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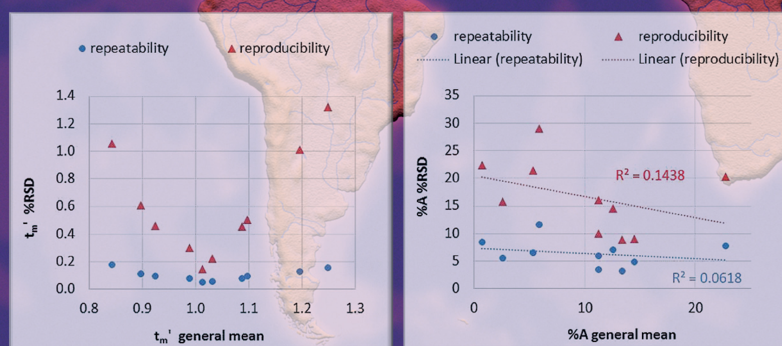

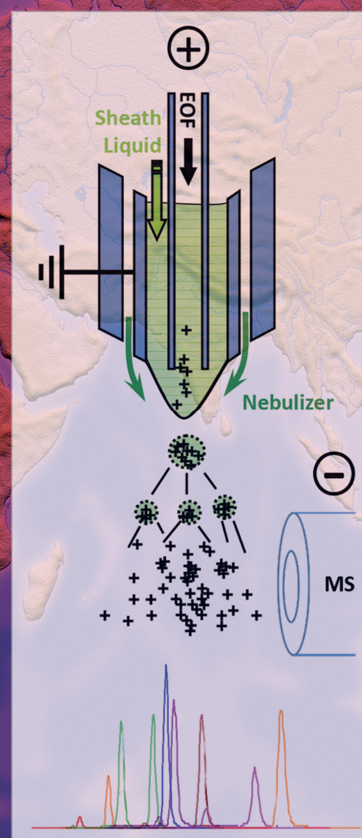

CE-MS interlaboratory study

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Research Article

Screening anti-tumor compounds from *Ligusticum wallichii* using cell membrane chromatography combined with high-performance liquid chromatography and mass spectrometry

Tyrosine 367 Cysteine-fibroblast growth factor receptor 4 cell membrane chromatography combined with high-performance liquid chromatography and mass spectrometry was developed. Tyrosine 367 Cysteine-HEK293 cells were used as the cell membrane stationary phase. The specificity and reproducibility of the cell membrane chromatography was evaluated using 1-*tert*-butyl-3-{2-[4-(diethylamino)butylamino]-6-(3,5-dimethoxyphenyl) pyrido[2,3-d]pyrimidin-7-yl}urea, nimodipine and dexamethasone acetate. Then, anti-tumor components acting on Tyrosine 367 Cysteine-fibroblast growth factor receptor 4 were screened and identified from extracts of *Ligusticum wallichii*. Components from the extract were retained on the cell membrane chromatographic column. The retained fraction was directly eluted into high-performance liquid chromatography with mass spectrometry system for separation and identification. Finally, Levistolide A was identified as an active component from *Ligusticum wallichii* extracts. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan colorimetric assay revealed that Levistolide A inhibits proliferation of overexpressing the mutated receptor cells with dose-dependent manner. Phosphorylation of fibroblast growth factor receptor 4 was also decrease under Levistolide A treatment. Flex dock simulation verified that Levistolide A could bind with the tyrosine kinase domain of fibroblast growth factor receptor 4. Therefore, Levistolide A screened by the cell membrane chromatography combined with high-performance liquid chromatography and mass spectrometry can arrest cell growth. In conclusion, the two-dimensional high-performance liquid chromatography method can screen and identify potential anti-tumor ingredients that specifically act on the tyrosine kinase domain of the mutated fibroblast growth factor receptor 4.

Keywords: Cell membrane chromatography / Fibroblast growth factor receptor 4 / High-performance liquid chromatography with mass spectrometry / Levistolide A / Mutations
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1 Introduction

Fibroblast growth factors receptors (FGFRs) belong to the tyrosine kinase receptor family. They mediated the fibroblast growth factor (FGF) signal from the outgoing cytoplasmic membrane to nuclear transferring. The signaling pathway for FGFs is through FGFRs that regulate fundamental physiological processes controlling a wide range of biological functions [1, 2] that include the regulation of embryonic development [3], wound repair, and cell proliferation and survival. Previous studies reported that FGF signaling plays an important role in tumorigenesis [4, 5]. FGFR4 high expression of breast cancer cells resistant to doxorubicin and cyclophosphamide is because of FGFR4 activating MAPK pathway, inhibiting cell apoptosis [6]. Tyrosine kinase receptor inhibitor such as Sunitinib and Lapatinib could inhibit P-gp, MRP, and BCRP activity function, increased the effective drug concentration within the tumor cells, induce apoptosis [7–9].

