Journal of Chromatography A, 1218 (2011) 6433–6438

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Direct screening of G-quadruplex ligands from *Kalopanax septemlobus* (Thunb.) Koidz extract by high performance liquid chromatography

Ge Bai[a], Xueli Cao[a,*], Hong Zhang[b,c], Junfeng Xiang[b], Hong Ren[a], Li Tan[b], Yalin Tang[b,**]

[a] *Beijing Key Laboratory of Plant Resources Research and Development, Beijing Technology and Business University, Beijing 100048, China*
[b] *Beijing National Laboratory for Molecular Sciences, Center for Molecular Sciences, State Key Laboratory for Structural Chemistry of Unstable and Stable Species, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China*
[c] *Graduate University of the Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 21 May 2011
Received in revised form 5 July 2011
Accepted 11 July 2011
Available online 20 July 2011

Keywords:
G-quadruplex ligands
High-performance liquid chromatography (HPLC)
High-speed countercurrent chromatography (HSCCC)
Kalopanax septemlobus (Thunb.) Koidz
Antitumor activity

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, high-performance liquid chromatography (HPLC) was used for fast screening of G-quadruplex ligands from the crude extract of *Kalopanax septemlobus* (Thunb.) Koidz, a traditional Chinese medicine. Four potent G-quadruplex ligands were firstly selected through HPLC by comparing the peak profiles and absorption intensity of the crude sample before and after interaction with G-quadruplex DNA. Then the target compounds were isolated and purified by high-speed countercurrent chromatography (HSCCC) for further confirmation of their stabilities of G-quadruplex by temperature-dependent circular dichroism (CD). Four compounds were isolated and identified as 2,4-dihydroxybenzoic acid (I), chlorogenic acid (II), caffeic acid (III) and 5-feruloylquinic acid (IV) each by MS and NMR. Finally, compound I, II, III were each proved to be potent G-quadruplex ligands by decreasing the peak intensity in HPLC chromatogram after complexation with G-quadruplex, which stabilize G-quadruplex by $7 \pm 2\,^{\circ}C$, $10 \pm 2\,^{\circ}C$, and $3 \pm 2\,^{\circ}C$ respectively, based on CD analyses. However, compound IV showed no G-quadruplex stability. The decrease of peak absorption intensity in HPLC chromatogram is the most important signal to find G-quadruplex ligands. This provides a very promising strategy for fast screening G-quadruplex ligands from natural plant extracts.

1. Introduction

Recently, cancer researches have pointed out that unlimited proliferative ability of cancer cells depends on telomere maintenance, and telomeric G-quadruplex ligands could interfere with telomere replication by blocking the elongation step catalyzed by telomerase and act as antitumor agents [1]. Thus, the development of fast screening methods of G-quadruplex ligands is of great significance.

Several methods have been reported for screening potential G-quadruplex ligands, including competition dialysis [2], equilibrium dialysis combined with electrospray ionization mass spectrometry [3], and the telomeric repeat amplification protocol [4], which mainly focused on screening molecule(s) with known structure. However recent reports by our group [5–7] have shown that nuclear magnetic resonance (NMR) can be used for screening potential G-quadruplex ligands directly from the crude plant extracts, making the fast screening of G-quadruplex ligands possible. Meanwhile, this led to our further development of a simpler fast screening method using HPLC, which has been employed to study the interaction of TBA (thrombin-binding aptamer) G-quadruplex with ligand TmPyP4 based on the change of retention time and absorption intensity before and after interaction [8].

In this study, HPLC was used to screen possible G-quadruplex ligands directly from the crude extract of *Kalopanax septemlobus* (Thunb.) Koidz (*K. septemlobus*), a traditional Chinese medicine, which has been proved to show anti-tumor activity and stabilize G-quadruplex [9]. Four possible G-quadruplex ligands were firstly selected through HPLC analysis by comparing the peak profiles and absorption intensity of the crude sample before and after interaction with G-quadruplex. Then the target compounds were isolated by high-speed countercurrent chromatography (HSCCC) and characterized by electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). Temperature-dependent circular dichroism (CD) was employed in evaluating the stability of G-quadruplex by each compound and crude sample.

