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Live cell extraction and HPLC–MS analysis for predicting bioactive components of traditional Chinese medicines

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Abstract

A novel strategy for predicting bioactive components in traditional Chinese medicines (TCM) using live cell extraction and high performance liquid chromatography–diode array detection–mass spectrometry (HPLC–DAD–MS) analysis was proposed. The hypothesis is that when cells are incubated together with the extract of TCM, the potential bioactive components in the TCM should selectively combine with the cells, and the relative concentrations of the cell-combining components in the suspension medium should decrease, while the cell-combining components would be detectable in the extract of denatured cells. The identities of the cell-combining components could be determined by HPLC–DAD–MS analysis. Using the proposed approach, the potential bioactive components of Danggui Buxue decoction, a commonly used TCM for anaemia, and its compositions, Radix Angelica Sinensis and Radix Astragli for endothelial cells, were investigated. Six compounds in the extract of Danggui Buxue decoction were detected as the components selectively combined with endothelial cells, among them two were contributed by Radix Angelica Sinensis, and four by Radix Astragli. The identities of four of the six potential bioactive compounds were elucidated as ononoside, calycosin, 3-butylphthalide and ligustilide by HPLC–DAD–MS analysis. The results indicate that the proposed approach may be applied to predict the bioactive candidates in TCM.

Keywords: Live cell extraction; HPLC–DAD–MS; Bioactive components predicting; Danggui Buxue decoction; Radix Astragli; Radix Angelica Sinensis

1. Introduction

Traditional Chinese medicines (TCM) are natural therapeutic remedies used under the guidance of traditional Chinese medical philosophy and have been prescribed by TCM practitioners in China and Chinese community worldwide for thousands of years. TCM are mostly used in combination, in which the composite formulae will produce a synergistic effect or antagonistic action [1]. In most cases, bioactive components of TCM are not or only partially known. Therefore, the screening and analysis of bioactive components of TCM is very important not only for elucidating the therapeutic principles but also for their quality control.

The traditional procedures for finding bioactive components of TCM are extraction and purification of constituents from TCM followed by in vitro or in vivo pharmacological screening of the purified compounds. Although possibly a limited number of compounds are responsible for pharmaceutical effects of TCM, the bioactive compounds are usually among complex mixtures containing up to hundreds or even thousands of different components, which makes the purification and screening of bioactive components extremely difficult.

Based on the hypothesis that the active components should appear in blood and urine with appropriate blood concentration and urinary excretion rates after the administration of TCM, Homma et al. introduced the concept of pharmacokinetics into their study to discover bioactive components in TCM [2]. This was indeed a strategy for discovering absorbable bioactive candidates with a higher probability. But absorbable compounds do not guarantee bioactivity. Modern pharmacological studies have revealed that the important step of a drug action is binding with some receptors and (or) channels and (or) enzymes on cell membranes, and (or) inside cells. Therefore, the ability to interact with cells is very important for the biological

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activities of a drug. Based on this principle, some approaches called cell membrane chromatography (CMC) [3] and frontal affinity chromatography (FAC) [4], in which entrapped cells membrane and immobilized receptors or antibodies were used as the stationary phase of a liquid chromatography, were developed for online pharmacological studies and as rapid screens for the isolation and identification of lead drug candidates from complex biological or chemical mixtures [3,5–9]. Recently these methods were employed as promising approaches for the efficient screening of lead compounds or drug candidates from natural resources including TCM [3,5,10–12]. However, the life of immobilized receptors or entrapped cell membrane, etc., was very short (1 day–1 week) [3], and the entrapment or immobilization procedure must be adapted to the kind of material that is to be analyzed and to the kind of gel matrix used [13]. In addition, the affinity interaction between components of TCM and receptors or biomembrane is rarely compatible to their chromatographic separation. Moreover, CMC could not be directly connected to mass spectrometry at present because of the presence of a high concentration of inorganic salts in eluting solvents, resulting in a difficulty of online identity elucidating of components with significant retention on CMC.

