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Combined fibroblast growth factor receptor 4 cell membrane chromatography online with high performance liquid chromatography/mass spectrometry to screen active compounds in *Brassica albla*

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ABSTRACT

We investigated an analytical method for the recognition separation, and identification of active components from the traditional Chinese medicinal plant *Brassica albla* L. using fibroblast growth factor receptor 4 cell membrane chromatography (FGFR4/CMC) with high performance liquid chromatography/mass spectrometry (HPLC/MS). HEK293-FGFR4 cells were obtained by stable transfection of the HEK293 cell line with pcDNA3.1 vector containing the FGFR4 gene. Crude extracts of *B. albla* L. were firstly subjected to FGFR4/CMC column, and the retain contents on the FGFR4/CMC column were then transferred and enriched using a pre-column, and a ten port column switcher were used between FGFR4/CMC column and HPLC. The retained components on FGFR4/CMC column were then directly delivered to the HPLC/MS system for separation and identification. Gefitinib, nicotine, atenolol, and nimodipine were used in order to verify FGFR4/CMC-HPLC/MS system specificity. Subsequently, we investigated the reproducibility and reliability of the FGFR4/CMC-HPLC/MS system. Finally, sinapine was identified as an active component of *B. albla* L. The MTT colorimetric assay revealed sinapine could inhibit the proliferation of HEK293-FGFR4 cells with dose dependence. Competitive displacement assay approved getitinib could occupy binding site of sinapine with competition way. And FleX dock simulation further exhibited sinapine and gefitinib could bind with the FGFR4 tyrosine active domain. Thus, sinapine is a potential tumor antagonist that acts on the tyrosine kinase domain.

1. Introduction

Fibroblast growth factor receptors (FGFRs) belong to the tyrosine kinase receptor family. The signaling pathway for fibroblast growth factors (FGFs) is through FGF receptors (FGFRs) that regulate fundamental physiological processes controlling a wide range of biological functions [1] that include the regulation of embryonic development [2], wound repair, and cell proliferation and survival; thus FGF signaling plays an important role in tumorigenesis [3,4]. Recently, emerging research reports have demonstrated that FGFR4 plays a very important role in cancer cell proliferation [5], metastasis [6], and resistance to chemotherapy drug sensitivity [7]. Therefore, FGFR4 is a potentially important drug target that enables the screening of leading FGFR4 antagonist compounds.

Sinapine, an important natural product, is often obtained from cruciferous plants. Many biological characteristics, such as antioxidant, substance of oil-bearing crop, poor palatability for animal feed, etc., draw many scientists attention to study the distribution, content, metabolic pathway of sinapine [8–11]. In Chinese Pharmacopoeia, the seed of *Brassica albla* L. in the Crucifer family is a traditional Chinese medicine (TCM) called Bai jiezi (BJZ) in Chinese [12]. The key component of BJZ is sinapine and the pharmacological effects of BJZ have been demonstrated in previous studies, such as the inhibition of prostatic hyperplasia, antitussive capabilities, and expectorant, anti-asthmatic, anti-inflammatory, and analgesic properties, etc. [13–18]. However, few studies have been conducted that screen for the active compounds among the complex metabolized products that could potentially serve as an anti-tumor drug [19]. In our study, we aim to use HEK293-FGFR4 to screen for the active components in BJZ.

Cell membrane chromatography (CMC), a type of biological affinity chromatography, has been demonstrated to serve as an effective method for separating active components that act on

Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBST, phosphate buffered saline–Tween-20; CMSP, cell membrane solid phase; ODS, ctadecyl silane; CDS, coding sequence; TCM, traditional Chinese medicine; RSD, relative standard deviation.

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a specific membrane receptor [20–24]. In our previous studies, we developed a cell membrane chromatography (CMC) method in combination with over-expressed membrane receptor and successfully applied this method to screen for active components in TCMs [25–28].

In this study, we successfully developed a two-dimensional (2D) liquid chromatography method by combining the FGFR4/CMC model with an HPLC/MS system and implementing an online column switching technique. For the first dimension of the novel method (FGFR4/CMC system), the active components from the extract of BJZ are recognized, and separated; the second dimension of the method (HPLC/MS) directly identifies the active components that are retained in the first dimension. This method potentially enables the collection of important evidence for anti-tumor drug discovery in TCMs.

2. Materials and methods

2.1. Chemicals and materials

Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China) and was activated at 105 °C. Sinapine cyanide sulfonate was purchased from the National Institute for the Control of Pharmaceuticals and Biological (Lot Number: 111702-200501, Beijing, China). BJZ was purchased from the TCM Store (Xi'an, China), and it was authenticated by the Department of Pharmacognosy, Xi'an Jiaotong University (Xi'an, China). Gefitinib was purchased from Nanjing Ange Pharmaceutical Co., Ltd. (Nanjing, China). Nimodipine (Lot Number: N149), Nicotine (Lot Number: N0267), atenolol (Lot Number: A7655) were all from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Ammonium acetate and Acetic acid was obtained from Fuchen chemical agent Co., Inc. (Tianjin, China). Acetonitrile (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, USA). All aqueous solutions were prepared using ultrapure water which is produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Instruments

