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Short Communication

Tracking antiangiogenic components from *Glycyrrhiza uralensis* Fisch. based on zebrafish assays using high-speed countercurrent chromatography

Natural products are some of the most important sources of lead compounds for drug discovery. The advanced isolation technique of lead compounds of natural origin using therapeutically relevant bioassays is capable of enhancing work efficiency from complex multiconstituent extracts. In the present study, a bioassay-guided isolation strategy combined with bioactivity screening was used to identify novel angiogenesis inhibitors from licorice (*Glycyrrhiza uralensis* Fisch.) based on the zebrafish model and rapid preparative separation by high-speed countercurrent chromatography. Zebrafish embryos at 24 h postfertilization were chosen as the angiogenesis inhibition model for bioactivity screening. A solvent system (*n*-hexane–ethyl acetate–methanol–water) with different ratios was optimized and applied in the high-speed countercurrent chromatography separation of two fractions, Fr5 and Fr6, from the ethyl acetate extract of licorice. Blood circulation and vascular outgrowth in intersegmental vessels were found to be simultaneously inhibited by isoliquiritigenin and isolicoflavonol in a dose-dependent manner. Thus, these two compounds were identified and considered as active inhibitors against angiogenesis. These experimental results indicate that zebrafish bioassays combined with high-speed countercurrent chromatography may provide an alternative pathway for the rapid isolation of bioactive natural products.

Keywords: *Glycyrrhiza uralensis* Fisch. / High-speed countercurrent chromatography (HSCCC) / Isolicoflavonol / Isoliquiritigenin / Licorice / Zebrafish
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1 Introduction

Natural products are a significant reservoir of unexplored diverse chemicals for early-stage drug discovery. The target bioassay-guided isolation of natural angiogenesis inhibitors has received considerable attention worldwide. However, the establishment of a low-cost ideal bioassay-guided isolation with a rapid preparation method for a large amount of compounds and a reliable biomedical screening model remains a great challenge.

The conventional bioassay-guided isolation of plant extract complexes is time consuming, laborious, and expensive. Compared with other separation methods such as silica gel, preparative high-performance liquid chromatography (HPLC), and thin layer chromatography (TLC), high-speed countercurrent chromatography (HSCCC) is an optimal choice. The method has the advantages of no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity, low cost, and proven successful application in the isolation and purification of many bioactive compounds from natural products [1, 2].

Thus far, a report on using HSCCC for the isolation and purification of angiogenic inhibitors from natural sources has not been seen. Thus, in the current paper, a novel systematic separation method was established based on the zebrafish model and HSCCC for the first time. Zebrafish blood vessels form by angiogenic sprouting and appear to use the same pathways as mammals for blood vessel growth [3]. Zebrafish has become an ideal *in vivo* model for the systematic identification of bioactive natural products with antiangiogenic potential [4]. Thus, it could enable the high-throughput, low-volume screening of natural products.

In the course of our preliminary studies on large-scale drug screening for antiangiogenic components from traditional Chinese medicines and herbs, the ethyl acetate-soluble fraction (100 μg/mL) of the root of licorice (*Glycyrrhiza uralensis* Fisch.) was found to have the potential of inhibiting angiogenesis in zebrafish embryos.

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Abbreviations: **HSCCC**, high-speed countercurrent chromatography; **ISF**, isolicoflavonol; **ISL**, isoliquiritigenin; **ISV**, intersegmental vessel

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Therefore, some novel antiangiogenic components may exist in licorice.

2 Experimental

2.1 Apparatus

The preparative HSCCC instrument used was a TBE-300 HSCCC (Shanghai Tauto Biotech Co. Ltd., Shanghai, China) with three polytetrafluoroethene preparative coils (diameter of tube = 2.6 mm, total volume = 300 mL). The revolution speed of the apparatus can be regulated using a speed controller within the range of 0–1000 rpm. The HSCCC system was also equipped with a TBP-50A constant flow pump (Shanghai Tauto Biotech Co., Ltd.) and a model 8823B-UV monitor (Bingdayingchuang Sci & Tech, Beijing, China). The data were collected using a model N2000 chromatography workstation (Zhejiang University, Hangzhou, China). The HPLC apparatus used was an L-2000 HPLC system (Hitachi, Japan) equipped with an L-2130 pump, L-2400 UV detector, a model 7725i injection valve with a 20 μL loop, and a D-2000 ChemStation (Hitachi).

