Determination of Anti-Cancer Constituents in *Oplopanax horridus* and *Oplopanax elatus*

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Summary: A rapid and reliable RP-HPLC method for the analysis of 3α-hydroxylup -20(29)-ene-23,28-dioic acid(**1**) and 3α-hydroxylup-20(29) -ene-23, 28-dioic acid-3-O-β-D-glucoside (**2**) in leaves of *Oplopanax horridus* and *O. elatus* was established, and their contents changes between species and different cultivated places were compared. The established analysis method presented good results and could be used as a method for the quality control of leaves of *O. horridus* and *O. elatus*. Meanwhile, the inhibitory effect of **1** and **2** on human hepatoma carcinoma cell (HepG-2) were examined and their IC_{50} were determined to be 41.15 μM and 120.06 μM indicating that the anti-cancer activity of **1** was stronger than that of its glycoside. Moreover, the inhibitions of **1** on human colon cancer cell line (HCT116), human lung carcinoma cell line (NCI-H460) and human gastric cancer cell line (MGC803) were further tested, and the IC_{50} were determined to be 21.40 μM, 22.80 μM and 21.26 μM, respectively. While the inhibition of **1** on human pancreatic cancer cell line (PANC-1) was insignificant, indicating **1** possessed selectivity for the anti-cancer activity.

Key words: *Oplopanax horridus*, *Oplopanax elatus*, anti-cancer, content determination

Introduction

Oplopanax horridus (also known as Devil's club), a common deciduous shrub mainly distributed in the Northern Pacific Coast of North America (Alaska) and British Columbia. The inner bark of its roots and stems has been used by indigenous people to treat a variety of ailments including colds, fever, psoriasis and diabetes [1]. Phytochemical research has revealed that this plant had anti-fungal, anti-viral, antibacterial, anti-mycobacterial, anti-proliferative, antioxidant and anti-tuberculosis activities [2-6]. The constituents of volatile oil from the leaves and stem bark of *O. horridus* were studied by our group [7, 8]. As a continual study of nonvolatile substance, 3α-hydroxylup-20(29)-ene-23, 28-dioic acid (**1**) and 3α-hydroxylup-20(29)-en-23,28-dioic acid- 3-O-β-D-glucopyranoside (**2**) together with other two triterpenoids were isolated from *O. horridus* [9, 10]. *O. elatus* (Chinese name Ci-Ren-Shen), mainly distributed in Changbai Mountain district, is a Chinese traditional herb whose roots possess tonic, antitussive, fever eliminating and blood pressure regulating effects [11]. Previously, to exploit the natural resources and find the anti-psoriasis constituents from *O. elatus*, the chemical constituents of which were studied [12]. And the comparison of HPLC fingerprints of *O. horridus* and *O. elatus* was conducted by our group [13-15]. However, this is the first report on the content determination of anti-cancer constituents of *O. horridus* and *O. elatus*.

Results and Discussions

An HPLC method for determining contents of **1** and **2** in leaves of *O. horridus* and *O. elatus* was established. Their structures are shown in Fig. 1. Separation conditions were optimized in order to achieve satisfactory resolution.

The method was validated by linearity, precision, repeatability, stablity and recovery and all were up to the demands for HPLC.

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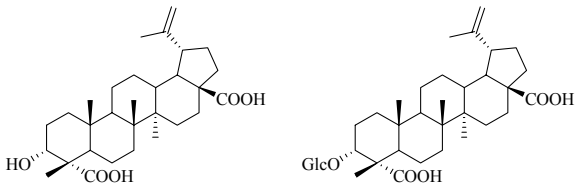
Table-1: Validation of the HPLC method.

compound	Linear range (μg)	Regression equation	Correlation Coefficient, r	Repeatability (RSD %)	Precision (RSD %)	Stability (12 h, RS%)	Recovery (RSD %)
1	0.20~1.00	y=37.84x+123.83	0.9997	0.93	0.79	1.67	100.5 (1.60)
2	4.00~19.98	y=1423.60x+965.81	0.9995	1.30	0.77	0.62	101.0 (1.90)



1 2

Fig 1: Chemical structures of 1 and 2.

