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Separation of the potential G-quadruplex ligands from the butanol extract of *Zanthoxylum ailanthoides* Sieb. & Zucc. by countercurrent chromatography and preparative high performance liquid chromatography☆

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ABSTRACT

G-quadruplex DNA structure is considered to be a very attractive target for antitumor drug design due to its unique role in maintaining telomerase activities. Therefore, discovering ligands with high stability of G-quadruplex structure is of great interest. In this paper, pH-zone refining counter current chromatography (CCC) and preparative high performance liquid chromatography (HPLC) were employed for the separation of potent G-quadruplex ligands from the *n*-butanol fraction of the crude extract of *Zanthoxylum ailanthoides,* which is a traditional Chinese medicine recently found to display high inhibitory activity against several human cancer cells. The 75% aqueous ethanol extract of the stem bark of *Z. ailanthoides* and its fractions with petroleum ether, ethyl acetate and *n*-butanol displayed almost the same G-quadruplex stabilization ability. Here, pH-zone refining CCC was used for the separation of the alkaloids from the *n*-butanol fraction by a seldom used solvent system composed of dichloromethane-methanol-water (4:1:2.5) with 10 mM TEA in the organic stationary phase as retainer and 10 mM HCl in the aqueous mobile phase as eluter. Compounds I, II and III were obtained, with purity greater than 95%, in the quantities of 31.2, 94.0, and 26.4 mg respectively from 300 mg of lipophilic fraction within 80 min, which were identified as three tetrahydroprotoberberines isolated for the first time in this plant. In addition, a phenylpropanoid glycoside compound IV (Syringin), an isoquinoline (Magnoflorine, V), and two lignin isomers (+)-lyoniresiol-3α-*O*-β-D-glucopyranoside (VI) and (−)-lyoniresinol −3α-*O*-β-D −glucopyranoside (VII) were isolated by traditional CCC together with preparative HPLC. Compounds IV, V, VI and VII were obtained, with purity greater than 95%, in the quantities of 4.0, 13.2, 6.7, and 6.5 mg respectively from 960 mg of hydrophilic fraction. Among the seven isolated compounds, tetrahydroprotoberberine I, II and III were found to display remarkable stabilization effects on G-quadruplex by increasing G-quadruplex's T_m approximately 10 °C, which may be the most potent G-quadruplex ligands in *Z. ailanthoides*.

1. Introduction

The G-quadruplex (G4) is a four-stranded DNA with stacks of G-quartets formed by four guanines in a planar structure through Hoogsteen base pairing in the presence of K+ or Na+ [1,2]. Small molecules that can facilitate the formation or stabilize the G-quadruplex structures have the potential for the arrest of cancer-cell growth through disruption of telomere maintenance or alteration of oncogene expression levels, and may be potentially valuable as antitumor drugs [3–5]. There is, thus, a considerable interest in discovering G-quadruplex ligands that could ultimately be used against cancer cells [6,7]. Plant-derived agents play an important role in pharmaceutical research, due to their diversity in structure and bioactivity and low toxicity.

In China, the stem bark of *Zanthoxylum ailanthoides* is used as a traditional medicine "Hai-Tong-Pi" for the treatment of eczema, rheumatism and dredging meridians, and also as an antipyretic-analgesic. Our recent study found that the ethanol extract of the stem bark of *Z. ailanthoides* from Zhejiang province displayed high inhibitory activity against A-549 human cancer cells, HCT-8 colon cell and Bel-7402 liver cell. One of our previously published papers reported the isolation and identification of four compounds from the petroleum ether fraction of the crude extract of *Z. ailanthoides*

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Table 1
Precipitation reaction of different fractions with three precipitants.

Sample	Precipitants		
	Wagner's reagent (I_2+ KI)	Modified Dragendorff's reagent ($KBiI_{4+KI}$)	Bertrad's reagent (Silicotungstic acid)
75% ethanol extract	+	+	+
petroleum ether fraction	–	–	–
ethyl acetate fraction	+	+	–
n-butanol fraction	++	++	++
water fraction	+	–	–

++ Remarkable precipitation reaction; + Observed precipitation reaction; – No precipitation reaction.

Table 2
Kacid and Kbase produced by different solvent systems for pH-zone refining CCC*.