2.2 Reagents and materials

All organic solvents used for the HSCCC were analytical grade and purchased from Yongda Chemicals, Tianjin, China. The methanol used for HPLC analysis was chromatographic grade and purchased from Tedia, USA. PTK787 (*N*-(4-chlorophenyl)-4-(pyridin-4-ylmethyl)phthalazin-1-amine succinate) was purchased from Sigma, USA. The licorice roots were purchased from a local drug store and identified as *G. uralensis* Fisch. by Professor Song Guang-Yun (Biology Institute, SDAS).

2.3 Sample preparation of licorice extract

Dry licorice powder (1 kg) was refluxed twice with 70% methanol. After concentration under reduced pressure, the 70% methanol extract was sequentially extracted with petroleum, acetic acetate, and *n*-butanol. The acetic acetate fraction was evaporated in a vacuum yielding a brown crude solid (30 g), which was filtered through a polyamide resin column (60–80 mesh, 5 cm × 100 cm). The filtrate was gradient eluted with distilled water as well as 30, 50, 70, and 95% ethanol. All eluates were concentrated and combined based on TLC analysis. Seven fractions, namely, Fr1 (1.82 g), Fr2 (2.44 g), Fr3 (5.44 g), Fr4 (4.16 g), Fr5 (6.25 g), Fr6 (3.47 g), and Fr7 (2.36 g), were obtained and stored in a refrigerator.

2.4 HSCCC separation procedure

The composition of the two-phase solvent system was determined according to the partition coefficient *K* and peak resolution of the target compounds. *K* was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase by HPLC [5]. Preparative HSCCC was performed as follows. The coiled column was entirely filled with the upper (stationary) phase, and the apparatus was rotated at 850 rpm. In the meantime, the lower (mobile) phase was pumped into the column in the head-to-tail elution model. The mobile phase was 2.0 mL/min for the separation of Fr5 and 2.5 mL/min for Fr6. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 5 mL of the sample solution was injected into the head of the column through the injection valve. The effluent of the column was monitored using a UV detector at 254 nm. Peak fractions manually collected according to the elution protocol were evaporated under reduced pressure and stored at −20°C before HPLC and nuclear magnetic resonance (NMR) analyses.

2.5 HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis of the crude extracts and HSCCC peak fractions were performed using a reverse phase Apollo C_{18} (5 μm, 250 mm × 4.6 mm id; Alltech) column. The mobile phase consisting of methanol (A) and 0.5% acetic acid in water (B) was programmed a gradient elution of 65–95% A from 0 to 40 min for analyzing Fr5, and 60–95% A from 0 to 50 min for analyzing Fr6. The flow rate was 1.0 mL/min and the UV detection wavelength was 254 nm.

The HSCCC peak fractions were identified by ^{1}H- and ^{13}C-NMR experiments using a Varian NOVA-600 (Varian Corporation, Palo Alto, CA, USA) NMR spectrometer with tetramethylsilane as the internal standard.

2.6 Zebrafish assays

The AB line zebrafish used in the current study was obtained from Harvard Medical School (Boston, MA, USA). Zebrafish husbandry, embryo collection, as well as embryo and larvae maintenance were performed as described in an earlier report [6, 7].

The test samples, including crude extracts, eluted fractions, and purified compounds, were dissolved in dimethyl sulfoxide (DMSO) stock solution. Zebrafish assays were performed in 24-well microtiter plates using 8–10 embryos per well in 2 mL of system water. Embryos were exposed to sample solutions at 24 h postfertilization (hpf) prior to the initiation of intersegmental vessel (ISV) outgrowth, and scored for relative vascular outgrowth at 48 hpf. PTK787 was used as a positive control. The relative vascular outgrowth of control group was scored 100%. The ISV outgrowth was indicated by endogenous alkaline phosphatase (EAP) staining as previously described [8]. Images were taken using a SZX-16 Stereo Microscope equipped with DP2-BSW software (Olympus, Japan).