More recently, Dong et al. [14] proposed a new approach for screening and analyzing bioactive components in TCM using cell membranes extraction and high performance liquid chromatography–mass spectrometry (HPLC–MS) analysis, which was successfully applied to predict the potential bioactivities of multiple compounds in *Angelica sinensis* simultaneously. The exciting results indicate that this method has advantages over conventional methods for investigation of potential bioactive components of TCM in that: first, through screening by this method, the numbers of compounds interested will be extremely reduced compared with that in the original complex mixtures of TCM, which will make the further investigation more cost-effective; second, HPLC–MS analysis has not only high resolution for compounds separation, but also has the capacity of identities elucidation of components interested; third, this approach avoids entrapment or immobilization procedures of cell membranes, which are professional, time-consuming and arduous work. But how about the results if the lysed cell membranes are substituted by live cells, as the circumstances of live cells are more similar to that of cells in organisms than that of lysed cell membranes, so that the cell-compound interactions more resemble that in organisms, leading to the bioactive candidates with high probability.

Therefore, in present study, a novel strategy for screening and analyzing bioactive components in TCM using live cell extraction and high performance liquid chromatography–diode array detection–mass spectrometry (HPLC–DAD–MS) analysis was proposed. The hypothesis is that when cells are incubated together with the extract of TCM, the potential bioactive compounds in the TCM should selectively combine with the cells, the relative concentrations of the cell-combining components in the suspension medium should decrease and the cell-combining components would be detectable in the extract of denatured cells. The identities of the cell-combining components could be determined by HPLC–DAD–MS analysis. Using the proposed approach, the bioactive components of Danggui Buxue decoction, a commonly used combined prescription of Radix Angelica Sinensis and Radix Astragli (5:1, w/w) for anaemia, were investigated.

2. Experimental

2.1. Herbal materials and chemicals

Radix Angelica Sinensis and Radix Astragli were, respectively, collected from Minxian County of Gansu Province and Hunyuan County of Shanxi Province, China, and were authenticated by Professor Ping Li. Ferulic acid was obtained from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Ligustilide and Calycosin were isolated and purified from Radix Angelica Sinensis and Radix Astragli, respectively, in our laboratory. The identities of all these reference compounds were confirmed by IR, MS, ^{1}H- and ^{13}C-NMR. Buffer A (pH 7.4) is 5 mM sodium phosphate buffer. Methanol for LC was purchased from Jiansu Hanbon Sci. & Tech. Co. Ltd. (Jiansu, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). All other chemicals were analytical grade. All solvents and samples were filtered through 0.45 μm nylon membranes before use.

2.2. Sample preparation

Extract A: Thirty grams mixed powders of Radix Astragli:Radix Angelica Sinensis (5:1, w/w) were immersed in 240 ml 75% ethanol for 1 h and were refluxed for 1 h, then the solvent was removed with rotary evaporation at 40 °C under vacuum, and the residue was dissolved in 30 ml buffer A and filtered through a 0.22 μm membrane, the filtrate was used as a sample for HPLC–DAD–MS analysis and live cell extraction.

Extract B: Twenty-five grams powders of Radix Astragli were treated as the procedures for the extract A.

Extract C: Five grams powders of Radix Angelica Sinensis were treated as the procedures for the extract A.

2.3. Cell culture

Human umbilical vein endothelial cells were harvested from human umbilical vein using 0.05% trypsin with 0.02% EDTA and plated on 0.1% gelatin-coated dishes (Becton-Dickinson) in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 10 μg L^{-1} basic fibroblast growth factor (bFGF), 100 kU L^{-1} penicillin (Sigma) and 100 mg L^{-1} streptomycin sulphate (Sigma). Then the cells grown in a humidified atmosphere of 5% CO_2–95% air at 37 °C were dissociated with 0.125% trypsin–EDTA and subcultured on gelatin-coated polyester sheet (250 ml, Plastic Suppliers) at a seeding density of 2.0×10^6 to 3.0×10^6 per sheet. The cells were cultured until they reached confluence (2–3 days after seeding), and then

digested with 0.125% trypsin. After centrifuged at 1000 × *g* for 2 min, the deposited cells were suspended with buffer A for live cell extraction.

