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Screening, analysis and in vitro vasodilatation of effective components from *Ligusticum Chuanxiong*

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Abstract

Effective components, ligustilide and butylidenephthalide, from *Ligusticum Chuanxiong* (*Ligusticum wallichii* Franchat, Umbelliferae) were screened and identified by using a cell membrane chromatography (CMC) and a gas chromatography/mass spectrometry (GC/MS). The components showed the effects of inhibiting vasoconstriction in vitro on rat abdominal aorta segments. The screening procedure was performed in a rat artery CMC column (50 mm × 2.0 mm I.D.) with a sodium phosphate buffer (pH 7.4) as mobile phase at 37 °C. The identification was accomplished by a DB-5MS 30 m capillary column (0.25 mm I.D., 0.25 μm film thickness) with helium as carrier gas operating under program control temperature and electron impact ionization mass spectrometer in a scan mode. Results demonstrated that ligustilide and butylidenephthalide can act on rat artery cell membrane similar to verapamil in CMC system. They significantly inhibited the vasoconstrictions induced by norepinephrine bitartrate (NE) and calcium chloride ($CaCl_2$). The relaxing effect of ligustilide on the NE- and $CaCl_2$-induced constrictions is more potent than that of butylidenephthalide. Ligustilide and butylidenephthalide seem to be the two main effective components of *Ligusticum Chuanxiong* as a traditional Chinese medicine for treating blood vessel diseases.

Keywords: Ligustilide; Butylidenephthalide; Vasodilatation; *Ligusticum Chuanxiong*; GC/MS; CMC

Introduction

A lot of traditional Chinese medicines (TCM) are widely used for treating various kinds of chronic diseases, such as cardiovascular system diseases, in China (Gong and Sucher, 1999; Kaneko et al., 2001; Melanie and Peter, 2003), Japan (Kazuo et al., 1998), Korea (Robert and Yuan, 2000), and Singapore (Soon et al., 2004), etc. As well known, it is very important to sort out effective components in the TCM. Molecular bio-chromatography (Kong et al., 2000; Mao et al., 2002, 2003; Wang et al., 2000; Xu et al., 2004), chromatographic fingerprinting (Brian et al., 2003; Gong et al., 2003, 2004), and capillary electrophoresis (Kua et al., 2003) are most common methods for screening and analyzing bioactive components of TCM.

Ligusticum Chuanxiong, as a TCM native to China, is commonly used in the prescriptions to treat cardiovascular diseases in clinic (Hou et al., 2004a,b; Tsai et al., 2002). Chuanxiongzine, also called tetramethylpyrazine, has been reported to be an effective component of *Ligusticum Chuanxiong* affecting heart, brain and vessel diseases (Cui et al., 2003; Huang et al., 1998; Li et al., 2000). It was purified from *Ligusticum Chuanxiong* by high-speed counter-current chromatography (Li and Chen, 2004). Some of organic acids, including ferulic acid, sedanonic acid, folic acid, vanillic acid and caffeic acid, were isolated and identified from *Ligusticum Chuanxiong* by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry (Chen et al., 2004). There were also two phthalides separated from it (Naito et al., 1996). In addition, butylidenephthalide and ligustilide in volatile oil from *Ligusticum Chuanxiong* showed a long-term effect on pentobarbital sleep in mice, and was suggested the role involved in central noradrenergic and/or $GABA_A$ system (Matsumoto et al., 1998). But their effects on blood vessel maintain still obscure.

We have firstly prepared a cell membrane stationary phase (CMSP) and established a cell membrane chromatography (CMC) as a chromatographic system of bionics (He et al., 1999, 2001) to have screened effective components from

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Angelica sinensis, *Herba eplmedii*, *Leontice robustum* (Gao et al., 2003; Liang and He, 2004; Zhao et al., 2000, 2002). In this study we used rat artery CMC system to screen effective components from *Ligusticum Chuanxiong* and demonstrated ligustilide and butylidenephthalide inhibiting vasoconstriction of rat abdominal aorta segments.

