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A combined A431 cell membrane chromatography and online high performance liquid chromatography/mass spectrometry method for screening compounds from total alkaloid of *Radix Caulophylli* acting on the human EGFR

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ABSTRACT

We have developed an online analytical method that combines A431 cell membrane chromatography (A431/CMC) with high performance liquid chromatography and mass spectrometry (LC/MS) for identifying active components from *Radix Caulophylli* acting on human EGFR. Retention fractions on A431/CMC model were captured onto an enrichment column and the components were directly analyzed by combining a 10-port column switcher with an LC/MS system for separation and preliminary identification. Using Sorafenib tosylate as a positive control, taspine and caulophine from *Radix Caulophylli* were identified as the active molecules which could act on the EGFR. This A431/CMC-online-LC/MS method can be applied for screening active components acting on EGFR from traditional Chinese medicines exemplified by *Radix Caulophylli* and will be of great utility in drug discovery using natural medicinal herbs as a source of novel compounds.

1. Introduction

The epidermal growth factor receptor (EGFR) is a kind of transmembrane phosphoglycoprotein that has been identified in almost all adult tissues with the exception of hematopoietic cells, and over expression and constitutive activation of EGFR as a hallmark of several solid human cancers, including squamous cell carcinoma [1–4]. The activation of EGFR would promote tumor cell proliferation [5], angiogenesis [6], metastasis, and inhibit apoptosis [7]. Hence, the EGFR is considered to be a primary target for antitumor treatment strategies [8]. Many known EGFR agonists including sorafenib [9], cetuximab [10], gefitinib [11] and the others [12–14] are selective for EGFR. EGFR gene was found in human epidermal squamous cells (A431 cells) as early as in 1984 [15]. The high expression of EGFR is currently recognized in A431 cells [16,17]. Therefore, A431 cell line will be an effective target cells enriched EGFR for screening the leading compounds of EGFR antagonists.

Since the 21st century, a variety of screening methods or techniques, such as a direct drug screen method based on target receptors or enzyme and virtual screen using computer-aided drug design [18,19], have been used for investigating leading compounds or drugs. However, there is little information about the screening models for directly finding out active compounds or components from a complex sample such as medicinal herbs. Since the 1990s, LC/MS methods have been widely used in life science to analyze complex samples [20]. With an increasing analytical requirement, the multi-dimensional HPLC (MD-HPLC), such as the common two-dimensional HPLC, has been established and used to separate medicinal herbs [21,22]. In our previous studies, we developed a cell membrane receptor affinity chromatography method known as cell membrane chromatography (CMC) [23–25] useful for studying drug–receptor interactions [26–29] and for investigating active components from TCMs [30–32], and interactions between drug and membrane receptor [30–32].

Radix Caulophylli is a kind of traditional medicinal herb and the alkaloids from *Radix Caulophylli* are known to have specific pharmacological activities [33]. In our previous works, we found that magnoflorine and caulophine from *Radix Caulophylli* alkaloids were identified as the active molecules acting on the α_{1A}AR [34]. For further study of the *Radix Caulophylli* alkaloids, the present work therefore aimed to establish an A431 cell membrane chromatography (A431/CMC) system for screening alkaloids from *Radix Caulophylli*. This was achieved by combining the CMC system with a two-dimensional liquid chromatography technique. The A431/CMC system was first validated using a known selective EGFR antagonist. A column switching technique was then used to combine the A431/CMC system with LC/MS analysis for the direct

