves has a different and complex chemical composition in CS extract. Presumably, no effective HPLC method has been reported to evaluate the quality of *S. hexaphylla* leaves. Popularizing this newly developed functional food in the global market requires a quality control system. Therefore, this study established a simple and efficient HPLC-UV method to control CS extract quality by quantitatively determining bioactive compounds.

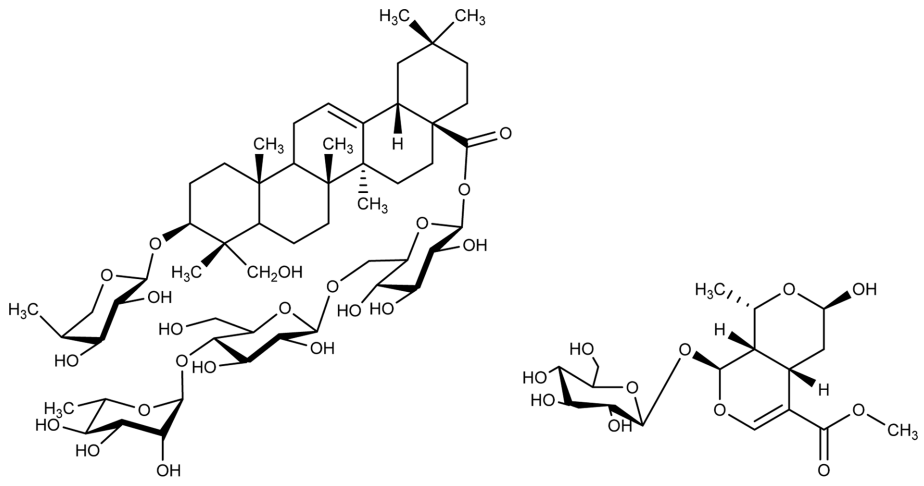


*Fig.* 1. Chemical structure of morroniside and hederacoside D: (a) hederacoside D, (b) morroniside.

## 2. Experimental

### 2.1. Chemical and reagents

Two reference internal standards: morroniside and hedercoside D (*Fig.* 1) were bought from ChemFaces (Wuhan, China). Reagents including acetonitrile, methanol, and ethanol were purchased from Burdick & Jackson (Muskegon, MI, USA); formic acid and acetic acid (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Distilled water was purified using a Milli-Q system (Sinhan, Seoul, Korea).

### 2.2. Preparation of CS extract

The CS extract was obtained from Chong Kun Dang Healthcare (Seoul, Korea). The samples of *S. hexaphylla* plants were harvested in the area of Goheung-gun, Jeollanam-do, South Korea, and authenticated by professor Young Ho Kim and Jong Seong Kang. Voucher specimens (CNU 17002) were deposited at the Herbarium of Chungnam National University. The leaves of *S. hexaphylla* were dried at 60 °C for 12 h. After drying, the samples were extracted with 70 % ethanol at 75 °C for 12 h, and then concentrated and dried to obtain extract powder. The dried fruit of *Cornus officinalis* Siebold & Zucc fruit was collected in the areas of Gurye-gun, Jeollanam-do, Republic of Korea. Then, dried samples were extracted with 70 % ethanol at 75 °C for 12 h and then concentrated and dried to achieve extract powder.

### 2.3. Apparatus and chromatographic condition

Qualitative and quantitative analysis of CS extracts was performed by HPLC system (Shimadzu LC-20A series system, Shimadzu Corporation, Kyoto, Japan) equipped with SPD-20A ultraviolet-visible (UV/VIS) detector, a SIL-20A auto-sampler, two LC-20AD liquid chromatography, one CTO-20A column oven, a CMB-20A communication bus module and DGU-20A degasser. The chromatographic separation was implemented using A Hector $C_{18}$ column (250 mm × 4.6 mm, 5 μm, RStech, Daejeon, Korea). The optimum resolution was obtained using the mobile phase comprising (A) 0.1 % formic acid and (B) acetonitrile-formic acid (99.9:0.1) with a gradient elution of 12 %-40 % B at 0-50 min with a column temperature of 30 °C and a flow rate of 1.0 mL/min.

