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Establishment of the model of vascular endothelial cell membrane chromatography and its preliminary application

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A model of vascular endothelial cell membrane chromatography was established by using an ECV304 cell membrane stationary phase (ECV304 CMSP) prepared by immobilizing the ECV304 cell membrane onto the surface of silica carrier. The surface and chromatographic characteristics of ECV304 CMSP were studied. The active component from *Caulophyllum robustum* was screened by using the model of vascular endothelial cell membrane chromatography. The interaction between the active component and membrane receptor was determined by using a replace experiments. The effect of the active component was tested by using tube formation of ECV304 cell. The results indicated that the model of ECV304 cell membrane chromatograph (ECV304 CMC) can stimulate the interaction between drug and receptor *in vitro* and the retention characteristics of taspine as active component was similar to that of model molecule in the model of ECV304 CMC. And therefore, taspine acted on VEGFR2 and inhibited the tube formation of ECV304 cell induced by VEGF. This model can be used to screen definite active component as a screening model.

vascular endothelial cell, cell membrane chromatography, angiogenesis

Radioligand binding assays are the traditional methods for studying the affinity of drug and receptor. Cell membrane chromatography (CMC), a new bio-affinity chromatography technique pioneered by He et al.[1−3], can be used to effectively study the interaction between drug and receptor by the chromatographic characteristics of drug on the stationary phase prepared by immobilizing cell membrane onto the surface of silica carrier. Zhang et al.[4−7] have demonstrated that CMC method can be used to evaluate the affinity of drug and receptor, and the affinity of drugs is similar to and correlates well with that obtained from radioligand binding assays. At present, CMC method has been successfully used to screen the active component from nature product[8].

Tumor growth and metastasis are angiogenesis-dependent[9−11]. Although tumor angiogenesis is a complex process, pro-angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin (Ang) are one of the important factors that initiate angiogenesis[12−15]. During angiogenesis, VEGF from tumor cell selectively binds to the receptor expressed by endothelial cell, and then receptor tyrosine kinase is activated and angiogenesis is started[16−18]. Therefore, it is a meaningful pathway of finding angiogenesis inhibitor to screen the compound that can bind to vascular endothelial growth factor receptor (VEGFR).

In this study, we developed a new model of vascular endothelial cell membrane chromatography by means of the CMC methods and anti-VEGFR1 antibody and anti-VEGFR2 antibody as model molecule. It was used to screen the active component from *Caulophyllum robustum* located at Shannxi province. And the effects of

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the active component were tested.

1 Experimental

1.1 Instruments and materials

The chromatographic system consisted of a SPECTRA SERIE P200 chromatographic pump, a SPECTRA 100 detector (Thermo Separation Products, USA), a 7125 hand sampling valve (Rheodyne, USA), and an ANASTAR chromatographic work station (AOTAI Tequenology Co. limited, Tianjin). Also employed in this study were an HERMLE ZK-401 high speed freezing centrifugal machine (Germany), a Heraeus CO_2 incubator (Germany), an XDS-1B inverted microscope (China), a Philips XL series 7XL20 scanning electron microscope and an EDAX DX-4 energy spectrometer (Holland).

An endothelial cell line derived from human umbilical vein endothelial cell (HUVEC), ECV304, was purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences (Shanghai, China).

Fetal bovine serum was purchased from Hangzhou Sijiqing Serum Works. RPMI1640, DMEM and trypsin were purchased from Sigma (USA). Anti-VEGFR1 antibody and anti-VEGFR2 antibody were purchased from Santa Cruz (USA). Matrixgel and VEGF were purchased from BD (USA). DAB immunohistochemistry kit (Wuhan Boshide Bioengineering Institute, China) and protein determination kit (Nanking Jiancheng Bioengineering Institute, China) were used. Na_2HPO_4 was of analytical grade (Xi'an Chemical Reagen Factory, China). Column chromatography silica gel (Qingdao Ocean Chemical Plant, China) and macroporous ball silica (3—5 μm, 100 Å) (Institute of Chemistry of the Chinese Academy of Sciences, China) were used. *C. robustum* was identified by Pharmacognostical Lab of College of Pharmacy, Xi'an Jiaotong University.

