RESEARCH ARTICLE

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 (Tozaburo et al., 1979; Liang et al., 1990; Milovanovic et al., 1996; Ikeda et al., 1998; Bos et al., 2002; Koulman 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

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or an Avance III 600 spectrometer with TMS as an internal standard using $CDCl_3$ 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% H_2SO_4 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 to recovery cells.

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 (3×5 L), chloroform (3×5 L), ethyl acetate (3×5 L), and n-Butyl alcohol (3×5 L), to yield petroleum ether soluble (102.9 g), chloroform-soluble (9.3 g), ethyl acetate-soluble (43.4 g), and n-Butyl alcohol-soluble (112.1 g) fractions, respectively. The petroleum ether soluble (92.0 g) was 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 (1.9 g). 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).

^1H and ^{13}C NMR spectra for compounds 1-9

3-Methoxy-4, 5-methylenedioxybenzaldehyde: $C_9H_8O_4$, white powder. ^1H NMR (600MHz, DMSO): 9.78 (1H, s, -CHO),7.30 (1H, s, arom. H), 7.09 (1H, s, arom. H), 6.14 (2H, s, $-OCH_2O-$), 3.89 (3H, s,$-OCH_3$) (Kozawa et al., 1978b).

β-Sitosterol: $C_{29}H_{50}O$, white needle ccvrystal, was elucidated by comparing with authentic compound by TLC means.

(Z)-2-angeloyloxymethyl-2-butenoic acid: $C_{10}H_{14}O_4$, yellow grease. ^1H NMR (600 MHz, $CDCl_3$): 6.52 (1H, q, J=13.8, 6.6Hz, olefinic H), 6.06 (1H, q, J=13.2, 6.0Hz, olefinic H), 4.82 (2H, brs, $-OCH_2-$), 2.11 (3H, d, J=7.2 Hz, $-C=C-CH_3$), 1.96 (3H, m, $C=C-CH_3$), 1.88 (3H, brs, $-C=C-CH_3$) (Wang et al., 2009).

(-)-(R)-Carveol: $C_{10}H_{16}O$, yellow grease, 13C NMR (150 MHz, $CDCl_3$): 135.8 (s, C-8), 134.7 (s, C-1), 127.7 (d, C-2), 117.3 (t, C-9), 63.5 (d, C-5), 58.6 (d, C-4), 31.8 (t, C-6), 29.1 (t, C-3), 22.6 (q, C-7), 14.1 (q, C-10) (Elamparuthi et al., 2012).

Margaric acid triglycerides: $C_{20}H_{40}O_4$, white grain, ^1H NMR (400MHz, $CDCl_3$): 4.31 (s, OH), 4.30 (s, OH), 4.29 (d, 2H), 4.28 (tt, 1H), 4.15 (d, 2H), 2.31 (t, 2H), 1.61 (tt, 2H), 1.53 (tt, 2H), 1.26-1.29 (24H, m, 5-16-CH2), 0.88 (q, 3H).

^{13}C NMR (150 MHz, $CDCl_3$): 173.26 (s, C=O), 69.03 (t, C-1'), 62.10 (t, C-3'), 34.23 (t, C-2), 34.06 (t, C-3), 31.92 (t, C-4), 29.12-29.69 (t, C-5-13), 24.91 (t, C-14), 24.87 (t, C-15), 22.67 (t, C-16), 14.08 (q, C-17).

5-(3-Methoxy-1-propenyl)-1,3-benzodioxole: $C_{11}H_{12}O_3$, colorless needle crystal, ^1H-NMR (400MHz, CDCl3): 6.61 (d, J=1.5Hz, 1H), 6.53 (d, J=1.5Hz, 1H), 6.50, 6.47 (brd, J=15.5Hz, 1H), 6.18-6.22 (ddd, J=15.5, 6.0, 6.0 Hz, 1H), 5.95 (s, OCH_2O, 2H), 4.29, 4.28 (dd, J=6.0, 1.5 Hz, 2H), 3.90 (s, OCH_3, 3H).

^{13}C NMR (150 MHz, $CDCl_3$): 149.18 (s, C-5'), 143.58 (s, C-3'), 135.06 (s, C-4'), 131.70 (d, C-1), 130.96 (s, C-1'), 127.32 (d, C-2), 106.9 (d, C-2'), 101.49 (t, OCH2O), 100.09 (d, C-6'), 63.60 (t, C-3), 56.60 (q, OCH_3) (Ikeda et al., 1998).

Anthricin: $C_{22}H_{22}O_7$, white grain, developing solvent: petroleum ether / acetone (2:1), Rf=0.40. Anthricin was verified by TLC method comparing with the synthetic anthricin, whose structure had been elucidated through spectral date.

Isoan-thricin: $C_{22}H_{22}O_7$, white needle crystal, 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=7.3 Hz, 2H), 4.44 (t, J=7.3 Hz, 2H), 3.91 (dd, J=10.3, 8.4 Hz, 2H), 3.87 (dd, J=10.3, 8.4 Hz, 2H), 3.79 (s, 3,5-OMe, 3H), 3.74 (s, 4-OMe, 3H), 3.08 (dd, J=15.8, 4.8 Hz, 2H), 3.05 (dd, J=13.0, 5.1 Hz, 1H), 2.77 (dd, J=15.8, 11.7 Hz, 2H), 2.73 (m, 1H).

