

Isolation and Purification of Antibacterial Components in Cortex *Phellodendri*

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황백나무로부터 항균성분의 분리 및 정제

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국문요약

황백 껍질은 황백나무(*Phellodendron amurense*)의 건조된 수피로부터 얻어진다. 이 수피는 한국의 전통 한약제로서, 설사, 황달, 무릎과 발의 통증, 요도관 및 피부 감염증에 폭넓게 사용되어 왔다. 본 연구는 황백나무의 메탄올 추출액으로부터 항균성 화합물 분리를 위해 CPC 방법으로 효과적으로 수행하였다. 두 용매의 CPC 최적조성은 n-butanol: acetic acid:water(4:1:5 v/v/v)이었다. 이동상의 유속은 1,000 rpm 회전력에서 상승법으로 분당 3 ml 속도로 전개시켰다. CPC에서 분리된 분획분은 prep-HPLC로 정제하였다. 분리된 palmatine은 ¹H, ¹³C-NMR, ESI-MS 데이터 분석으로 확인하였다.

Key words: CPC, HPLC, palmatine, Cortex *Phellodendri*

INTRODUCTION

Many efforts have been made to discover new antimicrobial compounds from various natural sources such as soil, microorganisms, animals and plants. One such resource is folk medicines and their systematic screening can result in the discovery of novel effective compounds. We have intensively searched for new effective medicines among natural products, particularly oriental herbal medicines (OHM). OHM have played an important role in clinical therapy in Korea for thousands of years and have been utilized widely in the healthcare system. They have been the major foundation for preventing and treating many diseases for centuries.

Cortex *Phellodendri* (CP) is derived from the dried bark of

Phellodendron amurense. It has been widely used as a drug in traditional Korean medicine for treating diarrhea, jaundice, swelling pains in the knees and feet, urinary tract infections and infections of the body surface (Lee et al. 2005; Min et al. 2008).

The diverse geographical sources of this plant result in large variations in alkaloid content such as berberine, jatrorrhizine, and palmatine, which are quite different from each other. Thus, it is necessary to determine the species of CP as well as the actual total alkaloid content used in the raw material because of different doses for different medical uses (Chen et al. 2008).

Advances in biological and separation sciences have facilitated the transition between the use of crude herbal medicines and the exploitation of purified active herbal constituents (Chen et

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al. 2010; Li et al. 2009).

Many analytical methods have been utilized to study CP, such as thin-layer chromatography, silica gel and octadecyl (C18) gel column chromatography. Centrifugal partition chromatography (CPC) is a liquid-liquid partition chromatography technique that does not use a solid support matrix, resulting in no irreversible adsorption of the sample onto the solid matrix and less peak tailing and contamination (Poucault AP 1994). High selectivity is obtained by careful choosing the biphasic solvent system; thus, allowing separation of compounds with very similar structures. This method has been widely applied for preparative separation of various natural products such as alkaloids, flavonoids and hydroxyanthraquinones (Yin et al. 2009; Zhang et al. 2011). In this study, the antibacterial activities of methanol extracts from 11 kinds of OHMs were screened *in vitro* using the plate disk assay method. Among them, The methanol extracts of CP (stem of *Phellodendron amurense*) showed remarkably potent antibacterial activities.

We report the results of screening for antibacterial activities in a CP extract. Isolation of the active constituents of CP was performed by CPC.

MATERIALS AND METHODS

1. Apparatus

Preparative CPC was performed using a LLB-M high performance CPC (Sanki Instruments Ltd, Tokyo, Japan). The CPC system was equipped with a 321 pump (Gilson, Middleton, WI, USA) and a UV-VIS detector (S-3702 Soma, Sōma City, Japan), a fraction collector (FC-203B, Gilson) and a 1 mL sample loop. The analytical high performance liquid chromatography (HPLC) system consisted of a binary Gilson 305 pump, a UV detector (M720 Youngin, Seoul, South Korea) and a 506C interface module (Gilson). Nuclear magnetic resonance (NMR) (300 MHz for ^1H -NMR and 225 MHz ^{13}C NMR) spectra were measured in methanol-D₄ (MeOD, 99.9%) using a Bruker Biospin DSX-300 spectrophotometer (Billerica, MA, USA) and mass spectra and the molecular weights of the compound were measured by positive electrospray ionization mass spectroscopy (ESI-MS) (Applied Biosystems, Foster City, CA, USA) at the Korea Basic Science Institute (Daejeon, South Korea).