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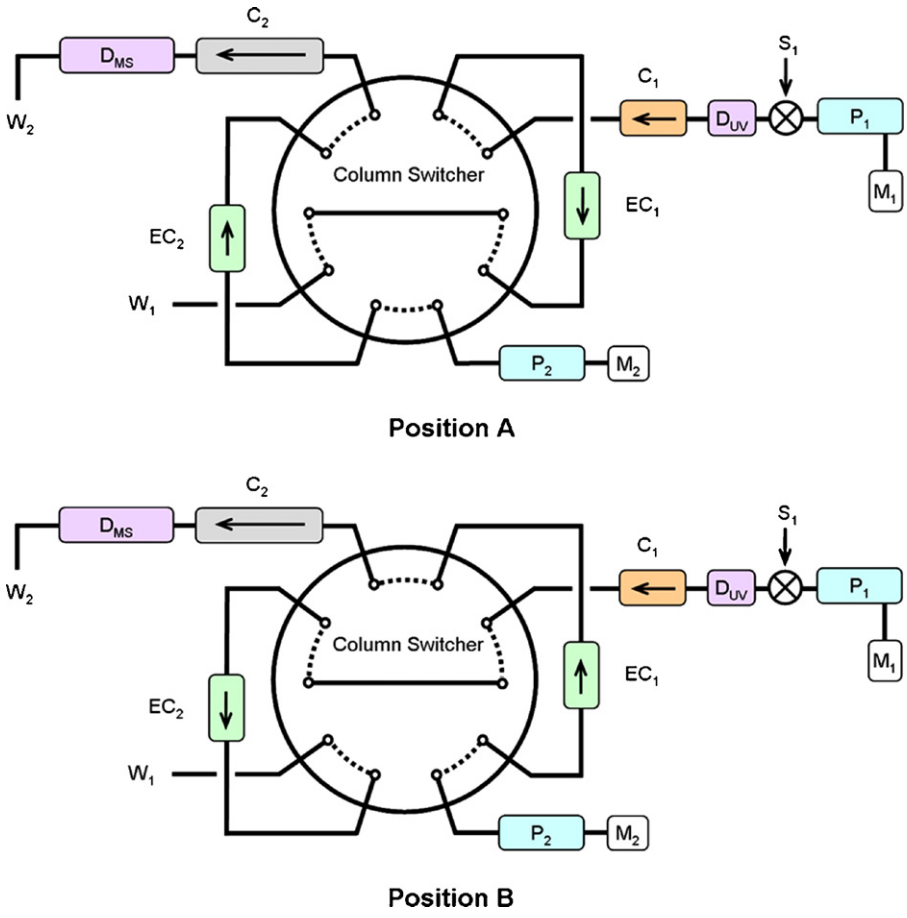

Fig. 1. Outline of the combined A431/CMC-online-LC/MS method. Position A: Affinity procedure using the A431/CMC system. The first retention fraction was captured into the first enrichment column (EC_1) after the A431/CMC column (C_1) from a complex sample (S_1); establishing the equilibrium procedure of LC/MS system from the second enrichment column (EC_2) to an analytical column (C_2) with mobile phase of second D. Position B: Analytical identification procedure using LC/MS. The first captured fraction was analyzed using C_{18}-LC/MS to separate and analyze their structural characteristics; the second retention fraction (if any) was captured into the second enrichment column (EC_2) after the A431/CMC column (C_1) from the same sample (S_1). D_{UV}, UV/Vis detector; D_{MS}, DAD and MS detector; P_1 and P_2, pumps.

separation and identification of active components. This method was successfully applied to the screening of active compounds present in a total *Radix Caulophylli* alkaloid preparation.

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). Sorafenib tosylate (SFR) was provided by Nanjing Ange Pharmaceutical Co. Ltd. (Nanjing, China). Nitrendipine (NIT), decaesadril (DEC), and tamsulosin hydrochloride (TAM) were purchased from National Institute of the Control of Pharmaceutical and Biological Products (Beijing, China). Taspine (TSP) and caulophine (CLP) were from Research and Engineering Center for Natural medicine, Xi'an Jiaotong University. A431 cell line was purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). GIBCO F12 medium was purchased from Invitrogen Corporation (Grand Island, NY, USA), 10% fetal calf serum was ordered from Lanzhou Minhai Co. Ltd. (Lanzhou, China), dimethylsulfoxide (DMSO), MTT and trypsin were from Sigma (Saint Louis, MO, USA). HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). *Radix Caulophylli* was collected in the Qinba Mountains area (Shaanxi province, China) in October 2008 and dried at room temperature. Herbs were authenticated by the Department of Pharmacognosy at 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 HPLC mass spectrometry system (LC/MS, Shimadzu Corporation, Kyoto, Japan) included three LC-20AD pumps, a DGU-$20A_3$ degasser, a SIL-20A autosampler, a CTO-20A column oven, a SPD-20A UV/VIS detector, a SPD-M20A diode array detector, a LCMS2010EV mass spectrometer and a LCMS solution workstation.

