Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1063–1069

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

An online coupled cell membrane chromatography with LC/MS method for screening compounds from *Aconitum carmichaeli Debx.* acting on VEGFR-2

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ARTICLE INFO

Article history:
Received 3 April 2010
Received in revised form 5 July 2010
Accepted 12 July 2010
Available online 16 July 2010

Keywords:
Cell membrane chromatography
High performance liquid chromatography mass spectrometry
Online analysis
VEGFR
Aconitum carmichaeli Debx

ABSTRACT

An online analytical method coupling high expression vascular endothelial growth factor receptor (VEGFR) cell membrane chromatography (VEGFR-CMC) with high performance liquid chromatography mass spectrometry (LC/MS) for screening and identification of active component from traditional Chinese herb *Aconitum carmichaeli Debx.* acting on VEGFR-2 was established. Through a 10-port column switcher, factions separated by VEGFR-CMC column (first dimension) were transferred and were adsorbed on an enrichment column. Then, these fractions were sent into LC/MS system (second dimension) immediately and directly for separation and preliminary identification, respectively. Sunitinib malate (SN) was used as positive control, while nifedipine (NF), dexamethasone acetate (DX), methoxyamine hydrochloride (MT) and atenolol (AT) as negative controls. The specification of this VEGFR-CMC-online-LC/MS method was validated by competitive displacement test. As a result, mesaconitine (MSC), aconitine (AC), and hypaconitine (HPC) were identified as the active constituents acting on VEGFR-2. The in vitro inhibition activity of starting extract of *Aconitum carmichaeli Debx.*, MSC, AC, and HPC on HEK293/VEGFR cell viability by MTT test, separately. The in vitro inhibition activity of MSC, AC, and HPC on vascular endothelial growth factor (VEGF) secretion of HEK293/VEGFR cell was tested by VEGF-ELISA assay. The screening results given by the system offered additional exemplification supporting this online coupling method and gave new evidence to the development of anti-tumor drug from natural products.

1. Introduction

Vascular endothelial growth factor receptor (VEGFR) is a subgroup of receptors belonging to receptor tyrosine kinase (RTK) family. VEGFR mainly expressed on vascular endothelial cell (EC) and bone marrow-derived cell [1]. VEGFR-2, also named KDR or flk-1, is a subtype of VEGFR, and it is known as the primary mediator in angiogenesis by promoting the mitoschisis, permeability and proliferation of ECs in pre-existing vessels under physiological and some pathological conditions, such as the growth of tumor [2,3]. As a key receptor contributing to the progression of tumor, VEGFR-2 has been currently an important target for anti-tumor therapeutics research [4–6]. Some small-molecule agents against the neovascularization by blocking the signal transduction pathway of VEGFR-2 have shown potent clinical effects, such as sunitinib and sorafinib. However, new drug against the same target with lower toxicity and better therapeutic effect is still in demand. In order to improve the maneuver of ligands–receptor interaction research and existed drug screening strategy aiming at specific receptor, some biological affinity chromatography with better specification on methods have been developed. Wainer et al. established the immobilized artificial membrane liquid chromatography for determination of ligand binding affinities, which well improved the methodology of affinity chromatography [7,8]. In 1996, He and his colleagues introduced cell membrane chromatography (CMC), which has been verified to be an effective chromatographic technique for the study of active components acting on specific receptor [9–13]. Separation technology coupled with structure analysis function has been developed for decades and it well met the further requests of instant identification for the fractions being focus on after separation. In our laboratory, artificial cultures highly expressing VEGFR-2 on their cell surface named HEK293/VEGFR cell lines were constructed in HEK293 engineering cell line. The CMC established based on HEK293/VEGFR cell line was considered to be a model that could underline the performance of specific interaction between VEGFR-2 and ligands. Online combined CMC-LC/MS method, firstly reported by Wang et al., was used for studying of drugs acting on specific receptor, and could preliminarily identify the drugs with mass-spectrometer [14]. In this study, we used a modified CMC-online-LC/MS system, which loaded the cell membrane of HEK293/VEGFR as stationary phase in a VEGFR-CMC model, to screen and identify compound from *Aconitum carmichaeli Debx.* acting on VEGFR-2, hoping to give some evidence to the anti-tumor drug discovery from natural products.