Materials and methods

Reagents and chemicals

HPLC grade acetonitrile, methanol, *n*-hexane, ethyl acetate (AcOEt) and dimethyl sulphoxide were purchased from Fisher Scientific (Pittsburg, PA, USA). Helium (purity, 99.999%) was from Xi'an Analytical Instrument Factory (Xi'an, P.R.China). Silica Gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Haiyang Chemical Co. Ltd (Qingdao, P.R.China). Norepinephrine bitartrate (NE) was purchased from Wuhan Pharmaceutical Factory (Wuhan, PR China). Verapamil (Ver) was from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used were analytical grade.

Extraction and isolation

Ligusticum Chuanxiong was purchased from the TCM Store (Xi'an, PR China) and was ground to 40 mesh. The volatile oil of *Ligusticum Chuanxiong* was prepared by using a typical extraction apparatus. 1 kg ground sample was transferred to a holder where 5 L of distilled water was added and extracted by steam distillation for 4 h. 8 mL volatile oil was collected.

The volatile oil was systematically separated by column chromatographic methods. Firstly, 7 g volatile oil sample was subjected to a normal phase column chromatography (CC, Silica Gel, 3×200 g) using a mixture solution of *n*-hexane/AcOEt from (95:5) to (70:30) as a mobile phase, which gave 10 fractions (5×100 mL each fraction) after recombination (**1**–**10**). Fraction **3** (2.7 g) was subjected to a reverse phase CC (C_{18}, 20 μm, 5×100 g) with a mixture solution of methanol/water (65:35) as a mobile phase, to afford 13 fractions after recombination (2×100 mL each fraction). From fraction **3**–7 we obtained a pure compound A (1.1 g, purity: 100% by TLC and >99% by HPLC). After a new reverse phase column in the same conditions as above, 13 fractions were separated from fraction **5** and fraction **5**–3 was identified to be a pure compound (compound B, 0.3 g, purity: 100% by TLC and >99% by HPLC).

CMC screening

A stationary phase of rat artery cell membrane was prepared by the previously described method (He et al., 2001). Enzymatic bioactivity of the stationary phase as a special cell membrane preparation was determined according to references (Lowry et al., 1951; Xu et al., 2002). The chromatographic conditions as followed: rat artery cell membrane column (50 mm × 2.0 mm i.d.) was used. Mobile phase was 50 mmol·L^{-1} sodium phosphate buffer (pH 7.4) with flow rate of 0.5 mL·min^{-1} and the detection wavelength was at 236nm under the column temperature at 37 °C. The volatile oil and the isolated compounds of *Ligusticum Chuanxiong* were screened by CMC system under the conditions above.

GC/MS procedure

The volatile oil, compounds A and B were analyzed by a capillary gas chromatography/mass spectrometry (Model GCMS-QP2010, Shimadzu, Kyoto, Japan). The gas chromatographic conditions were as followed: GC oven fitted with a DB-5 MS 30 m capillary column (0.25 mm I.D., 0.25 μm film thickness, Aglient, Palo Alto, CA, USA) with carrier gas helium at flow rate of 1.2 mL min^{-1}, operating under a initial temperature at 80 °C for 2 min up to 250 °C in ramp rate of 10 °C min^{-1}, then held for 11 min. 1.0 μL sample solution was injected into the system with a split ratio of 1:50 for analysis. The electron impact ionization mass spectrometer was operated with an ionization voltage of 70 eV and an ion source temperature at 200 °C, using a scan mode and measuring total ions chromatogram (TIC) under a mass range of 50.0–350.0. The datum analysis was performed on a NIST library (Shimadzu, Kyoto, Japan).

In vitro pharmacology

SD rats, weighing 180–220 g, were anaesthetized with ether and sacrificed by decapitation. The abdominal aorta were immediately removed and immersed in cold (4 °C) standard buffer solution composed of 119 mM NaCl, 15 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.5 mM $CaCl_2$ and 5.5 mM glucose. The abdominal aorta was dissected free from adherent tissue and cut into 1 mm long circular segment under cold conditions. Vasomotor reactivity was analyzed in temperature-controlled (37 °C) tissue baths containing the buffer solution (1 mL). The solution was continuously gassed with 5% CO_2 in O_2 resulting in a pH of 7.4. The segment was mounted on two L-shaped metal prongs. One prong was connected to a force–displacement transducer (FT-03, Grass Instr., Quincy, USA) attached to a PowerLab unit (AD Instrument, Hastings, UK) for continuous recording of isometric force by means of the Chart® software (AD Instruments, Hastings, UK). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs. A passive force of 5 mN was applied to the segment before the specimen was allowed to stabilize at this level of tension for 1 h. The contractile capacity of each segment was tested by exposure to a potassium-rich buffer solution (60 mM), which had the same composition as the standard solution except that NaCl was replaced for an equimolar concentration of KCl. The segments were used only if potassium elicited reproducible responses over 1.0 mN. When two reproducible contractions induced by potassium-rich buffer solution had been obtained, the segment was used for further studies. Concentration–response curves for the compounds in different concentrations were obtained by cumu-

