

## Screening and Purification of an Anti-Prostate Cancer Compound, Deoxypodophyllotoxin, from *Anthriscus sylvestris* Hoffm

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The prostate cancer is the critical health problem, increasing of its related death in worldwide. Unfortunately present surgery and chemotherapeutic choices seem to be impossible in curing or controlling prostate cancer, because metastasis occasionally advances even after these potentially curative therapies. Therefore, there is immediate need to alternative chemoprevention and chemotherapeutic agents. Over one hundred species of dried medicinal herbs were tested for proliferation inhibitory effects on prostate cancer cell line, PC-3. One of them, *Anthriscus sylvestris* was selected because of potent anti-proliferation effect. The dried root of *A. sylvestris* was extracted with 100% methanol for 2-3 days and its extract was fractionated by using ethyl acetate. And ethyl acetate layer was subjected to column chromatographies on silica gel, reverse phase-18 (RP-18) and Sephadex LH-20, in turn. Finally, the pure compound was obtained by crystallization in methanol at 4°C for overnight and identified as deoxypodophyllotoxin by NMR spectrosopic and physico-chemical analyses. In addition, it was confirmed that deoxypodophyllotoxin clearly inhibits the proliferation of PC-3 cells in a dose- and time-dependent manner.

**Key words** : Chromatography, deoxyphyllotoxin. prostate cancer, purification, screening

### Introduction

Modern men are confronted with an increasing incidence of cancer and cancer deaths annually. Statistics indicates that men are largely taken by lung, colon, rectal, and prostate cancer, while women increasingly suffer from breast, colon, rectal, and stomach cancer [1,8]. The prostate cancer is a critical health problem in worldwide and it has been a recent trend in Asia towards increasing incidences of prostate cancer, such as Korea and Japan, reporting a more rapid increase than high risk countries [2]. Prostate cancer is the second leading cause of cancer-related deaths among men in the United States and many western countries [2,8]. In the United States only, one of nine men over the age of 65 years is diagnosed as prostate cancer.

Currently although potentially therapeutic options including surgic prostatectomy, radiotherapy and hormonal therapy for the treatment of prostate cancer are used in clinical, the general survival treatment of patients is still unsatisfactory once the disease is metastatic [18,19]. Because the

molecular mechanism of prostate cancer is yet not defined and metastasis problem occasionally happen after curative therapies. Androgen ablation is a frequently treated option for prostate cancer [13]. However, this treatment has limited scope, especially, for hormone-refractory cancers. Moreover, chemotherapy and radiation therapy are largely ineffective against advanced prostate cancer that fails to respond to hormone ablation therapy [6,20].

So many efforts have been focused on developing new cancer targeted agents [4,11,12]. Many natural products are available as chemoprotective agents against commonly occurring cancer types [15,17]. Apoptosis mechanism is a target for reducing prostate cancer by chemopreventative and anti-cancer agent from natural products [3,14,16]. These natural products were discovered from vegetables, fruits, plant extracts, tea and herbs through a routine examination of terrestrial plants and microorganisms. Natural products and theirs structural derivatives accounted for nearly 50% of agents used in cancer chemotherapy [15,17]. Although the mechanism of anti-cancer agent effect is not clear but unique chemical structures of natural products is lead to anti-cancer activity against tumor. A number of naturally occurring compounds from herb, tea and plants applied to chemoprevention properties against carcinogenesis. In the

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present study, methanol extracts of various medicinal plants were used to screen for anti-proliferation and anti-apoptotic agents. We selected *A. sylvestris* because it showed potent growth inhibitory effect on human prostate cancer cell line, PC-3.

## Materials and Methods

### Materials

Air-dried root of *A. sylvestris* was purchased from a Korean medicinal market, Busan, Korea. Silica gel (Kiesel gel 60, particle size: 0.045-0.063 mm), a precoated silica gel plate 60F<sub>254</sub> (0.25 mm in thickness) and an ODS RP-18 F<sub>254</sub> (25 DC-Platten 5×10 cm) were purchased from Merck Co. (Darmstadt, Germany). Also the ODS RP-18 gel (ODS-A, 120A, S-5 mm) was purchased from the YMC-Gel Co. (Tokyo, Japan). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### Tumor cell lines and cell culture

Human prostate carcinoma PC-3 cell line (androgen independent cells) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained and cultured in DMEM medium (Hyclone Co., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Co.), 100 units/ml of penicillin and 100 mg/ml of streptomycin (Hyclone Co.). Cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Preparation and screening of methanol extracts

Over one hundred plants were extracted with methanol at room temperature for 2-3 days. PC-3 cells were cultured for overnight in 48-well plates at a density of 1×10<sup>4</sup> cells/well. The cells were treated with an appropriate concentration of each methanol extract for 48 hr at 37°C in 5% CO<sub>2</sub> incubator. After that, MTT solution of final concentration 0.5 mg/ml in phosphate-buffered saline was added and incubated for 3-4 hr at 37°C [5,21]. After the medium was removed, dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan complex. The formazan complex was analyzed at 540 nm in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). The cell viability was observed as percentage of control, comparing with one of the untreated cells.