The HPLC mass spectrometry system (HPLC/MS, Shimadzu Corporation, Kyoto, Japan) included three LC-20AD pumps, a DGU-20A_3 degasser, a SIL-20A autosampler, a SPD-20A UV/VIS detector, a SPD-M20A diode array detector, a CTO-20A column oven, a LCMS-2010EV mass spectrometer and a HPLC/MS solution workstation. The first dimensional FGFR4/CMC column (10.0 mm × 2.0 mm I.D., 5 μm) was packed using a RPL-10ZD column loading machine from Dalian Replete Science and Technology Co., Ltd. (Dalian, China). A VICIAG 10G-0911V 10 port 2-pos valve (Valco Instrument Co. Inc., Houston, USA) was used as the column switcher and one Shim-pack VP-ODS pre-column (10 mm × 2.0 mm I.D., 5 μm, Shimadzu Corporation, Kyoto, Japan) was used as the enrichment column. An FGFR4/CMC column (10 mm × 2.0 mm I.D., preparation method of FGFR4/CMC column was listed in Section 2.8 was used as the first dimension column and a Shimadzu Shim-pack VP-ODS column (150 mm × 4.6 mm I.D., 5 μm, Kyoto, Japan) represented the second dimension column.

2.3. Preparation of the standard solution and sample preparation

Standard stock solutions of gefitinib, nimodipine, nicotine, and atenolol were separately prepared in methanol (1 mg/mL). A mixed standard solution containing gefitinib, nimodipine, nicotine, and atenolol was prepared in methanol.

Total extract of BJZ was prepared following a previously described method [19]. Briefly, the dried seeds of BJZ were ground into powder, placed into a drying oven at 60 °C for 1 h, and extracted using an eight-fold volume of 50% (v/v) ethanol for 2 h; this process was repeated two separate times, total extract were filtered by Buchner funnel under conditions of reduced pressure. And the filtrates were combined into a round bottom flask. The filtrates were then concentrated to obtain a dark brown viscous mass under conditions of reduced pressure at 60 °C. A sample of extract was then dissolved in ethanol under ultrasonic conditions and diluted with mobile phase.

2.4. Plasmid construction

The gene encoding the FGFR4 protein was amplified by polymerase chain reaction (PCR) using a pOTB7/FGFR4 plasmid obtained from Open Biosystem (Huntsville, USA). Oligonucleotide primer sequences for the CDS of FGFR4 were as follows: forward primer is 5′-AGTAAGCTTATGCGGCTGCTGCTGGCCCT-3′, and reverse primer is 5′-GATCTCGAGTCATGTCTGCACCCCAGACC-3′. The entire CDS length of FGFR4 was directly ligated into the expression vector pcDNA3.1(+) containing a pCMV promoter site (Invitrogen, Carlsbad, USA). The 0.2 μg DNA of the construct was transformed into competent *E. coli* DH5α at 42 °C for 90 s. Next, the transformed cells were plated onto solid LB medium with 100 μg/mL ampicillin. After 16 h, the clones were selected and transferred to LB medium for the propagation of reconstructed plasmid. The plasmid was then purified using a plasmid midi kit from Qiagen (Hilden, Germany). Insertion of the desired gene into the plasmid was verified using the 3730XL automated DNA sequencer from Applied Biosystems (Carlsbad, USA).

2.5. Cell culture, stable transfection

The human embryonic kidney 293 (HEK293) cell line was obtained from the American Type Tissue Collection (Manassas, USA) and maintained in Dulbecco's modified Eagle's medium from Gibco-BRL (Rockville, USA) containing 4.5 g/L glucose and supplemented with 10% fetal calf serum from Gibco-BRL (Rockville, USA) supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin. Dimethyl sulfoxide (DMSO), MTT, and trypsin were also obtained from Sigma.

The HEK 293 cell line was transfected with recombinant pcDNA3.1(+)/FGFR4 using the Lipofectamine™ 2000 reagent kit from Invitrogen (Carlsbad, USA) in accordance with the manufacturer's instructions. The procedure was as follows: 2 μg of plasmid was diluted in 200 μL of Opti-MEM® I Medium and incubated for 10 min at room temperature. At the same time, 7 μL of Lipofectamine™ 2000 was added to 200 μL of Opti-MEM® I Medium for 10 min. Next, the diluted DNA was gently mixed with the diluted Lipofectamine™ 2000 and incubated for 20 min at room temperature. The complex was then added to each well containing cells and medium. The plates were then mixed gently by rocking back and forth, the cells were incubated at 37 °C in a CO_2 incubator for 4–6 h, and the medium was replaced with fresh medium lacking Geneticin for 48 h. The stable transfectants were selected in Geneticin (G418, 800 μg/mL) for 20 days. The clones were transferred to 24-well plates to amplify the stable G418-resistant clones at a concentration of 300 μg/mL.

2.6. Cell lysis and immunoblotting

Cells of the selected clones were washed with PBS and incubated at 4 °C with lysis buffer (50 mM HEPES/NaOH, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 200 μM DTT) supplemented with protease inhibitor (1 mM PMSF) for 30 min. Cellular debris was removed by centrifugation at 12,000 rpm. The protein concentration was confirmed using a BCA Protein Assay Kit from Pierce (Rockford, USA). The samples were suspended in 5× SDS

sample buffer and boiled for 10 min, and 20 μg protein was assayed by 10% SDS-PAGE. For western blot analysis, protein samples were transferred to PVDF membranes and incubated with FGFR4 antibody (Santa Cruz 1:2000). The PVDF membranes were then incubated with horseradish root peroxidase-conjugated AffiniPure goat anti-rabbit antibody (Jackson ImmunoResearch 1:25,000). Signals were assessed using an enhanced chemiluminescence detection system from Pierce (Rockford, USA).