2.4. Endothelial cells extraction

Above obtained endothelial cells mixed with 1 ml extract A were incubated at 37 °C for 6 h. The suspension was then centrifuged at 1000 × *g* for 5 min and the obtained supernatant was filtered with 0.45 μm membranes for HPLC–DAD–MS analysis. The deposited cells were washed four times by 2 ml of PBS each time with subsequent centrifugation at 1000 × *g* for 5 min to remove the possible non-selectively combining components. The eluates were discarded except the last one that was collected as contrast for HPLC–MS analysis. The deposited endothelial cells were then denatured and extracted with 2 ml of 75% ethanol. After centrifugation at 1000 × *g* for 5 min, the supernatant was filtered through 0.45 μm membranes for HPLC–DAD–MS analysis. The cell-containing buffer A was substituted with blank buffer A to prepare the control sample using the same procedures described above. By comparing the chromatograms of sample derived from extract A treated with endothelial cells with that of control sample, the analytes which had obviously decreased peak areas and (or) detectable in the extract of denatured deposited cells were considered to be the potential bioactive candidates of Danggui Buxue decoction for endothelial cells. The same procedures were performed for extracts B and C, respectively, to confirm the contributions of peaks concerned in Danggui Buxue decoction.

2.5. HPLC analysis

Chromatographic analysis was performed on an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a dual pump, a Rheodyne7125i injection valve with a 20 μl sample loop and a DAD detector, data were acquired and processed by an HP ChemStation. A ZORBAX ODS C18 column (4.6 mm × 250 mm i.d., 5 μm) and a ZORBAX ODS C18 guard column (4.6 mm × 12.5 mm i.d., 5 μm) were employed, the column temperature was set at 25 °C. The mobile phase consisted of (A) 0.5% aqueous acetic acid and (B) methanol. The gradient elution conditions were: 0–10 min, 20–40% B; 10–40 min, 40–65% B; 40–55 min, 65–85% B. The flow-rate was 1 ml/min, and the injection volume was 20 μl. Peaks were monitored at 254, 280 and 320 nm, respectively.

The high resolution MS analysis was performed on an Agilent G1969 LC/MSD TOF system (Agilent Technologies) equipped with an ESI source. Data were acquired and analyzed by LC–MS TOF Software Ver. A. 01.00 (Agilent Technologies) and PE Sciex Analyst QS 1.1. The operating conditions for the ESI source were as follows: the capillary temperature, 300 °C; capillary voltage, 3500 V; drying gas (N_2) flow, 9 L/min; nebulizer (N_2) pressure, 35 pis. Full scan data acquisition was performed from *m/z* 50 to 3000 in MS scan mode.

3. Results and discussions

3.1. Bioactive candidates of Danggui Buxue decoction and its compositions for endothelial cells

Danggui Buxue decocotion, discovered historically by Dongyuan Li of the Jingyuan Dynasty of China, is a combined prescription of Radix Astragli and Radix Angelica Sinensis (5:1, w/w), and has been used to treat anaemia in TCM practice for thousands of years. In vitro pharmacological studies showed that the extracts of this combined prescription could inhibit the enhanced endothelial cells permeability induced by histamine [15] and promote the proliferation of human umbilical vein endothelial cells and increase the expression of intercellular adhesion molecule-1 in human umbilical vein endothelial cells [16], but the relevant bioactive components are still not known.

The chromatograms of Danggui Buxue decoction monitored at 320 nm are shown in Fig. 1. It was found that over 30 peaks were detected in Danggui Buxue decoction under the present chromatographic conditions (Fig. 1A), among which five (peaks 1–5) were determined with peak area decreasing after the extracts were incubated with endothelial cells (Fig. 1B). On the other hand, five peaks were detected in the extract of denatured endothelial cells (Fig. 1D), with the same retention times of peaks 1–5, respectively, of the control sample (i.e. without treatment of endothelial cells) of Danggui Buxue decoction (Fig. 1A). Meanwhile, no peaks were detected in the fourth eluate of the endothelial cells (Fig. 1C). Fig. 2 showed the chromatograms of Danggui Buxue decoction with or without endothelial cells treatment monitored at 280 nm (Fig. 2A and B) and 254 nm (Fig. 2C and D). It could be found that except peaks 1–5, no other peaks with obviously decreased peak areas were determined. All these results suggested that at least five components in extracts of Danggui Buxue decoction have the interactions with endothelial cells. And the interaction could be deduced as non-physical adsorption of, but bio-selective binding with, or permeating into endothelial cells. Therefore, these