### Purification of *A. sylvestris*

The dried *A. sylvestris* (500 g) was soaked in methanol at room temperature for 2-3 days and then extracted 3 times. The methanol extract was evaporated, resuspended and extracted in water and ethyl acetate. And ethyl acetate layer was then evaporated *in vacuo* and concentrated to yield a crude extract. The ethyl acetate extract was chromatographed on a silica gel column (Ø 2.5×12 cm, 70-230 mesh) using gradient solvent of hexane/ethyl acetate (3/1→1/1). The fractions with growth inhibitory activity were pooled and subjected to a reverse phase-18 (RP-18) column (Ø 1.5×10 cm, 230-400 mesh). And each fraction was eluted by using 60% methanol. The mixture of active fractions was further chromatographed on a Sephadex LH-20 using 100% methanol. If necessary, high-performance liquid chromatography (HPLC) was applied to purify it. Finally pure compound (50 mg) was obtained by crystallization in methanol at 4°C for overnight.

### Instrumental analysis

Silica gel TLC plate (silica gel 60F<sub>254</sub>) or RP-18 gel TLC plate (RP-18 F<sub>254</sub>) were developed with hexane/ethyl acetate (1/1, v/v) and 80% aqueous methanol, respectively. The plates were colorized by heating for 10 min at 110°C after spraying with anisaldehyde-sulfuric acid. HPLC was carried out on a SCL-10A controller unit that includes SPD-M10A UV, LC-10AT, FCV-10AL, and DGU-14A (Shimadzu Scientific Co., Tokyo, Japan) using an YMC-PACK ODS C18 (Ø 4.6×250 mm, YMC-Gel Co., Tokyo, Japan). The injection volume was 10 µl and the flow rate was 0.5 ml/min using the solvent system of 60% aqueous methanol. The <sup>1</sup>H- or <sup>13</sup>C-NMR spectra were recorded on a JEOL Eclipse NMR spectrometer (JEOL, Tokyo, Japan) at the frequency of 500 MHz or 125 MHz, respectively. Proton or carbon chemical shifts were relative to tetramethylsilane (TMS) as an internal standard.

### Statistical analysis

All experiments were conducted in duplicate and standard deviations were indicated in the figures with bars.

## Results and Discussion

Screening of growth inhibitory medicinal extracts on human prostate cancer cells

The growth inhibition of medicinal methanol extracts on

PC-3 cells was determined by MTT assay. In order to screen for anti-proliferation and anti-cancer agents, PC-3 cells were treated with medicinal methanol extracts for 48 hr. As a result, the methanol extract of the root of *A. sylvestris* showed most potent inhibition on the growth of PC-3 cells, as compared with control, as shown in Fig. 1. *A. sylvestris* is a perennial herb that grows in Europe, Caucasus, Siberia, Kamchatka, Kuriles, Sakhalin, China, Manchuria, and Korea [9]. The root of *A. sylvestris* has been used as therapy in coughing, asthma, laryngitis and anti-cancer. Also, it has been reported to exhibit anti-tumor activity, anti-platelet aggregation activity, anti-viral activity, anti-proliferation activity and broad insecticidal activity [9].

Purification and identification of deoxypodophyllotoxin from *A. sylvestris*

At first, to determine which fraction is active, the methanol extract of *A. sylvestris* was resuspended in water and extracted with organic solvents. And then inhibitory activity of each layer was evaluated by MTT assay. As a result, the ethyl acetate layer from methanol extract of *A. sylvestris* was highest inhibitory activity on the growth of PC-3 cells (Fig. 2). Its active compound in *A. sylvestris* was purified by the methods of methanol extraction, ethyl acetate extraction and column chromatography on silica gel, reverse phase-18 (RP-18) and Sephadex LH-20, etc., as shown in Fig. 3. The methanol extract of *A. sylvestris* was extracted with water and ethyl acetate to fractionate ethyl acetate layer. The