2.7. Immunofluorescent staining of FGFR4

HEK293-pcDNA3.1 and HEK293-FGFR4 cells were cultured on coverslips in six-well plates. When cell coverage reached 60% confluence, cells were fixed in 4% paraformaldehyde (Lot Number: 41533, Sinopharm Chemical Reagent Co. Ltd.) for 15 min at room temperature, permeabilized with PBS containing 0.1% Triton X-100 for 3 min at room temperature, blocked in 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and then incubated 2 h at room temperature with FGFR4 antibody (Santa Cruz, 1:200; sc-124). After 3× 10 min PBST wash steps, cells were incubated with FITC-Conjugated AffiniPure goat anti-rabbit IgG (H + L) antibodies (ZSGB-BIO, 1:100; ZF-0311). Cells were washed thoroughly with PBS and then mounted onto microscope slides. Finally, samples were analyzed using a regular fluorescence microscope and Nikon Ti-u from Nikon (Tokyo, Japan).

2.8. Preparation of FGFR-4/CMC column

HEK293-FGFR4 cells (7×10^6) were washed three times with phosphate buffered saline (pH 7.4; 50 mM) by centrifugation at 2000 × *g* for 10 min at 4 °C. Next, HEK293-FGFR4 cells were ruptured under ultrasonic conditions for 30 min in Tris–HCl (pH 7.4; 50 mM) buffer. The cell membrane suspension was centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g* for 20 min at 4 °C. The precipitate was resuspended in 10 mL phosphate buffered saline (pH 7.4; 50 mM), and the suspension was again centrifuged at 12,000 × *g*. Finally, we obtained a cell membrane suspension in 5 mL phosphate buffered saline (pH 7.4; 50 mM). The cell membrane suspension was slowly added to activate the silica (0.05 g silica at 105 °C for 30 min) under vacuum and agitation at 4 °C for 30 min. Next, the mixture was left at 4 °C overnight. Finally, the FGFR4/CMSP was packed into the FGFR4/CMC column (10 mm × 2.0 mm I.D.) using the column-loading machine according to the wet packing procedure. The FGFR4/CMC column was installed onto the CTO-20A column oven at a temperature of 37 °C. The mobile phase consisted of aqueous ammonium acetate buffer solution (pH 7.4; 5 mM) at a flow rate of 0.2 mL/min. Data acquisition was executed using an ultraviolet detector at a wavelength of 326 nm.

2.9. FGFR-4/CMC-online-HPLC/MS system

The FGFR4/CMC model was combined with the HPLC/MS system through the 10 port column switcher (Fig. 1). Any retention fraction in the FGFR4/CMC system was enriched onto the ODS pre-column (PC), and the enriched fraction was then eluted into the HPLC/MS system for further analysis. The procedure was conducted as follows: initially, the columns in both of the two dimensions were equilibrated with the column switcher in position A. The sample was then injected into the first dimension FGFR4/CMC system. When the fraction separated by the FGFR4/CMC was eluted, the column switcher immediately altered to position B and then the fraction will be adsorbed on the pre-column. Then, the column switcher altered back to position A. The fraction that retained on the pre-column was eluted into the HPLC/MS system for further separation and identification, and the entire system returned to a state of equilibration poised for the next cycling operation.

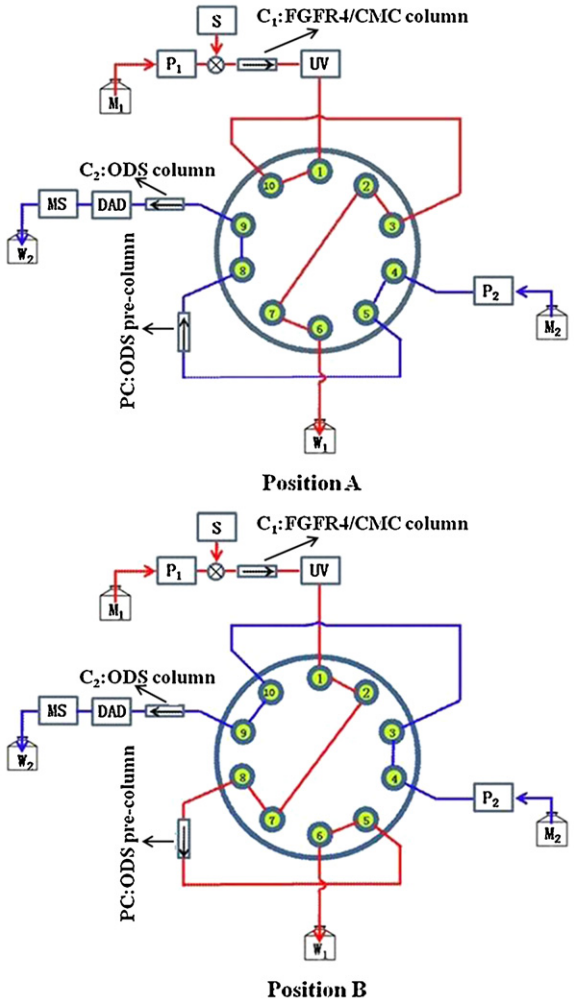

Fig. 1. Scheme of FGFR4/CMC online HPLC/MS method. C_1, FGFR4/CMC column; C_2, ODS column; PC, ODS pre-column; M_1, mobile phase of FGFR4/CMC column; M_2, mobile phase of the second dimension; P_1, pump of the first dimension; P_2, pumps of the second dimension; S, sample; UV, ultraviolet detector; DAD, diode array detector; MS, LCMS-2010EV mass spectrometer; W_1 and W_2, waste.