* Corresponding author. Tel.: +86 10 68984898; fax: +86 10 68984898.
** Corresponding author. Tel.: +86 10 62658184; fax: +86 10 62569564.
E-mail addresses: caoxl@th.btbu.edu.cn (X. Cao), tangyl@iccas.ac.cn (Y. Tang).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.07.028

2. Experimental

2.1. Materials and reagents

The stem barks of *K. septemlobus* were purchased from Beijing Tong Ren Tang Pharmacies (originated in Zhejiang Province, China) and identified in Institute of Chinese Medicine, China Academy of Chinese Medicine Sciences.

G-quadruplex used in this paper was c-kit sequence (5′-AGGGAGGGCGTGGGAGGAGGG-3′), which was purchased from Tsingke Biotechnology Ltd. (Beijing, China) and purified by C18 column. The solution was prepared in potassium buffer (PBS-K+: 17.20 mM K_2HPO_4/KH_2PO_4, pH = 7.00) and kept overnight at 4 °C before measurement.

All solvents used for extraction and HSCCC separation were of analytical grade and purchased from Beijing Chemical Reagent Factory (Beijing, China). Acetonitrile (Merck, Germany) and Ammonium acetate (NH_4AC) (Tianjin Guangfu, China) used for HPLC were of chromatographic grade, and water was from ultrapure water system in our lab.

2.2. Apparatus

The HPLC analyses were performed on an Agilent 1100 HPLC system equipped with a quaternary pump, an auto-sampler, and a diode array detector (DAD). An Agilent ZORBAX SB-C_{18} column (150 mm × 4.6 mm i.d., 5 μm) was used for analysis.

A TBE-300A HSCCC system (Shanghai Tauto Biochemical Technology, China), was employed for separation of potential G-quadruplex ligands. The coiled column was manufactured by winding 1.6 mm i.d. PTFE tubing around three spool-shaped column holders with a total capacity of 280 mL.

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

2.3. Sample preparation

The stem barks of *K. septemlobus* (7 kg) were extracted with 75% aqueous ethanol (1:5, w/v) by maceration for three times at room temperature, 72 h each time. After combination and filtration, the total extract was evaporated under reduced pressure to yield a non-alcoholic aqueous solution, which was then subjected to successive extraction (1:1, v/v) with petroleum ether (b.p.: 60–90 °C), ethyl acetate and *n*-butanol three times for each solvent. After concentration and dryness, 50 g of petroleum ether extract, 192 g of the ethyl acetate extract and 100 g of n-butanol extract were obtained respectively. They were stored at −4 °C for future use.

2.4. Temperature-dependent CD experiments

Since G-quadruplex structures have distinctive CD spectra, temperature dependent changes in CD have often been used to determine G-quadruplex stability and their interaction with potential ligands. All CD spectra were baseline-corrected for signal contributions from the buffer. The temperature for each measurement was increased from 20 to 90 °C with a heating rate of 2 °C/min. The final concentration of c-kit was 1 μM. The melting temperature (T_m) values of c-kit in the absence and presence of potential ligand were determined by monitoring the CD signal at 262 nm. The changes of melting temperature (ΔT_m) enable a comparison of the relative stabilities of c-kit before and after interaction with ligands [10,11].

Table 1
Stability of G-quadruplex by different solvent fractions.

Sample (50 μg/mL)	Changes of CD melting temperature (ΔT_m, °C)
Ethanol crude extract	6 ± 2
Petroleum ether fraction	−5 ± 2
Ethyl acetate fraction	5 ± 2
n-Butanol fraction	−5 ± 2

The crude ethanol extract, different solvent fractions, and following purified target compounds were all tested by CD experiments for their stabilities of G-quadruplex.

2.5. HPLC screening of G-quadruplex ligands from ethyl acetate extracts

Based on the results of CD experiments, the ethyl acetate fraction was targeted to screen possible G-quadruplex ligands by HPLC, The G-quadruplex solution (c-kit sequence), ethyl acetate fraction of *K. septemlobus*, and their mixture were each analyzed by HPLC. The mobile phase was composed of 0.1 M NH_4Ac aqueous solution (A) and acetonitrile (B) and eluted in gradient as follows: 0–5 min isocratic 95% A, 5–15 min gradient to 90% A, at a flow rate of 0.5 mL/min at 25 °C. All chromatograms were recorded at 262 nm by a DAD. An aliquot of 10 μL solution was injected, where the concentration of G-quadruplex and extract sample was 20 μM and 1.5 mg/mL respectively. The concentration of each component in their mixture sample solution was the same as in their parent sample solutions.