lative application of NE (Cao et al., 2004; Sebastian et al., 1997, 2002).

In "calcium withdrawal" experiments, the rings were incubated in Ca^{+}-free buffer solution, in which the composition was the same as the standard solution except that $CaCl_2$ was exchanged for an equimolar concentration of NaCl, and cumulated $CaCl_2$ (final concentration 10 mmol L^{-1}) was added to make the ring contractile. The other process was the same as above.

Statistics

Data were presented as means ± S.E. The concentration–response curves were obtained by cumulative application of NE or $CaCl_2$. The contractile responses were expressed as percentage of the maximum contraction induced by NE (or $CaCl_2$) without BDI and BI (mean ± S.E.), and n stood for the number of rat abdominal aorta ring segments. The pD_2' value of each compound was calculated from pD_2 values of different concentrations. Statistical analysis was performed by GraphPad Prism 4.0 software and $p < 0.05$ is considered as significant.

Results

Fractions from volatile oil

Volatile oil and other isolated samples (fractions and compounds) from *Ligusticum Chuanxiong* were obtained by combining silica gel and C_{18} CC separation with the rat artery CMC screen. Firstly, the volatile oil of *Ligusticum Chuanxiong* were separated into 10 fractions by using silica CC. Fraction **3** and fraction **5** were determined to have bioactive effects on CMC system, and a further isolation was preformed for obtaining pure components. Secondly, there were 13 fractions separated from fraction **3** and 12 fractions separated from fraction 5. Among the total, the seventh fraction from fraction **3** and the third fraction from fraction **5**, expressed as fraction **3**–7 (compound A) and fraction **5**–3 (compound B) respectively, were found to be the active components on artery cell membrane. As shown in Fig. 1, they were the main components in volatile oil of *Ligusticum Chuanxiong* and pure enough for structure identification and pharmacological test.

Table 1
Retention features in the rat artery CMC system

Solutes	log k'
Verapamil	1.35
Volatile oil	1.06
Fraction **3**	1.05
Fraction **5**	1.03
Compound A	1.07
Compound B	1.01

Chromatographic conditions: rat artery cell membrane column (50 mm × 2.0 mm i.d.) was used. Mobile phase was 50 mmol·L^{-1} sodium phosphate buffer (pH 7.4) with flow rate of 0.5 mL·min^{-1}, and detection wavelength was at 236 nm under the column temperature at 37 °C.

Retention features in CMC system

The rat artery CMC system was performed for screening bioactivity of the components. Result showed that the components had a similar retention feature as verapamil. An affinity between solute (or compound) and stationary phase (or rat artery cell membrane) are commonly expressed in the logarithm of retention factor (log k') of the solute as following equation:

$$\log k' = \log[(t_R - t_0)/t_0]$$

where t_R is the retention time of the solute and t_0 is the void time of the solvent which will not act on rat artery cell membrane in CMC system.