Recently, Oncogenome screening has identified Y367C mutation in FGFR4 in MDA-MB-453 cell line [10]. The FGFR4 mutant elicits constitutive phosphorylation leading to an

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Abbreviations: FGF, fibroblast growth factor; **FGFR4**, fibroblast growth factor receptor 4; **FRS2**α, FGFR substrate protein-2α; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan; **Y367C**, Tyrosine 367 Cysteine; **PD173074**, 1-*tert*-butyl-3-{2-[4-(diethylamino) butylamino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-d]pyrimidin-7-yl}urea; **CMC**, Cell membrane chromatography

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activation of the mitogen-activated protein kinase cascade. Thus, Y367C-FGFR4 is a potentially important drug target that enables the screening of binding with FGFR4 antagonist compounds.

Ligusticum wallichii belongs to Umbelliferae *artemisia*, and is well-known for its hematopoietic, antioxidant, immunoregulatory, and anti-cancer activities. Phytochemical investigations on this plant led to alkaloids, volatile oil, organic acid, lactone, and other metabolites [11]. 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 in *Ligusticum wallichii*. In our study, we aim to applying Y367C-FGFR4 cell membrane chromatography method to screen Y367C-FGFR4 inhibitor in *Ligusticum wallichii*.

CMC is a type of biological affinity chromatography and has been demonstrated to serve as an effective method for separating active component that act on a specific membrane receptor [12–16]. In our previous studies, we developed a CMC method in combination with over-expressed membrane receptor for screening active components in traditional Chinese medicine [17–20].

In the present study, we successfully developed a 2-D LC method by combining Y367C-FGFR4-CMC with HPLC–MS through a column switcher. For the first dimension of Y367C/FGFR4-CMC, the active components from the extract of *Ligusticum wallichii* are retained; the second dimension of HPLC–MS directly identifies the active components that are retained on the first dimension. This method potentially can collect important evidence for anti-tumor drug discovery in traditional Chinese medicines (TCMs).

2 Materials and methods

2.1 Chemicals and materials

Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical (Qingdao, China) and was activated at 105°C. Ligusticum wallichii was from Xi 'an medicine market. Levistolide A was from Shanghai sonon lai treasure biological technology, Shanghai (Shanghai, China). Nimodipine (lot number: N149), PD173074 was from SelleckChemicals (Lanmu chemical technology, Shanghai, China). Dexamethasone acetate was from Shanghai high gen chemical technology (Shanghai, China). Methanol was from American Burdick & Jackson. $Na_2HPO_4{\cdot}12H_2O$ and HCl were from Xi 'an chemical reagent factory. All aqueous solutions were prepared using ultrapure water that is produced by MK459 Millipore Milli-Q Plus ultra-pure water system.

2.2 Instruments

The HPLC–MS system (Shimadzu Corporation, Kyoto, Japan) included three LC-20AD pumps, a DGU-20A3 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 LC solution workstation. The first dimensional Y367C-FGFR4-CMC column (10.0 × 2.0 mm I.D. 5 μm) was packed using a RPL-10ZD column loading machine from Dalian Replete Science and Technology (Dalian, China). A VICIAG 10G-0911V ten-port two-position valve (Valco Instrument, 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, Kyoto, Japan) was used as enrichment column. An Y367C-FGFR4-CMC column (10.0 × 2.0 mm I.D. 5 μm) 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) represented the second dimension column.

2.3 Preparation of the standard solution and sample preparation

Standard solution of nimodipine was prepared in methanol (1 mg/mL). PD173074 was prepared in DMSO and then obtained standard stork solution of 10 mM. Standard solution of Levistolide A was prepared in DMSO (final concentration 20 mM).

Ligusticum wallichii was ground into power, 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; total extract were filtered by Buchner funnel. Then, the filtrates concentrated to dark brown viscous mass under conditions of reduced pressure at 60°C. The extracted was then dissolved in methanol as the sample and diluted with mobile phase for usage.