2. Crude Extract Sample Preparation

Dried CP and OHMs were obtained from a Wonju Oriental

medicine market (Kwangwon Province, Korea). Dried CP (400 g) was extracted with 2 L of 80% methanol for 48 hr. The extract was concentrated in a rotary vacuum evaporator to obtain the crude extract (40 g). The extract was dissolved in 100 mL water and extracted with butanol. A portion of the residue obtained after evaporation of the butanol was dissolved as the lower layer in the CPC solvent system.

3. Antimicrobial Assay

Antibacterial activities were assessed using the disc diffusion method (Seneviratne et al. 2007). The test bacteria was *Staphylococcus aureus* KCTC 1621. OHMs (5 g) were extracted with 100 mL 80% methanol in an ultrasonic apparatus at room temperature and concentrated *in vacuo*. The OHMs were dissolved in sterilized distilled water to achieve a final volume of 1 mL (10 mg/mL) Mueller–Hilton agar plates were prepared, and the inocula were seeded by the spread plate method. Sterile discs (diameter, 7 mm) were loaded with 200 μL of OHM methanol extract and introduced in sterile medium with the test organism. The plates were incubated at 37°C for 24 hrs. Activity was evaluated by measuring the diameter of the inhibition zone.

4. CPC Separation Procedure

The two-phase solvent system was composed of n-butanol: water (1:1). The solvent mixture was mixed vigorously in a separatory funnel and equilibrated at room temperature to obtain the upper and lower phases. The lower phase (stationary phase or aqueous phase) of the two-phase solvent system was pumped into partition cells in the ascending mode at a flow rate of 15 mL/min without rotation. When all cells were completely filled with the stationary phase, the upper phase was pumped at a flow rate of 3 mL/min at a rotor speed of 1,000 rpm. After equilibrium was established, as indicated by the glow of the mobile phase solvent from the CPC system outlet, a water sample solution was injected into the CPC system (Poucault AP. 1994). The eluate was monitored at 254 nm and each fraction was collected in an 10 mL/tube with a Gilson FC 203B fraction collector.

5. HPLC Analysis

The CPC-separated fraction and crude extract were analyzed by HPLC. A J'sphere ODS-H80 column (4 μm particle size, 120Å, 150×4.6 mm, YMC Co. Ltd., Tokyo, Japan) was used. The mobile phase was composed of 25% acetonitrile in

0.1% aqueous trifluoroacetic acid (TFA) in a gradient system. The flow rate was 1 mL/min with UV absorbance detection at 280 nm. Preparative HPLC was performed using a reversed phase column (Gemini 5 μ m, 110 Å, 100×21.28 mm, Phenomenex, Torrance, CA, USA) with 25% acetonitrile in 0.1% aqueous TFA at a flow rate of 4 mL/min and monitoring at 321 nm.

RESULTS AND DISCUSSION

1. Screening and Antibacterial Activities

Methanol extracts of roots, fruit, leaves, radix and stems of 11 species of OHM were examined for antibacterial activity. *Staphylococcus aureus* KTCC 1621 was used to assess the growth inhibitory effects by the paper disc diffusion method. CP showed the highest inhibitory activity against *S. aureus*, followed by *Rheum palmatum*, *Glycyrrhiza uralensis*, *Schisandra chinensis*, *Scutellaria baicalensis*, *Cornus officinalis* and *Cnidium officinale* (Table 1).