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doi:10.1016/j.jpba.2010.07.011

2. Experimental

2.1. Chemicals and materials

Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). The VEGFR-2 high expression cell lines were constructed with engineering cell line named HEK293 in our laboratory. Sunitinib malate was from Nanjing Ange Pharmaceutical Co., Ltd. (Nanjing, China). Nifedipine, dexamethasone, atenolol, mesaconitine, aconitine, and hypaconitine were supplied by National Institute for the Pharmaceutical and Biological Products of China (Beijing, China). Methoxyamine hydrochloride was supplied by Sigma–Aldrich Inc. (St. Louis, USA). DMEM was from Invitrogen Corporation (Grand island, USA). HPLC grade methanol and acetonitrile were from SK Chemicals Co., Inc. (Ulsan, Korea). Aqueous ammonia (25–28%) was from Sanpu Chemicals Co., Inc. (Xi'an, China). Analytical pure grade diethyl ether were from Fuyu Chemicals Co., Inc. (Tianjin, China). Ammonium acetate was from Fuchen chemical reagent Co., Inc. (Tianjin, China). *Aconitum carmichaeli Debx*. purchased from TCM store in Xi'an was authenticated by the Department of Pharmacognosy, Xi'an Jiaotong University (Xi'an, China). A reference sample has been deposited at the Specimen Laboratory, Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University (Xi'an, China).

2.2. Instruments

The first dimensional VEGFR-CMC column (10.0 mm × 2.0 mm I.D., 5 μm) was packed by the RPL-10ZD column loading machine from Dalian Replete Science and Technology Co., Ltd. (Dalian, China). A model E36-230, 10-port 2-position switch valve made by Valco Instruments Co., Inc., (Houston, USA) was used as the column switcher and one Shimadzu VP-ODS guard column (10.0 mm × 1 mm I.D., 5 μm) made by Shimadzu Corporation (Kyoto, Japan) for HPLC was used as enrichment column. The LC/MS system was also from Shimadzu Corporation, including three LC-20AD pumps, a DGU-20A_3 degasser, a SIL-20A autosampler, a CTO-20A column oven, a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm I.D., 5 μm) as the second dimensional column, a SPD-20A UV/VIS detector, a SPD-M20A diode array detector, an LCMS-2010EV mass spectrometer, and an LCMS Solution workstation software. Model 680 microplate reader was from Bio-Rad Laboratories, Inc. (CA, USA).

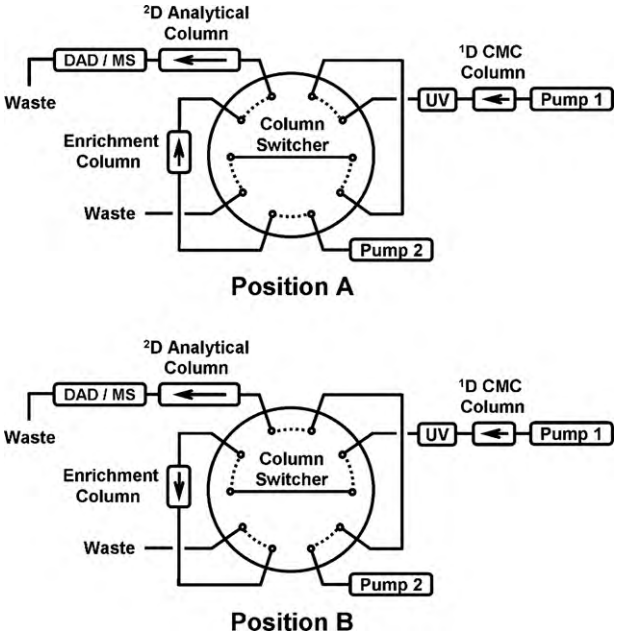

Fig. 1. The schematic diagram of VEGFR-CMC-online-LC/MS system. UV: ultraviolet detector; DAD/MS: diode array detector/mass spectrometer detector; ^{1}D CMC column: the VEGFR-CMC column playing as the first dimensional column. ^{2}D analytical column: the VP-ODS column playing as the second dimensional column.

2.3. Sample preparation

SN, NF, DX, MT, AT, MSC, AC and HPC were resolved in methanol to prepare 1 mg/mL standard stock solution, respectively. All of them were stored at −20 °C in dark.

The starting extract of *Aconitum carmichaeli Debx.* was prepared as follows: 10 g ground material was extracted with 50 mL aether-aqueous ammonia (4:1, v/v) by supersonic vibration for 2 h. Then the supernatant was evaporated to dryness under N_2 flow and redissolved in 1 mL acetonitrile, followed by centrifugation (12,000 × *g*, 10 min). The supernatant was collected as working solution of sample, which was stored at −20 °C in dark.