Solvent systems (v/v)	Compounds	Kacid (10 mM HCl)	Kbase (10 mM TEA)
CH_2Cl_2-methanol-water			
4:3:2	I	0.17	4.35
	II	0.86	50
	III	1.23	50
4:2:2	I	0.04	2.13
	II	0.78	9.09
	III	0.90	10
4:1.5:2	I	0.03	1.23
	II	0.19	2.78
	III	0.25	3.54
4:1:2.5	I	0.02	3.70
	II	0.08	14.28
	III	0.11	14.28

*Kacid and Kbase were all calculated as $K_{L/U}$, the concentration in lower phase divided by that in upper phase.

and their cytotoxic activities against A-549 cancer cells were also evaluated [8]. Further investigation revealed that ethyl acetate and *n*-butanol fractions displayed higher inhibitory activity than other non-aqueous fractions, while all three fractions displayed almost the same G-quadruplex stabilization ability. The precipitation chromogenic test indicated the existence of alkaloids in the *n*-butanol fraction. Thus, targeting the alkaloids, here we report the isolation and identification of potential active compounds from the *n*-butanol fraction of the crude extract by pH-zone refining counter-current chromatography (CCC) and preparative high performance liquid chromatography (HPLC). The stability of G-quadruplex by each isolated compounds was tested by temperature-dependent circular dichroism (CD).

2. Experimental

2.1. Materials and reagents

The stem bark of *Z. ailanthoides* was purchased from Beijing Tong Ren Tang Pharmacies (origin in Zhejiang Province, China); identity was confirmed at the Beijing University of Chinese Medicine by microscopy. All solvents used for extraction and CCC separation were of analytical grade and purchased from Beijing Chemical Reagent Factory (Beijing, China). Methanol (Tedia, USA) used for HPLC analysis was of chromatographic grade. Water for HPLC was prepared with a Millipore purifier (Millipore, USA) in our laboratory.

G-quadruplex used in this paper was H22 DNA (5′-AGGGTTAGGGTTAGGGTTAGGG-3′) which was purchased from Invitrogen Biotechnology Ltd. (Shanghai, China). The DNA solution was prepared in potassium buffer (PBS-K+: 6.09 mM K_2HPO_4/3.90 mM KH_2PO_4, pH = 7.00) in the concentration of 77.1 μmol/mL and kept overnight at 4 °C before measurement. The sample solution was prepared by dissolving 3 mg sample in 1 mL PBS-K+ buffer making a concentration of 3 mg/mL.

Three different precipitation reagents including Wagner's reagent (I_2 and KI), Modified Dragendorff's reagent (bismuth subnitrate and KI), and Bertrad's reagent (silicotungstic acid) were prepared in our laboratory.

2.2. Apparatus

The CCC separation was performed on a Spectrum HPCCC centrifuge (Dynamic Extraction, UK). The apparatus was equipped with two columns: analytical (0.8 mm i.d. tubing, 22.5 mL) and preparative (1.6 mm i.d., 133.5 mL). The β value varied from 0.64 to 0.81 and 0.52–0.86 for the analytical and preparative columns, respectively. The maximum rotational speed was 1600 rpm. ASH150-1500 constant temperature regulator (Lab Tech, Beijing, China) was used to maintain the temperature at 30 °C. The HPCCC system was equipped with a KNAUER Smartline HPLC system (Berlin, Germany). The effluent was continuously monitored with a UV-2500 detector and a pH detector (Sensorex Model S450C) combined with a flow cell (Sensorex Model FC49 K, CA, USA).

The preparative HPLC system used was an Amen Spot Prep system (Armen, France) equipped with Armen V2 gradient pumps and a variable wavelength UV detector; A Calesil A-120 preparative column (250 × 21.2 mm i.d., 10 μm, Shanghai Sunyear Co. Ltd.) was used in separation.

The HPLC analyses were performed on an Agilent 1260 HPLC system equipped with G1311A QuatPump, G1315 B UV–vis photodiode array detector, G1329 B autosampler with a 20 μL loop, a G1332 degasser and Agilent HPLC workstation. An Agilent ZORBAX SB-C18 column (150 × 4.6 mm i.d., 5 μm) was used for analysis.

NMR analyses were conducted on a Bruker AV 600 MHz spectrometer. Electrospray ionization mass spectrometry (ESI–MS) experiments were performed on a Shimadzu LCMS-2010 (Japan). Circular dichroism (CD) spectra were recorded on a Jasco-815 spectrometer (Japan).

2.3. Preparation of the crude extract and fractions

The stem bark of *Z. ailanthoides* was extracted with 75% aqueous ethanol (1:5, m/v) three times at room temperature. After combination and removal of the ethanol under reduced pressure, the aqueous extract solution was subjected to a series of solvent extractions (1:1, v/v) with petroleum (b.p. 60–90 °C), ethyl acetate and finally *n*-butanol. After concentration and freeze-drying, 42 g of petroleum ether extract, 180 g of the ethyl acetate extract and 90 g of *n*-butanol extract were obtained respectively. They were stored at −4 °C for future use.

Each fraction was subjected to three different precipitation tests for the detection of alkaloids as described below. A small amount of crude extract and its fractions were dissolved in acidified water (pH = 2) separately with each in three duplicates, and then several drops of the above precipitation reagents were added to each solution. Only the fractions with three positive precipitation chromogenic reactions confirmed the existence of alkaloids.