2.6. HSCCC separation of the target compounds

After screening possible G-quadruplex ligands by HPLC, the extract of *K. septemlobus* was subjected to HSCCC separation for the target compounds. In each separation, a suitable solvent system was prepared and thoroughly equilibrated in a separatory funnel and the two phases was separated shortly before use. The coiled column was first entirely filled with the upper stationary phase and then the lower mobile phase was pumped into the column at a flow-rate of 2 mL/min while the column was rotated at 850 rpm. After the mobile phase front emerged and hydrodynamic equilibrium was established, the sample solution was injected through the sample loop. The separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 254 nm. Each peak fraction was manually collected according to the chromatogram and further evaporated under reduced pressure. The residuals were redissolved in methanol for subsequent HPLC analysis.

The chemical structures of the isolated compounds were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR.

3. Results and discussion

3.1. Stability of G-quadruplex by different solvent fractions

The crude extract of *K. septemlobus* and different solvent fractions were firstly subjected to CD test. As illustrated in Table 1, the ethanol crude extract can stabilize G-quadruplex by 6 °C. Among the three solvent fractions, only the ethyl acetate fraction showed the stability of G-quadruplex by 5 °C, and which were investigated further accordingly.

3.2. Four possible G-quadruplex ligands recognized by HPLC-DAD

The ethyl acetate extract of *K. septemlobus,* G-quadruplex solution (c-kit sequence) and their mixture were analyzed separately

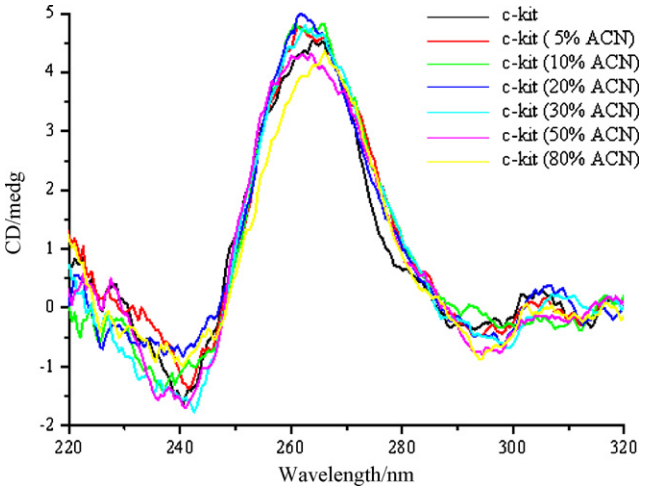

Fig. 1. CD spectra of c-kit in PBS-K+ buffer solution in the absence and presence of different concentration of acetonitrile (monitored at 262 nm).

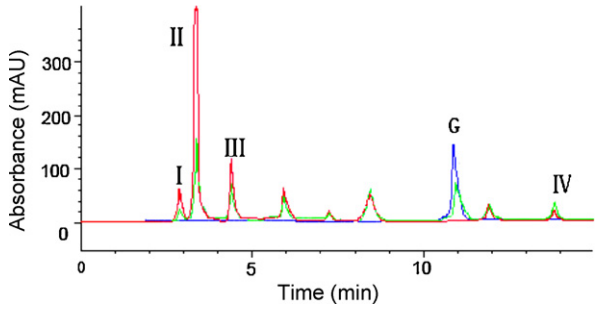

Fig. 2. HPLC chromatograms for c-kit sequence (blue line), extract of *K. septemlobus* (red line), and their mixture (green line). Conditions: column: Zorbax SB-C18 (150 mm × 4.6 mm i.d., 5 μm); mobile phase: 0.1 M NH_4AC aqueous solution (A), acetonitrile (B), 0–5 min isocratic 95% A, 5–15 min directly switch to 90% A; flow-rate: 0.5 mL/min; detection: 262 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

by HPLC under the same condition. The stability of G-quadruplex in HPLC mobile phase was confirmed by CD (Fig. 1), which indicated G-quadruplex could maintain the stable structure under HPLC condition. As shown in Fig. 2, the chromatogram of the *K. septemlobus* extract showed eight well resolved peaks (red line), and as expected a single peak was observed in that of G-quadruplex (blue line). When the complex mixture of *K. septemlobus* extract (1.5 mg/mL) with 20 μM c-kit sequence was analyzed, nine peaks were detected in its chromatogram (green line), which correspond to the eight peaks in *K. septemlobus* extract and G-quadruplex peak without any drift in retention time, meanwhile, no additional peaks were observed. However, after complexation, the intensity of peaks I, II and III decreased significantly while that of peak IV increased slightly. For G-quadruplex peak, its intensity decreased in all cases. This result has been repeated for several times, and the systematic deviation can be excluded. This decrease in peak intensity may suggest the interaction of compound I, II, and III with G-quadruplex.

