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Efficient Isolation of Dihydrophaseic acid 3'-*O*-β-D-Glucopyranoside from *Nelumbo nucifera* Seeds Using High-performance Countercurrent Chromatography and Reverse-phased High-performance Liquid Chromatography

Taewoong Rho and Kee Dong Yoon*

College of Pharmacy and Integrated Research Institute of Pharmaceutical Sciences, The Catholic University of Korea, Bucheon14662, Korea

Abstract – High-performance countercurrent chromatography (HPCCC) coupled with reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to isolate dihydrophaseic acid 3'-*O*-β-D-glucopyranoside (DHPAG) from the extract of *Nelumbo nucifera* seeds. Enriched DHPAG sample (2.3 g) was separated by HPCCC using ethyl acetate/*n*-butanol/water system (6:4:10, v/v/v, normal-phase mode, flow rate: 4.0 mL/min) to give 23.1 mg of DHPAG with purity of 88.7%. Further preparative RP-HPLC experiment gave pure DHPAG (16.3 mg, purity > 98%). The current study demonstrates that utilization of CCC method maximizes the isolation efficiency compared with that of solid-based conventional column chromatography.
Keywords – *Nelumbo nucifera,* Dihydrophaseic acid 3'-*O*-β-D-glucopyranoside, High-performance countercurrent chromatography, Isolation efficiency

Introduction

Nelumbo nucifera Gaertner, belongs to Nymphaceae, is anaquatic rhizomatous plant which is widely distributed and consumed especially in Asian region including Australia, India, Korea, China and Japan.[1] In Korea, the rhizome of *N. nucifera* have been used as an edible crop, and seeds have been used as a traditional medicine to treat nervous disorder, insomnia and cardiovascular disorder.[2] Many reports have published concerning pharmacological activities of *N. nucifera* such as antioxidant,[3-6] skin protective,[7] anti Alzheime's diseases,[8,9] hepatoprotective effects.[10] As for phytochemicals, diverse constituents including alkaloids, flavonoids, phenolics, sesquiterpenoids, essential oils have been reported from the seeds and leaves of *N. nucifera*.[1,11] During HPLC analysis to find marker compound characteristic to *N. nucifera* seeds, we found that an abscisic acid derivative, dihydrophaseic acid 3'-*O*-β-D-glucopyranoside (DHPAG, Fig. 1), was present in the seeds of *N. nucifera* with observable level than previously reported alkaloids and flavonoids. The presence of dihydrophaseic acid, the aglycone of DHPAG, already has been identified from several plants including *N. nucifera*, *Persea americana*, *Phaseolus vulgaris*, *Pyrus communis, Spinacia oleracea*, *Vigna unguiculate and Zea mays*,[12] and DHPAG and its stereoisomer were found from seeds of *N. nucifera*, *Zizyphus jujube*, barks of *Ginkgo biloba* and cortex of *Lyciumchinensis* with osteoblast differentiation effect.[13,14] Although DHPAG also have been found in other plants, but it has been steadily discovered in *N. nucifera seeds,* which suggests that DHPAG has sufficient potential as a standard marker compound of *N. nucifera* seeds.

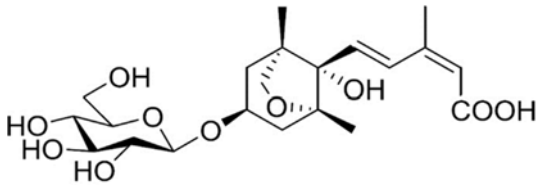

Fig. 1. Chemical structure of dihydrophaseic acid 3'-*O*-β-D-glucopyranoside (DHPAG).

Countercurrent chromatography (CCC) is a type of liquid-liquid chromatography which do not employ solid based absorbent such as silica gel. The big advantages of CCC are no target loss during isolation process, rapid and

*Author for correspondence
Kee Dong Yoon, College of Pharmacy and Integrated Research Institute of Pharmaceutical Sciences, The Catholic University of Korea, Bucheon14662, Korea
Tel: +82-2-2164-4091; E-mail: kdyoon@catholic.ac.kr

simple experimental step and employment of large sample mass. Due to the aforementioned merits, CCC methods are widely utilized for natural products isolation.[15]

To date, several CCC methods have been proposed to separate flavonoid and alkaloid from plumule and petals of *N. nucifera*,[16-18] and a paper described the utilization of CCC method as anisolation step for DHPAG from avocado seeds (*Persea americana*).[19] However, there is no report concerning selective isolation of DHPAG from *N. nucifera* seeds. In the current study, CCC method was developed to isolate DHPAG from the extract of *N. nucifera* seeds, and the isolation efficiencies of DHPAG between CCC and conventional column chromatography methods were concisely compared.