2.4 Preparation of Y367C-FGFR4-CMC column

Y376C-FGFR4 HEK293 cell was constructed in our laboratory [1]. $7{\times}10^6$ cells were cultured in DMEM containing 10% fetal bovine serum at 37°C in a humidity atmosphere with 5% CO_2. Cells were harvested and washed three times with phosphate buffered saline (PH 7.4; 50 mM) at 4°C. Then, cells were ruptured under ultrasonic conditions for 30 min in Tris-HCL (pH 7.4; 50 mM) buffer. Repeat for two times. The cell membrane suspension was centrifuged at 1000×g for 10 min at 4°C, and then the supernatant was centrifuged at 12 000×g for 20 min at 4°C. The precipitate was resuspended in 5 mL phosphate buffered saline (pH 7.4; 50 mM) and then we obtained the cell membrane suspension in 5 mL phosphate buffered saline (pH 7.4; 50 mM). Second, the cell membrane suspension was slowly added to activate the silica (0.05 g silica at 105°C for 1 h) under vacuum and agitation at 4°C for 30 min, and then left at 4°C overnight. Finally, the Y367C-FGFR4/CMSP was packed into the Y367C-FGFR4-CMC column (10 mm × 2.0 mm I.D., 5 μm) using the column-loading machine according to the wet packing procedure. The chromatographic conditions were the flowing: column oven at a temperature of 37°C, velocity of flow is 0.2 mL/min, SPD-M20A diode array detector. Data acquisition was executed using an UV detector at a wavelength of 326 nm.

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2.5 Y367C-FGFR4-CMC–HPLC–MS system

Y367C-FGFR4-CMC was combined with the HPLC–MS through a 10 port column switcher. Retained fraction on Y367C-FGFR4-CMC was enriched into ODS pre-column (PC), and then eluted into HPLC–MS for further separation and identification. The procedure was conducted follows previous study [20]. Briefly, two dimensions of the column were equilibrated with the column switcher in position A. The first dimension Y367C-FGFR4-CMC system was injected the sample, when the fraction separated by the Y367C-FGFR4-CMC was eluted, the column switcher immediately altered to position B and then fraction will be absorbed on the pre-column, where after the column switcher changed to position A. Secondly, the fraction of the pre-column was eluted into the HPLC–MS system for further separation and identification. Finally, the system returned to an equilibrium state for the next cycling operation.

The HPLC conditions were as follows: the first dimension conditions contain: Y367C-FGFR4-CMC column; mobile phase, phosphate solution (1 mM, pH7.4); flow rate, 0.2 mL/min; wavelength, 254 nm; temperature, 37°C. The second dimension conditions contain: Shim-pack VP-ODS reversed-phase column (150 × 2.0 mm I.D. 5 μm); mobile phase, methanol/water (65:35, v/v); flow rate, 0.2 mL/min; detector, SPD-M20A diode array detector and LCMS2010EV mass spectrometer. MS conditions were as follows: Ionization mode, ESI; nebulizer gas (N2, purity > 99.999%); flow rate, 1.5 L/min; drying gas (N2, purity > 99.999%); pressure, 0.1 MPa; curved desolvation line temperature, 250 °C; heat block temperature, 200 °C; interface temperature, 250 °C; detector voltage, 1.5 kV; curved desolvation line voltage, 10 V; positive ionization scan mode, scanning from m/z 200 to 800.

2.6 Validation of Y367C-FGFR4-CMC–HPLC–MS

The specificity of Y367C-FGFR4-CMC column was investigated. The standard solution of PD173074 (FGFR4 inhibitor), dexamethasone acetate (glucocorticoid receptor inhibitor), Nimodipine (calcium channel blockers) were analyzed. Reproducibility of HEK293-FGFR4-CMC–HPLC–MS was studied using PD173074. The RSD of retention times was represented as an important guideline. A standard PD173074 solution (5 μL, 1 mM) was injected into the same Y367C-FGFR4-CMC columns within 24 h five times. Additionally, we investigated the retention time for PD173074 on three separate Y367C-FGFR4-CMC columns.