^{13}C NMR (150 MHz, $CDCl_3$): 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-6'), 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), 56.14 (q, 3,5-OCH3), 47.41 (d, C-8), 43.67 (d, C-7), 33.05 (t, C-7'), 32.70 (d, C-8').

Deoxypicropodophyllotoxin: $C_{22}H_{22}O_7$, colorless grease, 1H-NMR (400MHz, CDCl3): 6.68(s, 1H), 6.47 (s, 1H), 6.36 (s, 1H), 5.93 (d, J=1.4Hz, 2H), 5.92 (d, J=1.4Hz, 2H), 4.18 (dd, J=9.2, 7.3 Hz, 2H), 4.17 (d, J=3.1Hz, 2H), 4.15 (dd, J=9.2, 3.1 Hz, 2H), 3.85 (dd, J=9.5, 3.1 Hz, 1H), 3.82 (s, 4-OMe, 3H), 3.75 (s, 3,5-OMe, 3H), 2.86-2.92 (m,1H), 2.61 (dd, J=15.3, 6.3 Hz, 2H), 2.51 (dd, J=15.3, 5.4 Hz, 2H).

^{13}C NMR (150 MHz, $CDCl_3$): 178.46 (s, C-9), 153.31 (s, C-3,5), 147.96(s, C-3'), 146.44 (s, C-4'), 137.03 (s, C-1), 133.32 (s, C-4), 131.56 (s, C-6'), 121.54 (s, C-1'), 108.78 (d, C-5'), 108.32 (d, C-2'), 106.36 (d, C-6), 101.09 (t, C-10'), 71.16 (t, C-9'), 60.85 (s, 4-OMe), 56.14 (s, 3,5-OMe), 46.47 (d, C-8), 41.07(d, C-7), 38.37 (d, C-8'), 35.28 (t, C-7') (Kuo et al., 1993).

These compounds were elucidated on the basis of spectral data and comparison with published literatures. The structures of compounds 1-9 were showed 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% CO_2 (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 1×10^5 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% CO_2 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 IC_{50} 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

Table 1. Cytotoxicity of Compounds 1, 6 - 8, 9 and Different Polar Fractions of A. Sylvestris Extract.

Sample	IC50 [µ g/ml][a]			
	HepG2	MG-63	B16	HeLa
1	n.a.	n.a.	n.a.	n.a.
6	>150	n.a.	n.a.	>150
7	12.24	14.37	43.25	18.72
8	>150	34.68	65.72	>150
9	70.91	n.a.	n.a.	118
Petroleum ether fraction	36.53	>150	>150	18.25
Chloroform fraction	40.62	>150	>150	45.66
Ethyl acetate fraction	n.a.	n.a.	n.a.	n.a.
n-Butyl alcohol fraction	n.a.	n.a.	n.a.	n.a.
Cisplatinb	3.45	2.85	28.38	16.2

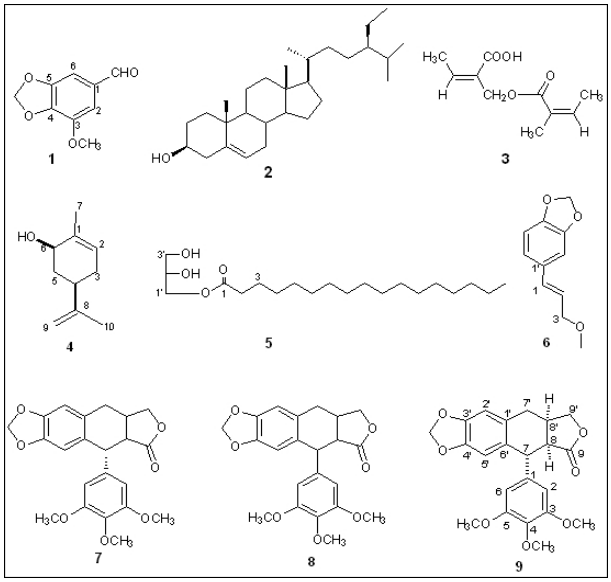

Figure 1. The Structures of Compound 1-9

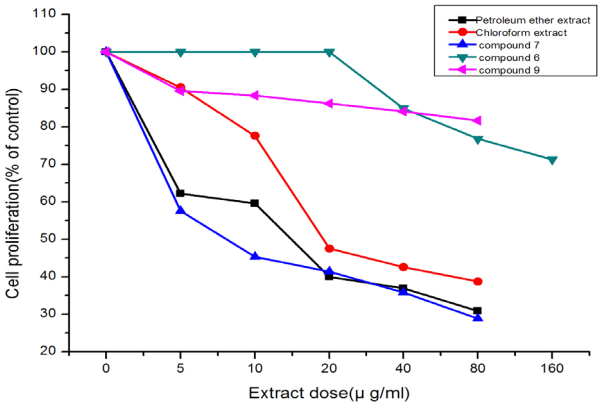

Figure 2. Cell Proliferation of Petroleum Ether Fraction, Chloroform Fraction and Compound 6, 7, 9 with Different Doses Against Hela Cells