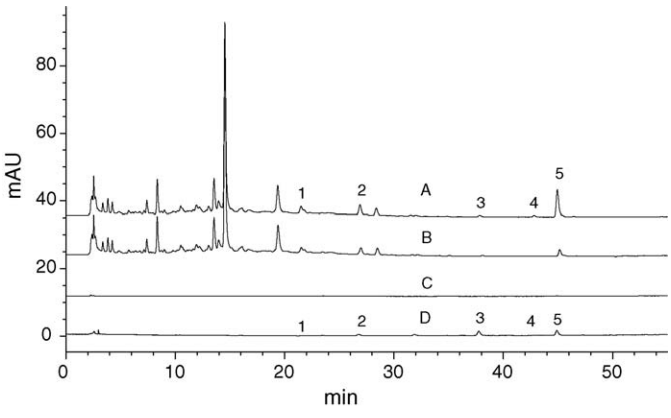

Fig. 1. Chromatograms of Danggui Buxue decoction monitored at 320 nm. (A) Extract of Danggui Buxue decoction treated with blank buffer A; (B) extract of Danggui Buxue decoction treated with endothelial cells; (C) the fourth eluate of deposited endothelial cells; (D) extract of denatured deposited endothelial cells.

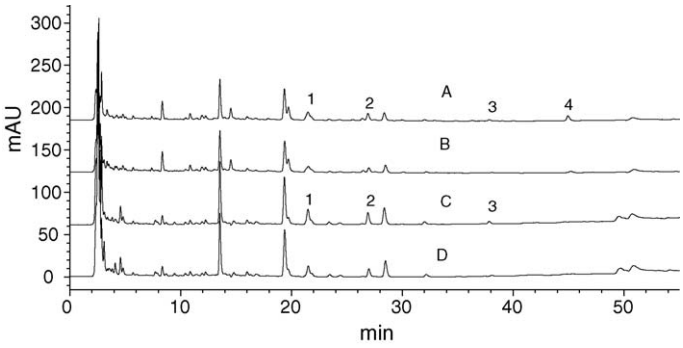

Fig. 2. Chromatograms of Danggui Buxue decoction monitored at 280 and 254 nm. (A and C): Extract of Danggui Buxue decoction treated with blank buffer A; (B and D) extracts of Danggui Buxue decoction treated with endothelial cells: (A and B) monitored at 280 nm: (C and D) monitored at 254 nm.

cells-interacting compounds could be regarded as the potential bioactive candidates of Danggui Buxue decoction for endothelial cells.

Radix Angelica Sinensis and Radix Astragli, the compositions of Danggui Buxue decoction, were investigated in the same way as described above for Danggui Buxue decoction to confirm the contributions of the five area-changing peaks in Danggui Buxue decoction. As shown in Fig. 3, two peaks (peaks 1 and 2) were determined as the area-changing peaks in Radix Angelica Sinensis, which had the same retention times of peaks 4 and 5, respectively, in Danggui Buxue decoction (Fig. 1A). Similarly, four peaks (peaks 1–4) were analyzed as area-changing peaks in Radix Astragli (Fig. 4), which had the same retention times of peaks 1–4, respectively, in Danggui Buxue decoction (Fig. 1A). These results indicated that peaks 1, 2 and 3 in Danggui Buxue decoction (Fig. 1A) were contributed by peaks 1, 2 and 3, respectively, of Radix Astragli (Fig. 4), while peak 4 in Danggui Buxue decoction (Fig. 1A) was contributed by peak 1 of Radix Angelica Sinensis (Fig. 3), and peak 5 in Danggui Buxue decoction (Fig. 1A) was contributed by both peak 2 of Radix Angelica Sinensis (Fig. 3) and peak 4 of Radix Astragli (Fig. 4).

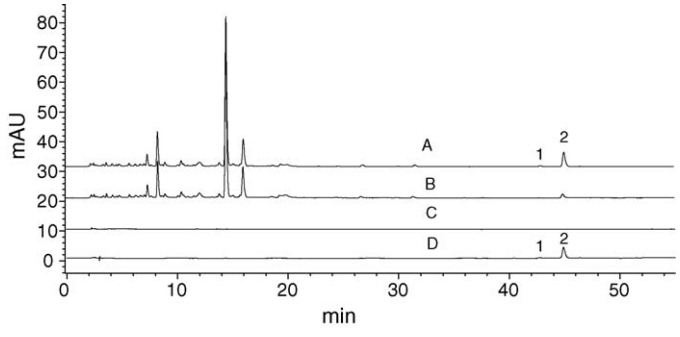

Fig. 3. Chromatograms of Radix Angelica Sinensis monitored at 320 nm. (A) Extract of Radix Angelica Sinensis treated with blank buffer A; (B) extract of Radix Angelica Sinensis treated with endothelial cells; (C) the fourth eluate of deposited endothelial cells; (D) extract of denatured deposited endothelial cells.