The contents of **1** and **2** in leaves of *O. horridus* and *O. elatus* cultivated at different places were evaluated by HPLC. The results are showed in Table-2 and Fig. 2(a)(e).

Table-2: Contents of 1 and 2 in leaves of *O. horridus* and *O. elatus* cultivated at different places (Mean±SD, μg/g)

	O. hurridus (Alaska, America)	*O. elatus* (Benxi, China)	*O. elatus* (Jilin, China)
1 (μg/g)	8103.60±112.90	1791.40±15.10	4811.20±21.40
2 (μg/g)	618.80±6.09	591.50±9.33	900.16±9.12

The results revealed that the contents of **1** and **2** varied greatly between different species and even between the same species collected in different areas. The highest content of **1** was detected in samples collected from American Homer AK, followed by those collected from Yanji, Jilin (China), and samples from Benxi, Liaoning (China) showed the lowest content. But the content of **2** did not show obvious difference. However, it displayed that **1** and **2** were abundant in leaves of both *O.horridus* and *O. elatus*.

Anti-Cancer Activity

The anti-proliferation activities of **1** and **2** on HepG-2 *in vitro* were investigated. The results indicated that both **1** and **2** exhibited inhibitory activity on HepG-2 to some extent, especially **1** with IC_{50} 20 μg/mL, at which content the inhibition of 5-FU was 87%. Their IC_{50} were shown in Table-3.

Table-3: Results of inhibitory activities of 1 and 2 on HepG-2

Substance	IC_{50} (μg/mL)	IC_{50} (μM)
1	20.00	41.15
2	77.97	120.06

In view of the strong inhibitory on HepG-2, **1** was subjected to cytotoxic study on HCT116, NCI-H460, PANC-1 and MGC803 *in vitro*. Table-4 displayed **1** with a cell-selective anti-cancer activity and its strong activity on inhibiting proliferation of HCT116, NCI-H460 and MGC803.

Table-4: IC_{50} (μg/mL) of inhibitory activities of 1 on HCT116, NCI-H460, PANC-1, MGC803

Cell	IC_{50} (μg/mL)	IC_{50} (μM)
HCT116	10.40	21.40
NCI-H460	11.08	22.80
PANC-1	—	—
MGC803	10.33	21.26

In respect of the anti-cancer activity of **1** and **2**, *O. horridus* could be used as substitute for *O. elatus*.

Experimental

Plant Material

Leaves of *O. horridus*, collected from American Homer AK in Aug, 2007, was identified by David C Smith. and Stoney J Wright., Director of Alaska Plant Material Center, Butte, Alaska, USA. Leaves of *O. elatus* were collected from Benxi, (Liaoning province) and Yanji, (Jilin province), China, and identified by Professor Wang Bin, College of Pharmacy, Liaoning University of Traditional Chinese Medicine. The vouchers were deposited in the College of Pharmacy, Liaoning University of Traditional Chinese Medicine (voucher no. Oh-20070914, Oe-20090908 and Oe-20090903 for *O. horridus, O. elatus* from Benxi and Yanji respectively)

Chemicals:

Solvents for HPLC analyses were purchased from Kermel Chemical Co. (Tianjin, China) and filtered through 0.45 μm organic membranes prior to use. Water was purified using Mill-Q-plus filter systems (Millipore, Bedford, MA, USA). **1** and **2** were isolated and identified from leaves of *O. horridu*. All other reagents were analytical or HPLC grade.