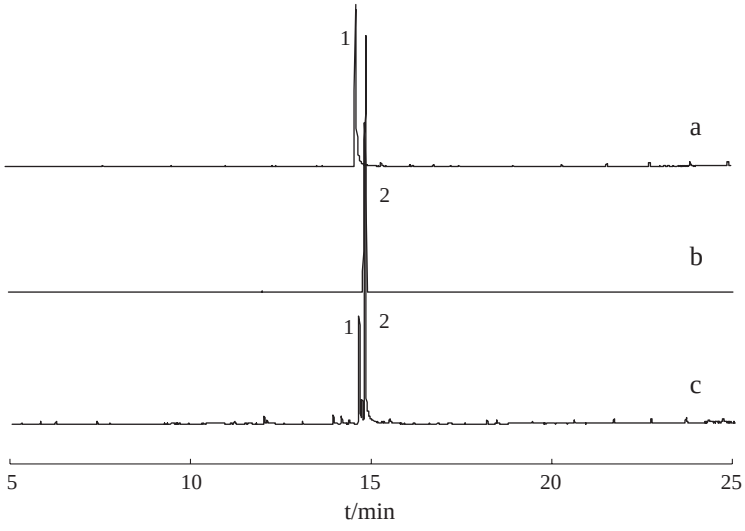

Fig. 1. GC/MS total ion chromatograms of compound B (a), compound A (b) and volatile oil from *Rhizoma chuanxiong* (c) on a DB-5MS capillary column. Gas chromatographic conditions: GC oven fitted with a DB-5MS 30 m capillary column (0.25 mm I.D., 0.25 μm film thickness) with carrier gas helium at flow rate of 1.2 mL min^{-1}, operating under a initial temperature at 80 °C for 2 min up to 250 °C in ramp rate of 10 °C min^{-1}, then held for 11 min. Injection volume was 1.0 μL with a split ratio of 1:50. (1, compound B; 2, compound A).

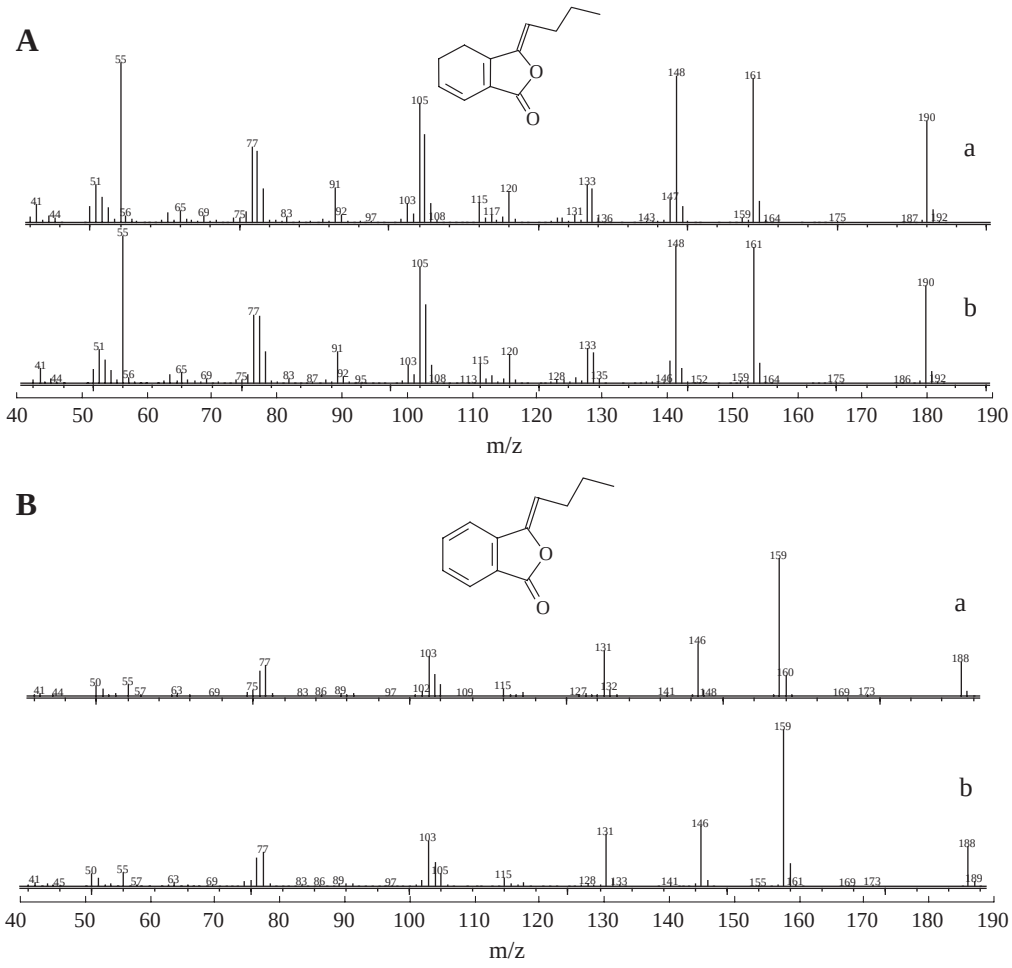

Fig. 2. MS chromatograms of compound A, compound B and the same peak in volatile oil. EIMS conditions: ionization voltage of 70 eV, ion source temperature at 200 °C, using a scan mode and measuring total ions chromatogram (TIC) under a mass range of 50.0–350.0. (A): compound A (a) and the same peak in volatile oil (b); (B): compound B (a) and the same peak in volatile oil (b).