1.2 Establishment of the model of ECV304 CMC

(i) Cell culture. The ECV304 cell derived from human umbilical vein endothelial cell were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 100 $IU \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, and 200 $mmol \cdot L^{-1}$ L-glutamine (pH 7.4) in a humidified incubator under 37℃ and 5% CO_2[19,20]. The cells were routinely passaged at 90% confluence.

(ii) Immunohistochemistry. After ECV304 cells were maintained in exponential growth, the cells were digested by 0.25% trypsin and prepared to single cell suspension. The cells were seeded onto cover slip (7×22 mm) which was put into 12-well plates and incubated for 2 d. After washing with 0.1 mol/L PBS, the cover slips were fixed in 0.25% glutaraldehyde for 30 min at 4℃, and then were blocked for 10 min in 3% H_2O_2 in distilled water at room temperature for the inactivation of endogenous peroxidase. For immunostaining, blocking was carried out with 1∶10 normal serum confining liquid in PBS for 20 min at room temperature. The cover slips were then incubated with anti-VEGFR1 antibody and anti-VEGFR2 antibody (1∶100) for 2 h at 37℃. The cover slips were washed with 0.1 mol/L PBS and reacted with biotin-conjugated polyclonal goat antimouse immunoglobuline and SABC fluid. DAB was used for staining. The cover slips were washed with distilled water, dehydrated in a graded series of alcohol, and then cleared in xylene. They were covered with cover slips.

(iii) Preparation and chromatographic characteristics of ECV304 CMSP. After ECV304 cells were cultured until 80% confluent, the cells were digested with 0.25% trypsin and then the cells suspension was centrifuged at 1000 *g* for 10 min. The precipitate was resuspended in deionize water and stood for 30 min, and then sonicated for 10 min and centrifuged at 2000 *g* for 20 min. The supernatant was centrifuged at 12000 *g* for 20 min. The precipitate was ECV304 cell membrane and all procedures were performed at 4℃[21].

0.3 g activated silica was added to a 10-mL reaction tube and then the suspension solution of ECV304 cell membrane was slowly added to it under the evacuation condition at 4℃. The adsorption of the cell membranes on the activated silica surface was taken for 1 h until equilibrium was reached. Then, the supernatant in the tube was removed by centrifugation and the ECV304 CMSP was washed with Tris-HCl buffer until there was no residually free cell membrane on it.

The surface characteristics of ECV304 CMSP were analyzed by a scanning electron microscope and a surface energy spectrometer.

Chromatographic conditions. The ECV304 CMSP dispersed in a buffer was packed into the column (30 mm×2 mm, I.D.) by slurry method under a low pressure. Mobile phase was a 50 $mmol \cdot L^{-1}$ phosphate buffer solution (pH 7.4) with a 0.5 $mL \cdot min^{-1}$ flow rate at 37℃ column temperature. The detection wavelength was 254 nm. It took 3—4 h to establish equilibrium of the chromatographic system before injection. 5μL anti-VEGFR1

antibody and anti-VEGFR2 antibody were injected separately, and the pure silica stationary phase was used as negative control.

The amount of membrane protein of ECV304 cell membrane stationary phase was measured by Lowry et al.'s method[22] after it had been used for 3 d in order to investigate the stability of ECV304 cell membrane stationary phase column. In this experiment, five similar columns packed with the same ECV304 CMSP were tested by dividing the ECV304 CMSP in one column into two parts (pre-half part and post-half part).

1.3 Screening of the model of ECV304 CMC

(i) Preparation and screening of analytical sample. The total alkaloids from *C. robustum* were prepared according to ref. [23]. The total alkaloids from *C. robustum* were subjected to column chromatography over silica gel using a gradient of chloroform, methanol and ammonia water (from 10∶1∶0.1 to 2∶1∶0.1) as eluent. The eluate was collected by each 100 mL and detected with TLC. The eluates containing the same components were combined. The total alkaloids from *C. robustum* were isolated into 24 fractions labeled HMQTA 01－24 respectively. HMQTA05 was concentrated to offer a white solid substance, which was recrystallized repeatedly to give a white amorphous substance: m.p. 372－374℃; UV($\lambda_{max}^{methanol}$): 245, 285, 333, 348; IR(KBr)ν: 3066, 2941, 2764, 1730, 1597, 1472, 1436, 1288, 1136, 1092 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$)δ: 8.21(*d*, J=8.8 Hz, 1H), 7.31(*d*, J=8.8 Hz, 1H), 7.20 (s, 1H), 4.10 (s, 6H), 3.53 (*t*, J=7.6 Hz, 2H), 2.68 (*t*, J=7.6 Hz, 2H), 2.41 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 158.7, 157.7, 151.2, 151.0, 144.3, 137.9, 136.8, 126.9, 119.1, 118.5, 116.5, 113.6, 111.6, 109.2, 60.3, 56.6, 56.5, 45.3, 33.0; MS $C_{20}H_{20}NO_6$ (M + H) 370.1291. The above data agreed with the data of taspine in literature report[24,25]; thus the compound was confirmed as taspine.