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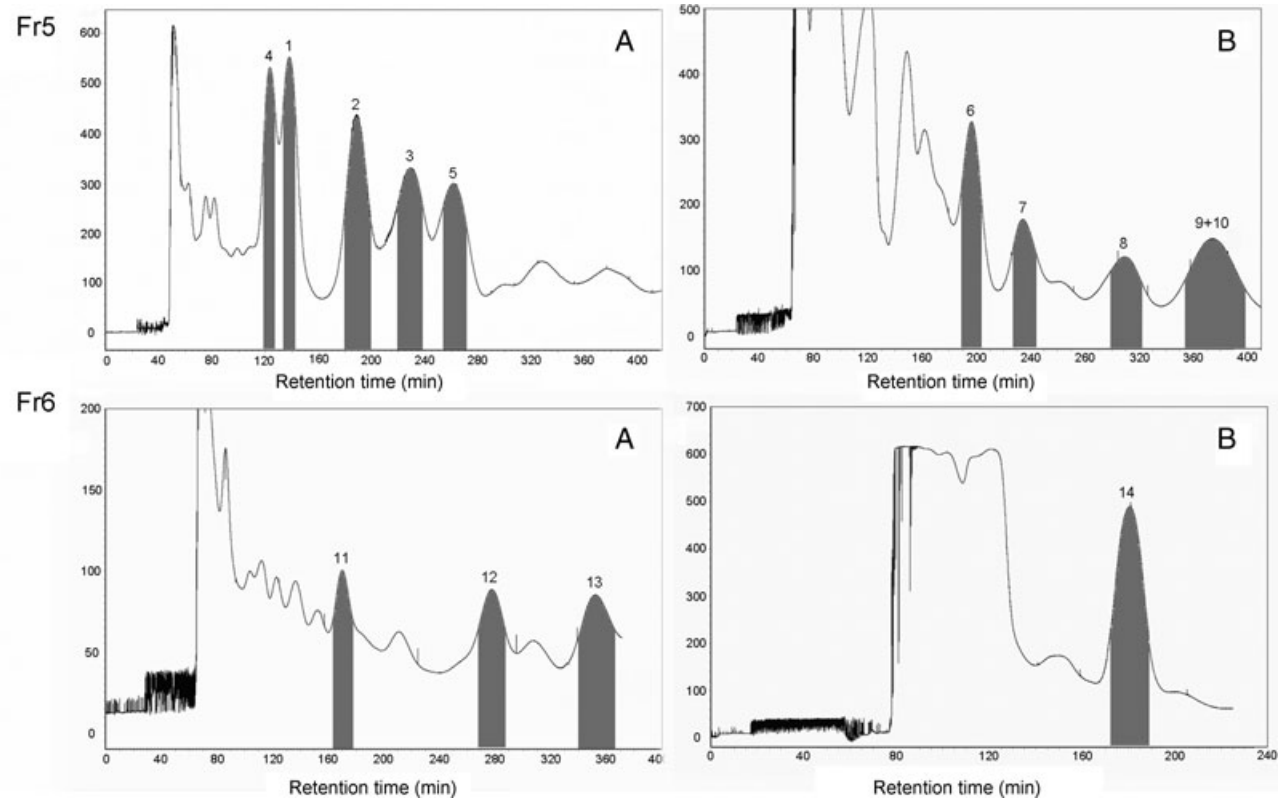

Figure 1. Chromatogram of two-step preparative HSCCC separation of Fr5 and Fr6. Fr5 (A), step-1; Fr5 (B), step-2. Solvent system: Fr5 (A), *n*-hexane–ethyl acetate–methanol–water (5:6:5:5, v/v/v/v) with retention percentage of the stationary phase of 57%; Fr5 (B), *n*-hexane–ethyl acetate–methanol–water (6:5:6:5, v/v/v/v) with retention percentage of the stationary phase of 62%. Fr6 (A), step-1; Fr6 (B), step-2. Solvent system: Fr6 (A), *n*-hexane–ethyl acetate–methanol–water (5: 5:5:6, v/v/v/v) with retention percentage of the stationary phase of 61%; Fr6 (B), *n*-hexane–ethyl acetate–methanol–water (7:5:7:5, v/v/v/v) with retention percentage of the stationary phase of 70%. Sample amount: Fr5, 70 mg; Fr6, 80 mg. Flow rate: Fr5, 2.0 mL/min; Fr6, 2.5 mL/min. Stationary phase: upper phase; mobile phase: lower phase; resolution speed: 850 rpm. UV detection wavelength: 254 nm. Peak numbers are corresponding to the peak numbers in HPLC chromatogram.