A $VICI_{AG}$ 10G-0911V 10 port 2-pos valve (Valco Instrument Co. Inc., Houston, USA) was used as the column switcher and two Shim-pack VP-ODS pre-columns (10 × 2.0 mm I.D., 5 μm, Shimadzu Corporation, Kyoto, Japan) were used as the enrichment columns. An A_{431}/CMC column (10 mm × 2.0 mm I.D.) was used as the first dimension column and a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm I.D., 5 μm, Kyoto, Japan) as the second dimension column, respectively.

2.3. Preparation of standard solutions

Standard stock solutions of SFR, NIT, DEC, and TAM (1 mg/mL each) were separately prepared in methanol. Standards solution (0.01 mg/mL) of SFR and a mixed standards solution (0.01 mg/mL) of SFR, NIT, DEC, and TAM were prepared in 5 mmol/L ammonium acetate water solution. Standard stock solutions of TSP, and CLP

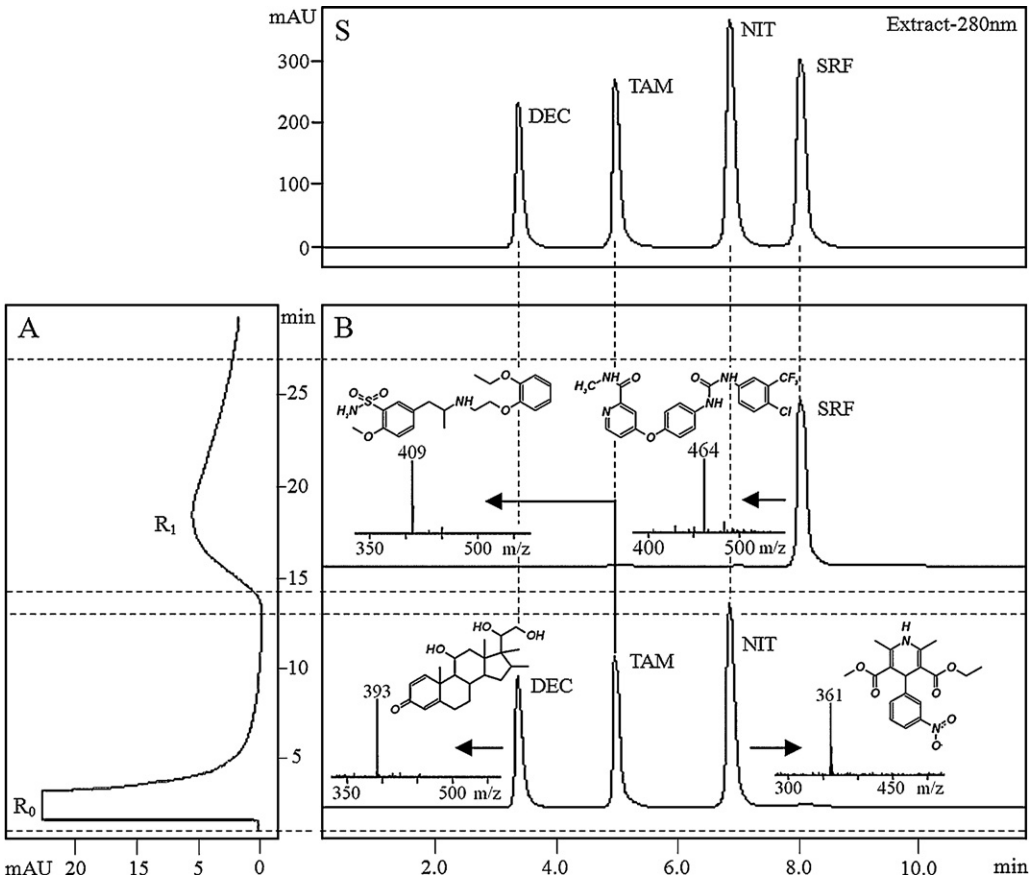

Fig. 2. Chromatograms of mixed standards using the combined A431/CMC-online-LC/MS method. (A) A431/CMC chromatogram of the mixed standards including R_0 and R_1 fractions (between two dotted lines). (B) LC/MS chromatograms of the fractions. (S) LC/MS chromatograms of mixed standards directly analyzed by LC/MS. DEC: decaesadril, TAM: tamsulosin hydrochloride, NIT: nitrendipine, and SFR: Sorafenib tosylate.