Using anti-VEGFR1 antibody and anti-VEGFR2 antibody as a control, 5 μL of analytic sample solutions of HMQTA 01－24 were injected into the column of ECV304 cell membrane stationary phase. Chromatographic conditions had been described in (iii) of subsec. 1.2.

(ii) Replacement experiment. Taspine was added to a 50 mmol·L^{-1} phosphate buffer solution (pH 7.4) to prepare taspine solutions of different concentrations. Using these solutions as mobile phases[26], after ECV304-CMC system achieved equilibrium, 5 μL anti-VEGFR1 antibody and anti-VEGFR2 antibody were injected into ECV304-CMC system separately and the capacity factor (k') of anti-VEGFR1 antibody and anti-VEGFR2 antibody were determined.

$$k' = (t_R - t_0)/t_0$$

t_R is the retention time of solute, and t_0 is the elution time of no-retention solvent.

1.4 Tube formation assay

A 96-well plate coated with 0.1 mL matrigel per well was allowed to solidify at 37℃ for 1 h. Each well was seeded with 1×10^4 ECV304 and cultured in DMEM containing 5 ng·mL^{-1} VEGF and various concentrations of taspine or 0.1% DMSO (v/v) for 24 h. The enclosed networks of tubes were photographed from five randomly chosen fields under a microscope. The total length of the tube structures in each photograph was measured using Adobe Photoshop software[27]. Inhibition of tube formation was calculated as [1–(tube $length_{treated}$/tube $length_{control}$)]×100%.

2 Results and discussion

2.1 The characteristic of ECV304 CMSP

(i) ECV304 cell expressed VEGFR1 and VEGFR2. As shown in Figure 1(a), ECV304 cell was confluent and formed a consecutive cell monolayer which arranged like "pebble path". As shown in Figure 1(b)－(d), ECV304 cell expressed VEGFR1 and VEGFR2. According to immunology theory, the results indicated that anti-VEGFR1 antibody and anti-VEGFR2 antibody can specially bind to VEGFR1 and VEGFR2 and there is special affinity between antibody and receptor.

(ii) The surface characteristic of ECV304 CMSP. The results, shown in Figure 2(c) and (d), indicated that the surface of silica carrier was completely covered and integrated with ECV304 cell membrane and the surface of ECV304 CMSP was very different from that of pure silica carrier. Therefore, under the above conditions, the surface of silica carrier was adsorbed and completely covered with ECV304 cell membrane to form ECV304 CMSP. As shown in Figure 2(a) and (b), from the surface energy spectrum of the silica carrier there were a low peak of oxygen (O) and a high peak of silica (Si). But from that of the ECV304 CMSP there were not only a new peak of carbon (C) but also the silica (Si) peak just disappearing. The results further demonstrate that