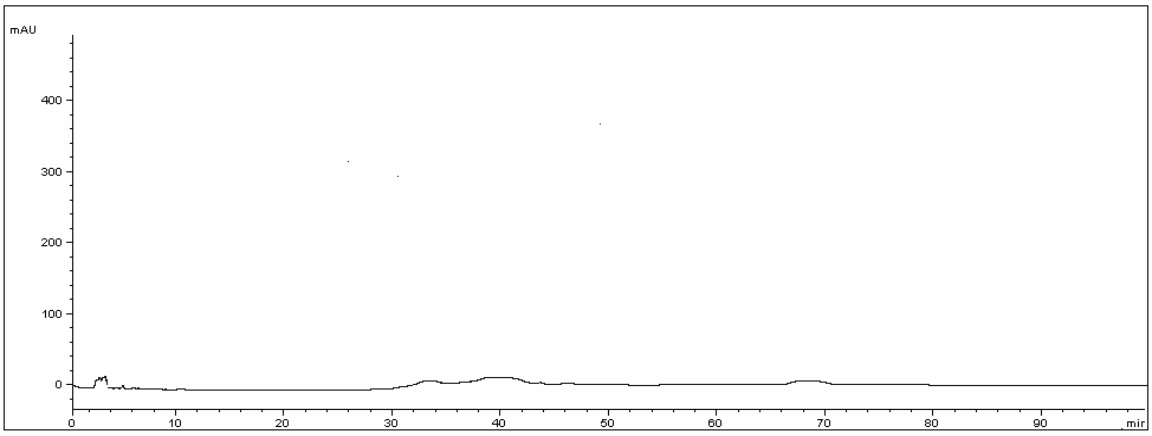


Fig 2(a): HPLC chromatogram of blank control

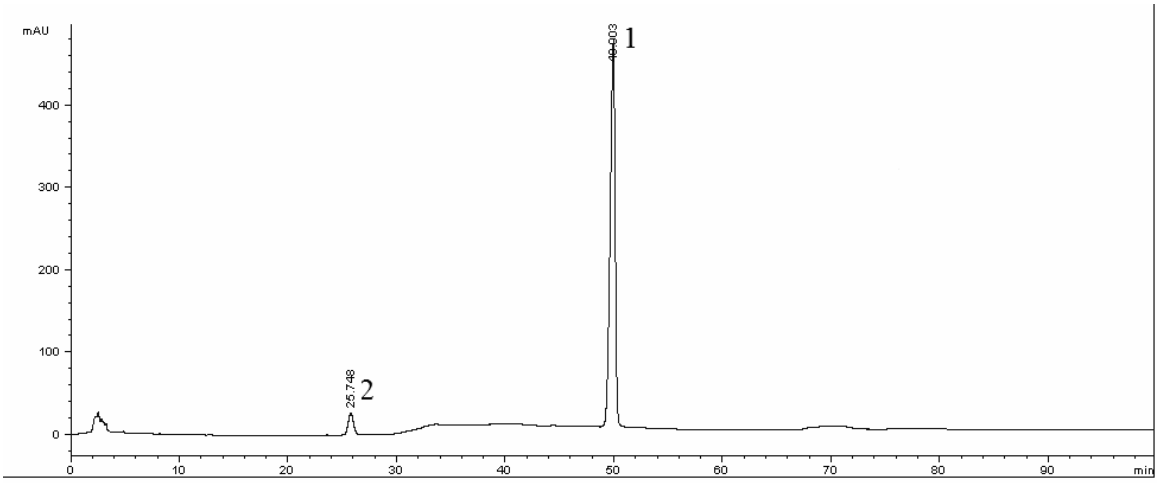


Fig 2(b): HPLC chromatogram of reference substance.