In this rat artery CMC system, verapamil as control had a stronger affinity with a retention factor of 22.39. Under the same conditions, volatile oil of *Ligusticum Chuanxiong*, the fraction **3** and fraction **5** almost had the similar retention factors of 11.48, 11.22 and 10.72 respectively. Retention factors for compound A and compound B were 11.75 and 10.23 respectively (shown in Table 1). Other isolated fractions and compounds had no retention features in the CMC system. Vitamin C, which has no interaction with rat abdominal aorta was chosen as a negative control did not show any retention (data not shown). The log k' values of the solutes in CMC system were calculated with the equation above and listed in Table 1.

Chemical structure of compounds A and B

Compound A and compound B were identified to be ligustilide (3-butylidene-4, 5-dihydro-isobenzofuranone, BDI) and butylidenephthalide (3-butylidene-isobenzofuranone, BI) in comparison with standard MS data and data in NIST

Table 2
E_{max} and pD'_2 values of BDI on the NE- and $CaCl_2$-induced contractions of rat abdominal aorta segments

	Concentration, μM	NE			$CaCl_2$		
		E_{max} (%)	pD_2	pD'_2	E_{max} (%)	pD_2	pD'_2
Control	–	100±0	6.34±0.11	–	100±0	3.51±0.07	–
Verapamil	0.08	62±5**	6.23±0.06	6.68±0.04	48±5**	2.99±0.08	7.12±0.08
	0.6	91±8*	6.55±0.21		89±3*	3.16±0.06	
BDI	1.8	69±4**	6.32±0.16	5.30±0.15	54±6**	3.07±0.04	5.54±0.07
	5.4	49±6**	5.95±0.12		39±2**	2.82±0.03	

The rat abdominal aorta segment contractions were analyzed under conditions as described in in vitro pharmacology. Concentration–response curves for BDI in different concentrations were obtained by cumulative application of NE in standard buffer solution or $CaCl_2$ in Ca^+-free buffer solution. The maximum contraction values are expressed as mean±S.E. ($n=8$). **$p<0.01$, *$p<0.05$, compared with the maximum contraction values of verapamil in the same condition.

Table 3
E_{max} and pD'_2 values of BI on the NE- and $CaCl_2$-induced contractions of rat abdominal aorta segments

	Concentration, μM	NE			$CaCl_2$		
		E_{max} (%)	pD_2	pD'_2	E_{max} (%)	pD_2	pD'_2
Control		101±0	6.73±0.11	–	100±0	3.33±0.02	–
Verapamil	0.08	62±5**	6.23±0.06	6.89±0.10	48±5**	2.99±0.08	7.13±0.08
	2.0	78±7**	6.28±0.12		87±3**	3.29±0.03	
BI	6.0	67±5**	6.29±0.13	4.85±0.09	67±3**	3.17±0.04	5.03±0.04
	18.0	44±2**	6.06±0.05		30±2**	2.88±0.11	

The rat abdominal aorta segment contractions were analyzed under conditions as described in in vitro pharmacology. Concentration–response curves for BI in different concentrations were obtained by cumulative application of NE in standard buffer solution or $CaCl_2$ in Ca^{+}-free buffer solution. The maximum contraction values are expressed as mean±S.E. (n=8). **p<0.01, *p<0.05, compared with the maximum contraction values of verapamil in the same condition.