Experimental

General experimental procedure – The CCC instrument was a Spectrum HPCCC (Dynamic Extractions, Berkshire, UK).The Spectrum HPCCC was equipped with a 1525 binary HPLC pump (Waters, MA, USA), a 2487 dual λ absorbance detector (Waters, MA, USA)and a Foxy® R2 fraction collector (Teledyne Isco, NE, USA). Internal temperature of HPCCC was maintained at 30 °C by a CCA-1111 circulatory temperature regulator (Eyela, Tokyo, Japan). MPLC system was composed of an IOTA S 300 pump (ECOM, Prague, Czech Republic), 2487 dual λ absorbance detector (Waters, MA, USA) and a Foxy® R2 fraction collector (Teledyne Isco, NE, USA). The qualitative and quantitative HPLC analyses were performed by an Alliance HPLC system (Waters, MA, USA). Solvents for HPCCC and MPLC were obtained from Daejung-Chemical and Metals Co. Ltd. (Kyunggi-Do, Korea). Deionized water was provided by a Millipore Milli-Q water purification system (Millipore, USA). NMR spectra were measured by Bruker Ascend TM 500 spectrometer (Bruker, Germany).

Preparation of sample material – Seeds of *Nelumbo nucifera* (500 g) were purchased from Humanherb (Deagusi, Korea), and the voucher specimen (CU-NeNu-180212) was deposited at the herbarium of the College of Pharmacy, The Catholic University of Korea. Seeds of *N. nucifera* (500 g) was extracted with 25% aqueous ethanol to give crude 25% ethanol extract (NNE, 51.7 g). The crude extract was sequentially partitioned by organic solvents to give ethyl acetate (NNE-E, 1.4 g), *n*-butanol (NNE-B, 8.2 g) and water-soluble extract (41 g). The enriched DHPAG sample (En-DHPAG) was prepared by solid phase extraction method. NNE-B (8.0 g) was absorbed to 40 g of silica gel which was packed in a MPLC column and it was eluted with $CHCl_3$-MeOH (CM) mixture (20:1 →7:1 →3:1 MeOH, v/v, each 1.0 L). A fraction eluted with CM (3:1, v/v) was selected as an En-DHPAG sample (2.3 g) using HPLC analysis. All *N. nucifera* samples were stored at a refrigerator (-20 °C) prior to experiment.

Qualitative HPLC condition – HPLC analyses for *N. nucifera* samples (NNE-B and En-DHPAG), and DHPAG from HPCCC experiment were performed by an INNO Column C18 (250 × 4.6 mm I.D., YoungJinBiochrom Co. Ltd, Seung-Nam, Korea). The flow rate was 1.0 mL/min and the detection wavelength was 265 nm. The mobile phase A was a MeCN acidified with 0.01% TFA, and the mobile phase B was an aqueous 0.01% TFA. The mobile phase gradient was 5% A (0 – 5.0 min), 5 – 55% A (5 – 50 min).

Quantitative HPLC procedure – In-house HPLC quantification for DHPAG was performed using authentic compound (purity > 98%) which was isolated from preliminary experiment.[11] The authentic DHPAG (2.0 mg) was dissolved in 2.0 mL of methanol to prepared stock solution at 1.0 mg/mL. The stock solution was diluted sequentially to give six concentration levels (0.4, 2.0, 10.0, 50.0, 250.0, 500.0 μg/mL) and to build a calibration curve. Sample concentration were 20.0, 5.0 and 0.5 mg/mL for NNE-B, En-DHPAG and a fraction from CCC experiment, respectively. The injection volume was 20.0 μL. The linear regression equation for DHPAG was $y = 21.749x - 49.709$ ($r^2 = 0.9991$) (x: concentration; y: peak area).

HPCCC procedure – The ethyl acetate/*n*-butanol/water (E/B/W, 6:4:10, v/v/v, normal-phase mode) system was used for HPCCC experiment. Using this solvent condition, the K value of DHPAG was 2.78. En-DHPAG was dissolved in a 1:1 mixture of the upper and lower phases of E/B/W (6:4:10, v/v/v) condition, and sample solution was subjected to HPCCC instrument with normal-phase mode. The HPCCC parameters are as follows; flow rate: 4.0 mL/min; rotational speed: 1500 rpm; detection wavelength -265 nm).The HPCCC experiment for En-DHPAG was repeated four times, and the net loading amount of En-DHPAG was 2.3 g (600 mg, 600 mg, 600 mg and 500 mg, respectively).

Preparative RP-HPLC condition – The preparative RP-HPLC for HPCCC fraction of DHPAG (24.1 mg) was performed using MeCN-Water mixture (15:85, v/v) with a Luna C18 column (250 × 21.2 mm I.D., Phenomenex). The flow rate was 5.0 mL/min and detection wavelength was 265 nm.