2.4. Integration of the VEGFR-CMC module

HEK293/VEGFR cells (7×10^6) were washed for 3 times with phosphate buffer saline (pH 7.4; 50 mM) and then centrifuged

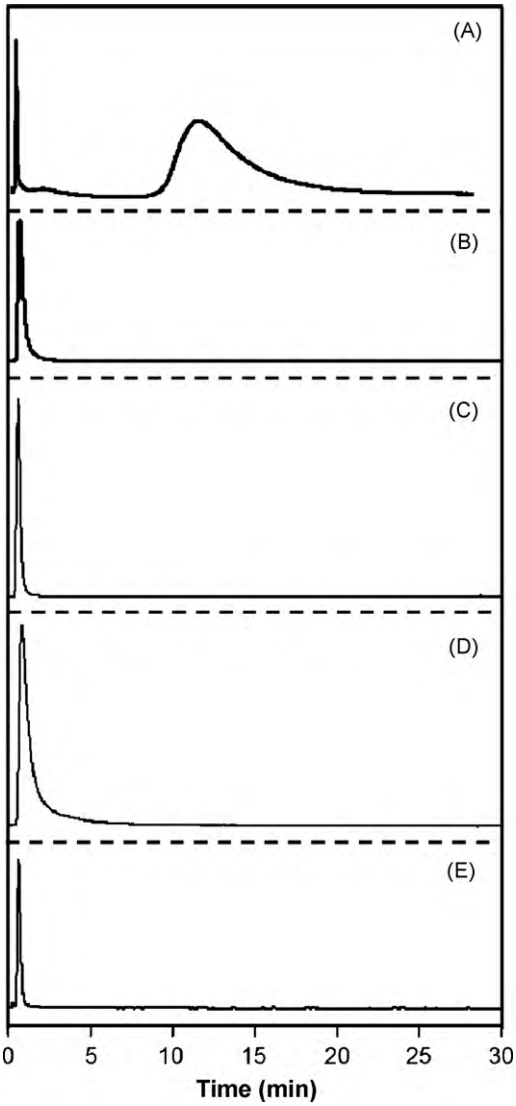

Fig. 2. Chromatograms of the positive control (SN) and negative controls (NF, DX, MT and AT) on the VEGFR-CMC. (A) SN; (B) NF; (C) DX; (D) MT; (E) AT.

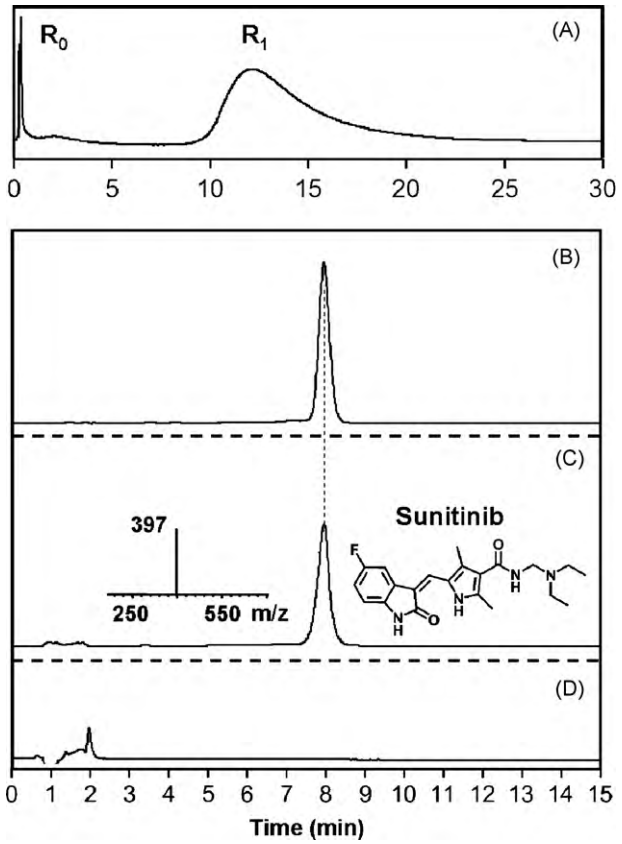

Fig. 3. Chromatograms of SN analyzed by the coupled VEGFR-CMC-online-LC/MS system. (A) Chromatogram of the SN on the VEGFR-CMC. (B) Chromatogram of the SN on LC/MS. (C) Chromatogram and mass-spectrogram of the fraction retained by the VEGFR-CMC on the LC/MS (R_1). (D) Chromatogram of the non-retained fraction eluted from the VEGFR-CMC on the LC/MS (R_0). (R_0) The fraction non-retained on the VEGFR-CMC. (R_1) The fraction retained on the VEGFR-CMC.