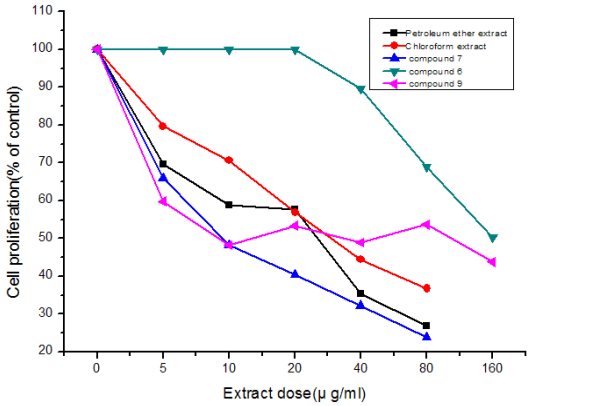

Figure 3. Cell Proliferation of Petroleum Ether Fraction, Chloroform Fraction and Compound 6, 7, 9 with Different Doses Against Hepg2 Cells

growth of the four tumor cells with the IC_{50} 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

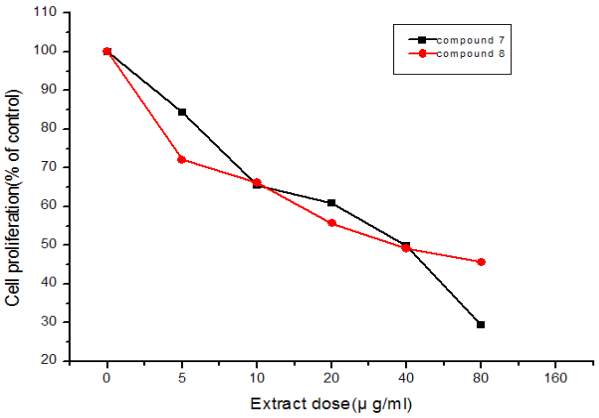

Figure 4. Cell Proliferation of Compound 7, 8 with Different Doses Against B16 Cells

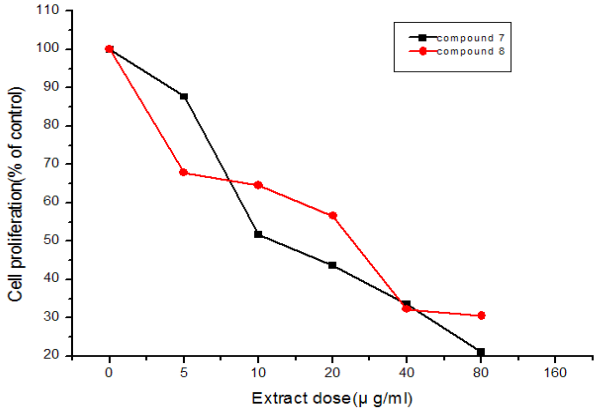

Figure 5. Cell Proliferation of Compound 7, 8 with Different Doses Against MG-63 Cells

with different doses.

Petroleum ether fraction, chloroform fraction, compounds 6, 7, and 9 on the proliferation of HeLa cells in culture (48 h treatment), in which the petroleum ether fraction and compound 7 showed the strongest inhibitory effect (see Figure 2). For HepG2 cells, only the inhibition of compound 9 is not dosage dependent among petroleum ether fraction, chloroform fraction, compounds 6, 7 and 9 (see Figure 3), And control tests were conducted by using different fractions and compounds to detect its inhibition at different concentration for B16 and MG-63 cells. It was found that only compounds 7 and 8 showed remarkable inhibitory activity, and the inhibition is dosage dependent (see Figure 4 and 5). Cell proliferation (% of control) equals the MTT value of extract-treated culture divided by that of control.

Discussion

Among all compounds, only compounds 7, 8 and 9 showed relatively potent antineoplastic activity. Compounds 7, 8 and 9 are all arylnaphthalenes, so it could be supposed that arylnaphthalenes are the key compounds in *A. sylvestris* against the four cancer cells. The series of these compounds are valuable for further investigation. The structures of compounds 7, 8 and 9 were similar, but the antitumor activities of them differ widely. Compare compound 7 with compound 8, it could be supposed that 7-benzene were very important to the antitumor activities and seemed to be active groups. For compound 7 and 9, different conformations of 8, 8'-H showed different antitumor activities. This information could offer some clues for chemical modification and structure transformation of compounds 7, 8 and 9. The fractions of petroleum ether and chloroform small-polar show strong antitumor activities, but ethyl acetate and n-butyl alcohol fractions are mostly inactive. These results illustrate that petroleum ether fraction of *A. sylvestris* is 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_{50} 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_{50} 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.

Acknowledgements

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