Structure elucidation of DHPAG – The Structure of

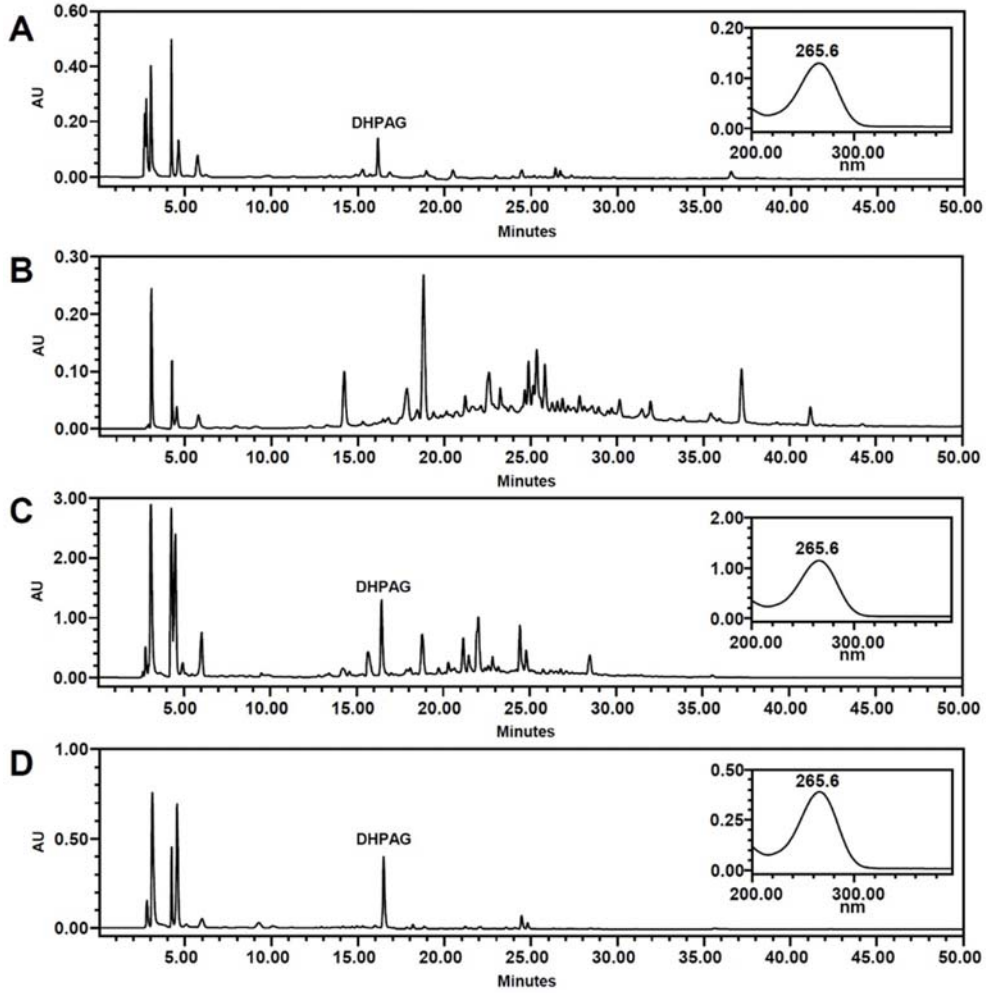

Fig. 2. HPLC analyses of NNE samples. (A) 25% ethanol extract, (B) ethyl acetate soluble extract, (C) *n*-butanol soluble extract and (D) enriched DHPAG extract.

DHPAG from HPCCC experiment was determined by HPLC analysis and spectroscopic data which was compared to authentic DHPAG obtained from previous experiment.[11]

Result and Discussion

HPLC analysis of NNE samples for DHPAG detection – The HPLC analysis showed that DHPAG was observed in 25% ethanol extract as a main peak and it was concentrated in *n*-butanol soluble extract (NNE-B, Fig. 2A – 2D), which indicated that DHPAG possesses a relative hydrophilic property. The content of DHPAG was estimated to be 4.25 mg/g of dried NNE-B and the total DHPAG was estimated to be 34.0 mg in 8.0 g of NNE-B sample. In general, the low amount of target compound in sample extract absolutely results in lowering the separation efficiency. To improve separation efficiency and exclude intervening impurities, enriching process for target compound is necessary for CCC experiment. In this study, enriched DHPAG sample (En-DHPAG) was simply prepared by solid phase extraction (SPE) method using silica gel as a solid absorbent. As shown in Fig 2D, DHPAG was highly concentrated in a fraction [eluent: $CHCl_3$-MeOH (3:1, v/v)] and impurity peaks close to DHPAG were successfully removed. This fraction was selected asan En-DHPAG sample (2.3 g), and the content of DHPAG was 9.89 mg/g of dry weight of En-DHPAG. The total amount of DHPAG in 2.3 g of En-DHPAG was calculated to be 22.8 mg (Table 1).The quantitative HPLC analysis indicated that approximately 23% of DHPAG was lost during a SPE process.

