Antitumor Constituents from *Anthriscus Sylvestris* (L.) Hoffm

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Abstract

Bioassay-guided chemical investigation of the roots of *Anthriscus sylvestris* (L.) Hoffm. resulted in the isolation of nine compounds, whose structures were determined by spectroscopic methods. Compound 1 was isolated from this plant for the first time and compounds 3 and 9 were first found from this genus. Different polar fractions of *A. sylvestris* extract and compounds 1, 6-8 and 9 were evaluated for antitumor activities against HepG2 (human hepatocellular carcinoma), MG-63 (human osteosarcoma cells), B16 (melanoma cells) and HeLa (human cervical carcinoma cells) lines by the MTT method. The petroleum ether fraction of *A. sylvestris* extract exhibited excellent inhibitory activity with an IC\(_{50}\) value of 18.3 μg/ml. Among the isolates from the petroleum ether fraction, compound 7 showed significant inhibition against the growth of the four tumor cells with IC\(_{50}\) values ranging from 12.2-43.3 μg/ml.

Keywords: *Anthriscus sylvestris* - anthricin - constituents - antitumor activities

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Introduction

*Anthriscus sylvestris* (L.) Hoffm. (Umbelliferae) is a perennial herb that grows in Europe and in parts of North America, Africa, Asia and New Zealand (Jeong, 2007; Oktavia, 2011). The roots of *A. sylvestris* have been used as antitussive, antipyretic, analgesic, diuretic, and cough remedy in Chinese traditional medicine, and the young aerial part of this plant is used for food (Kozawa et al., 1978a; Wang et al., 1982; Yang et al., 2010). This plant has a lignan named deoxypodophyllotoxin (Noguchi et al., 1940), which is known to have many bioactivities such as antitumor activity (Kozawa et al., 1978b; Ayres et al., 1990; Lim et al., 1999), anti-platelet aggregation activity, antiviral activity, antiproliferative activity, broad insecticidal activity, inhibition of passive cutaneous anaphylaxis reactions (Lin et al., 2004), liver protective action (Kiso et al., 1982), and anti-inflammatory activity (Lee et al., 2004). *A. sylvestris* contains highly lignans, phenylpropanoids, flavonoids, coumarins, organic acids, and so on (Tozabcuro et al., 1979; Liang et al., 1990; Milovanovic et al., 1996; Ikeda et al., 1998; Bos et al., 2002; Koultman et al., 2007). Umbelliferae has high research value of anti-tumor activity. There were many reports. For example, the alcoholic extracts and seed oil of *Petroselinum sativum* (Umbelliferae) induced cell death in MCF-7 cells (Farshori et al., 2013).

In course of searching for anti-tumor agents from medicinal herbs, the MeOH extract of the roots of *A. sylvestris* was found to be active against human chronic myelogenous leukemia cell K562 (Lim et al., 1999). In recent years, there is a trend to explore antitumor activities of some plants like *A. Sylvestris*, which are known for its food and medicinal properties, expected to find some drugs with relatively low toxicity and high activity. *Oroxylum indicum* (L.) can effectively target ER-negative breast cancer cells to induce apoptosis, without harming normal cells by cancer-specific cytotoxicity (Kumar et al., 2012). *Syzygium aromaticum* L showed inhibition activity of some cancer cell lines like HeLa (Dwivedi et al., 2011). In addition, some compounds like oleanolic acid and ursolic acid from food and medicinal herbs had relatively low toxicity and antitumor activities (Gayathri et al., 2009; George et al., 2012). In this study, the bioassay-guided chemical investigation of the roots of *A. sylvestris* resulted in the isolation of nine known compounds, and their antitumor activities were tested.

Materials and Methods

Materials

The roots of *A. sylvestris* were purchased at Emei Mountain, Sichuan Province, China in October, 2012, and identified by Prof. Liang-Ke Song, Southwest Jiaotong University. The specimen (CE20121210) was deposited at the Specimens laboratory of Southwest Jiaotong University.

General

NMR spectra were recorded on a Bruker AV-400 MHz
or an Avance III 600 spectrometer with TMS as an internal standard using CDCl₃ as solvent. Silica gel (200-300 mesh, Qingdao Marine Chemical Inc., People's Republic of China), Sephadex LH-20 (Pharmacia) were used for column chromatography. Silica gel (Qingdao Marine Chemical Inc., the types of G and GF254) were used for TLC. Fractions were monitored by TLC and spots were visualized by heating after spraying with 10% H₂SO₄ in ethanol. HepG2, MG-63, B16, HeLa cells were bought from National Key Laboratory of Biotherapy for Human Diseases, West China Hospital of Sichuan University. MTT and DMSO were produced in Sigma Company, America. PRMI-1640 (Gibco Company, America.) and FBS (fetal bovine serum) (Hyclon Company, America.) were used.