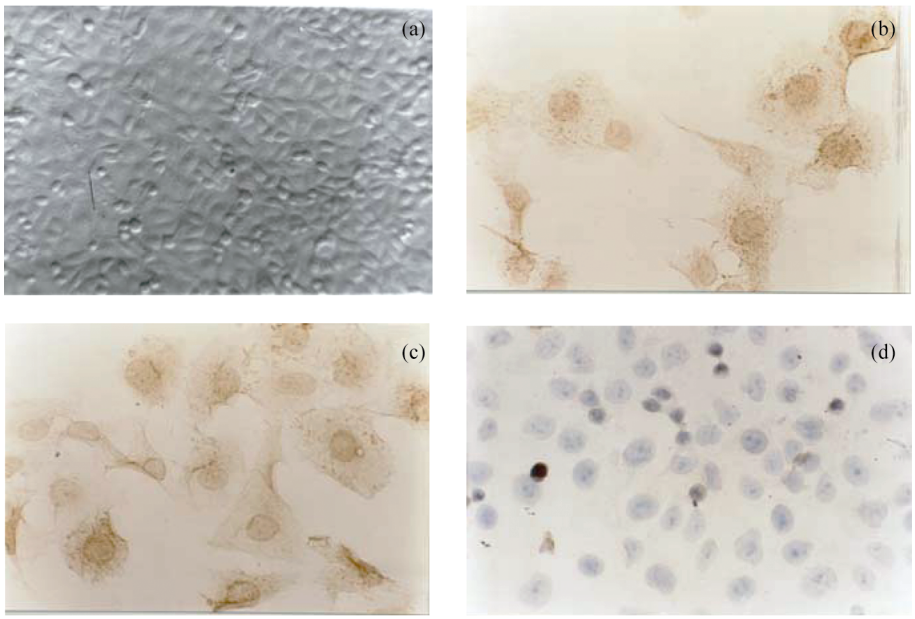

Figure 1 ECV304 cell and immunohistochemistry figure. (a) Human umbilical vein endothelial cell ECV304 (10×10); (b) ECV304 expressed VEGFR1 (S_{ABC}20×10); (c) ECV304 expressed VEGFR2 (S_{ABC}20×10); (d) negative control (S_{ABC}20×10).

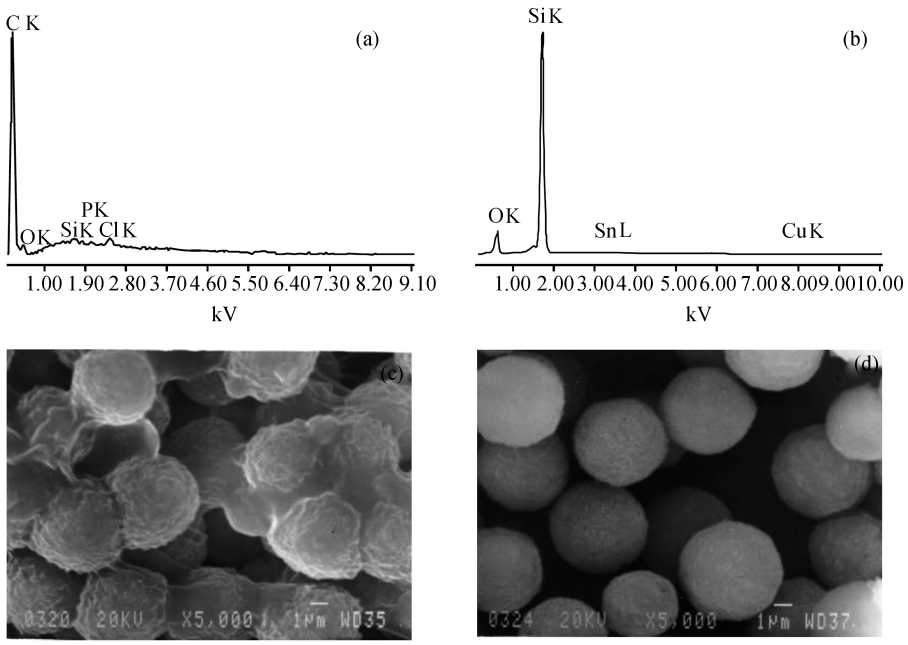

Figure 2 The surface characteristic of ECV304 cell membrane stationary phase. (a) Energy spectrum of ECV304 cell membrane stationary phase; (b) energy spectrum of pure silica carrier; (c) scanning electron micrograph of ECV304 cell membrane stationary phase (×5000); (d) scanning electron micrograph of pure silica carrier (×5000).

the surface of silica carrier was completely covered by the cell membranes.

Five similar columns packed with the same ECV304 CMSP were tested by dividing the ECV304 CMSP in one column into two parts (pre-half part and post-half part). The average amount of membrane protein from five measurements was 26.7±3.0 $mg \cdot g^{-1}$ for the pre-half part and 28.3±3.8 $mg \cdot g^{-1}$ for the post-half part of the ECV304 CMSP. There was no significant difference between two parts of the ECV304 CMSP in the column. The results indicated that the cell membrane on ECV304 CMSP was stable enough not to be eluted from the

pre-tip to the post-end of the column with flowing mobile phase.