These four compounds were then separated by HSCCC for structure elucidation and further confirmation of their interaction with G-quadruplex.

3.3. HSCCC separation of the target compounds I, II, III, and IV

An optimized two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:5:0.5:5, v/v) was employed for the first separation of the crude extract. The upper phase was used as the stationary phase and the lower phase as the mobile phase during 0–180 min, then the mobile phase was changed to the lower phase of *n*-hexane–ethyl acetate–methanol–water (1:5:2:4, v/v) during 180–230 min (Fig. 3A). Further purification was carried out for each compound using different solvent systems: compound I with ethyl acetate–methanol–water (5:1:4, v/v), compounds II and IV with *n*-butanol–ethyl acetate–water (1:4:5, v/v), and compound III with *n*-butanol–ethyl acetate–methanol–water (2:4:1:4, v/v), where lower phase was used as mobile phase in all cases (Fig. 3B–E). Finally, 4.8 mg of compound I, 91.2 mg of compound II, 12.5 mg of compound III, and 8.8 mg of compound IV were obtained from 1 g of crude ethyl acetate extract of *K. septemlobus* at the purities of 98.4%, 98.6%, 98.9% and 97.7%, respectively, as determined by HPLC analyses (Fig. 4 with their UV spectra).

3.4. Identification the four isolated compounds

The structures of four isolated compounds were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR. The results were summarized in

Table 2
ESI-MS, ^{13}C NMR and ^{1}H NMR data of compound I, II, III, and IV (I and III in MeOD; II and IV in d_6-acetone).

	Compound I		Compound II		Compound III		Compound IV	
m/z [M-H]$^-$	152.9		353.1		179.0		367.1	
NMR (ppm)	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ
C1		122.47		75.26		126.5		75.36
C2		144.65	1.96–2.27 *m*	36.95	7.04 *d J* = 1.8 Hz	113.7	1.96–2.28 *m*	36.95
C3	7.44 *d J* = 1.8 Hz	116.34	5.32 *m*	70.40		145.5	5.39 *m*	70.41
C4		150.12	3.79 *m*	72.54		148.0	3.78 *m*	72.54
C5	7.42 *dd J* = 7.8, 1.8 Hz	114.35	4.22 *m*	70.65	6.78 *d J* = 8.4 Hz	115.1	4.24 *m*	70.80
C6	6.78 *d J* = 7.8 Hz	121.75	1.96–2.27 *m*	37.24	6.94 *dd J* = 1.8, 8.4 Hz	121.41	1.96–2.28 *m*	37.24
C7		168.80		174.09	7.53 *d J* = 15.7 Hz	145.3		174.18
C8					6.2 *d J* = 16.2 Hz	114.5		
C9						169.9		
C1′				126.79				126.60
C2′			7.15 *d J* = 2 Hz	115.03			7.28 *d J* = 4.2 Hz	115.14
C3′				144.84				144.88
C4′				147.74				147.74
C5′			6.86 *d J* = 8 Hz	115.45			6.87 *d J* = 7.8 Hz	115.19
C6′			7.04 *dd J* = 8, 2 Hz	121.62			7.14 *dd J* = 7.8, 4.2 Hz	123.09
C7′			7.57 *d J* = 16 Hz	145.31			7.60 *d J* = 15.6 Hz	149.15
C8′			6.26 *d J* = 16 Hz	114.28			6.38 *d J* = 15.6 Hz	110.40
C9′				166.21				166.31
OCH_3							3.93 *s*	55.47