2. Choice of Two-phase Solvent System

Purification procedures were carried out by CPC and preparatory HPLC to identify the CP antibacterial compounds. The 80% methanol extract of CP was concentrated *in vacuo* at 50°C, and the mother liquor was extracted with butanol. A portion of the residue obtained after evaporating the butanol was dissolved

Table 1. Antimicrobial activities of methanol extracts of oriental herbal medicines

Name of herbal medicines	Inhibition zone(mm)
<i>Angelica tenuissima</i>	3
<i>Cnidium officinale</i>	5
<i>Cornus officinalis</i>	7
<i>Glycyrrhiza uralensis</i>	10
<i>Phellodendron amurense</i>	15
<i>Rheum palmatum</i>	11
<i>Rubus coreanus</i>	4
<i>Schisandra chinensis</i>	9
<i>Scutellaria baicalensis</i>	8
<i>Eucommiae cortex</i>	5
<i>Patrinia scabiosaefolia</i>	3

An antibacterial activity was assessed using the disc diffusion method.

The tested bacterium was *Staphylococcus aureus* KCTC 1621.

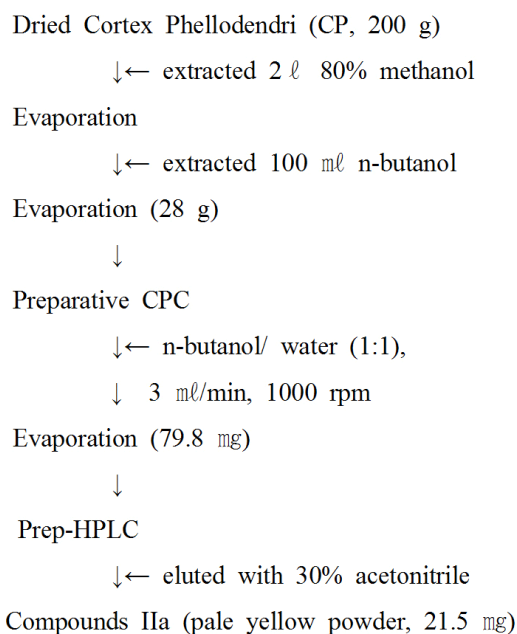


Fig. 1. Purification scheme of the dried Cortex Phellodendri methanol extract.

in the lower layer of the solvent system and then pumped into the column at 3 mL/min. The crude methanol extract was purified by the procedure summarized in Fig. 1.

A successful separation by CPC depends on selecting an optimum two-phase solvent system with an ideal partition coefficient (K) range for the target compound of 0.2 and 2 (Kim et al. 2006; Kim et al. 2007; Osamu et al. 2008; Kim et al. 2010; Kim JB 2011). If K value is higher than 10, the eluted peaks are broad and excessive mobile phase volume and time were required to complete a CPC run. The compounds do not separate under K conditions < 0.2. Several two-phase solvent systems were tested and their K values were measured. A two-phase solvent system composed of n-butanol: acetic acid: water showed appropriate K values. As a result of the partitioning behavior of peaks IIa, the two-solvent system composed of n-butanol: acetic acid: water (4:1:5 v/v/v) resulted in a favorable partitioning value of $K = 1.05$, between the two layers (Table 2).

Table 2. The K (partition coefficient) values of palmatine in the two-phase solvent system

Solvent system	Volume ratio	K value
n-Butanol : water	5 : 5	0.67
n-Butanol : iso-butanol : water	4 : 1 : 5	0.33
n-Butanol : acetic acid : water	4 : 1 : 5	1.05

3. CPC Separation

The dried CP methanol extract was separated and purified by CPC with a n-butanol: acetic acid: water (4:1:5) solvent system. As shown in Fig. 2, the fractions were collected for

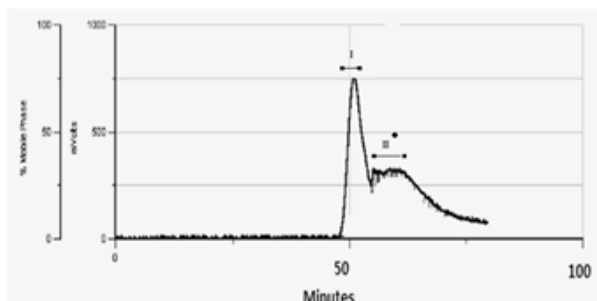


Fig. 2. Preparative centrifugal partition chromatography (CPC) separation of the methanol Cortex Phellodendri extracts. Solvent system: n-butanol/acetic acid/water (4:1:5), flow rate 3 ml /min; rotation speed, 1,000 rpm; detection, 280 nm