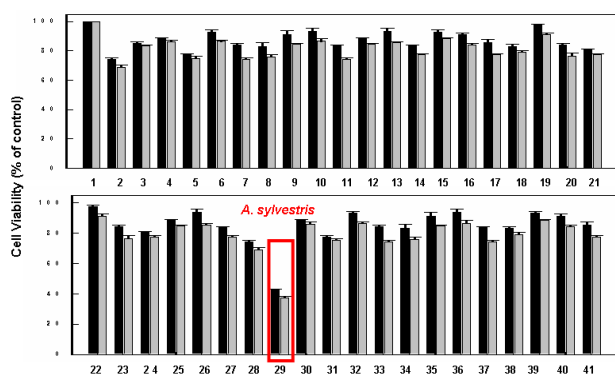


Fig. 1. Screening of medicinal methanol extract exhibiting the growth inhibition of PC-3 cells. PC-3 cells were cultured for overnight in 48-well plates at a density of  $1 \times 10^4$  cells/well overnight. The cells were treated with 10  $\mu$ g/ml (■) and 30  $\mu$ g/ml (□) of each methanol extract for 48 hrs at 37°C in 5% CO<sub>2</sub> incubator. Inhibitory activity of methanol extracts was determined by MTT assay. 1: Control, 2-41: medicinal methanol extracts.

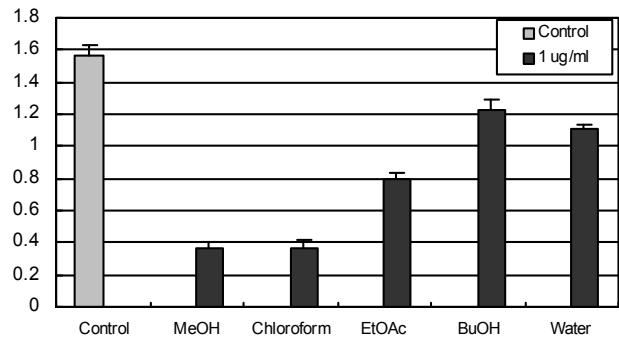


Fig. 2. Inhibitory effect of solvent extracts of *Anthriscus sylvestris* on the growth in PC-3 cells. PC-3 cells were cultured for overnight in 48-well plates at a density of  $1 \times 10^4$  cells/well overnight. The cells were treated with 1  $\mu$ g/ml of each solvent extract for 48 hr at 37°C in 5% CO<sub>2</sub> incubator. Inhibitory activity of solvent layer was determined by MTT assay.

ethyl acetate extract of *A. sylvestris* was subjected to column chromatography on silica gel to give 7 fractions using hexane/ethyl acetate as an elution solvent. Active fractions with strong inhibitory activity were pooled and further purified by using RP-18 gel with 60% aqueous methanol and Sephadex LH-20 column chromatography with 100% methanol. The each fraction was monitored on normal or reverse phase TLC analysis by using a developing solvent (hexane/ethyl acetate=1/1 or 80% aqueous methanol, respectively). Active fractions with inhibitory activity were further purified by HPLC (YMC-Gel C18,  $\emptyset$  4.6 $\times$ 250 mm), if necessary, which the active compound was eluted at retention time of approximately 14 min (Fig. 4). Final pure

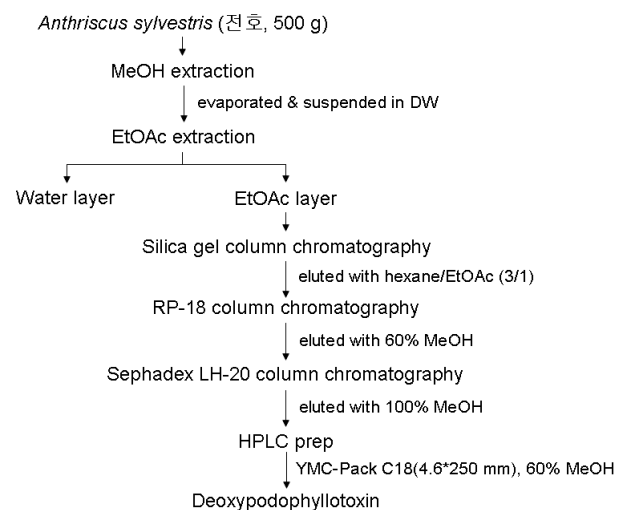


Fig. 3. Purification scheme of deoxypodophyllotoxin from methanol extract of *A. sylvestris*.