library, respectively (shown in Fig. 2). According to EI/MS chromatograms, the main fragments and split rules of compound A shown as following (m/z, intention): 190 (M^+, 66.3), 161 (M^+-C_2H_5, 95.4), 148 (M^+-C_3H_6, 94.9), 133 (M^+-C_3H_5O, 24.1), 105 (M^+-$C_4H_6O_2$, 76.5), and 77 (M^+-$C_6H_9O_2$, 46.5); whileas those of compound B (m/z): 188 (M^+, 24.1), 159 (M^+-C_2H_5, 100.0), 146 (M^+-C_3H_6, 38.0), 131 (M^+-C_3H_5O, 34.6), 103 (M^+-$C_4H_6O_2$, 30.3) and 77 (M^+-$C_6H_9O_2$, 23.8). Compound A and compound B showed quasi-molecular ions corresponding to the molecular formula $C_{12}H_{14}O_2$ and $C_{12}H_{12}O_2$, respectively.

Effects of BDI and BI on rat abdominal aorta

Both of BDI and BI showed the inhibitory effects on NE- and $CaCl_2$-induced contractions of rat abdominal aorta segments in a concentration-dependent manner. As listed in Table 2, BDI with 0.6, 1.8 and 5.4 μM concentrations depressed NE-induced contraction from 100% of control to 91±8%, 69±4% and 49±6%, and $CaCl_2$-induced one from 100% to 89±3%, 54±6% and 39±2%, respectively. In addition, BI with 2.0, 6.0 and 18.0 μM was from 100% to 78±7%, 67±5% and 44±2% by NE-induced, and was from 100% to 89±3%, 54±6% and 39±2% by $CaCl_2$-induced as listed in Table 3. The contraction–response curves were shifted toward the right in a nonparallel manner with a pD_2 value of 5.30±0.15 (NE-induced) and 5.54±0.07 ($CaCl_2$-induced) for BDI, and that of 4.85±0.09 and 5.03±0.04 for BI (listed in Tables 2 and 3). The vasodilatation of BDI was higher in the pD'_2 values than that of BI.

Discussion

In this study, we have found that the rat artery CMC system are very effective for screening active components in mixture species such as TCM. In fact, the CMC system acts just as same as a bio-affinity chromatography. The stationary phase of rat artery cell membrane has characteristics of both bioactive and chromatographic separation and is capable to recognize the components from mixture samples. The bioactive components will have the retention time in CMC system. In order to determine the bio-affinity of the CMC system, a control drug (it has usually a selective effect on the cell membrane) should be used to test the system. The log k' values of the drug and components will reflect the interaction between the solute and the cell membrane. In this scheme, the target components acted on the cell membrane would be easily found in a mixture after it is isolated by the CC separation. Through the procedure, we have demonstrated that there are two components acted on rat artery cell membrane from the volatile oil taking verapamil as control. However, log k' values of BDI and BI as the target components are lower than that of verapamil (as shown in Table 1), indicating that the bio-affinity of BDI and BI to artery cell membrane will be lower than that of verapamil.

We have also found that the screening results in rat artery CMC system are closely correlative to pharmacological effects in vitro. The contractile response indicated that in NE- and $CaCl_2$-induced vasoconstrictions the inhibitory of BDI and BI are similar to that of verapamil, a vasodilator acted on both coronary and peripheral arteries. In addition, a potent sequence–verapamil>BDI>BI–is obviously correlative to that of log k' values on rat artery CMC system for BDI and BI (shown in Tables 1–3). The correlation coefficient between the log k' value and the pD'_2 value is 0.9983 for NE-induced vasoconstriction, and 0.9975 for $CaCl_2$-induced vasoconstriction. The properties of BDI and BI as vascular selective agents are dominated by vasodilating activity similar to calcium antagonist. From those data, we believed that the BDI and BI are main effective components of *Ligusticum Chuanxiong* as a TCM for treating cardiovascular diseases in clinic. Therefore, the contents of BDI and BI in *Ligusticum Chuanxiong* and its prescription should be strictly controlled in order to keep the therapeutic process available.

The results strongly support our earlier suggestion that the CMC system can be used effectively to screen active components from a complex sample or mixture, such as traditional Chinese herbs. CC, GC/MS and HPLC/MS techniques might be further applied for isolation and identification of the components. Sequentially, pharmacological effects of the components might be investigated on special organ and tissue by functional tests in vitro. This is a systematic pattern to study effective components from TCM in molecular and tissues levels. However, in vivo effects of effective components from TCM still need to be investigated further.

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