2.7 Application of Y367C-FGFR4-CMC-HPLC–MS

Rhizoma *Ligustici wallichii* extract was screened on Y367C-FGFR4-CMC. As shown in Fig. 2, there is obvious retained peak on cell membrane column. the retention time was about 14.28 min. Combined with previous research that *Ligustici wallichii* has certain anti-tumor activity [11], we put rhizoma *Ligustici wallichii* for further analysis.

2.8 Cell viability assay

Drug sensitivity was analyzed using MTT colorimetric assay. Levistolide A was filtered through 0.22 μm micropore filter and stored at –20°C. The MDA-MB453 cells were cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum at 37°C in a humidity atmosphere with 5% CO_2. Cells were plated into 96-well plates with 8000 cells/well. When the cell confluences reach to 90%, different doses of Levistolide A were incubated for 24 h, respectively. Then, the medium was replaced by 200 μL MTT (5 mg/mL) diluted with fresh DMEM medium. Then, the plate was further incubated for 4 h at 37°C. The formazan crystals were dissolved in 150 μL of DMSO for determination absorbance at 490 nm by using microplate reader (Bio-rad, Carlsbad, CA).

2.9 FGFR4 activity detection

After incubation with different concentrations of Levistolide A for 24 h, the cells were washed twice with cold PBS and were incubated with cold lysis buffer (50 mM Tris/HCl [pH, 7.4], 150 mM of NaCl, 1 mM of EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% Nonidet-P40, and 0.1% SDS) supplemented with 0.1 mM DTT, protease inhibitor, and phosphatase inhibitor (Roche applied science, Mannheim, Germany) on ice for 30 min. 20 μg proteins were assayed using a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Chalfont, UK). After blocking with 5% non-fat milk, the membranes were washed using TBS containing 0.02% Tween 20 and probed with primary antibodies for p-FRS2α (1:1000) and GAPDH (1:100) followed by incubation with their respective horseradish peroxidase-conjugated secondary antibodies. Signals were assessed using an enhanced ECL chemiluminescence detection system (Pierce, Rockford, USA).

2.10 Molecular docking simulation

Molecular docking and virtual screening on account of molecular docking have become an integral part of many modern structure-based drug discovery efforts [21–23]. Protein-ligand docking is widely used in predicting interaction of ligand with protein [24]. Therefore, molecular level of protein-ligand docking of Levistolide A with FGFR4 tyrosine kinase domain was performed using SYBYL-X 1.1 to identify the protein-binding mode. Using the Sybyl/Sketch module constructed the substrate and Powell's method. Using the Tripos force filed with the convergence criterion performed the minimization set at 0.005 kcal/(Å mol) and the maximum set at 1000 iterations and Gasteiger–Hückel charges. To take into consideration the intermolecular interaction,

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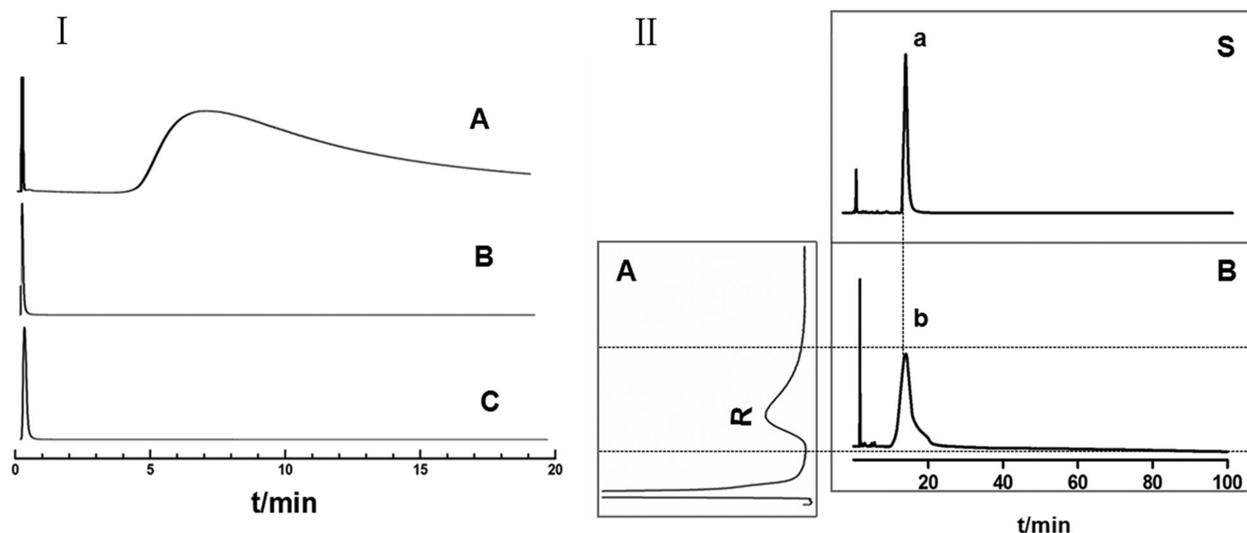