**Extraction and isolation**

The dried and shattered roots of A. sylvestris (8.0 kg) were extracted with 95% EtOH by percolation. The extract was concentrated and suspended in water (2 L) followed by successive partition with petroleum ether (3x5 L), chloroform (3x5 L), ethyl acetate (3x5 L), and n-Butyl alcohol (3x5 L) to yield petroleum ether soluble (102.9 g), chloroform soluble (9.3 g), ethyl acetate soluble (3x5 L), and n-Butyl alcohol (3×5 L), to yield petroleum ether soluble (3×5 L), chloroform (3×5 L), ethyl acetate (3×5 L), and n-Butyl alcohol (3×5 L), to yield petroleum ether soluble (112.1 g) fractions WA-WF, and the fraction WF was then purified followed by successive partition with petroleum ether / acetone stepwise gradient solvent system of petroleum ether/acetone (100:1-50:1) to afford fractions A-Z and to yield compounds 1 (102.9 g), 2 (43.4 g), and 3 (150.1 mg), 4 (13 mg). Compounds 6 (21.3 mg) and 9 (11.1 mg) were separated by silica gel and Sephadex LH-20 to obtain compounds 3 (7.3 mg), 4 (13 mg). Compounds 6 (21.3 mg) and 9 (11.1 mg) were separated by silica gel column (200-300 mesh) using a gradient solvent system of petroleum ether/acetone (100:1-0:1) to afford fractions A-Z and to yield compounds 1 (17 mg) and compounds 2 (19 mg). The fraction W was chromatographed on silica gel eluting with a petroleum ether / acetone stepwise gradient (60:0-0:1) to afford fractions WA-WF, and the fraction WF was then purified by silica gel and Sephadex LH-20 to obtain compounds 3 (7.3 mg), 4 (13 mg). Compounds 6 (21.3 mg) and 9 (11.1 mg) were isolated from sub-fraction WB by silica gel and Sephadex LH-20. The fraction Y was separated by silica gel eluting with a petroleum ether/acetone stepwise gradient (10:1-0:1 to afford compounds A-Z and to yield compounds 1 (17 mg) and compounds 2 (19 mg). The fraction W was chromatographed on silica gel eluting with a chloroform/ethyl acetate stepwise gradient (60:0-0:1) to afford fractions WA-WF, and the fraction WF was then purified by silica gel and Sephadex LH-20 to obtain compounds 3 (7.3 mg), 4 (13 mg). Compounds 6 (21.3 mg) and 9 (11.1 mg) were isolated from sub-fraction WB by silica gel and Sephadex LH-20. The fraction Y was separated by silica gel eluting with a petroleum ether/acetone stepwise gradient (10:1-0:1) to afford compounds 7 (39.3 mg) and 8 (179.5 mg). Compounds 5 (7.8 mg) was obtained from fraction K by chromatographed on silica gel eluting with a petroleum ether/ethyl acetate stepwise gradient (60:1-0:1).

**H and ¹³C NMR spectra for compounds 1-9**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Spectral Data</th>
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| 3-Methoxy-4-5-methylenedioxybenzaldehyde | C₂₀H₂₂O₂ | ¹H NMR (600 MHz, CDCl₃): 6.64 (s, 1H), 6.51 (s, 1H), 6.31 (s, 1H), 5.94 (d, J=1.4Hz, 2H), 5.91 (d, J=1.4Hz, 2H), 4.60 (d, 1H), 4.46 (t, J=7.3Hz, 2H), 4.44 (t, J=7.3Hz, 2H), 3.91 (dd, J=10.3, 8.4Hz, 2H), 3.87 (dd, J=10.3, 8.4Hz, 2H), 3.79 (s, 3-O-Me, 3H), 3.74 (s, 4-O-Me, 3H), 3.08 (s, J=15.8, 4.8Hz, 2H), 3.05 (s, J=15.8, 5.1Hz, 1H), 2.77 (dd, J=15.8, 11.7Hz, 2H), 2.73 (m, 1H).

**β-Sitosterol** | C₃₀H₅₀O | ¹H NMR (400MHz, CDCl3): 6.66 (s, 1H), 6.51 (s, 1H), 6.31 (s, 1H), 5.94 (d, J=1.4Hz, 2H), 5.91 (d, J=1.4Hz, 2H), 4.60 (d, 1H), 4.46 (t, J=7.3Hz, 2H), 4.44 (t, J=7.3Hz, 2H), 3.91 (dd, J=10.3, 8.4Hz, 2H), 3.87 (dd, J=10.3, 8.4Hz, 2H), 3.79 (s, 3-O-Me, 3H), 3.74 (s, 4-O-Me, 3H), 3.08 (s, J=15.8, 4.8Hz, 2H), 3.05 (s, J=15.8, 5.1Hz, 1H), 2.77 (dd, J=15.8, 11.7Hz, 2H), 2.73 (m, 1H).