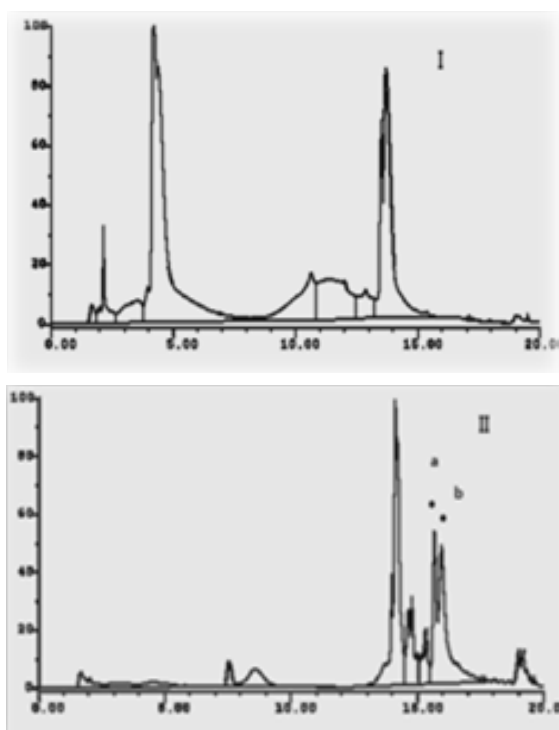


Fig. 3. Centrifugal partition chromatography (CPC) chromatogram of a Cortex Phellodendri methanol extract together with high performance liquid chromatography (HPLC) chromatograms of CPC peak fractions I and II. Column, J'sphere ODS-H80, 4 μ m particle size, 120 \AA , 150 \times 4.6 mm, YMC Co. Ltd.; elution solvent, 0~3 min 15% acetonitrile, 3~10 min 15~50% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) in a gradient system. Elution time, 20 min; detection, 280 nm

about 80 min and were grouped as I and II.

The separated fractions were analyzed by HPLC and the results indicated that the extract contained several compounds including peaks IIa and IIb (retention times, 15.6 and 16.2 min, respectively) and some unknown compounds (Yang et al. 2010). The peak IIb is in the process of structural analysis. Fig. 3 shows typical HPLC profiles of the CPC separated fractions. Fig. 3 shows that purification of IIa and IIb was achieved in only 80 min.

4. Structural Elucidation of Peaks IIa

The structural identification of peak fractions IIa in Fig. 4 was performed by ^1H NMR and ^{13}C NMR.

The area under the peaks showed that the resonances at δ 3.79, 3.84, 3.97, and 4.07 ppm corresponded to three ($-\text{CH}_3$) protons, whereas those at δ 3.06 and 4.69 ppm corresponded to two protons ($-\text{CH}_2$). All other resonances corresponded to one proton ($-\text{CH}$) (Tripathi et al. 2008; Li et al. 2009).

When these ^1H NMR, ^{13}C NMR, and ESI-MS data were compared to those reported in the literature (Lee et al. 2005;

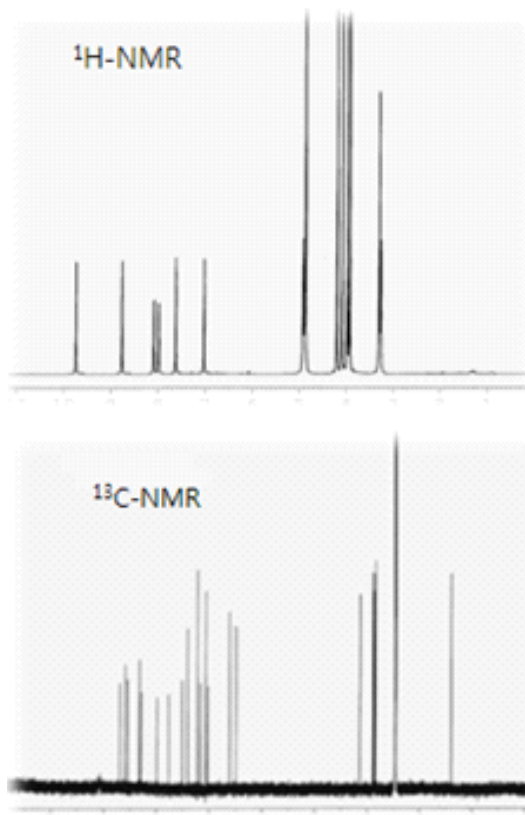


Fig. 4. ^1H -Nuclear magnetic resonance (NMR) and ^{13}C -NMR spectra of peak IIa.