HPCCC experiment on En-DHPAG – Under an E/B/W condition (6:4:10, v/v/v, normal phase mode), the *K* value of DHPAG was 2.78. As shown in Fig. 3A, En-DHPAG (600 mg) was separated by HPCCC yielding 6.5

Table 1. Content of dihydrophaseic acid 3'-*O*-β-D-glucopyranoside (DHPAG) in each NNE sample.

Sample	Content	Sample mass	Total amount of DHPAG in sample extract	Recovery (%) of DHPAG
NNE-B	4.25 mg/g	8.0 g	34.0 mg[a]	-
En-DHPAG	9.89 mg/g	2.3 g	22.8 mg[b]	67.1%[d]
CCC fraction of DHPAG	0.887 mg/mg	23.1 mg	20.4 mg[c]	89.5%[e]

[a, b, c] content ×sample mass; [d](b ÷ a) × 100; [e](c ÷ b) × 100

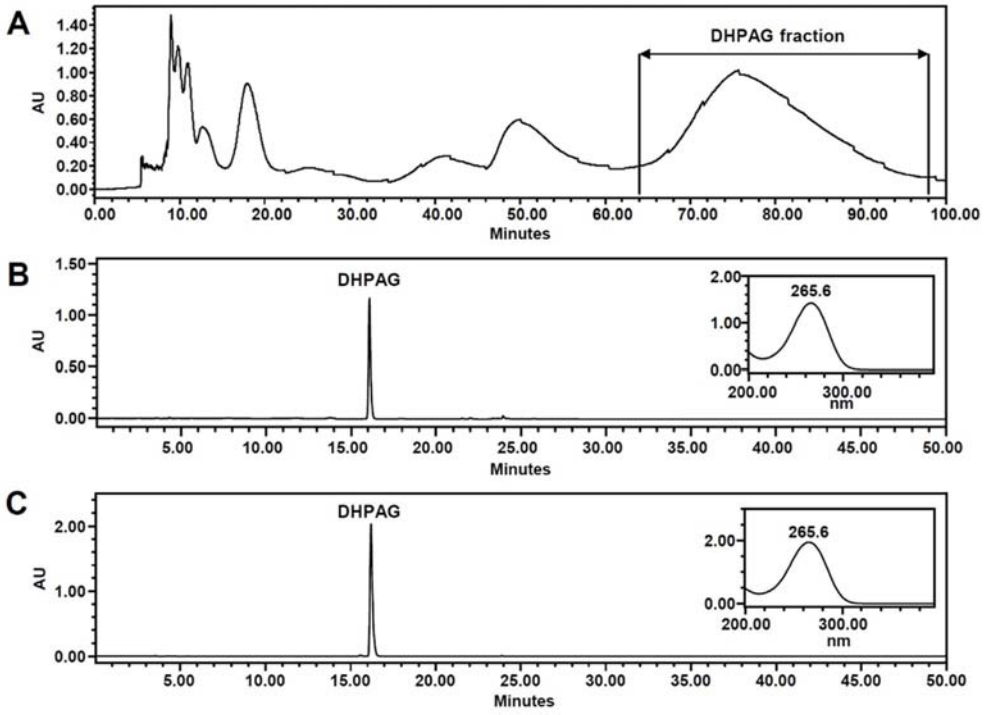

Fig. 3. HPCCC experiment on enriched DHPAG extract (A), HPLC analyses of target HPCCC fraction (B) and purified DHPAG by preparative RP-HPLC (C). HPCCC parameters are described in Experimental section.

mg of DHPAG, and totally 22.1 mg of DHPAG was isolated from 2.3 g of En-DHPAG with purity at 88.7%. Considering the yield and purity of isolated DHPAG, the amount of recovered DHPAG was calculated to be 20.4 mg showing 89.5% of DHPAG was recovered from En-DHPAG through HPCCC experiment. Finally, preparative RP-HPLC was used to improve the purity of DHPAG, which gave 16.3 mg of DHPAG with purity over 98%.

Comparison of the current study with previous report – The isolation efficiency of 16.3 mg of DHPAG from 500 g of *N. nucifera* seeds seems to be inefficient. Youn et al. reported the isolation and determination of DHPAG from 20 kg of *N. nucifera* seeds using conventional column chromatography method.[13] The yield of DHPAG was 4.0 mg which was estimated to be 0.00002% of mass of *N. nucifera* seeds. In this study, 16.3 mg of pure DHPAG was isolated from 500 g of *N. nucifera* seeds (0.0033%), which was 165-fold higher than that of previous report. Thus, it is concluded that the utilization of CCC method absolutely minimizes the loss of target molecules compared with solid based column chromatography method and is a powerful tool for the isolation of target compound from natural sources.

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