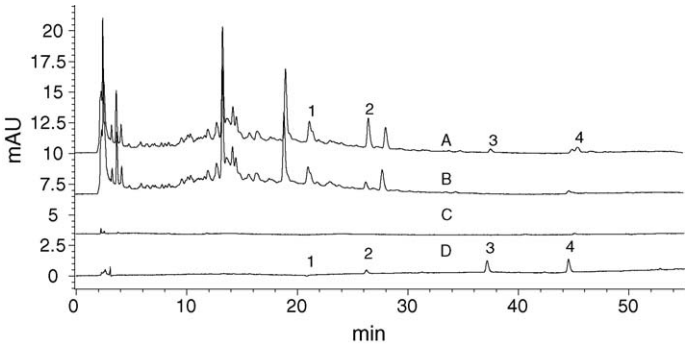

Fig. 4. Chromatograms of Radix Astragli monitored at 320 nm. (A) Extract of Radix Astragli treated with blank buffer A; (B) extract of Radix Astragli treated with endothelial cells; (C) the fourth eluate of deposited endothelial cells; (D) extract of denatured deposited endothelial cells.

3.2. Identification of the bioactive candidates in Danggui Buxue decoction and its compositions for endothelial cells

Up till now, at least five types of compounds were reported as the main ingredients of Danggui Buxue decoction or its compositions, i.e. polysaccharides, flavonoids (e.g. ononoside and calycosin), phthalides (e.g. ligustilide and 3-butylphthalide), saponins (e.g. astragaloside IV), phenolics (e.g. ferulic acid), etc. [17–22]. HPLC–DAD–MS hyphenated technique was used to elucidate the identity of the bioactive candidates of Danggui

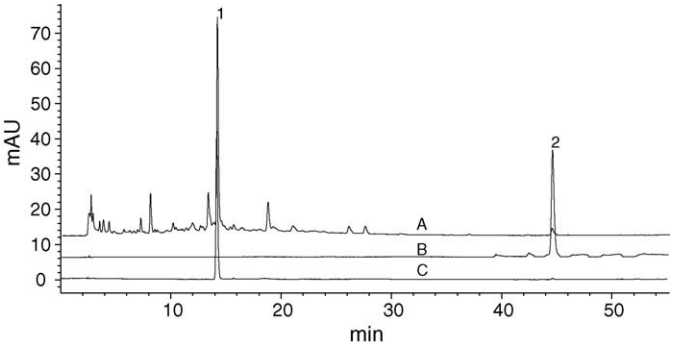

Fig. 5. Chromatograms of Danggui Buxue decoction and available reference compounds monitored at 320 nm. (A) Extract of Danggui Buxue decoction; (B) ligustilide; (C) ferulic acid.

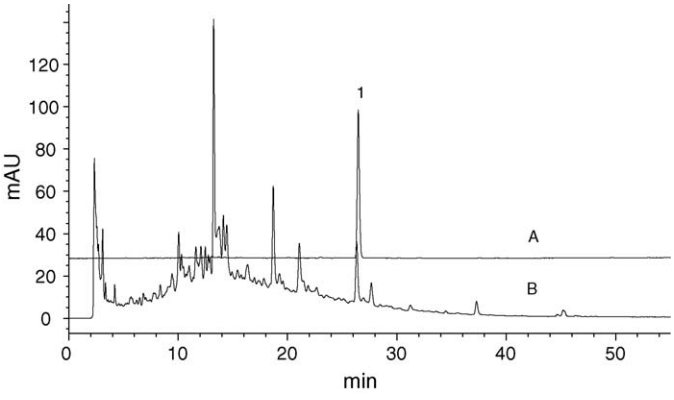

Fig. 6. Chromatograms of Radix Astragli and available reference compounds monitored at 320 nm. (A) Extract of Radix Astragli and (B) calycosine.