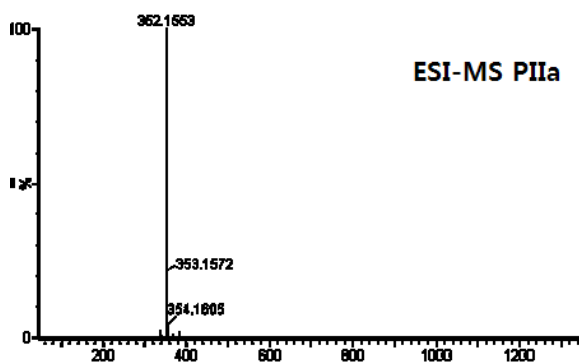
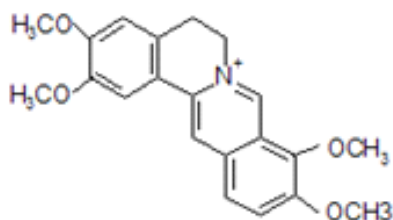


Fig. 6. Electrospray ionization mass spectroscopy(ESI-MS) chromatograms of palmatine(IIa).

Palmatine: ESI-MS m/z : 352.1553(M^+)

1H -NMR (900 MHz, MeOD) δ : 9.78 (1H, s, H-8), 8.88 (1H, s, H-13), 8.10 (1H, d, $J = 8.0$ Hz, H-11), 8.00 (1H, d, $J = 8.0$ Hz, H-12), 7.63 (1H, s, H-1), 7.03 (1H, s, H-4), 4.87 (2H, m, H-6), 4.19 (3H, s, 9-OCH₃), 4.08 (3H, s, 10-OCH₃), 3.97 (3H, s, 2-OCH₃), 3.92 (3H, s, 3-OCH₃), 3.52 (2H, m, H-5)

^{13}C -NMR (225 MHz, CD₃OD) δ : 152.6 (C-3), 150.7 (C-10), 149.7 (C-2), 145.2 (C-8), 144.5 (C-9), 138.6 (C-13a), 134.0 (C-12a), 128.9 (C-4a), 126.8 (C-12), 123.3 (C-11), 122.1 (C-13b), 120.1 (C-13), 119.3 (C-8a), 111.0 (C-4), 108.7 (C-1), 61.3 (C-9, OCH₃), 56.1 (C-2, OCH₃), 55.8 (C-3, OCH₃), 55.4 (C-6), 26.6 (C-5)



Palmatine

Fig. 7. The structures of palmatine(IIa).

Min et al. 2008; Deevanhxay et al. 2009; Hu et al. 2009; Ma et al. 2009) compounds IIa was identified as palmatine (Figs. 4~7).

Summary

Cortex Phellodendri (CP) is derived from the dried bark of *Phellodendron amurense*. It has been widely used as a drug in traditional Korea medicine for treating diarrhea, jaundice, swelling

pains in the knees and feet, urinary tract infections and infections of the body surface. In this study, preparative centrifugal partition chromatography (CPC) was successfully carried out to separate antibacterial compounds from a CP methanol extract. The optimum two-phase CPC solvent system was composed of n-butanol: acetic acid: water (4:1:5 v/v/v). The flow rate of the mobile phase was 3 ml/min in ascending mode with rotation at 1,000 rpm. The CPC-separated fraction and purification procedures were carried out by preparatory HPLC. Palmatine was identified by 1H , ^{13}C -nuclear magnetic resonance and electrospray ionization-mass spectroscopy spectral data analysis.

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