High-performance liquid chromatography coupled to photodiode-array and electrospray ionization mass spectrometry (HPLC-PDA-ESI-MS/MS) analysis was performed using a Shimadzu LCMS-8040 system (Kyoto, Japan) in the positive and negative modes. Electrospray ionization (ESI) mass spectrometry was operated under optimized conditions with an interface voltage of -3.5 kV for negative mode and 4.5 kV for positive mode. The conditions included nebulizing gas of 3 L/min, drying gas of 15 L/min, a desolvation line temperature of 250 °C, and a heat block temperature of 400 °C. Other analytical conditions were the same as those used for HPLC–UV analysis.

### 2.4. Sample and standard preparation

To prepare sample solutions, CS extract (2.5 g) was added to 50 mL of 50 % methanol in a conical flask. The *S. hexaphylla* leaves extracts (2.25 g) harvested at different times were dissolved in 50 mL of 50 % methanol. Then, solutions were treated by sonication (40 kHz, 280 W) at 50 °C for 60 min using a Mujigae ultrasonic machine (Seoul, Korea). The extract solution was then cooled to the indoor temperature. Standard mixtures of morroniside and hederacoside D were dissolved using methanol to final concentrations of 1 mg/mL and 2 mg/mL, respectively, and kept at 4 °C until use. All sample and standard solutions were filtered using a 0.22 μm syringe filter before HPLC injection.

### 2.5. Method validation

The developed method was validated by precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, stability, and specificity according to the guidelines of the International Council for Harmonization.[12] Accuracy and precision were evaluated by analyzing three concentrations (low, middle, and high) of two reference standards five times. They were expressed as relative standard deviation (RSD). The specificity of the analytical method was determined by comparing the retention time, UV, and mass spectra of morroniside and hederacoside D in the sample and standard. LOD and LOQ were calculated by setting up the

detector response signal to noise ratio of 3:1 and 10:1, respectively. The linearity of the peak area versus concentration of morroniside and hederacoside D was shown by building up a calibration curve with six concentrations under optimized analytical conditions. Repeatability was obtained by calculating the retention times and contents of the morroniside and hederacoside D in samples. For the recovery test, three concentrations (80 %, 100 %, and 120 %) of morroniside and hederacoside D stock solutions were added to the CS extract, and the solutions were analyzed five times and calculated as follows:

$$\text{Recovery (\%)} = (Cf - Co)/Cs \times 100$$

where Cf and Co are the found and original concentrations of marker compounds in the samples, respectively, while Cs is the spiked concentration of morroniside and hederacoside D in the standard solution.

For the stability test, the extract mixture was placed in an oven at 40 °C, and the concentration of the marker compounds was determined every 2 months for 6 consecutive months.

## 3. Results and Discussion

### 3.1. Selection of marker compounds and optimization of the chromatographic condition

Morroniside is a major component in *Cornus officinalis* not contained in *S. hexaphylla* leaves. It is also considered an active ingredient for treating prostate diseases in *Cornus officinalis*.[6] Hederacoside D is a characteristic compound in *S. hexaphylla* leaves not found in *Cornus officinalis*. It has a therapeutic effect on BPH through 5α-reductase inhibition and α-adrenergic receptor blockade.[10] Thus, morroniside and hederacoside D were selected as marker compounds for CS extract quality control.

To obtain rapid and simple chromatographic conditions, various HPLC parameters were compared and explored, including the composition of the mobile phase (acetonitrile-water and methanol-water containing different buffers, such as formic acid, acetic acid, and trifluoroacetic acid), the temperature of the column (25 °C, 30 °C, 35 °C, and 40 °C), and the flow rate of the mobile phase (0.7, 0.9, 1.0, and 1.2 mL/min). The UV spectra of morroniside and hederacoside D were characterized using the PDA for obtaining a satisfying resolution and separation. The starting detection wavelength was set at 240 nm, and the conversion to 205 nm was achieved within 35 min due to low UV absorption of hederacoside D. The most efficient separation was achieved with the mobile phase comprising 0.1 % formic acid (0.1:99.9, v/v; solvent A) and formic acid/acetonitrile (0.1: 99.9, v/v; solvent B). The gradient elution program was optimized to shorten the analytical time in 50 min (*Fig.* 2). Briefly, this optimized HPLC–UV method could be applied to the quality control of this functional food.