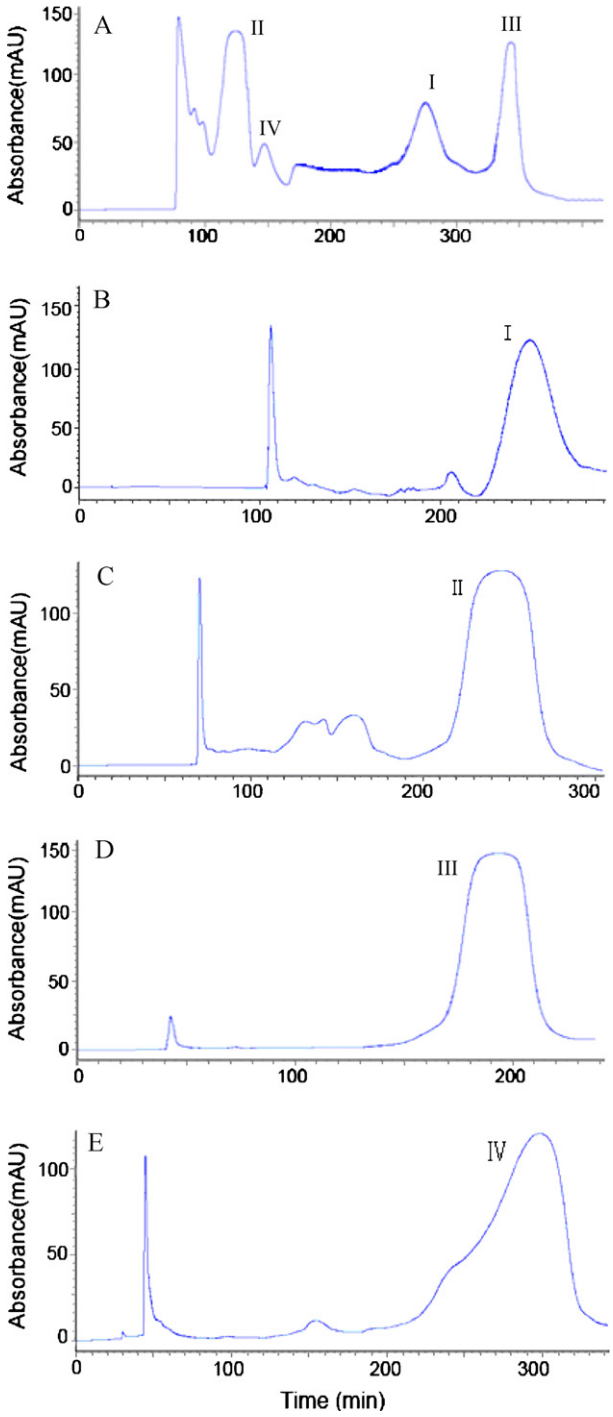

Fig. 3. HSCCC separation chromatograms. (A) Separation of the ethyl acetate extract of *K. septemlobus*. Solvent system: *n*-hexane-ethyl acetate–methanol–water (1:5:0.5:5, v/v); mobile phase: lower phase of *n*-hexane-ethyl acetate–methanol–water (1:5:0.5:5, v/v) during 0–180 min, then changed to the lower phase of *n*-hexane-ethyl acetate–methanol–water (1:5:2:4, v/v) gradiently during 180–230 min; sample weight: 500 mg. (B) Purification of peak I. Solvent system: ethyl acetate–methanol–water (5:1:4, v/v); sample weight: 20 mg. (C) Purification of peak II. Solvent system: *n*-butanol–ethyl acetate–water (1:4:5, v/v); sample weight: 190 mg. (D) Purification of peak III. Solvent system: *n*-butanol–ethyl acetate–methanol–water (2:4:1:4, v/v); sample weight: 30 mg. (E) Purification of peak IV. Solvent system: *n*-butanol–ethyl acetate–water (1:4:5, v/v); sample weight: 42 mg. Other conditions: mobile phase: lower phase; flow rate: 2 mL/min; rotation speed: 850 rpm; detection: 254 nm.