(1500 × *g*, 10 min, 4 °C). Tris–HCl (pH 7.4; 50 mM) was added to produce a cell suspension, which was ruptured by sonication (30 min), immediately. The resulting suspension was homogenated for 2 min and clarified by centrifugation (1500 × *g*, 10 min, 4 °C). The pellet was discarded, and the suspension was recovered by centrifugation (12,000 × *g*, 20 min, 4 °C). The pellet was resuspended in 5 mL phosphate buffer saline (pH 7.4; 50 mM) and cell membranes were again collected by centrifugation (12,000 × *g*, 20 min, 4 °C). The pellet was resuspended into 5 mL distilled water. The HEK293/VEGFR cell membrane stationary phase (CMSP) was prepared by adsorption of the cell membrane suspension (5 mL) onto activated silica (0.05 g) under vacuum at 4 °C with gentle agitation [12,13]. At last, The VEGFR-2 CMSP was packed into the VEGFR-CMC column using the column loading machine following a wet packing procedure. Distilled water was used as mobile phase. When VEGFR-CMC column was loaded onto the chromatography system at the temperature of 37 ± 0.5 °C, aqueous ammonium acetate buffer solution (pH 7.4; 5 mM) was pumped at a flow rate of 0.2 mL/min as the mobile phase. Data acquisition was executed by an ultraviolet detector (266 nm for SN, and 233 nm for the sample of *Aconitum carmichaeli Debx.*).

2.5. Validation of the specification of VEGFR-CMC module

Some usual specific drugs that did not act on VEGFR, such as NF (calcium channel blocker), DX (glucocorticoid), MT (α_{1A}-adrenoceptor agonist) and AT (β_1 receptor antagonist), were used as negative controls to validate specification of VEGFR-CMC module. Chromatographic condition parameters were the same as Section 2.4. The samples of positive and negative controls were