(1 mg/mL each) were separately prepared in methanol. The standard solutions (0.01 mg/mL) of TSP and CLP were prepared by diluting the standard stock solutions using 5 mmol/L ammonium acetate water solutions, respectively.

2.4. Sample preparation

Total alkaloid of *Radix Caulophylli* was prepared as references [34] as follows: air-dried root materials (3 kg) were ground and subsequently extracted with 95% aq. EtOH for 2 h and the process was repeated twice. The combined alcoholic extract was concentrated rotary evaporation under vacuum condition to obtain a dark brown viscous mass. The concentrated extract was extracted with 2% HCl overnight while being stirred and the extraction was repeated once. The combined acidic extract was passed through positive ion exchange resin (LSD001) column and was eluted with 4% NH_4OH–MeOH, following 2% HCl–MeOH until the elute was tested negative for alkaloids in the Dragendorff test. The elute was evaporated to dryness to yield total alkaloid of *Radix Caulophylli*. Sample solutions of *Radix Caulophylli* total alkaloids (1 mg/mL) were prepared in methanol and stored at 4 °C in the dark. Working solutions (0.1 mg/mL or 0.01 mg/mL) were diluted with mobile phase on the day of the experiment.

2.5. A431/cell membrane chromatography-online-LC/MS

The A431 cells were cultured in minimal essential medium (MEM; contained 10% fetal bovine serum). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells from exponentially growing cultures were used in all experiments. Cells were harvested using trypsin and incubated for 10 min at 4 °C. The harvested A431 cells (7×10^6) were washed three times with distilled water by centrifuging at 110 × *g* for 10 min at 4 °C. Tris–HCl (50 mM, pH 7.4) was added to produce an A431 cell suspension; the cells were then ruptured by ultrasonic procedure for 30 min. The resulting homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g* for 20 min at 4 °C. The precipitation was resuspended in 10 mL Tris–HCl (50 mM, pH 7.4), and the suspension was centrifuged at 12,000 × *g* again. A431 cell membrane suspension in 5 mL distilled water was obtained. According to the previously reported methods [23], A431 cell membrane stationary phase (CMSP) was prepared by the adsorption of cell membrane suspension (5 mL) on the activated silica (0.05 g) under vacuum and agitation conditions at 4 °C. The A431 CMSP was then packed into the column (10 mm × 2.0 mm I.D.) using a wet packing procedure. Other chromatographic conditions are as follows, 5 mmol/L ammonium acetate as a mobile phase, 0.2 mL/min flow rate, and column temperature at 37 ± 0.5 °C.

The HPLC conditions were a VP-ODS column (150 mm × 2.0 mm I.D., 5 μm), a mobile phase of methanol–water–ammonia (31:69:0.1, v/v), with 0.2 mL/min flow rate and column temperature at 37 °C. MS conditions were: 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, detector voltage 1.25 kV, positive ionization mode, scan range, 0–800 *m/z*.