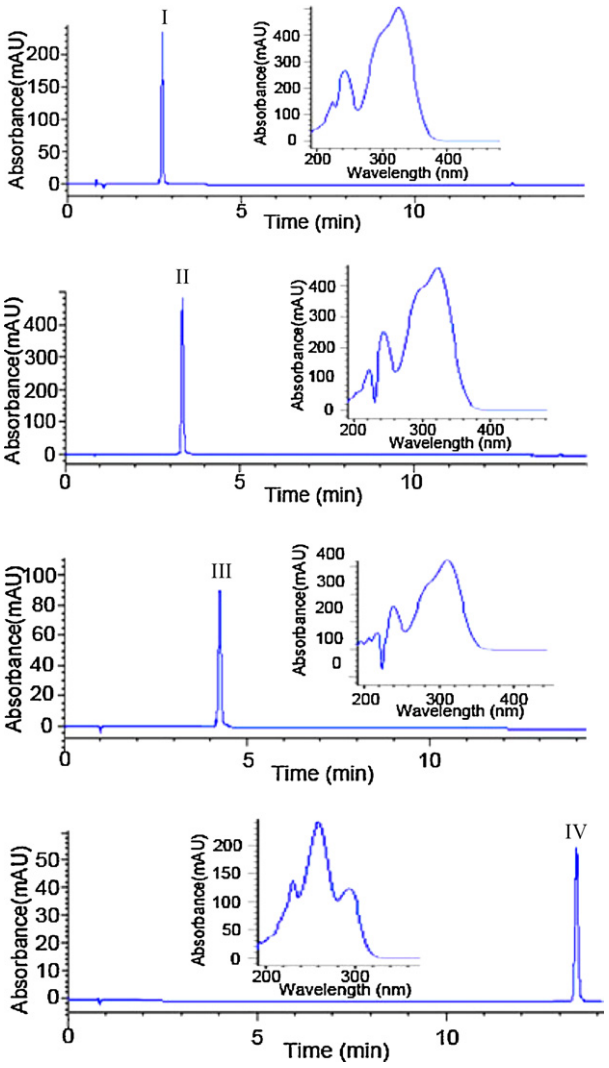

Fig. 4. HPLC analysis of four target peaks from the ethyl acetate extract of *K. septemlobus* by HSCCC. Conditions are the same as those in Fig. 1.

Table 2. According to the data in literatures [12–15], these compounds are identified as 2,4-dihydroxybenzoic acid (I), chlorogenic acid (II), caffeic acid (III), and 5-feruloylquinic acid (IV), and their chemical structures were presented in Fig. 5.

3.5. HPLC confirmation of individual G-quadruplex ligands

In order to confirm the interactions between each isolated compound and G-quadruplex, four HSCCC purified compounds was each mixed with G-quadruplex solution and their complex mixture were analyzed by HPLC. The results in Fig. 6(A–D) displayed the same phenomena as in Fig. 2. This indicates that the interaction of G-quadruplex with each compound in crude extract is reproduced with their purified compounds.

3.6. Stability of G-quadruplex by each compound

In order to confirm that these target compounds were G-quadruplex ligands, the temperature-dependent CD measurements have been carried out. Fig. 7 shows the melting profiles monitored at 262 nm for c-kit G-quadruplex in the presence and absence of four purified compounds. C-kit alone displayed a single transitional curve with a melting temperature of $59 \pm 1\,^{\circ}C$. The addition of compound I, II and III to the c-kit solution resulted

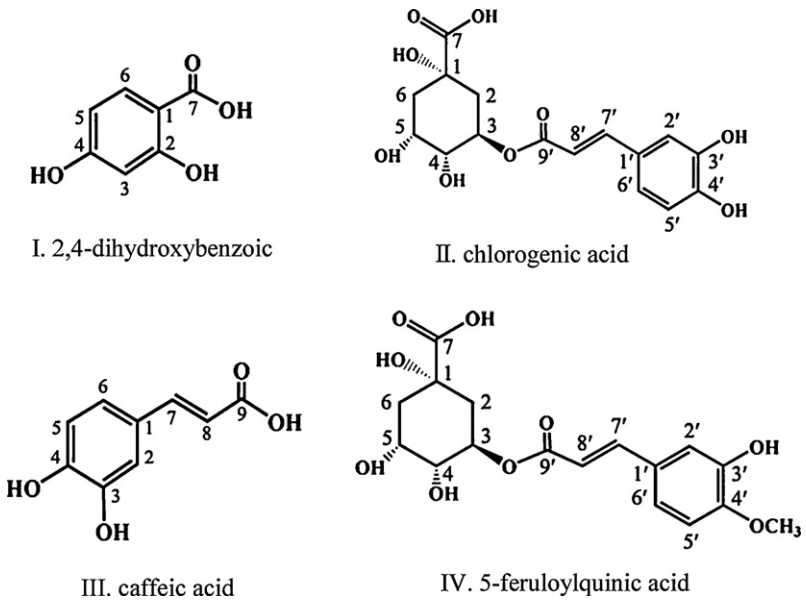

Fig. 5. Structure of four compounds isolated from the extract of *K. septemlobus*.