Figure 1. (I) Chromatography of PD173074, nimodipine and dexamethasone acetate on Y367C-FGFR4-CMC. (A) PD173074. (B) Nimodipine. (C) Dexamethasone acetate.
(II) Chromatography of PD173074 using Y367C-FGFR4-CMC–HPLC–MS method. (A) Y367C-FGFR4-CMC chromatogram of the positive medicine including R fractions. (B) HPLC–MS chromatogram of the positive medicine. (S) Reversed-phase separation chromatogram of positive medicine PD173074.

we adopted a non-bonded cut-off distance of 8 Å. Other docking parameters implied in the program were kept at default.

2.11 Statistical analyses

Using the GraphPad Prism 5.0 software plotted all data. Results are presented as mean±SD. The statistical significance of a result was determined to be $p < 0.05$.

3 Results and discussion

3.1 Characteristics of Y367C-FGFR4-CMC–HPLC–MS

The CMC column had biological activity. Therefore, the life time of CMC column should be taken into consideration. By repeated injecting PD173074 into CMC column, after 24 h use, PD173074 had a distinct retention on CMC column. Therefore, all of the CMC columns below were used only in 24 h after they were packed to ensure activity.

The Y367C-FGFR4-CMC-HPLC–MS method (a 2-D LC method) developed in this study was suitable for qualitative analysis of active components from the complex samples. When the first retention fraction recognized in the Y367C-FGFR4-CMC model was extracted onto an ODS pre-column, and then the extracted components were pumped into an ODS analytical column for qualitative analysis. At the same time, the second retention fraction was pumped onto another ODS pre-column and into it for analysis, alternately. This method is not only screening FGFR4 antagonists from the traditional Chinese medicine by the Y367C-FGFR4 model, but also can accurate identification by the HPLC–MS online system. The procedures of recognition, separation and identification for target components from complex sample can completed online. So, this method can effects screened active components and then were tested in cell level.

Specificity of Y367C-FGFR4-CMC was firstly investigated. PD173074 could be specifically retained and identified. PD173074 is a tyrosine kinase inhibitor for FGFR4 [10]. So, PD173074 used as positive controls and nimodipine and dexamethasone acetate as negative controls. The other components did not retain on the CMC column. The mixed standards solution was performed on the Y367C-FGFR4-CMC as shown in Fig. 1. PD173074 retained on Y367C-FGFR4-CMC and the retention time was about 7 min. Meanwhile, nimodipine and dexamethasone acetate cannot be retained (Fig. 1I). This indicated that Y367C-FGFR4-CMC could identify small molecular ingredients acting on tyrosine kinase domain of FGFR4.

The retained component on cell membrane chromatography column was enriched through pre-columns and then switched into HPLC–MS for separation and identification. The results indicated that PD173074 specifically retained by the Y367C-FGFR4-CMC column and could be further detected using the HPLC–MS system (Fig. 1II). This indicated that the Y367C-FGFR4-CMC–HPLC–MS was able to identify small molecular candidates that act on the tyrosine kinase domain of mutated FGFR4. We speculated that the Y367C-FGFR4-CMC column is a tyrosine kinase receptor specific affinity column that suitable for the screening of a potential tyrosine kinase inhibitor.