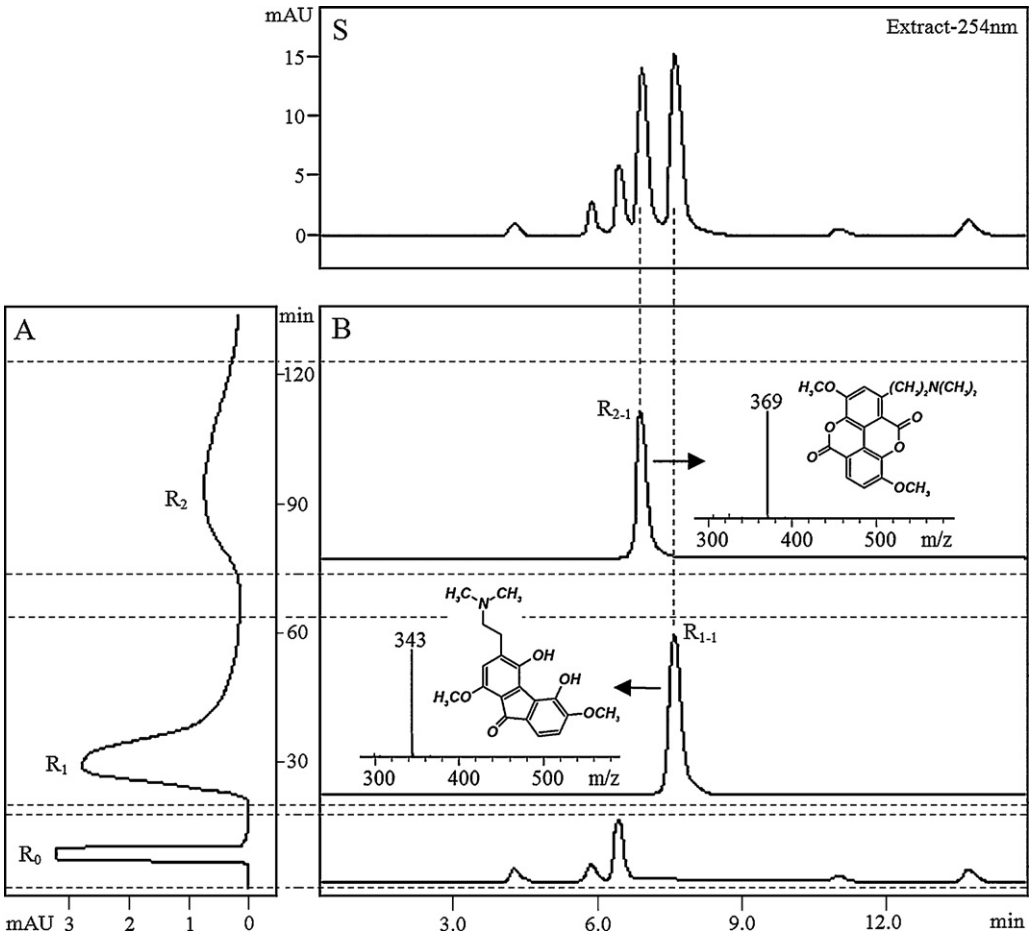

Fig. 3. Chromatograms of total alkaloids of *Radix Caulophylli* using the A431-CMC-online- LC/MS method. (A) A431/CMC chromatogram of total alkaloids. (B) LC/MS chromatograms of the fractions of R_0, R_1, and R_2 captured into the enrichment column (between two dotted lines). (S) LC/MS chromatogram of the total alkaloids directly analyzed by LC/MS.

2.6. Application of A431/cell membrane chromatography-online-LC/MS

The A431/CMC model was combined with HPLC/MS system by means of a 10-port column switcher in an online way. Any retention fraction on A431/CMC model was enriched onto a ODS pre-column (EC_1 or EC_2), and the enriched fraction was then eluted into HPLC/MS system for analysis. This A431/CMC-online-HPLC/MS method was used to screen EGFR antagonist using SFR as a positive control. Standard solutions and total alkaloids of *Radix Caulophylli* were analyzed separately.

2.7. Cell growth assay

The effect of SFR, CLP and TSP on A431 cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [35]. Briefly, exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well. After 24 h incubation at 37 °C, cells were treated with SRF, CLP and TSP at various concentrations for 48 h. 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 a microplate reader (Bio-RAD instruments, USA) at 490 nm and expressed as absorbance values.

2.8. FlexX dock

A molecular docking studies of SFR, TSP and CLP with EGF receptor tyrosine kinase (PDB ID: 1M17) and EGF receptor (PDB ID: 1VIO) were performed using SYBYL-X 1.1 to identify its binding mode with protein. The substrate was constructed with Sybyl/Sketch module and optimized using Powell's method. Energy minimization was performed using the Tripos force field with convergence criterion set at 0.005 kcal/(Å mol) and a maxiumum of 1000 iterations and Gasteiger–Hückel charges. A non-bonded cutoff distance of 8 Å was adopted to consider the intramolecular interaction [36]. The other docking parameters implied in the program were kept default.

3. Results and discussion

3.1. Combined A431/CMC-online-LC/MS

The A431/CMC-online-HPLC/MS method developed in this study was suitable for qualitative analysis of active components from complex samples. As shown in Fig. 1, at position A, the first retention fraction recognized by A431/CMC column (C_1) in the A431/CMC model was extracted onto an ODS pre-column (EC_1), and then at position B, the extracted components were pumped onto an ODS analytical column (C_2) for qualitative analysis. At the same time, the second retention fraction on C_1 was pumped onto another ODS pre-column (EC_2) and into the C_2 for analysis, alternately.