3 Results and discussion

3.1 Screening of angiogenesis inhibitors in different solvent fractions of licorice

Due to its potential to inhibit the angiogenesis of zebrafish embryos, the acetic acetate fraction of licorice was further separated by a polyamide resin column, yielding seven fractions Fr1–Fr7. Zebrafish assays were then performed to screen these fractions for antiangiogenic bioactivity. The ISVs of embryos treated with Fr5 and Fr6 were found to be significantly decreased compared with the vehicle control (0.5% DMSO). The relative ISV outgrowth of Fr5 and Fr6 were 36 and 24%, respectively, compared with the vehicle control ($P < 0.01$). Therefore, Fr5 and Fr6 were considered valuable angiogenesis inhibitors and further isolated by HSCCC.

3.2 Optimization of HSCCC conditions

There were ten main peaks in Fr5 (see Fig. 3A) and four main peaks in Fr6 (see Fig. 3B) in the HPLC chromatogram. Thus, these 14 compounds were selected as target compounds. A suitable two-phase solvent system is critical for successful isolation and separation by HSCCC. The two-phase solvent system should satisfy the following requirements: (i) short settling time (<30 s), (ii) sufficient sample solubility, (iii) suitable *K* values (usually between 0.5 and 2), and (iv) satisfactory retention of the stationary phase [9].

Two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water with different ratios (5:7:5:5, 5:6:5:5, 5:5:5:5, 6:5:6:5, and 7:5:7:5) were investigated for Fr5 separation. The basic two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water (5:5:5:5, v/v/v/v) yielded the following *K* values for the ten target compounds, respectively: 0.99, 1.94, 2.37, 1.28, 2.79, 5.67, 6.47, 3.44, 6.20, and 11.7. Hence, two different solvent systems were applied for the separation of compounds 1–5 and 6–10. When ethyl acetate was added to this solvent system, compounds 1–5 were more evenly distributed in the lower phase, and peak resolution improved. When the *n*-hexane–ethyl acetate–methanol–water (5:7:5:5, v/v/v/v) solvent system was used, the separation time was too long. Hence, the *n*-hexane–ethyl acetate–methanol–water (5:6:5:5, v/v/v/v) system had the best effect for separating compounds 1–5. When *n*-hexane and methanol were increased, the *K* values of compounds 6–10 decreased and the separation time decreased.