The VP-ODS column (150 mm × 4.6 mm I.D., 5 μm) from Shimadzu (Kyoto, Japan) was stationary in the CTO-20A column oven in the HPLC/MS system. We performed gradient elution using a flow rate of 1.0 mL/min with two different mobile phases. Mobile phase A was composed of acetic acid (2%, v/v), and mobile phase B was composed of acetonitrile. The following gradient was performed: *T* = 0–3 min, 95% A; *T* = 3–6 min, 95% A to 90% A; *T* = 6–10 min, 90% A; *T* = 10–13 min, 90% A to 80% A; *T* = 13–25 min, 80% A; *T* = 25–30 min, back to 95% A; *T* = 30–35 min, 95% A, *T* = 35 min, stop [29]. Because of fast flow rate of second dimension mobile phase (1.0 mL/min), a P470 high pressure graduated micro-splitter valve (Upchurch Scientific, Washington, USA) was used to split the mobile phase to be fit for the mobile phase flow rate of LCMS-2010EV mass spectrometer (0.2 mL/min). The 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; interface temperature, 250 °C; heat block temperature, 200 °C; curved desolvation line (CDL) temperature, 260 °C; detector voltage, 1.5 kV; CDL voltage, 10 V; negative ionization mode, scan *m*/*z* 200–800 [30].

2.10. Validation of FGFR-4/CMC-HPLC/MS system

Firstly, we investigated the difference in affinity between the HEK293/CMC column and the FGFR4/CMC column. A standard solution of gefitinib (5 μL, 0.1 mg/mL) was injected into the two CMC columns. All of the chromatographic conditions were identical between the CMC columns. In brief, a flow rate of 0.2 mL/min was used for the aqueous ammonium acetate buffer solution (pH 7.4; 5 mM). Data acquisition was executed at a column temperature of 37 ± 0.5 °C using an ultraviolet detector at the wavelength of 254 nm for gefitinib.

We also investigated the capability of the FGFR4/CMC column regarding drug selectivity. The mixed standard solution of atenolol (β-receptor antagonist), nicotine (nicotinic acetylcholine receptor agonist), nimodipine (calcium channel antagonist), and gefitinib (tyrosine kinase receptor inhibitor) was analyzed using this FGFR4/CMC-HPLC/MS system.

The reproducibility of the FGFR4/CMC column was investigated using gefitinib on FGFR4/CMC column. The RSD of retention times was represented as an important guideline. A standard gefitinib solution (5 μL, 0.1 mg/mL) was injected into the same FGFR4/CMC column within 24 h five times. Additionally, we investigated the retention time for gefitinib on three separate FGFR4/CMC columns.

2.11. Competitive displacement assay

A stoichiometric displacement model for the retention of solute in liquid chromatography was investigated in concordance with Geng and Regnier [31]. The active site of sinapine in the FGFR4 region was investigated by comparison with gefitinib. A competitive binding assay was studied by using gefitinib as a mobile phase additive at different concentrations changing from 1.186×10^{-7} to 1.898×10^{-6} mol/L, and sinapine was injected into the FGFR4/CMC column. The $\log k'$–$\log[G]_m$ curve of sinapine was obtained relative to the changing gefitinib concentration, k' was capacity factor and $[G]_m$ was the concentration of gefitinib in the mobile phase. In this study, the dissociation equilibrium constants of gefitinib and sinapine were obtained through the competitive displacement assay following Du et al. [32].

2.12. Molecular docking simulation

Molecular docking and virtual screening based on molecular docking have become an integral part of many modern structure-based drug discovery efforts [33–35]. Protein–ligand docking is widely applied to predict the structure of bound protein–ligand complexes and to predict the binding mode of certain ligand with protein [36]. Therefore, Molecular level of protein-ligand docking of sinapine with FGFR1 tyrosine kinase domain (PDB ID: 3C4F) was performed using SYBYL-X 1.1 to identify the protein-binding mode. The substrate was constructed with the Sybyl/Sketch module and optimized using Powell's method. Energy minimization was performed using the Tripos force field with the convergence criterion set at 0.005 kcal/(Å mol) and the maximum set at 1000 iterations and Gasteiger–Hückel charges. A non-bonded cut-off distance of 8 Å was adopted in order to take into consideration the intramolecular interaction [37]. Other docking parameters implied in the program were kept at default.

2.13. Cell viability assay

Drug sensitivity was analyzed using the MTT colorimetric assay. HEK293-pcDNA3.1 and HEK293-FGFR4 cells were seeded into 96-well plates with five replicate samples at 5×10^3 cells/well. After incubation with high glucose DMEM supplemented with 10% FBS and G418 (300 μg/mL) at 37 °C for 24 h, six different concentrations of sinapine were added. After 48 h of incubation, 20 μL of dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 490 nm using a Microplate Reader from Bio-rad (Hercules, USA).

2.14. Statistical analyses

All data were plotted using the GraphPad Prism 5.0 software. Results are presented as mean ± standard deviation (SD). The statistical difference between more than 2 groups was analyzed and one-way ANOVA followed by Tukey's multiple comparison tests was performed. The statistical significance of a result was determined to be $p < 0.05$.