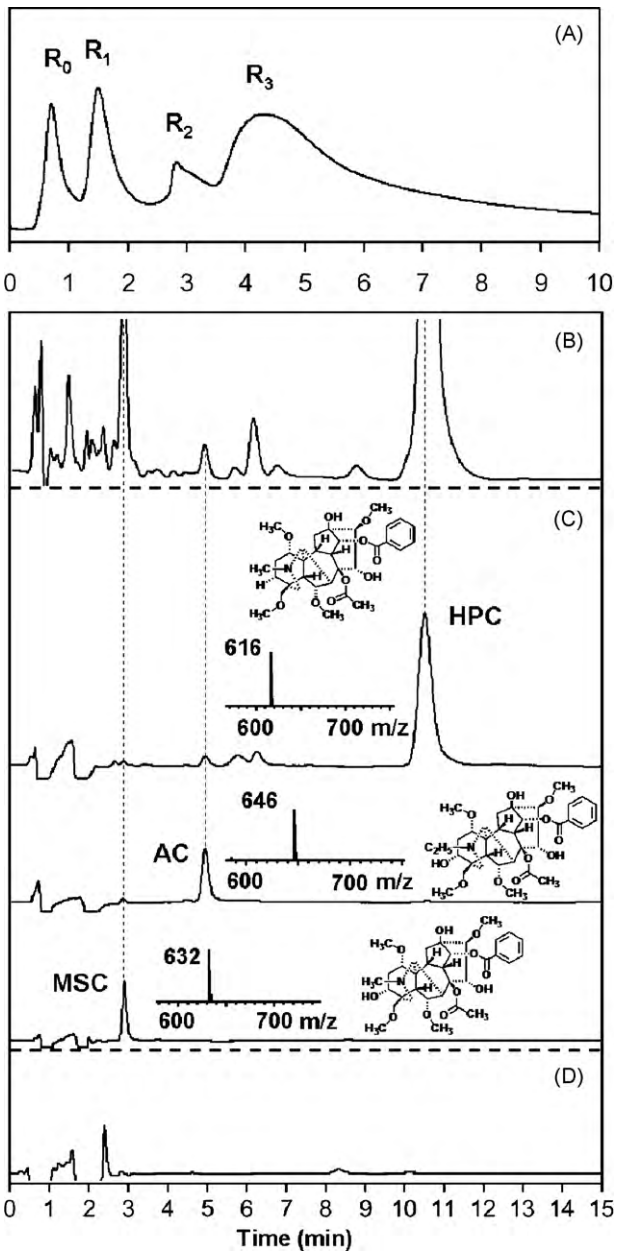

Fig. 4. Chromatograms of staring extract of *Aconitum carmichaeli Debx.* analyzed by the coupled VEGFR-CMC-online-LC/MS system. (A) Chromatogram of staring extract of *Aconitum carmichaeli Debx.* on the VEGFR-CMC. (B) Chromatogram of staring extract of *Aconitum carmichaeli Debx.* on the LC/MS. (C) Chromatograms and mass-spectrograms of the fractions retained by the VEGFR-CMC on the LC/MS. (D) Chromatogram of the non-retained fraction eluted from the VEGFR-CMC on the LC/MS. R_0: the fraction non-retained on the VEGFR-CMC; $R_{1,2,3}$: the fractions retained on the VEGFR-CMC; MSC: mesaconitine; AC: aconitine; HPC: hypaconitine.

injected into the VEGFR-CMC module and their retention characteristics were compared to validate the specification of screening on the VEGFR-CMC module for the compound acting on VEGFR-2.

2.6. LC/MS system

The VP-ODS column of LC/MS system was fixed in the same column oven as VEGFR-CMC column. The mobile phase was acetonitrile-aqueous triethylamine (0.1%; v/v) (50:50, v/v) with a flow rate of 0.2 mL/min. MS conditions were as follows: Ionization mode, ESI; nebulizer gas, N_2 (purity 99.999%); flow rate, 1.5 L/min; drying gas, N_2 (purity 99.999%); pressure, 0.1 MPa; inter-

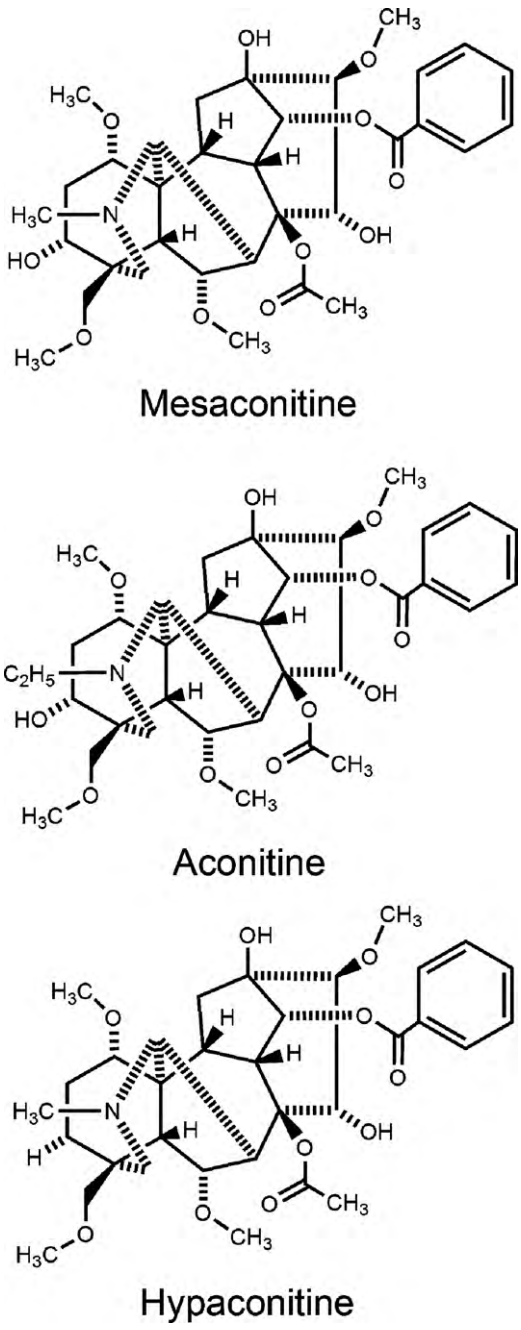

Fig. 5. Chemical structures of MSC, AC and HPC.

face temperature, 250 °C; heat block temperature, 200 °C; curved desolvation line (CDL) temperature, 260 °C; CDL voltage, 10 V; detector voltage, 1.5 kV; negative ionization mode, scanning from *m*/*z* 200 to *m*/*z* 1000.