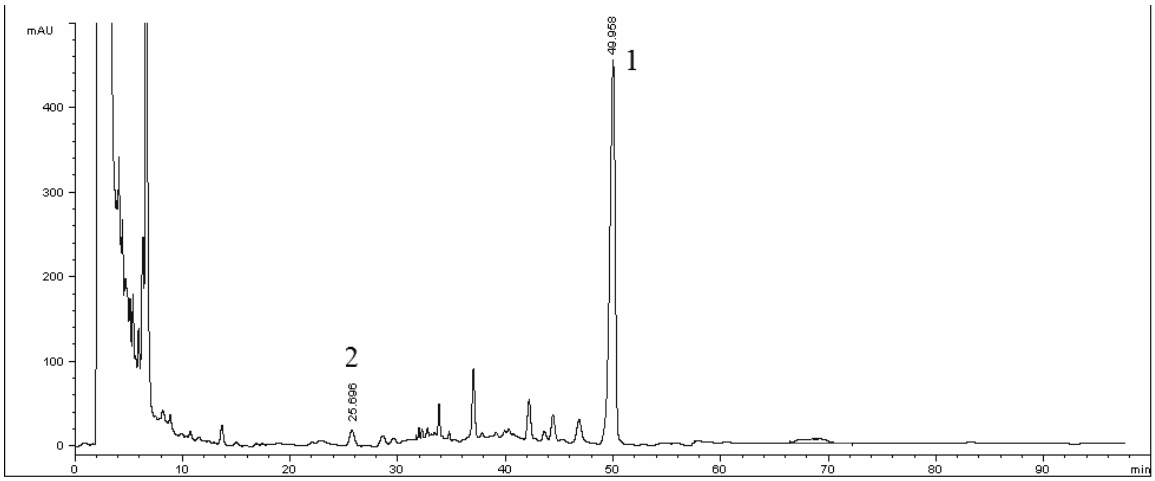


Fig. 2(c): HPLC chromatogram of leaves of *O. horridus* (Alaska, America**).**

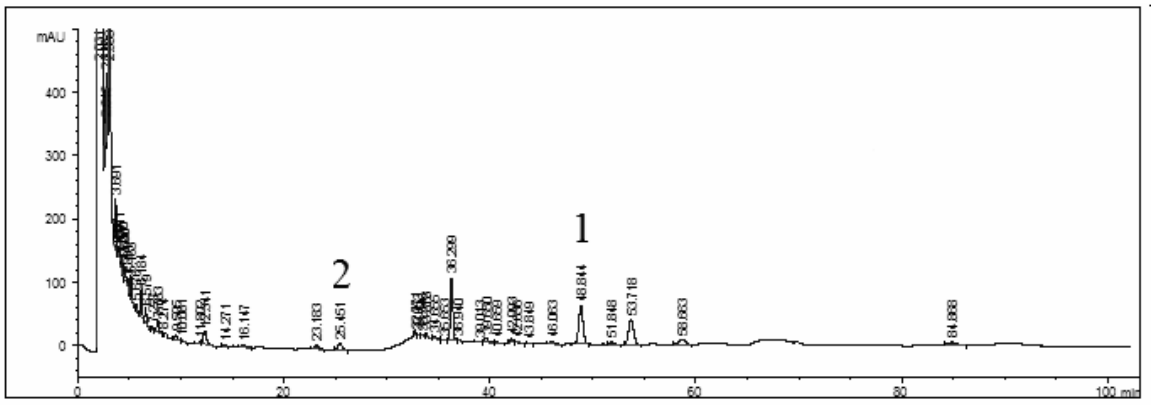


Fig. 2(d): HPLC chromatogram of leaves of *O. elatus* (Benxi, China).