3. Results and discussion

3.1. Expression and Location of FGFR4 in FGFR4-transfected HEK293 cells

The FGFR4 expression level was measured. The FGFR4 protein, with a molecular weight of 89 kDa, was highly expressed in HEK293-FGFR4 (F28 clone) (Fig. 2A) but not in HEK293-pcDNA3.1 cells (Fig. 2A).

The localization of the FGFR4 proteins was monitored utilizing indirect immunofluorescence. The results demonstrated that FGFR4 proteins were highly expressed in the cell membrane and cytoplast (Fig. 2B) in HEK293-FGFR4-F28 clone cells. Based on biochemical experiment results, HEK293-FGFR4 (F28 clone) serves well as the solid phase of CMSP in the FGFR4/CMC column.

3.2. Checking the FGFR-4/CMC-online-HPLC/MS system

Native HEK 293 cells (exhibiting low expression of FGFR4) were used to prepare a control CMC model in the same manner as FGFR4 high-expression cells. We used gefitinib as a positive control to determine the difference in retention times between the FGFR4/CMC column and HEK293/CMC column. The chromatographic conditions of the HEK293/CMC column were strictly consistent with the conditions of the FGFR4/CMC column. The retention time of gefitinib on the HEK293/CMC column was approximately 6 min, whereas the retention time on the FGFR4/CMC column was approximately 12 min (Fig. 3). Compared with the HEK293/CMC column, the FGFR4/CMC column exhibited longer retention time because of the greater number of drug binding sites compared to HEK293-FGFR4 with HEK293-pcDNA.

Moreover, the drug selectivity of the FGFR4/CMC-online-LC/MS system used gefitinib as the positive control and atenolol, nicotine, and nimodipine as the negative controls. Only gefitinib specifically interacts with the tyrosine kinase domain of FGFR4 among the four standard drugs. Chromatographic analysis of the mixed standards solution was performed on the FGFR4/CMC column. As shown in Fig. 4, two fractions (R_0 represent non-retained components and R_1 fractions represent retained components between the dotted lines) were sequentially enriched through pre-columns and then switched onto column C_2 for the second dimensional chromatographic separation (Fig. 1, position B) and identification using MS. The results demonstrated that, from the mixed standard solution, gefitinib was specifically retained by the FGFR4/CMC column and could be further detected using the HPLC/MS system (Fig. 4). This indicated that the FGFR4/CMC-online-HPLC/MS model was able to identify small molecular candidates that act on the tyrosine kinase domain of FGFR4.

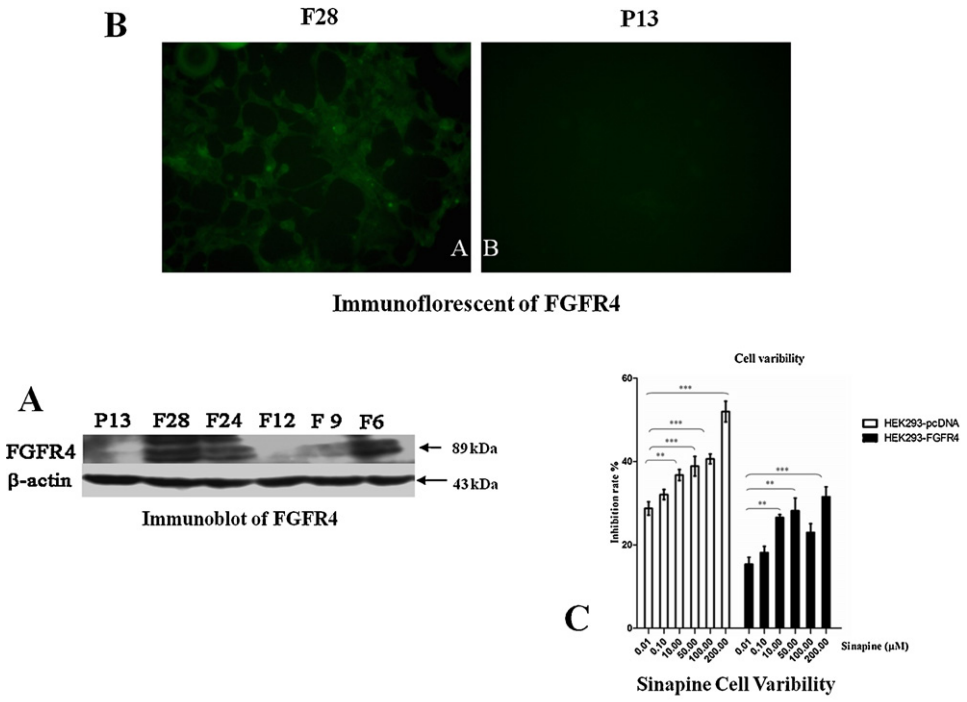

Fig. 2. Biological characteristics of HEK293-FGFR4 cells and HEK293-pcDNA cells. (A) Immunoblot analysis of stable transfected cells. HEK293-FGFR4 (clone F6, F9, F12, F24, F28), HEK293-pcDNA (clone P13). (B) Immunofluorescence of HEK293-FGFR4 (clone F28) and HEK293-pcDNA (clone P13). (C) The inhibition effect of sinapine screened in BJZ. Differences between groups were assessed by one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0001$.

Additionally, we investigated the stability of the FGFR4/CMC column using the positive drug gefitinib. The results demonstrated that the retention time exhibited by the FGFR4/CMC column was 11.373 ± 0.148 min ($n = 5$) RSD = 1.30%, and for different FGFR4/CMC columns it was 13.409 ± 0.391 min ($n = 3$) RSD = 2.92%.