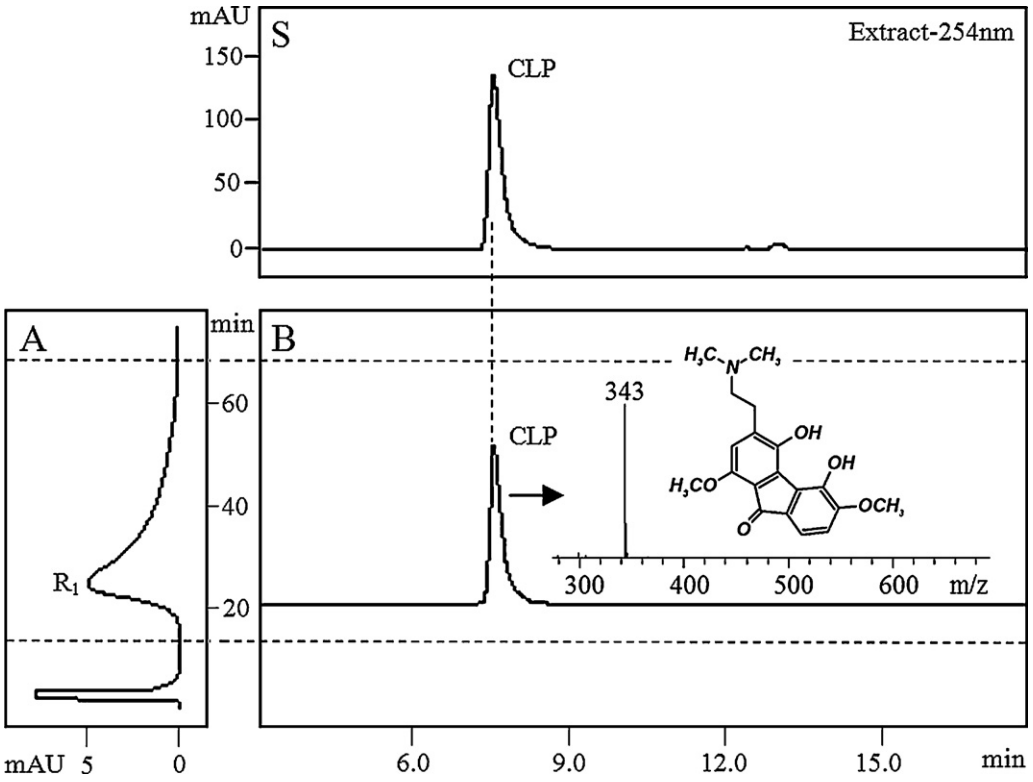

Fig. 4. Chromatograms of CLP standards using the combined A431/CMC-online-LC/MS method. (A) A431/CMC chromatogram of the CLP. (B) LC/MS chromatograms of the fraction R_1 captured into EC_1. (S) LC/MS chromatogram of the CLP directly analyzed by LC/MS.