2.7. The online coupling and application of the VEGFR-CMC and LC/MS system

The CMC-online-LC/MS method was firstly reported by Wang et al. to study the specific interactions between α1A-adrenoceptor and its ligands [14]. In this study, an enrichment column was used to replaced the capture loop of this system in order to reduce the expansion of retained fraction from VEGFR-CMC column the enrichment process before LC/MS analysis. As depicted in Fig. 1, the VEGFR-CMC system was online-coupled with LC/MS system through the 10-port 2-position column switcher, and an ODS C18

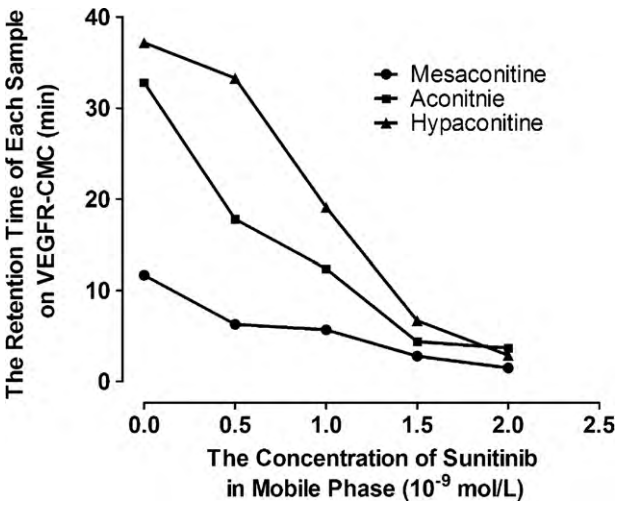

Fig. 6. The variation of retention time of MCS, AC, HPC on VEGFR-CMC with SN of gradient concentration dissolved in mobile phase.

guard column played as the enrichment column. At the very beginning, columns in both dimensions were equilibrated and the column switcher was in position A. Then sample was injected into the first dimensional VEGFR-CMC. When one of the fraction separated by the VEGFR-CMC was going to be eluted out from CMC column, the column switcher altered to position B immediately and guided this fraction to be adsorbed on the enrichment column. In the end, elution process of the separated fraction from VEGFR-CMC column was completed, and the column switcher altered back to position A. After the fraction stayed on the enrichment column was eluted into the LC/MS system for further separation and identification, the whole system returned to equilibration status and waited for next cycling operation. Screening for component(s) acting on VEGFR-2 from *Aconitum carmichaeli Debx*. was executed on this coupled VEGFR-CMC-online-LC/MS system, with SN as positive control.

2.8. Validation of the specification

To confirm that sunitinib and compound(s) screened from *Aconitum carmichaeli Debx*. were both active on the same site of VEGFR-2, competitive displacement test were performed. The compound(s) screened from *Aconitum carmichaeli Debx*. was injected into VEGFR-CMC with SN of gradient concentration dissolved in mobile phase (0, 0.5×10^{-9}, 1.0×10^{-9}, 1.5×10^{-9}, 2.0×10^{-9} M). Water was used as dissolvent of mobile phase. Other chromatographic condition parameters was as same as in Section 2.4. The variation of retention time was recorded for analysis.

2.9. Cell growth assay

The effects of starting extract *of Aconitum carmichaeli Debx*. and identified active compound(s) on HEK293/VEGFR viability were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells per well. After 24 h incubation at 37 °C for 48 h, cells were treated with the starting extract of *Aconitum carmichaeli Debx*. at concentrations of 0.24, 1.22, 6.12, 30.6 μg/mL, and the standard substance of identified active compounds at concentrations of 0.40, 2.00, 10.0, 50.0 μM, respectively. Then, 20 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. After the supernatant was discarded, 150 μL of DMSO was added to each well, and the optical density of cells was determined with microplate reader at 490 nm and expressed as absorbance values [15].