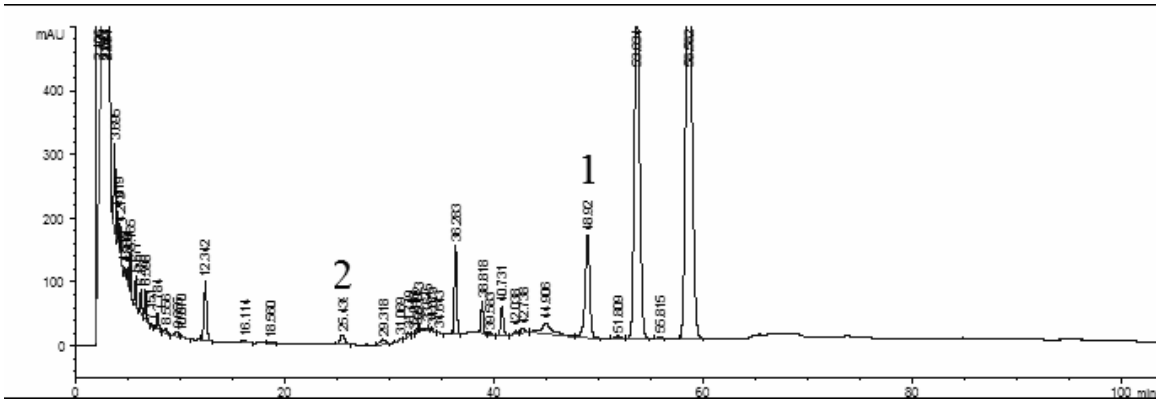


Fig. 2(e): HPLC chromatogram of leaves of *O. elatus* (Jilin, China).

1: 3α-hydroxylup-20(29)-ene-23, 28-dioic acid

2: 3α-hydroxylup-20(29)-ene-23, 28-dioic acid-3-O-β-D-glucoside

Procedure Of Measuring Inhibitory Activity On HepG-2, HCT116, NCI-H460, PANC-1 And MGC803

The cells were cultured in a growth medium (RPMI-1640, pH 7.4), supplemented with 10% fetal bovine serum (FBS). In brief, the cells were seeded into 4 wells of a 96-well microtiter plate (Costar 3599, Corning, NY, USA) at 5 × 104 cells per well with 200 μL RPMI-1640 growth medium and then incubated for 24 hours at 37□ under 5% CO_2 in a humidified atmosphere. Later, the medium was removed while fresh growth medium containing **1** and **2** at 25, 50, 100, 200 μg/mL was added. After 3 days of incubation, the medium was removed while 5 mg/mL MTT (200 μL) reagent was then added. After incubation for 4 hours at 37□, the MTT reagent was removed before adding 100 μL DMSO to each well and gently shaken. The absorbance was then determined by ELISA reader (Tecan Infinite M200) at 570 nm. Control wells received only the media without the tested samples. 5-FU, the conventional anticancer drug, was used as a positive control in this study. The inhibitions of cell growth by **1** and **2** was calculated as a percent anticancer activity using the following formula:

$$\text{Inhibitions (\%)} = 1 - \frac{As}{Ac} \times 100\%$$

As: the absorbance of the sample

Ac: the absorbance of the control

Preparation Of The Standard Solutions For Hplc Analysis

1 and **2** were dissolved in methanol, and the concentrations of stock solutions were 1.998 μg/mL and 1.004 μg/mL respectively.

Preparation Of Samples For 1 And 2 Content Analysis

Crude powder of dried leaves of *O. horridus* and *O. elatus* (1.0 g) were accurately weighed respectively and refluxed with 60% MeOH (50 mL) twice, each time 2 hours. The extract was then filtered,

and the filter liquor was evaporated to dry in vacuum. The residue was degreased with petroleum ether for 3 times, each time with 100 mL solvent and in 20 mins, the petroleum ether extract was abandoned. The residue was dried, then dissolved in methanol, transferred into 10 mL volumetric flask, and fixed to standard volume with methanol. Lastly, sample solution was filtered through 0.45 μm organic membranes prior to use.

*Chromatographic Condition***:**

Determination was achieved by using analytical HPLC in an Agilent 1100 series HPLC system with pump, Agilent model G1314A VWD, an Agilent reverse-phase Phenomenex 5μ C18 100A (4.6×150mm, 5μm particle size) protected by a pre-column purchased from the same company and thermostated at 30□. The column was eluted with water containing 0.5% phosphoric acid (A) and acetonitrile (B) in gradient at the flow rate of 1.0 mL/min. The solvent gradient consisted of 36% B at the beginning to 26 min, 49% at 30 min to 65 min, (run time, 65 min). UV absorption was determined at 203 nm. The injection volume was 10 μL. Peak identification was performed by comparison of retention times with standards.

Acknowledgments

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