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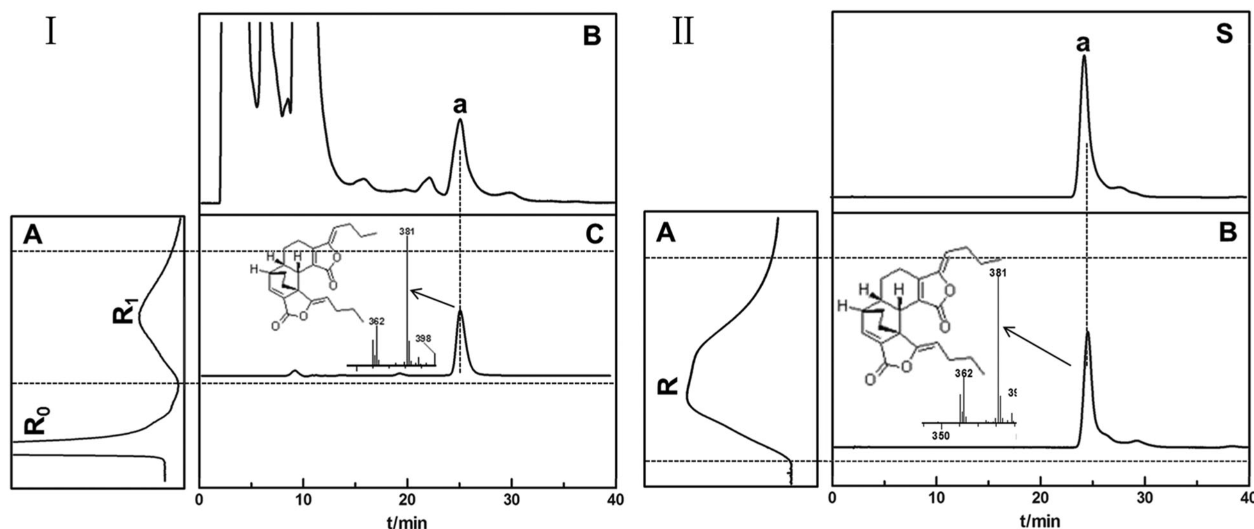

Figure 2. (I) Chromatography of Rhizome of *Ligusticum wallichii* extract using the combined Y367C-FGFR4-CMC–HPLC–MS method. (A) Y367C-FGFR4-CMC chromatogram of Rhizome of *Ligusticum wallichii* extract, retain components is R1. (B) HPLC–MS chromatogram of R1. (S) Reversed-phase separation chromatogram of Rhizome of *Ligusticum wallichii* extract was presumed to the Levistolide A. (II) Chromatography of the reference substance of Levistolide A using the combined Y367C-FGFR4-CMC–HPLC–MS method. (A) Y367C-FGFR4-CMC chromatogram of reference substance, retain components is R. (C) HPLC–MS chromatogram of R. (B) Reversed-phase separation chromatogram of *Ligusticum wallichii* extract is Levistolide A.

3.2 Screening components from *Ligusticum wallichii*

5 μL extracts of *Ligusticum wallichii* were injected into the Y367C-FGFR4-CMC–HPLC–MS system. One retention R1 (Fig. 2I) was observed on the chromatography, retention time of retained fraction was about 14.28 min, and further MS analysis revealed the retained component was supposed to be Levistolide A. To confirm the structure of R1, we investigated chromatogram of standard Levistolide A. First, the standard Levistolide A injected in the Y367C-FGFR4-CMC column to confirm whether Levistolide A could be retained on Y367C-FGFR4-CMC column. As the results shown in Fig. 2II, Levistolide A can be well retained on the Y367C-FGFR4-CMC–HPLC–MS column with a retention time similar to the total extract of *Ligusticum wallichii*. Then, HPLC–MS chromatogram of Levistolide A gave the one major chromatographic peak compare with those of the HPLC–MS chromatogram of the R1 fraction. It indicated that R1 fraction retained on CMC column was Levistolide A. According to Fig. 2, Y367C-FGFR4-CMC–HPLC–MS identifies Levistolide A from the extract of *Ligusticum wallichii*. Therefore, this method constructs a screen model for active components acting on mutated FGFR4 from medicinal herbs.

3.3 Inhibitory effects of Levistolide A

Levistolide A was screened and identified from *Ligusticum wallichii*. Thus, the biological activity was studied in present study. MTT was adopted to investigate the activity of Levistolide A on Y367C-FGFR4 overexpressing cells MDA-MB-453. As the results shown in Fig. 3, Levistolide A inhibits MDA-MB453 cell growth with dose dependent manner (Fig. 3I). The IC50 of Levistolide A is about 32.3 μM. This indicated that the method for screening active compounds from *Ligusticum wallichii* is feasible and efficient.