### 3.2. HPLC-ESI-MS/MS analysis of marker compounds

To confirm and identify the peaks of two marker

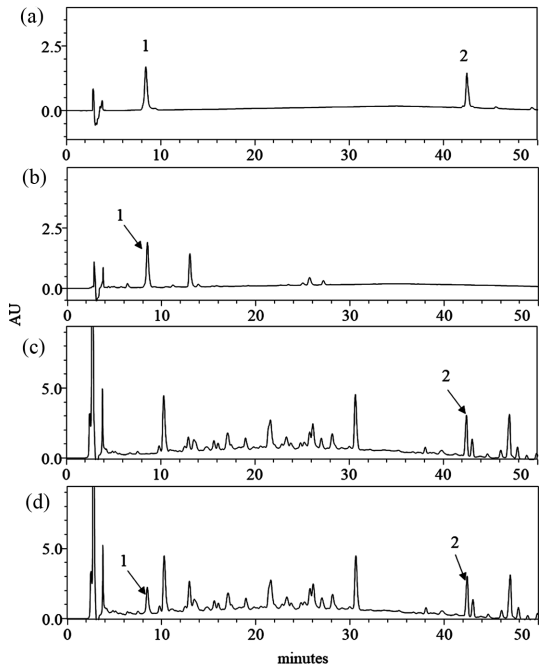


*Fig.* 2. The HPLC–UV chromatograms of mixed standards and the samples (a) mixed standards; (b) *Cornus officinalis* extract; (c) three-years-old *S. hexaphylla* leaves extract; (d) *Cornus officinalis* and *Stauntonia hexaphylla* leaves (radio is 9:1). 1: morroniside (Rt = 8.7 min); 2: hederacoside D (Rt = 42.3 min)

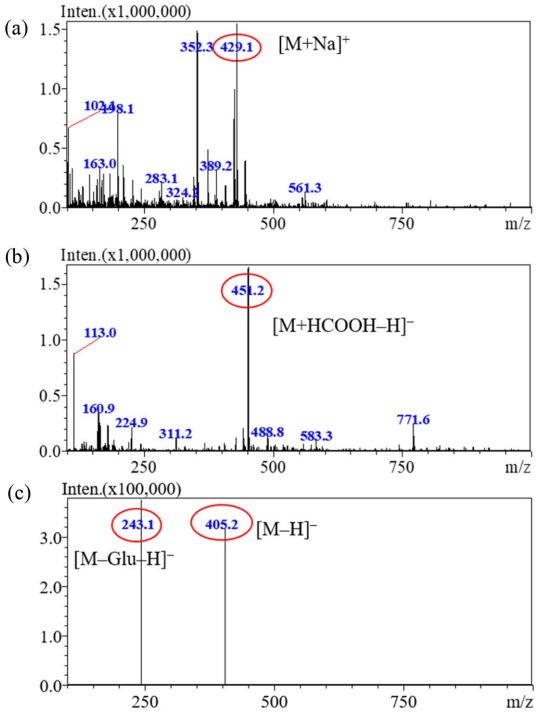


*Fig.* 3. $MS^1$ and $MS^2$ spectrum of morroniside: (a) $MS^1$ spectrum in positive mode; (b) $MS^1$ spectrum in negative mode; (c) $MS^2$ spectrum in negative mode; Glu: beta-D-glucopyranosyl.