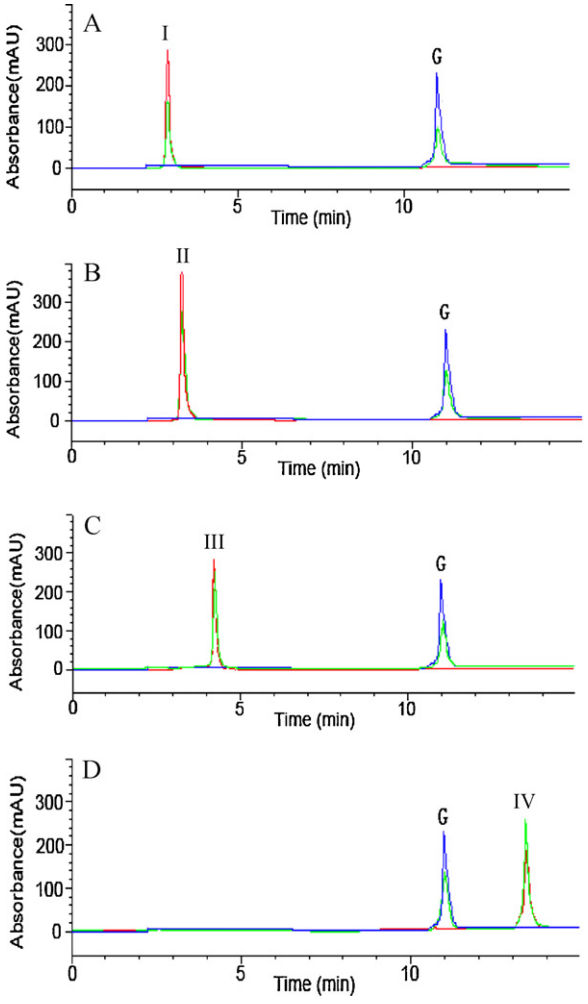

Fig. 6. HPLC chromatograms for c-kit sequence (blue line), four individual purified compounds (red line), and their complex mixture (green line). Conditions are the same as those in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in the change of the transition curve with melting temperature 66 ± 2 °C (I), 70 ± 2 °C (II) and 62 ± 2 °C (III) (Fig. 7A). Clearly, the presence of compounds I, II and III could increase G-quadruplex's T_m by 7 ± 2 °C, 10 ± 2 °C, and 3 ± 2 °C, respectively. These results indicate that compounds I, II and III can significantly increase the stability of G-quadruplex by acting as its ligands, and they could be potent anti-tumor compounds. However, the CD melting curve of G-quadruplex in the presence of compound IV was different from those of other three compounds, and no G-quadruplex's T_m can be

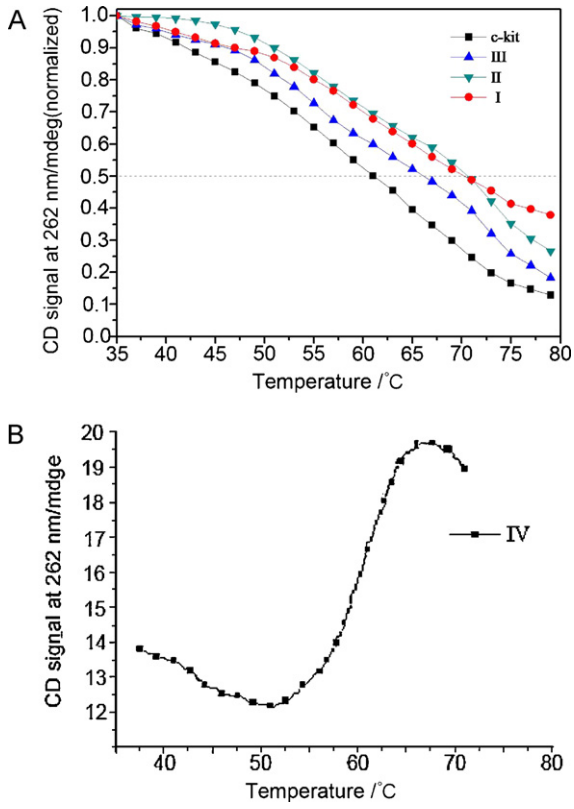

Fig. 7. The CD melting curves of 1 μM G-quadruplex in the absence and presence of compound I, II and III (A) and compound IV (B) (monitored at 262 nm).

obtained (Fig. 7B). The reason for this phenomenon is not clear at this stage.