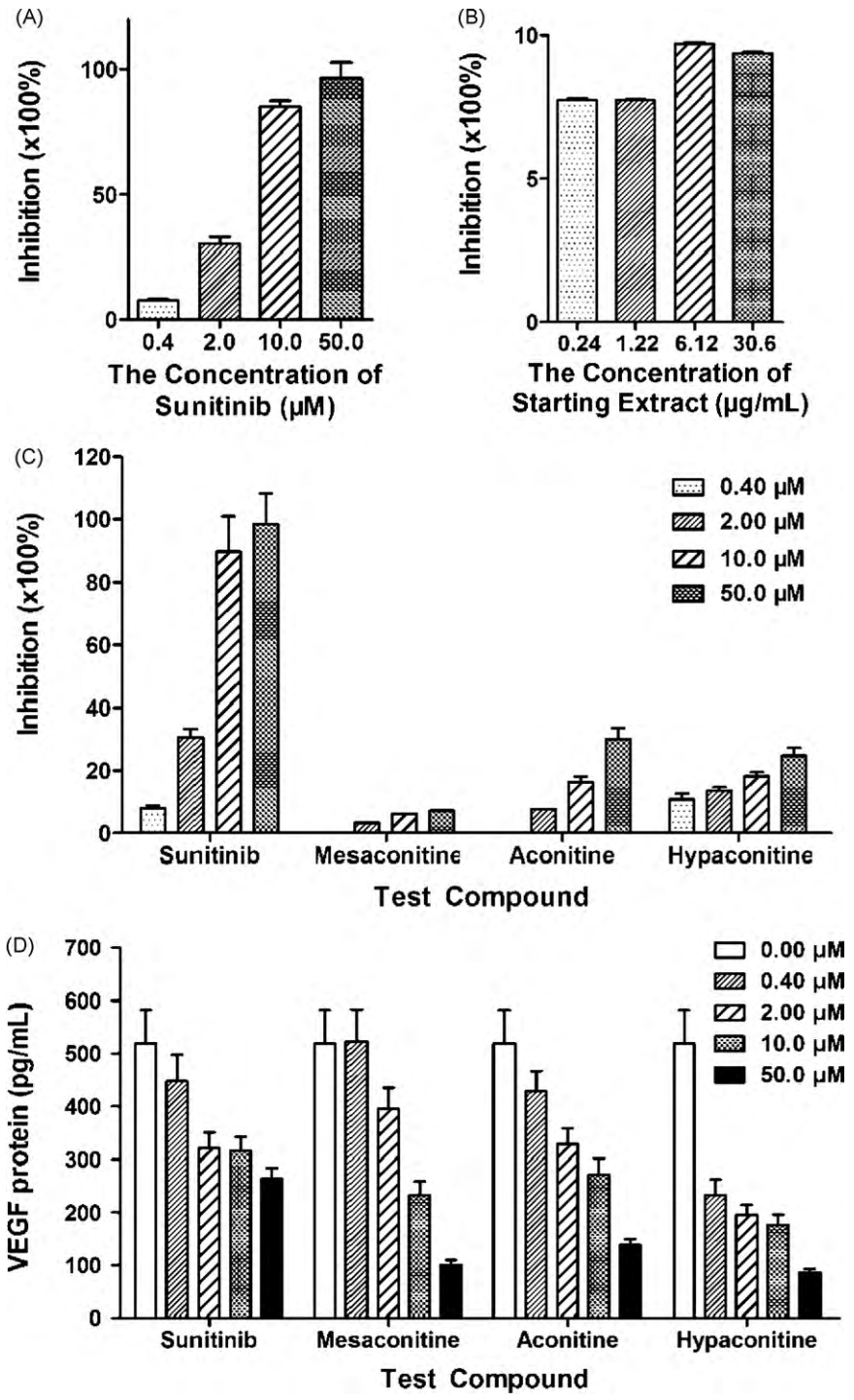

Fig. 7. The inhibition effect of staring extract of *Aconitum carmichaeli Debx*., MSC, AC, and HPC on HEK293/VEGFR cell. Values are expressed as means ± SD ($n = 5$). (A) The inhibition effect of SN on HEK293/VEGFR cell viability (postive control to Fig. 7B). (B) The inhibition effect of staring extract of *Aconitum carmichaeli Debx*. on HEK293/VEGFR cell viability. (C) The inhibition effect of MSC, AC, and HPC on HEK293/VEGFR cell viability. (D) The effect of MSC, AC, and HPC on VEGF secretion of HEK293/VEGFR cell.

2.10. VEGF secretion in vitro

HEK293/VEGFR (1×10^4 cells per well) cells were cultured in 24-well culture plates for 24 h. Then, the cells were incubated for another 24 h after the medium was changed to serum-free. When the standard substance of identified active compounds added to the well, the final concentrations for HEK293/VEGFR cells were 0.00, 0.40, 2.00, 10.0, 50.0 μM. The 48 h cultured cells were collected. VEGF protein concentrations were quantified by a commercially available VEGF-ELISA kit. Optical densities (ODs) were measured at 450 nm [16].

3. Results and discussion

3.1. Validation of VEGFR-CMC-online-LC/MS system

During the validation of retention characteristics and identification capability of this online-coupled system, SN was studied as

VEGFR-2 antagonist. Drugs supposed to have no specific interaction with VEGFR-2 (negative controls) and SN were injected into VEGFR-CMC module and their retention characteristics were compared in Fig. 2. Only SN could be retained on VEGFR-CMC. It confirmed the specification of interaction the sample and VEGFR-2 CMSP. Chromatogram of SN on VEGFR-CMC model (Fig. 3A) indicated the marked retention of sunitinib (R_1). After the switching of 10-port valve, the analysis of the non-retained fraction (R_0) and retained fraction (R_1) eluted from VEGFR-CMC column run on LC/MS, respectively. The chromatograms and mass-spectrogram of R_1 and R_0 shown in Fig. 3C and D confirmed that R_1 was sunitinib. Fig. 3B was the chromatogram of SN directly analyzed LC/MS model. According to the comparison of Fig. 3B and C, it was obvious that the retention characteristics of sunitinib in both LC/MS chromatograms were the same. Therefore, it confirmed that VEGFR-2 antagonist sunitinib could be recognized, captured and identified by this online-coupled method. Finally, we could draw the conclusion that the VEGFR-CMC-online-LC/MS could be used for the screening of compounds acting on VEGFR-2.