(iii) The biologic recognition of ECV304 CMSP. Under the identical chromatographic conditions, shown in Figure 3, anti-VEGFR1 antibody and anti-VEGFR2 antibody had chromatographic retention in ECV304 CMSP system, but they did not have chromatographic retention in pure silica stationary system. The results indicated that the affinity between antibody and receptor was exhibited by the chromatographic retention of antibody in ECV304 CMSP system and ECV304 cell membrane stationary phase had a bio-affinity.

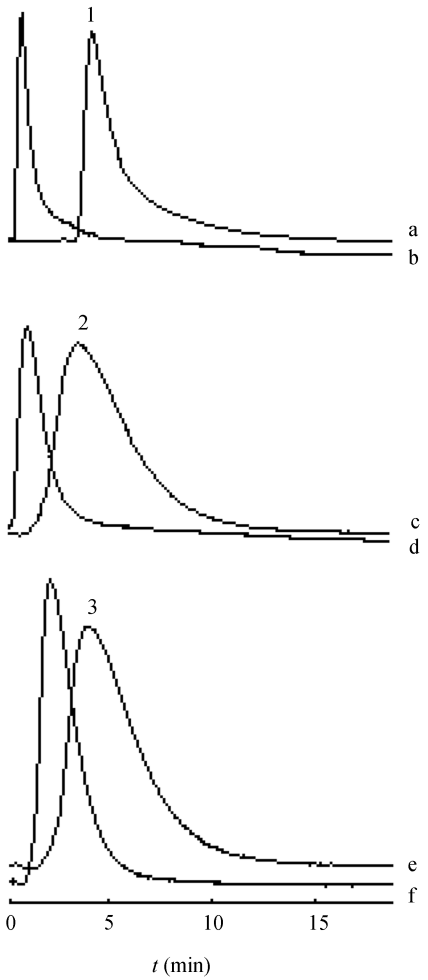

Figure 3 The chromatogram of anti-VEGFR1antibody, anti-VEGFR2 antibody and taspine. a, The chromatogram of anti-VEGFR1 antibody on ECV304 cell membrane stationary phase; b, the chromatogram of anti-VEGFR1 antibody on pure silica stationary phase; c, the chromatogram of anti-VEGFR2 antibody on ECV304 cell membrane stationary phase; d, the chromatogram of anti-VEGFR2 antibody on pure silica stationary phase; e, the chromatogram of taspine on ECV304 cell membrane stationary phase; f, the chromatogram of taspine on pure silica stationary phase. 1, Chromatographic peak of anti-VEGFR1 antibody; 2, chromatographic peak of anti-VEGFR2 antibody; 3, chromatographic peak of taspine.

2.2 The screening of the model of ECV304 CMC

(i) Screening of active component from the total alkaloids from *C. robustum*. The premise of drug's pharmacodynamic action is the interaction between drug and receptor. So drug does not have pharmacodynamic action unless drug binds to receptor. The model of CMC can screen the component binding to cell membrane and its receptor according to its chromatographic retention characteristic. Therefore, the model of CMC can be used to screen lead compound. Zhang et al.[4−8] have demonstrated that the chromatographic retention characteristic of active component in the model of CMC correlates with biological activity. The component that has chromatographic retention has some activity, but the component that does not have chromatographic retention does not have corresponding activity.

As shown in Figure 3, taspine had similar chromatographic retention characteristics as anti-VEGFR1 antibody and anti-VEGFR2 antibody in ECV304 CMC system, but did not have chromatographic retention in pure silica system. The result indicated that taspine can act on ECV304 cell membrane and its receptors. But other isolated fractions did not have retention in ECV304 CMC system, so the components of other isolated fractions cannot act on ECV304 cell membrane and its receptors. Therefore, taspine is the active component.

(ii) The comparison of interaction between taspine and ECV304 cell membrane and its receptors. Using taspine solutions of different concentrations as mobile phase, the capacity factor (k') of anti-VEGFR1 antibody and anti-VEGFR2 antibody are listed in Table 1. The values of k' of anti-VEGFR1 antibody hardly changed and the value of k' of anti-VEGFR2 antibody decreased when the concentration of taspine increased, indicating that the binding between anti-VEGFR2 antibody and VEGFR2 on the ECV304 cell membrane could be replaced by taspine. So taspine maybe acts on VEGFR2.