3.4 Inhibitory effect on phosphorylation of FGFR4

Whether cell growth arrest induced by Levistolide A is caused by FGFR4 was studied. Phosphorylation of the docking protein FRS2α contributes to FGFR-mediated cell proliferation and migration [25,26]. Levistolide A arrest phosphorylation of FRS2α in MDA-MB-453 cell (Fig. 3II). It indicated that Levistolide A have inhibitory effect on FGFR4-mediated signal transduction. Thus, we argued that Levistolide A could bind with FGFR4 for attenuating its catalytic activity.

3.5 Interaction simulation of Levistolide A with FGFR4

To investigate the possible binding modes of Levistolide A, SYBYL-X1.1 were performed the simulated docking of Levistolide A with FGFR4. The binding site of Levistolide A and PD173074 on FGFR4 were shown in Fig. 4. The docking results indicated that hydrogen bonds were highly correlated with the affinity of legend with FGFR4. Levistolide A and PD173074 docked into the cavity of the FGFR4 tyrosine kinase region. In proximity to the active site, Levistolide A showed a better affinity compared with PD173074. Therefore, we could predict that Levistolide A exhibits a good fit with the tyrosine kinas region of FGFR4. This binding hypothesis may provide valuable information for the structure-based design for

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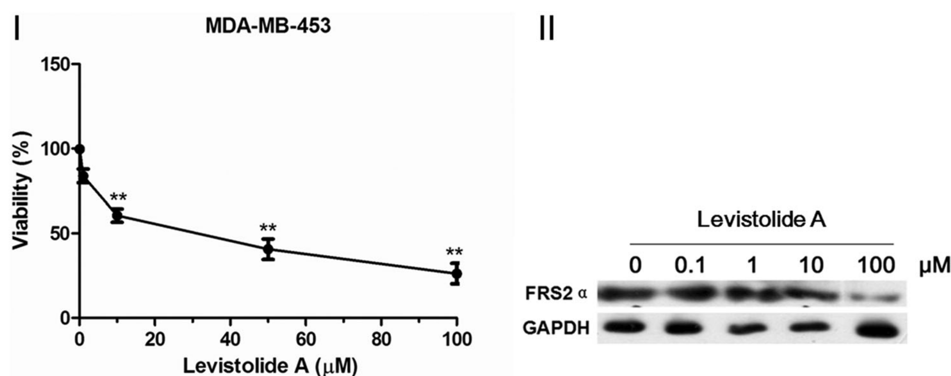

Figure 3. The biological effect of Levistolide A on MDA-MB-453 cell. (I) MTT assay for cell variability on MDA-MB-453 cell. Each value represents the mean ± SD, $n = 3$. ** represented as $p<0.01$ vs control group. (II) Western blot analysis of FRS2α phosphorylation level.

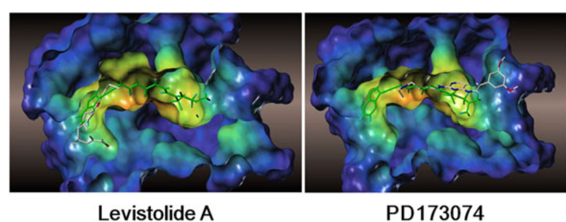

Figure 4. Surfex docked conformation of Levistolide A and PD173074 in the active site of (PDB ID: 4QRC). Binding model of Levistolide A was depicted by MOLCAD surface (left). (B) Binding model of PD173074 was depicted by MOLCAD surface (right).

Levistolide A derivatives acting as a potent small molecular tyrosine kinase inhibitor of FGFR4.

4 Concluding remarks

In summary, we established an Y367C-FGFR4-CMC–HPLC–MS method to screen anti-Y367C-FGFR4 antagonists from total *Ligusticum wallichii* extract. This method has the potential to improve the efficiency of the screening process by combining tyrosine kinase domain recognized Y367C-FGFR4-CMC column with HPLC–MS system. The active compound of total extract of *Ligusticum wallichii* was successfully distinguished through this Y367C-FGFR4-CMC–HPLC–MS system. Levistolide A was further screened and identified through a 2-D online CMC–HPLC–MS system. The flex dock simulation conformed that Levistolide A has same acting domain as PD173074 on tyrosine kinase domain of FGFR4. Meanwhile, using MTT certificated the inhibition activity of Levistolide A. The Y367C-FGFR4-CMC–HPLC–MS system could be used as a screening tool for active compound that could be interacted with Y367C-FGFR4 from natural medicinal herbs.

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The authors have declared no conflict of interest.

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