Overall results of our studies indicate that the decrease of peak absorption intensity in HPLC chromatogram is the most important signal to find G-quadruplex ligands.

4. Conclusion

In this paper, HPLC was used for fast screening of potent G-quadruplex ligands directly from the extract of *K. septemlobus*. Peak absorbance intensities of three compounds (I, II and III) in the crude extract were significantly decreased when the sample was mixed with G-quadruplex, indicating that they could be potent G-quadruplex ligands. This was further confirmed by CD test, while compound IV was proved to have no affinity to G-quadruplex. The above four compounds were purified by HSCCC and identified as 2,4-dihydroxybenzoic acid (I), chlorogenic acid (II), caffeic acid (III) and 5-feruloylquinic acid (IV), respectively, by MS and NMR. Although some of them are already commercially available, the separation of these compounds is not the focus of our studies. The purpose of the present studies is to demonstrate that HPLC can be efficiently used for fast screening of potent G-quadruplex ligands directly from crude plant extract. The present method can be applied for finding potential antitumor drugs from huge resources of medicinal natural products.

Acknowledgments

We acknowledge financial support of this work by the National Natural Science Foundation of China (Nos. 20877005 and 81072576) and Beijing Natural Science Foundation (No. 2102016).

References

[1] D. Sun, B. Thompson, B.E. Cathers, M. Salazar, S.M. Kerwin, J.O. Trent, T.C. Jenkins, S. Neidle, L.H. Hurley, J. Med. Chem. 40 (1997) 2113.
[2] P. Ragazzon, J.B. Chaires, Methods 43 (2007) 313.
[3] F. Rosu, E. De Pauw, L. Guittat, P. Alberti, L. Lacroix, P. Mailliet, J.F. Riou, J.L. Mergny, Biochemistry 42 (2003) 10361.
[4] S. Ladame, A.M. Whitney, S. Balasubramanian, Angew. Chem. 117 (2005) 5882.
[5] Q.J. Zhou, L. Li, J.F. Xiang, Y.L. Tang, H. Zhang, S. Yang, Q. Li, Q.F. Yang, G.Z. Xu, Angew. Chem. Int. Ed. 47 (2008) 5590.
[6] Q. Li, J.F. Xiang, X.D. Li, L.R. Chen, X.J. Xu, Y.L. Tang, Q.J. Zhou, L. Li, H. Zhang, H.X. Sun, A.J. Guan, Q.F. Yang, S. Yang, G.Z. Xu, Biochimie 91 (2009) 811.
[7] Q.J. Zhou, L. Li, J.F. Xiang, H.X. Sun, Y.L. Tang, Biochimie 91 (2009) 304.
[8] M. Toro, R. Gargallo, R. Eritja, J. Jaumot, Anal. Biochem. 379 (2008) 8.
[9] H. Zhang, J.F. Xiang, L. Tan, W. Dai, G. Bai, Y. Liu, X.L. Cao, Y.L. Tang, Acta Pharmaceutica Sinica 44 (2009) 1359.
[10] P.A. Rachwal, K.R. Fox, Methods 43 (2007) 291.
[11] B. Fu, J. Huang, L. Ren, X. Weng, Y. Zhou, Y. Du, X. Wu, X. Zhou, G. Yang, Chem. Commun. 31 (2007) 3264.
[12] B. Qin, R.H. Lu, H.Q. Wang, M. Wang, Nat. Prod. Res. Dev. 12 (2002) 41.
[13] H.A. Jung, J.C. Park, H.Y. Chung, J. Kim, J.S. Choi, Arch. Pharm. Res. 22 (1999) 213.
[14] K. Yuan, A. Jia, J.X. Zhu, Chin. J. Anal. Chem. 36 (2008) 47.
[15] J.H. Grabber, P.F. Schatz, H. Kim, F. Lu, J. Ralph, BMC Plant Biol. 10 (2010) 114.