The results indicated that the FGFR4/CMC column exhibited a high affinity with gefitinib, whereas this was not observed with the other columns, such as HEK293-α_{1A}/CMC column [38]. We theorize that the FGFR4/CMC column is a tyrosine kinase receptor specific affinity column that is suitable for the screening of a potential tyrosine kinase inhibitor.

3.3. Screening active components using the FGFR-4/CMC-online-HPLC/MS system

Many natural products have been used as potential new drug candidates. In this study, the FGFR4/CMC-online-LC/MS system was used to screen for active compounds from total BJZ extract. There was one retained fraction (R_1) detected in the chromatogram of analysis on FGFR4/CMC column (Fig. 5A), and the R_1 fraction was directly analyzed using HPLC/MS system for further separation and identification without retained components pretreatment. The chromatogram of the R_1 fraction was shown in Fig. 5C, and further mass spectrum analysis revealed the principal component was

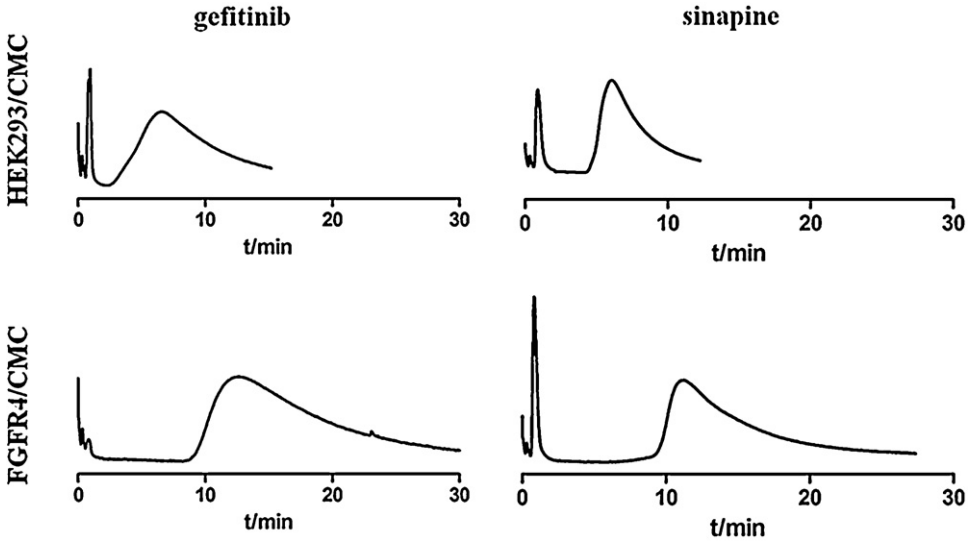

Fig. 3. Chromatography of gefitinib and sinapine on native HEK293/CMC and HEK293-FGFR4/CMC, respectively.

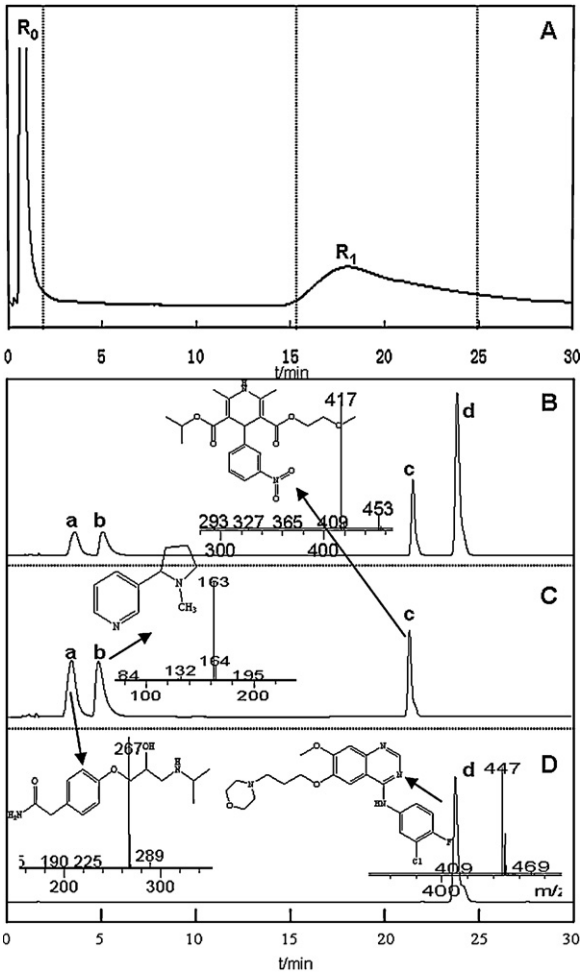

Fig. 4. Chromatograms of mixed drug standards using the combined FGFR4/CMC-online-HPLC/MS method. (A) FGFR4/CMC chromatogram of the mixed standards including R_0, and R_1 fractions (between the dotted lines). (B) HPLC/MS chromatograms of the mixed standards. (C) HPLC/MS chromatograms of the fractions (R_0). (D) HPLC/MS chromatograms of the fractions (R_1). a, atenolol; b, nicotine; c, nimodipine; d, gefitinib.