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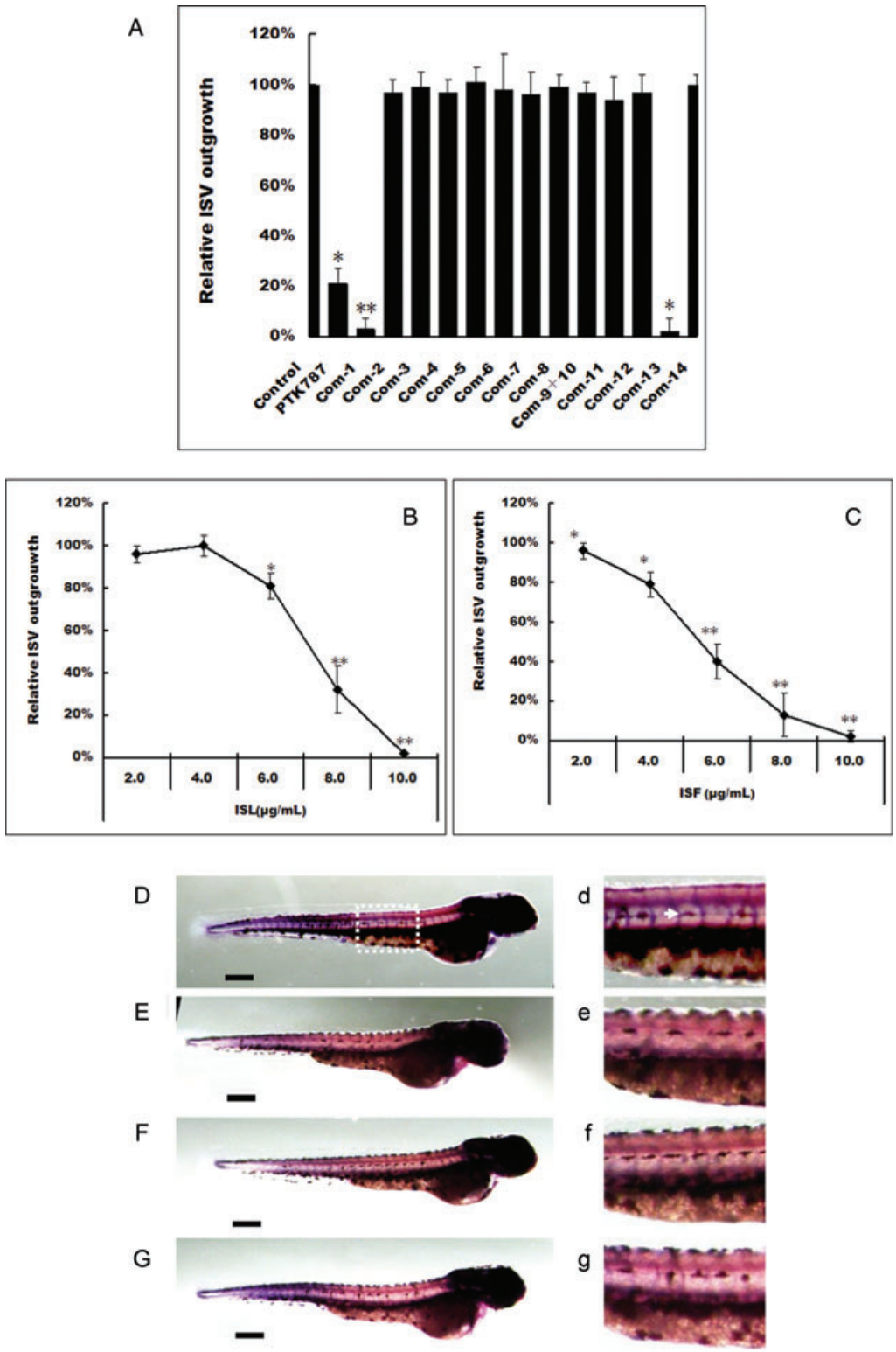

Figure 2. Antiangiogenic activities of the compounds obtained by HSCCC. (A) The concentration of test compounds was 10 μg/mL ($^{*}P < 0.05$ *versus* control; $^{**}P < 0.01$ *versus* control. (B and C) Dose-dependent effect of ISL and ISF on the growth of blood vessel of zebrafish embryos ($^{*}P < 0.05$ *versus* control; $^{**}P < 0.01$ *versus* control. (D–G) View of EAP-stained zebrafish embryos, The magnified views of broken white line parts of D–G are shown in d–g, respectively. (D, d) Vehicle-treated control at 48 hpf; (E, e) zebrafish larva treated with 2.0 μg/mL PTK787; (F, f) zebrafish larva treated with 10.0 μg/mL ISL; (G, g) zebrafish larva treated with 10.0 μg/mL ISF. Black scale indicates 200 μm. White arrow indicates ISVs.