Table 1 The comparison of interaction between taspine and ECV304 cell membrane and its receptors

Concentration of taspine(10^{-6} mol·L^{-1})	Capacity factor (k')	
	anti-VEGFR1 antibody	anti-VEGFR2 antibody
0	3.5	3.3
2.71	3.4	3.1
5.41	3.2	2.3
10.8	3.4	1.0
21.7	3.3	0.6

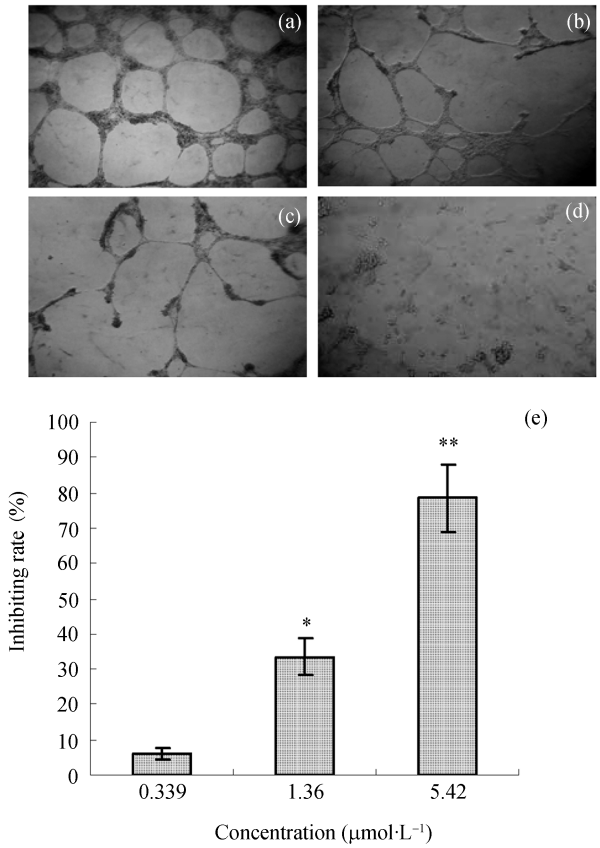

Figure 4 Taspine inhibited the tube formation of ECV304 cell. (a) Added to VEGF; (b) added to VEGF and 0.339 μmol · L^{-1} taspine; (c) added to VEGF and 1.36 μmol · L^{-1} taspine; (d) added to VEGF and 5.42 μmol · L^{-1} taspine; (e) inhibiting rate of tube formation of different concentration taspine. *, *t* test, $P<0.05$; **, *t* test, $P<0.01$.

2.3 Tube formation

As shown in Figure 4, under the inducement of VEGF, ECV304 incubated on matrigel for 24 h differentiate into an extensive and enclosed network of tubes. The amount of the tube structure decreased when the concentration of taspine increased. According to the calculation of the total length of the tube structures of each group and inhibiting rate, the inhibiting rate increased when the concentration of taspine increased. We observed significant, dose-dependent inhibition compared with the control group. The results indicated that taspine inhibited the tube formation of ECV304 cell under the inducement of VEGF.

The tube formation of vascular endothelial cell is a key step of angiogenesis. During angiogenesis, VEGF from tumor cell selectively binds to the receptor expressed by endothelial cell, and then receptor tyrosine kinase is activated and angiogenesis is started. It has been demonstrated that anti-VEGFR2 antibody blocked VEGFR2 and inhibited angiogenesis[28]. Taspine maybe acts on VEGFR2 and inhibits the tube formation of ECV304 cell induced by VEGF. Therefore, it was suggested that taspine maybe blocks VEGFR2 and inhibits the tube formation of ECV304. The molecular mechanism supporting this hypothesis using molecular biology method is being studied further in our laboratory.

In this study, taspine is the active component that can bind to ECV304 cell membrane and its receptors and inhibits the tube formation of ECV304 cell, so taspine is the lead compound through the preliminary screening. Other isolated fractions from *C. robustum* do not have pharmacodynamic action through binding to membrane receptor because they do not interact with membrane receptor. But whether other isolated fractions from *C. robustum* have biological activity through blocking signal transduction in cell or DNA systhesis, other corresponding screening models should be established and used to multiple model screening.

3 Conclusion

The model of vascular endothelial cell membrane chromatography was established using cell membrane of pure vascular endothelial cell cultured *in vitro*. The model can simulate the interaction between drug and membrane and its receptor *in vitro* and can be used to study the interaction between drug and vascular endothelial cell membrane and its receptor and to preliminarily screen the lead compound.

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