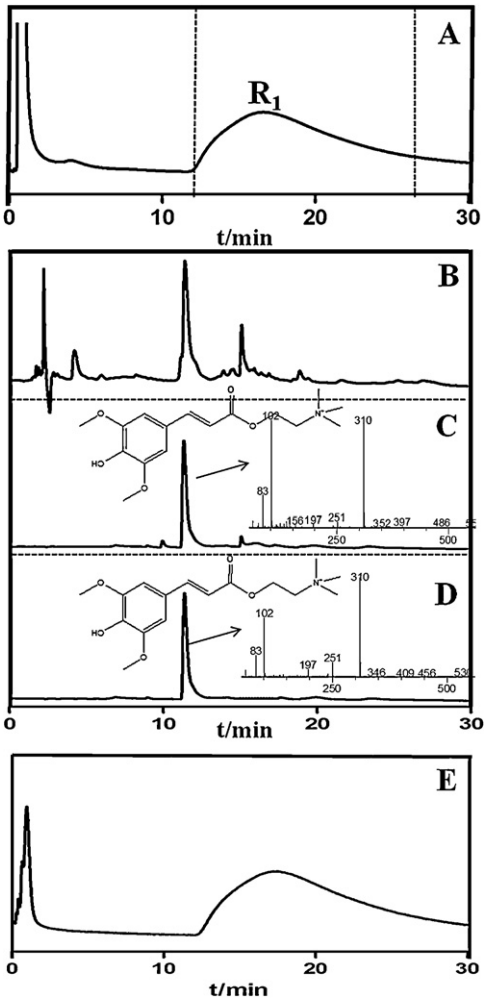

Fig. 5. Chromatograms of total extract of BJZ using the combined FGFR4/CMC-online-HPLC/MS method. (A) FGFR4/CMC chromatogram of the retained fraction between two dotted lines (R_1). (B) HPLC/MS chromatograms of the total extract of BJZ. (C) HPLC/MS chromatograms of the retained fraction. (D) HPLC/MS chromatograms of standard sinapine. (E) Chromatogram of sinapine on FGFR4/CMC column. FGFR4/CMC chromatogram of the retained fraction represented as (R_1).

supposed to be sinapine ($m/z = 310$). In order to confirm the structure of principal substance of R_1 fraction in Fig. 5A, we investigated chromatogram of standard sinapine. First, the standard sinapine injected in the FGFR4/CMC column to confirm whether sinapine could be retained on FGFR4/CMC column (Fig. 5E). As the results shown in Fig. 5E, sinapine can be well retained on the FGFR4/CMC column with a retention time similar to the total extract of BJZ. Then, the standard sinapine HPLC/MS chromatogram (Fig. 5D) gave the one major chromatographic peak compare with those of the HPLC/MS chromatogram of the R_1 fraction (Fig. 5C). Although several chromatographic peaks presented on chromatogram of total extract of BJZ using HPLC/MS (Fig. 5B), chromatographic peaks of R_1 fraction were substantially decreased because of specific FGFR4/CMC column affinity adsorption. According to Fig. 5, the FGFR4/CMC-online-HPLC/MS system could identify sinapine from total extract of BJZ. Therefore, this system built a rapid screen active component model which could be interacting with FGFR4 tyrosine kinase domain from total extract of TCM.

3.4. Potential pharmacological effects

In present study, the FGFR-4/CMC-online-HPLC/MS system successfully screened the active component sinapine from the BJZ. Thus, we further investigated the pharmacological activity of sinapine.

The MTT metabolization results demonstrated that sinapine (purchased from the National Institute for the Control of Pharmaceuticals and Biological) could inhibit HEK293-FGFR4 and HEK293-pcDNA cell growth (Fig. 2C). Both of the cell lines showed dose-dependent inhibition of activity. The HEK293-FGFR4 cells exhibited a lower drug sensitivity compared to HEK293-pcDNA cells. Therefore, FGFR4 plays an important role in preventing HEK293 cells from death when exposed to the drug. This result was in concordance with a previous study [7] reporting that FGFR4 plays an important role in chemo-resistance.

FGFR4, an oncogene encoded protein, has been investigated as a novel target for the therapy of tumor proliferation, metastasis, and chemo-resistance. The screened active compound, sinapine, exhibited a dose-dependent inhibition of HEK293-FGFR4 cell proliferation (Fig. 2C). Moreover, we confirmed that sinapine, screened form BJZ, acted as an antagonist for cell growth most likely via inhibition of ATP binding to the tyrosine kinase receptor domain.

Based on the pharmacological study, we demonstrated that sinapine screened from BJZ exhibited growth inhibition activity. Thus, we investigated the active domain of sinapine using the competitive displacement assay to elucidate the correlation with

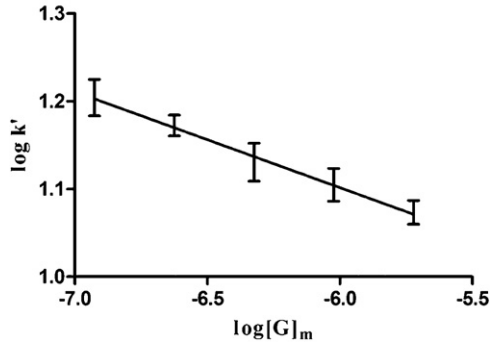

Fig. 6. A stoichiometric displacement model for the retention of solute in liquid chromatography. The *X*-axis represents the log of different concentrations of gefitinib. The *Y*-axis represents the log k' values of sinapine.

pharmacology. The retention time of sinapine on FGFR4/CMC column was decreased with an increasing concentration of gefitinib in the mobile phase (Fig. 6). This finding indicates that sinapine may have the same active domain on FGFR4. Meanwhile, both of the dissociation equilibrium constant of gefitinib ($K_{D[G]}$) and the dissociation equilibrium constant of sinapine ($K_{D[S]}$) were measured. The result was as follows: $K_{D[G]} = (5.75 \pm 0.23) \times 10^{-6}\,M^{-1}$ and $K_{D[S]} = (1.70 \pm 0.41) \times 10^{-5}\,M^{-1}$. These results corresponding with the retention time of gefitinib and sinapine on FGFR4 column.