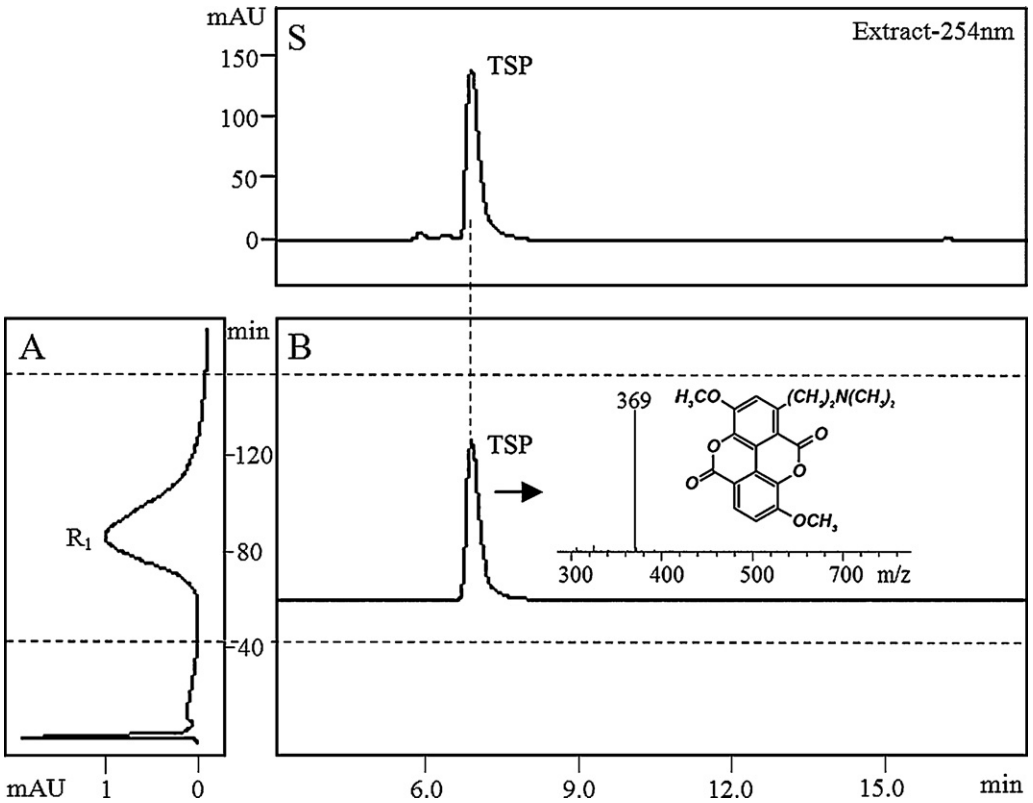

Fig. 5. Chromatograms of TSP standards using the combined A431/CMC-online-LC/MS method. (A) A431/CMC chromatogram of the TSP. (B) LC/MS chromatograms of the fraction R_1 captured into EC_1. (S) LC/MS chromatogram of the TSP directly analyzed by LC/MS.

A mixed standards solution containing SFR, NIT, MEC, and TAM was used to verify the specific selectivity of the A431/CMC-online-HPLC/MS system (Fig. 2). Of the four standard drugs, only SFR is a selective drug acted on EGFR. The chromatogram of the mixed standards solution on A431/CMC is presented in Fig. 2A. Two fractions (indicated by dotted lines) were sequentially extracted onto pre-columns and then switched onto column C_2 for chromatographic separation (Fig. 2B) and MS identification. This experiment demonstrated that SFR was specifically retained by the A431/CMC model from the solution of mixed standards and could simultaneously be analyzed by HPLC/MS system.

SFR standard solution was used to verify the sensitivity and stability of the A431/CMC-online-LC/MS system to "recognize" and identify target component. The retention time of SFR was 18 min. Repeatability and detection limit were studied as well. Intra-day and inter-day accuracy were studied by repeating injection of 5 μL 0.01 mg/mL SFR standard solution, retention time was used as indication. The results were 1.26% and 3.85% ($n = 5$), respectively. Detection limit was 0.1 μg/mL ($S/N = 3$).

3.2. Practical application

Alkaloids are the principal type of active components found in natural herbs. The A431/CMC-online-LC/MS method was applied to screen compounds from total alkaloids of *Radix Caulophylli* which can bind to EGFR. Chromatograms of total alkaloids obtained using the A431/CMC-online-LC/MS method is presented in Fig. 3. This revealed that there were two significant retention fractions (R_1 and R_2) (Fig. 3A); these fractions were captured and switched onto the LC/MS system online for further separation and identification. As shown in Fig. 3B, peak $R_{1\text{-}1}$ as a main component from R_1 fraction was identified as caulophine and peak $R_{2\text{-}1}$ from R_2 fraction was taspine. Compared with Fig. 3S, CLP and TSP were from the total alkaloids of *Radix Caulophylli*. The screening results on total alkaloids of *Radix Caulophylli* suggested that the main retention components belonged to the alkaloids of *Radix Caulophylli*.

In order to further verify the screening results above, finally, the standard solutions of CLP and TSP were analyzed using this A431/CMC-online-LC/MS method. As shown in Figs. 4 and 5, the main retention fractions on A431/CMC model were CLP and TSP.