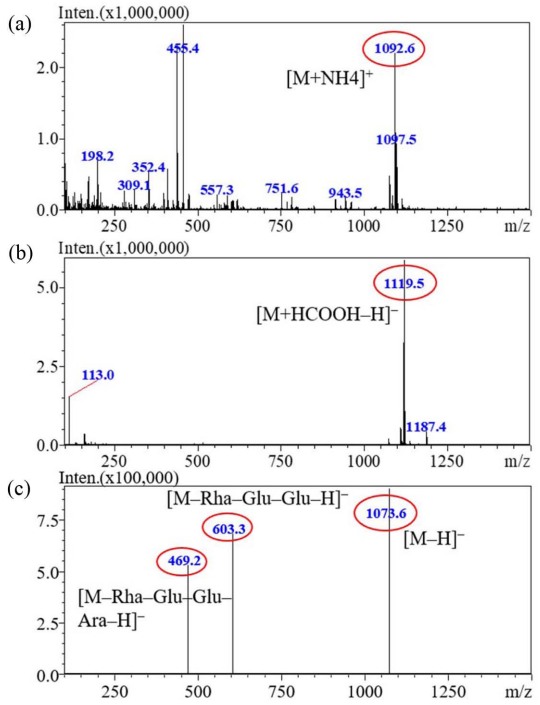


*Fig.* 4. $MS^1$ and $MS^2$ spectrum of hederacoside D (a) $MS^1$ spectrum in positive mode; (b) $MS^1$ spectrum in negative mode; (c) $MS^2$ spectrum in negative mode; Ara: alpha-L-arabinofuranosyl; Rha: alpha-L-rhamnopyranosyl..

compounds in CS extract, HPLC-PDA-ESI-MS/MS was conducted based on their precursor and product ions. For LC-MS analysis, hederacoside D and morroniside were detected at both positive and negative modes. For LC-MS/MS analysis, hederacoside D and morroniside were analyzed in the negative mode by considering the intensity of precursor ions. In the total ion current chromatograms, morroniside was observed in the deprotonated forms $[M+HCOOH–H]^-$ at 405 (*Fig*. 3). The product ions examined by LC-MS/MS were located at m/z 405 and 243 for morroniside (*Fig*. 3).

The MS spectra of hedercoside D yielded pseudo-molecular ions $[M+NH_4]^+$ at m/z 1093 and produced parent ions $[M+HCOOH–H]^-$ at m/z 1119 in the positive and negative mode, respectively (*Fig*. 4). For LC-MS/MS analysis, the fragmentation of $[M–H]^-$ formed daughter ions at m/z 603 because of the loss of 471 Da (Rha-Glu-Glu), which indicated that Rha-Glu-Glu was C-28 sugar chain of hedercoside D. The m/z 469 was observed in the mass spectrum, which was generated from m/z 603 by losing Ara connected to C-3 position (*Fig*. 4).

### 3.3. Validation of the method

#### 3.3.1. Linearity, LOD, and LOQ

*Table* 1 shows the acceptable results of the regression analysis, the correlation coefficients ($r^2$), LODs, and LOQs obtained for hederacoside D and morroniside. All calibration curves showed good linear regression ($r^2 > 0.9998$) within the test ranges; the LODs and LOQs of the morroniside and hederacoside D were 3.29-7.54 ng/mL and 9.98-22.84 ng/mL, respectively.

#### 3.3.2. Precision, repeatability, and recovery

The precision of intraday and interday were less than 3.6 % and the accuracy of this new analytical method ranged from 99.3 %-101.2 % with RSD less

*Table* 1. Validation data of morroniside and hederacoside D

| Parameters | Morroniside | Hederacoside D |
|---|---|---|
| Linearity range (μg/mL) | 25-800 | 400-2400 |
| Coefficient of determination ($r^2$) | 0.9998 | 0.9998 |
| Equation | y = 28.4x+181.9 | y =2.2x-129 |
| LOD (ng/mL) | 3.29 | 7.54 |
| LOQ (ng/mL) | 9.98 | 22.84 |
| Precision | | |
| Intra-day (%RSD) | 0.3-3.4 | 0.7-2.7 |
| Inter-day (%RSD) | 0.8-3.6 | 1.3-2.2 |
| Accuracy | | |
| Intra-day (%RSD) | 97.5-101.4 | 98.3-100.3 |
| Inter-day (%RSD) | 98.4-101.2 | 98.8-100.3 |
| Recovery (%) | 99.3-101.2 | 99.6-100.4 |
| Repeatability | | |
| Retention time (%RSD) | 0.1 | 0.1 |
| Content (%RSD) | 0.8 | 0.3 |
| Degration rate (%) | | |
| 0 month | 0 | 0 |
| 2 month | 0 | 0.9 ± 0.2 |
| 4 month | 1.8 ± 0.4 | 6.3 ± 1.4 |
| 6 month | 2.9 ± 0.8 | 11.8 ± 0.1 |

than 1.4 % (*Table* 1). The repeatability of retention time and concentration of morroniside and hederacoside D in the CS extract presented in *Table* 1 demonstrate that the developed assay was reproducible (RSD < 0.8 %).