3.2. Application of the VEGFR-CMC-online-LC/MS system

The screening study was executed on the VEGFR-CMC-online-LC/MS system. Fig. 4A was the chromatogram of starting extract of *Aconitum carmichaeli Debx*. on VEGFR-CMC, which had three retained fractions (R_1, R_2, and R_3). R_0 was supposed not to interact with VEGFR-2. These four fractions (R_0, R_1, R_2, and R_3) were sent to LC/MS for following analysis as the procedure described in section 2.7, respectively. The HPLC chromatograms and mass-spectrograms (showed in Fig. 4C and D) of the R_1, R_2, R_3 and R_0 were obtained and used to identify R_1 as MSC, R_2 as AC, and R_3 as HPC, by referring to reported ESI/MS data [17,18]. Fig. 4B was the chromatogram of starting extract of *Aconitum carmichaeli Debx*. analyzed by direct injection into LC/MS. The comparison of Fig. 4B and C confirmed that MSC, AC, and HPC were the major retained components in the extract of *Aconitum carmichaeli Debx*. on VEGFR-CMC column. The time range of an analysis process for one single fraction was not more than 30 min. Accordingly, the conclusion was that this VEGFR-CMC-online-LC/MS system could be used to screen compounds acting on VEGFR-2 efficiently from samples that had complicated constitutions. The chemical structures of MSC, AC and HPC were shown in Fig. 5.

3.3. Specification

The variation tendency of retention characteristic in competitive displacement test on VEGFR-CMC was shown in Fig. 6. As the concentration of SN in mobile phase increased (from 0 to 2.0×10^{-9} M), the retention time of MSC, AC and HPC decreased. It could be inferred that the more active points of VEGFR-2 on solid phase was occupied by sunitinib in mobile phase, the weaker the binding between MAC, AC, and HPC and VEGFR-2 would become. This result suggested that MSC, AC and HPC acted on the same site of VEGFR-2 as sunitinib.

3.4. The in vitro inhibition activity of starting extract of Aconitum carmichaeli Debx., MSC, AC, and HPC on HEK293/VEGFR

The inhibition activity of starting extract of *Aconitum carmichaeli Debx.*, MSC, AC, and HPC, on HEK293/VEGFR cell viability was determined by MTT assay, with SN as positive control (Fig. 7A–C). The inhibition activity of starting extract of *Aconitum carmichaeli Debx*. could be detected but low (Fig. 7B). It was necessary to screen the active constituent(s) from the starting extract which had definite inhibition activity. As depicted in Fig. 7C, In the range of dose from 0.40 to 50.0 μM, SN showed obvious and dose-depended inhibition activity. The inhibition activity of MSC, AC, and HPC could be detected but weaker than that of SN, and it also depended on does.

It was indicated in Fig. 7D that the secretion of VEGF by HEK293/VEGFR cell was inhibited by MSC, AC, and HPC compared with the untreated group, in the range of dose from 0.00 to 50.0 μM, respectively. And the inhibition activity depended on does.

In summary, the inhibition activity of MSC, AC, and HPC on HEK293/VEGFR cells was verified. The conclusion that active constituents had been distinguished from the starting extract of *Aconitum carmichaeli Debx*. by our online-coupled screening system was supported by the result of in vitro inhibition activity test.

4. Conclusions

On a newly integrated VEGFR-CMC-online-LC/MS system, the screening process of compounds acting on VEGFR-2 was validated to be efficient and specific. Three active components named mesaconitine, aconitine, and hypaconitine were successfully found out and accurately identified from the traditional Chinese medicine named *Aconitum carmichaeli Debx*. The inhibition activity of these three compounds and was confirmed by the MTT tests and the assays of VEGF secretion in vitro. Our research offered additional exemplification which supported this online-coupled method, and was supposed to give more evidence on researches of natural active substances with coupled bio-analysis and identification means.

Acknowledgement

This work was supported by National Natural Science Foundation of China (Grant Number 30730110).

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