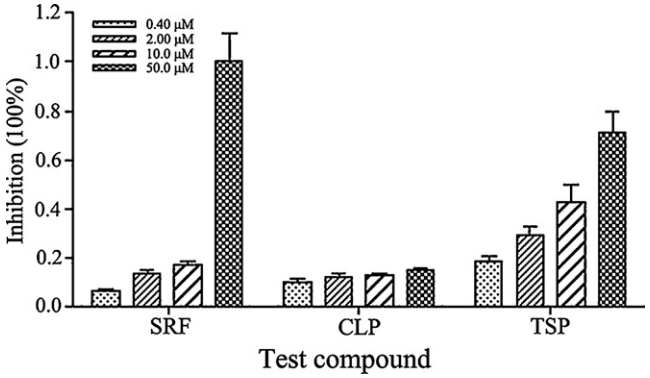

Fig. 6. The effect of SFR, CLP and TSP on A431 cell viability.

3.3. MTT assay

Inhibitory effects of CLP and TSP on A431 cell growth in vitro were tested. As shown in Fig. 6, TSP had inhibited A431 cell growth in a dose-dependent manner, although these actions were higher than that of SFR. However, CLP could not inhibit A431 cell growth. Retention time of TSP on A431/CMC was 92 min, compared with SFR of 18 min, which indicated a stronger interaction of TSP with EGFR. Retention behaviors of the two were correlated to their pharmacological activities.

3.4. Molecular docking assay

Docking of CLP and TSP in the active site of EGFR tyrosine kinase showed three H-bond interactions between oxygen of the inhibitor and residues of the active site. The result indicated that binding energy of CLP with EGFR tyrosine kinase was very low. By using the predicted binding energies of tyrosine kinase inhibitors as reference, CLP was predicted to target EGFR tyrosine kinase. Fig. 7 showed the docking model of SFR, CLP, and TSP into the EGFR tyrosine kinase and EGFR. Although the three compounds can bind to EGFR, but the binding sites of the compounds were different. This indicated why the activity of three compounds on A431 cell growth was different, and it needs further study.

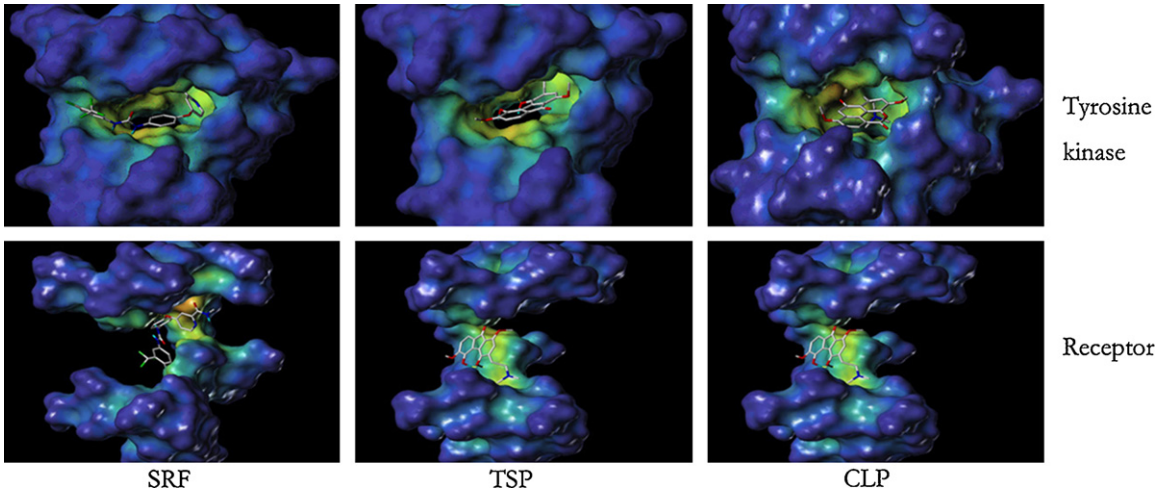

Fig. 7. SFR, TSP and CLP were docked into EGF receptor (PDB ID: 1IVO) and tyrosine kinase (PDB ID: 1M17).

4. Conclusion

In summary, we established an A431/CMC-online-LC/MS method for screening anti-EGFR antagonists from total alkaloids of *Radix Caulophylli*. This method can efficiently drive screening process by combining a specific recognition of A431/CMC model with an accurate identification of LC/MS online system. It will be a useful method in drug discovery with natural medicinal herbs as a leading compound resource.

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