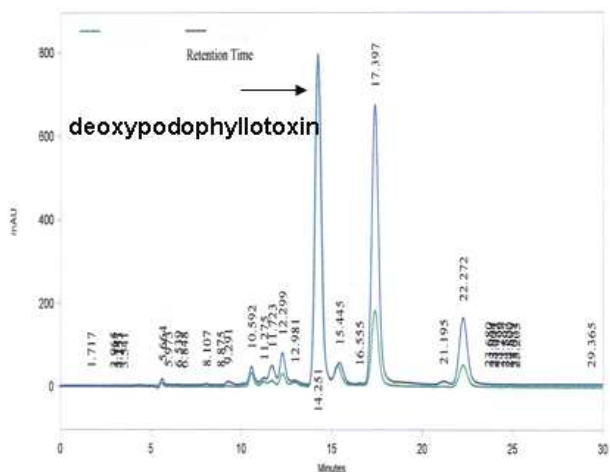


Fig. 4. High-performance liquid chromatography of active fractions eluted from Sephadex LH-20 column. Column: YMC-Gel C18, Ø 4.6×250 mm; mobile phase: 60% methanol; flow rate: 0.5 ml/min; UV wavelength: 240 nm; injection volume: 10 ml. The arrowed peak represents major active compound with retention time of about 14 min.

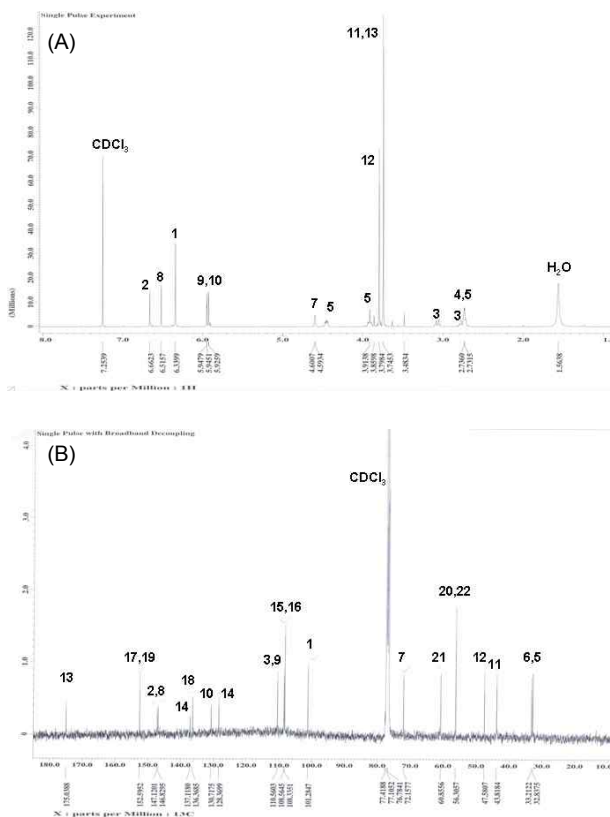


Fig. 5. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR analysis of deoxypodophyllotoxin purified from *A. sylvestris*. Spectra were taken at 500 MHz for proton (A) and 125 MHz for carbon (B). Chemical shifts were assigned on the basis of the relative shift to TMS as an internal standard.

compound (50 mg) was obtained as crystal form in methanol at 4°C for overnight. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were analyzed (Fig. 5) and chemical shift of the compound was assigned as ppm relative to internal standard TMS. Instrumental data were compared to previously reported results [7,9,10]. In addition, physico-chemical properties were analyzed to be colorless oil; UV λ<sub>max</sub> (in methanol), 241, 293 nm. Taken together, the crystal was identified as deoxypodophyllotoxin (Fig. 6), which has been reported to be anti-mitotic and anti-cancer activities in a variety of tumor cell lines.

Growth inhibitory activity of deoxypodophyllotoxin on human prostate cancer cells

Deoxypodophyllotoxin is a anti-tumor agent isolated from *A. sylvestris* which exhibits potent cytotoxicity against a wide variety of tumor cell line [7,9]. To assess growth inhibition of deoxypodophyllotoxin on PC-3 cells, the cells were treated with a different concentration (0.8-800 nM) of

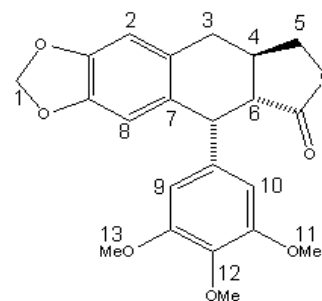


Fig. 6. Chemical structure of deoxypodophyllotoxin.