#### 3.3.3. Stability

Stability was evaluated by determining the content of hederacoside D and morroniside for 6 months (*Table* 1). While hederacoside D and morroniside decreased by 0.9 % and 1.8 % after 2 months, respectively, the degradation rate increased to 11.4 % and 11.8 %, respectively, after 6 months.

### 3.4. Comparison of hederacoside D in *S. hexaphylla* leaves from different harvest times

To control the raw material quality of this functional food formula, *S. hexaphylla* leaves of different ages and harvest seasons were evaluated. We first compared the contents of hederacoside D in *S. hexaphylla* leaves harvested in spring and autumn using this developed HPLC method. The results showed that the contents of hederacoside D in *S. hexaphylla* leaves harvested in autumn were more than 10 times those harvested in spring (*Fig*. 5), indicating that *S. hexaphylla* leaves were unsuitable for harvesting in spring. *S. hexaphylla* leaves of different ages were also analyzed. *Fig*. 5 shows that the contents of hederacoside D in *S. hexaphylla* leaves with different ages were significantly different. While the five-year-old *S. hexaphylla* leaves contained the highest amount of hederacoside D, the one-year-old *S. hexaphylla* leaves contained the least. Besides,

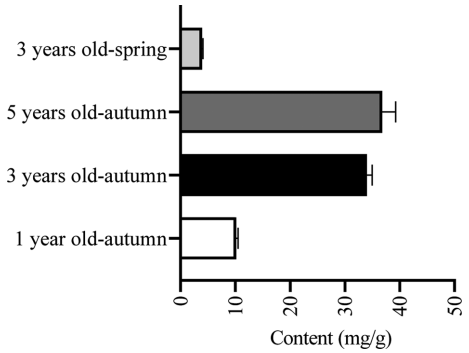


*Fig.* 5. The content of hederacoside D in *S. hexaphylla* leaves harvested from different times.

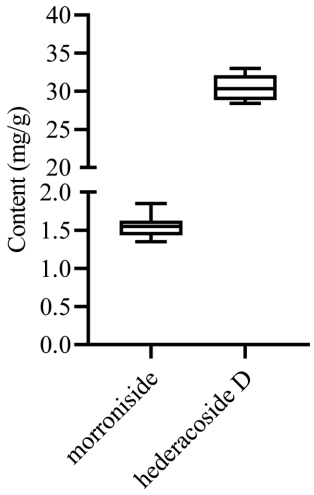


*Fig.* 6. Box plot of the content of morroniside and hederacoside D in 20 batches samples.

the t-test result showed no significant difference in the content of hederacoside D between the three-year-old and five-year-old *S. hexaphylla* leaves. Therefore, three-year-old *S. hexaphylla* leaves harvested in autumn was more suitable as the raw material of this functional food formula, and this rapid and simple HPLC analytical method could be used to control the quality of raw material of *S. hexaphylla* leaves.

### 3.5. Application to the analysis of 20 batches of CS extracts

The established method was successfully applied to simultaneously quantify hederacoside D and morroniside in 20 sample batches. *Fig*. 2 shows the representative chromatograms. The result showed that the content of morroniside in the CS extract ranged from 1.38-1.62 mg/g, and hederacoside D ranged from 28.42-32.02 mg/g (*Fig*. 6). Briefly, this study qualitatively and quantitatively measured two bioactive components in our developed functional food formula using the new HPLC method, which was further used to monitor CS extract quality in industrial routine analysis.

## 4. Conclusions

This is the first report on validating a simple and fast analytical method for qualifying and quantifying saponin and iridoid glucosides in CS extract. The contents of morroniside and hederacoside D in CS extract were quantified at 30.42 ± 2.42 mg/g and 1.5 ± 0.24 mg/g, respectively. Also, this reliable analytical method was successfully used to assess the quality of *S. hexaphylla* leaves from different harvest times, and the result revealed that three-year-old *S. hexaphylla* leaves harvested at autumn was most suitable for producing this functional food formula to improve prostate health.

## Disclosure Statement

The authors declare that there are no conflicts of interest.

## Acknowledgements

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