**Deoxypicropodophyllotoxin** | C₃₀H₅₀O | ¹H NMR (400MHz, CDCl3): 174.97 (s, C=O), 152.43 (s, C-3, 5), 146.97 (s, C-3'), 146.68 (s, C-4'), 136.88 (s, C-4), 136.28 (s, C-1), 130.55 (s, C-5'), 128.26 (s, C-1'), 110.41 (d, C-5'), 108.45 (d, C-2'), 108.13 (d, C-2, 6), 101.16 (t, OCH₂O), 77.35 (t, C-9'), 77.03 (t, C-9'), 76.71 (t, C-9'), 60.72 (t, C-4), 57.31 (s, 3-O-Me, 3H), 3.74 (s, 3-O-Me, 3H), 2.86-2.92 (m, 1H), 2.61 (dd, J=15.3, 6.3Hz, 2H), 2.51 (dd, J=15.3, 5.4Hz, 2H).

These compounds were elucidated on the basis of spectral data and comparison with published literatures. The structures of compounds 1-9 were shown in Figure 1.
Antitumor activities assay in vitro: The MTT bioassay determines the ability of viable cells to reduce the yellow tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] (MTT) to blue formazan crystals by mitochondrial enzymes. The in vitro antitumor activities or cytotoxicity of the samples were tested on HepG2 (human hepatocellular carcinoma), MG-63 (human osteosarcoma cells), B16 (melanoma cells) and HeLa (human cervical carcinoma cells) that were cultured on RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 25 cm² culture flasks at 37°C in humidified atmosphere with 5% CO₂ (Alabsi et al., 2013). The cells were harvested from the culture flasks at the exponential growth phase and resuspended in fresh medium at a cell density of 1x10⁵ cells ml⁻¹. The cell suspension was dispensed into a 96-well microplate at 100 μl per well and incubated in humidified atmosphere with 5% CO₂ at 37°C for 48 h, and then treated with the drugs (different polar parts of A. sylvestris and compounds 1, 6-8 and 9) at selected doses. Cell proliferation in the microplate was determined at various treatment intervals with the MTT assay. MTT colorimetric method is a kind of method of detecting the growth and survival of cells.

Results

The structures of compounds 1-9 were identified as 3-Methoxy-4,5- methylenedioxybenzaldehyde (1), β-sitosterol (2), (Z)-2-angeloyloxymethyl-2-butenoic acid (3), (-)-(R)-carveol (4), margaric acid triglycerides (5), 5-(3-Methoxy-1-propenyl)-1, 3-benzodioxole (6), anthricin (7), isoan-thricin (8) and deoxypicropodophyllotoxin (9), from which compound 1 was isolated for the first time from this plant and compounds 3 and 9 were found firstly from this genus.

The antitumor activities of A. sylvestris extract and compounds were evaluated against HepG2 cell line, MG-63 cell line, B16 cell line and HeLa cell line. The results are presented in Table 1. Petroleum ether and chloroform fractions exhibited excellent inhibitory activities with the IC₅₀ value in the range of 18.25-45.66 μg/ml. Ethyl acetate fraction had a weaker activity on B16, while n-butyl alcohol fraction had no inhibition. Among the isolations from the petroleum ether fraction, compound 7 exhibited significant inhibitory activities against the growth of the four tumor cells with the IC₅₀ value ranging from 12.24-43.25 μg/ml. And compound 8 also showed strong inhibitory activities against MG-63, B16 cells. Compound 6 and 9 showed weaker inhibition for HepG2, HeLa. Other compounds were weak cytotoxic agents or inactive. Figure 2-5 shows the relationship with the cell proliferation and different fractions of A. sylvestris extract growth of the four tumor cells with the IC₅₀ value ranging from 12.24-43.25 μg/ml. And compound 8 also showed strong inhibitory activities against MG-63, B16 cells. Compound 6 and 9 showed weaker inhibition for HepG2, HeLa. Other compounds were weak cytotoxic agents or inactive. Figure 2-5 shows the relationship with the cell proliferation and different fractions of A. sylvestris extract.
be supposed that 7-benzene were very important to the activity component and of which lignans especially arylnaphthalenes could be the most important compounds.

In conclusion, petroleum ether and chloroform fractions of the crude extracts exhibited remarkable inhibitory activities against HepG2 and HeLa cells with the IC<sub>50</sub> value in the range of 18.25-45.66 μg/ml. Furthermore, with the guide of antitumor inhibition, nine pure compounds with different antitumor inhibitory activity were isolated and identified. Especially, compound 7 showed remarkable inhibitory activity against tumor cells with the IC<sub>50</sub> value ranging from 12.24-43.25 μg/ml. It is first reported that control tests were conducted by compound 9 to detect its antitumor inhibition. We believe that our results have shown A. sylvestris to be a new source of antitumor. The isolated compounds could be used as lead compounds for drug screening.

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**References**