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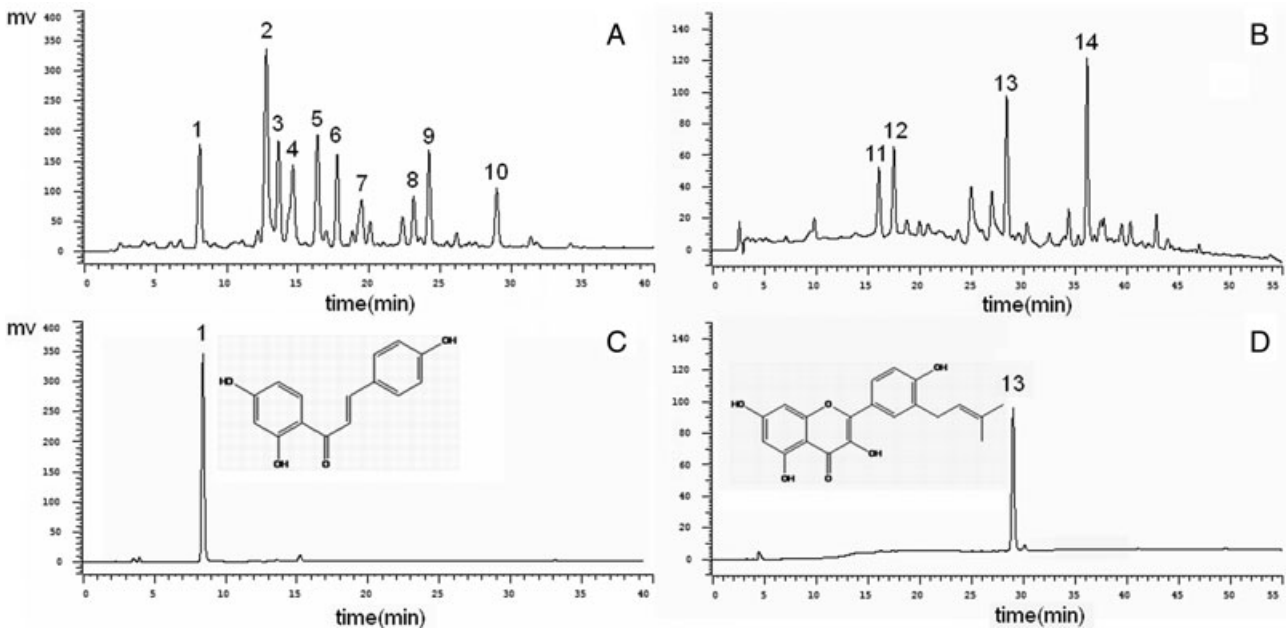

Figure 3. HPLC chromatogram of the Fr5, Fr6, and HSCCC peak fractions. (A) Fr5; (B) Fr6. (C) Compound 1; (D) compound 13. HPLC conditions: stationary phase, Apollo C_{18} (5 μm 250 mm × 4.6 mm id); mobile phase, A (methonal)-B (0.5% acetic acid in water); gradient program: (A, C) 0–40 min: 65% A -95% A; (B, D) 0–50 min, 60% A -95% A. Flow rate: 1.0 mL/min. UV detection wavelength: 254 nm.

However, the peak resolution worsened. Therefore, the *n*-hexane–ethyl acetate–methanol–water (6:5:6:5, v/v/v/v) system was selected to separate compounds 6–10 of Fr5 (Fig. 1, Fr5B).

Using the same optimized separation conditions, the *n*-hexane–ethyl acetate–methanol–water (5:5:5:6, v/v/v/v) system was successfully used to separate compounds 11–13; the *n*-hexane–ethyl acetate–methanol–water (7:5:7:5, v/v/v/v) system was used for the separation of compound 14 (Fig. 1, Fr6 (A) and (B)).

Under the optimized conditions, each compound obtained by HSCCC was collected and evaporated under reduced pressure. The following quantities were obtained in a single HSCCC separation: 0.8 mg of compound 4, 2.1 mg of compound 1, 3.3 mg of compound 2, 0.6 mg of compound 3, 1.2 mg of compound 5, 1.7 mg of compound 6, 1.4 mg of compound 7, 0.7 mg of compound 8, 2.8 mg of compound (9 + 10), 1.8 mg of compound 11, 2.6 mg of compound 12, 3.7 mg of compound 13, and 3.2 mg of compound 14.