3.5. Interaction simulation of sinapine with FGFR4

To investigate the possible binding modes of sinapine, the simulated docking of sinapine with FGFR4 was performed using SYBYL-X 1.1. Since the exact crystal structural of FGFR4 has not yet been identified, we selected FGFR1, another FGFR family member, which contains 820 residues and exhibits highly similar amino sequences compared with FGFR4, to predict the binding characteristics of sinapine with FGFR4. Sinapine was docked into the kinase domain of FGFR1 (PDB ID: 3C4F) (Fig. 7). Surflex-Dock method was based on various scoring algorithms such as crash, polar, *D*-Score, PMF-Score, *G*-Score, Chem-Score and CScore. Surflex-Dock scores (total scores) were expressed in $-\log_{10}(K_d)$ units to represent binding affinities. CScore (consensus score) was used for ranking the affinity of ligands bound to the active site of a protein. All stored poses were rescored using the CScore of SYBYL-X 1.1 comprising the following functions: Total Score, *D*-Score, *G*-Score, potential of mean force (PMF)-Score and Chem-Score function [39]. The binding site of sinapine on FGFR1 is shown in Fig. 7. The docking results indicated that hydrogen bonds were highly correlated with the affinity of ligand with FGFR1. Sinapine docks into the cavity of the FGFR1 tyrosine kinase region. According to docking simulation, the hydrogen atom of hydroxy forms one hydrogen bond interaction with ALA 564 with a distance of 2.05 Å. The oxygen atom of methoxyl also forms one hydrogen bond with ASN 568 with a distance of 1.98 Å. In general, protein pairs with a sequence identity higher than 35–40% are very likely to be structurally similar [40]. In proximity to the active site, homology of amino acids between FGFR1 and FGFR4 was 88% which suggest a highly structure similarity. Therefore, we were able to predict that sinapine exhibits a good fit with the tyrosine kinase region of FGFR4. The simulated binding mode was in concordance with experimental results of the pharmacological effects and displacement assay. This binding hypothesis may provide valuable information for the structure-based design for sinapine derivatives acting as a potent small molecular tyrosine kinase inhibitor of FGFR4.

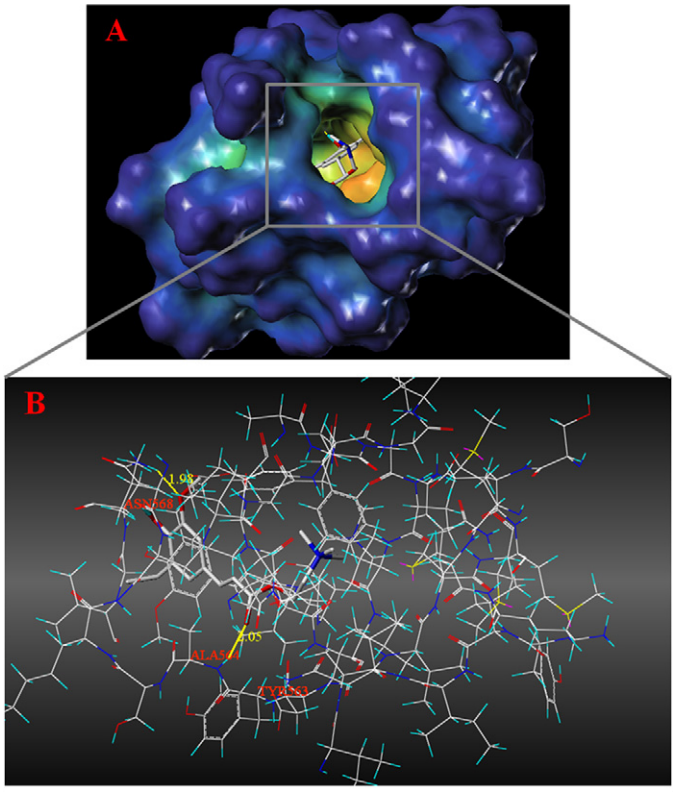

Fig. 7. Surflex docked conformation of sinapine in the active site of (PDB ID: 3C4F). (A) Binding model was depicted by MOLCAD surface. (B) Sinapine binding with the amino acid residues of the active site of FGFR1, hydrogen bonds between the ligand and residues are shown with yellow dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

In summary, we established a FGFR4/CMC-online-LC/MS method to screen anti-FGFR4 antagonists from total BJZ extract. This method has the potential to improve the efficiency of the screening process by combining tyrosine kinase domain recognized FGFR4/CMCcolumn with HPLC/MS system. The active compound of total extract of BJZ was successfully distinguished through this FGFR4/CMC-online-HPLC/MS system. And the sinapine was further screened and identified though a two-dimensional online CMC-HPLC/MS system. The competitive displacement assay and Flex dock simulation confirmed that sinapine has same acting domain as gefitinib on tyrosine kinase domain. Also, the inhibition activity of sinapine was studied using MTT. The FGFR4/CMC-online-HPLC/MS system could be used as a screening tool for active compound which could be interacted with FGFR4 from natural medicinal herbs.

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