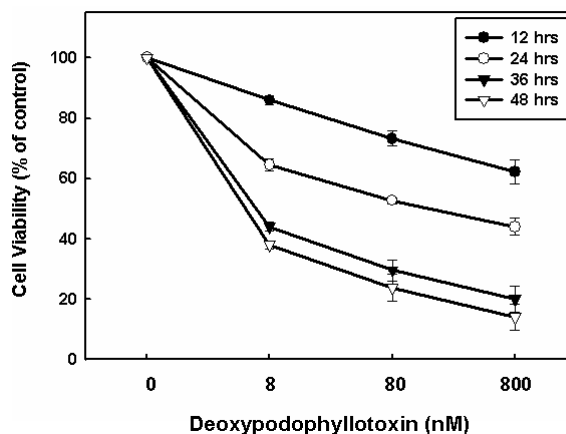


Fig. 7. Dose- and time-dependent inhibition of deoxypodophyllotoxin on the growth of PC-3 cells. The PC-3 cells were exposed with deoxypodophyllotoxin at a concentration of 0.8, 8, 80 and 800 nM for 12, 24, 36 and 48 hr. The cell viability was analyzed by MTT assay.

deoxypodophyllotoxin for 0-48 hr. As shown in Fig. 7, deoxypodophyllotoxin isolated from *A. sylvestris* significantly reduced cell proliferation in a dose-dependent manner. Its IC<sub>50</sub> (50% inhibitory concentration) was measured to be approximately 80 nM at 24 hrs treatment. Also, the anti-proliferation potential of deoxypodophyllotoxin was observed, depending on treatment time (Fig. 7). Remarkably, these results suggested that deoxypodophyllotoxin potently inhibits the proliferation of PC-3 cells, in a dose and time-dependent manner. In conclusion, our experiments demonstrated that deoxypodophyllotoxin exhibited significant anti-cancer activity in human prostate cancer cell, PC-3 cells.

Finally, deoxypodophyllotoxin might be useful in the search for new potential chemotherapeutic agents and understanding the molecular mechanisms of anti-cancer on human prostate cancers. Therefore, *in vivo* anti-cancer actions and clinical applications of the deoxypodophyllotoxin will be demanded for further investigation. In further study, anti-cancer effect of deoxypodophyllotoxin on human prostate cancer cell will be elucidated by investigating the apoptotic mechanisms on the prostate cancer cells by deoxypodophyllotoxin, such as proliferation, morphological changes, cell cycle progression and the expression of the apoptosis related proteins.

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초록 : 전호(*Anthriscus sylvestris* Hoffm)로부터 전립선 암세포 저해물질인 deoxypodophyllotoxin의 탐색 및 분리

조효진<sup>1</sup> · 유선녕<sup>1</sup> · 김광연<sup>1</sup> · 손재학<sup>3</sup> · 오현철<sup>3</sup> · 안순철<sup>1,2,\*</sup>

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전립선암은 현대 남성들에게 걸리기 쉬운 질병으로 나이가 들수록 발병의 위험이 증가하는 질환으로 현재 우리나라에서도 점점 증가하는 추세이다. 전립선암 치료법들은 치료영역이 제한적이고 재발할 가능성이 높아 근본적인 치료법으로는 사용되지 못하므로 새로운 전립선암 치료 방법이 필요하다. 이에 본 연구에서는 100 여 가지의 한약재 methanol 추출물을 이용하여 MTT 방법으로 전립선암 세포주인 PC-3 세포에 대한 항증식 효과를 탐색하였으며 그 결과, *A. sylvestris*가 가장 강한 항증식 활성을 보였다. *A. sylvestris*의 methanol 추출물로부터 저해물질을 분리하기 위하여 100% methanol에서 2-3일 추출하고 난 뒤, ethyl acetate로 추출하고 silica gel, reverse phase-18, Sephadex LH-20 등의 컬럼 크로마토그래피를 이용하여 분리하였다. 최종적으로 활성분획을 HPLC로 분리하고 4°C에서 methanol 용액에서 입방체 형태의 결정을 얻었으며 NMR 분광법과 이화학적 특성을 분석한 결과, deoxypodophyllotoxin으로 동정되었다. 순수 분리된 deoxypodophyllotoxin은 전립선암의 세포주 PC-3 세포에서 처리 농도와 처리 시간 의존적인 항증식 효과를 보였다.