3.3 Antiangiogenic activities of the obtained compounds

All obtained compounds were tested for antiangiogenic activity using zebrafish assays at concentrations of up to 10 μg/mL (Fig. 2A). The ISVs of embryos treated with compounds 1 and 13 were found to be significantly decreased than those of the control (0.5% DMSO) in a dose-dependent manner (Fig. 2B and C). When the dose of both compounds was 10 μg/mL, the ISV formation of embryos was almost entirely inhibited. From the photos of EAP staining, both compounds showed obvious inhibition effect on ISV formation at 10 μg/mL (Fig. 2D–G). The other tested compounds did not show any obvious antiangiogenic bioactivity.

3.4 Determination of peak purity and identification of the obtained compounds

The respective purities of compounds 1–8, 9 + 10, and 11–14, as determined by HPLC using the area normalization method (Fig. 3C and D) were as follows: 93.2, 93.7, 94.1, 90.8, 91.3, 89.6, 90.1, 87.5, 85.3, 93.2, 92.2, 93.7, and 98.4%.

The chemical structures of compounds 1–14 were determined based on their ^{1}H- and ^{13}C-NMR data. Due to the NMR data complexity of the different compounds, five known compounds were confirmed by comparing with literature. These compounds were isoliquiritigenin (ISL) (compound 1) [10], glyurallin A (compound 5), licocoumarone (compound 11), isolicoflavonol (ISF) (compound 13) [11], and glyurallin B (compound 14). More time is needed for the identification of the chemical structures of the other compounds. The NMR data for ISL and ISF, two angiogenesis inhibitors, were as follows:

Compound 1, yellow powder, ^{1}H-NMR (600 MHz, DMSO) δ: 8.16 (1H, d, J = 9.0 Hz, H-6′), 7.78 (2H, H-2,6), 7.75 (1H, H-α), 7.72 (1H, H-β), 6.82 (2H, d, J = 8.4 Hz, H-3,5), 6.40 (1H, dd, J = 9.0, 2.4 Hz, H-5′), 6.27 (1H, d, J = 2.4 Hz, H-3′). ^{13}C-NMR (600 MHz, DMSO) δ: 191.29 (C=O), 165.74

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(C-4′), 165.27 (C-2′), 160.21 (C-4), 144.06 (C-β), 132.71 (C-6′), 131.10 (C-2,6), 125.63 (C-1), 117.30 (C-α), 115.74 (C-3,5), 112.73 (C-1′), 108.16 (C-5′), 102.50 (C-3′).

Compound 13, yellow powder, ^{1}H-NMR (600 MHz, DMSO) δ: 7.89 (1H, dd, J = 1.8, 10.2 Hz, H-6′), 6.94 (1H, d, J = 8.4 Hz, H-5′), 6.41 (1H, d, J = 1.2 Hz, H-8), 6.18 (1H, d, J = 1.2 Hz, H-6), 5.30 (1H, t, J = 7.8 Hz, H-2″), 3.28 (2H, d, J = 7.2 Hz, H-1″), 1.72 (3H, s, H-4″), 1.71 (3H, s, H-5″). ^{13}C-NMR (600 MHz, DMSO) δ: 176.29 (C-4), 163.84 (C-7), 163.84 (C-8a), 157.43 (C-4′), 156.65 (C-5), 148.26 (C-2), 139.14 (C-3), 132.32 (C-3′), 129.57 (C-3″), 128.14 (C-6′), 127.48 (C-2′), 122.95 (C-1′), 122.13 (C-2″), 115.38 (C-5′), 98.78 (C-6), 93.93 (C-8), 28.60 (C-1″), 26.02 (C-5″), 18.21 (C-4″).

4 Concluding remarks

Using the zebrafish bioassay-guided natural product discovery and isolation strategy described in the present paper, 14 compounds were successfully discovered in licorice. Among these compounds, two were identified as antiangiogenesis compounds, namely, ISL and ISF. ISL has been documented as an angiogenic inhibitor by cell and chick embryo chorioallantoic membrane assays [12, 13], as well as the zebrafish assay in the present paper. However, no antiangiogenic activity of ISF has been reported to date. In the current work, we demonstrated the use of zebrafish bioassay-guided fractionation by HSCCC and further established zebrafish as an *in vivo* platform for the discovery of bioactive natural products. Compared with other chromatography techniques, HSCCC is more convenient for target isolation and the scale-up of the isolation process of natural candidate compounds.

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The authors have declared no conflict of interest.

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