Table 1
MS data of five bioactive candidates of Danggui Buxue decoction for endothelial cells

Peak no.	Ions (+ESI)	Ions (−ESI)	Elemental composition	Error[a] (ppm)	DBE[b]	Identity
1	431.1338 $[M+H]^+$	465.0957 $[M+Cl]^-$	$C_{22}H_{22}O_9$	0.3268 (+)	12	Ononoside
2	285.0764 $[M+H]^+$	283.0631 $[M+Cl]^-$	$C_{16}H_{12}O_5$	2.2795 (+)	11	Calycosine
3	269.0842 $[M+H]^+$	267.0687 $[M-H]^-$	$C_{16}H_{12}O_4$	1.7367 (+)	11	268.0766 (MW, Da)
4	191.1068 $[M+H]^+$	–	$C_{12}H_{14}O_2$	0.7519 (+)	6	3-Butylphthalide
5	191.1068 $[M+H]^+$	–	$C_{12}H_{14}O_2$	0.7519 (+)	6	Ligustilide

[a] (+) Calculated from positive ions.
[b] Double bond equivalence.

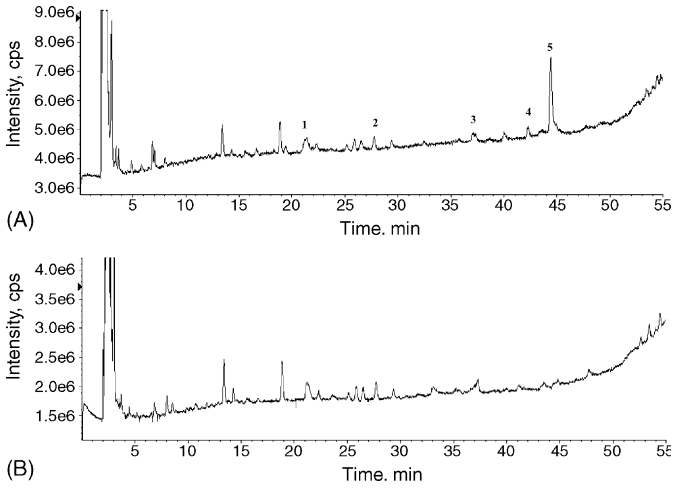

Fig. 7. The total ions chromatogram (TIC) of Danggui Buxue decoction in: (A) positive mode and (B) negative mode.

Buxue decoction for endothelial cells. The identities of the area-changing peaks were confirmed by the comparison of retention times and MS data with that of available references compounds or that in literatures.

The chromatograms of Danggui Buxue decoction and Radix Astragli, and the available reference compounds ferulic acid, ligustilide and calycosin monitored at 320 nm are shown in Figs. 5 and 6. The total ions chromatogram (TIC) of Danggui Buxue decoction is shown in Fig. 7. The MS data of five area-changing peaks are summarized in Table 1. The bioactive candidates of peaks 2 and 5 (contributed by Radix Angelica Sinensis) of Danggui Buxue decoction (Fig. 1A) were unequivocally identified as calycosin and ligustilide by comparison of retention times and MS data with that of reference compounds, while peaks 1 and 4 were tentatively assigned as ononoside and 3-butylphthalide by the comparison of their mass data with that of literatures [18–21] (Fig. 8), but for peak 3, only elemental composition and molecular weight were calculated, its identity is still under elucidation. Unfortunately the signal was not sensitive enough for the identification of peak 5 contributed by Radix Astragli under the present HPLC–DAD–MS conditions.

It has been reported that DL-3-butylphthalide could increase NO production in both bovine cerebral endothelial cells and bovine aortic endothelial cells, and selectively increase epoprostenol production in bovine aortic endothelial cells [23]. Calycosin could protect endothelial cells from hypoxia-induced barrier impairment by increasing intracellular energetic sources and promoting regeneration of the cAMP level, as well as improving cytoskeleton remodeling [24]. These findings indicate that 3-butylphthalide and calycosin did have bioactivities relevant to efficacy of Danggui Buxue decoction for anaemia treatment, and are with high probability the bioactive components of this combined prescription, which accordingly imply

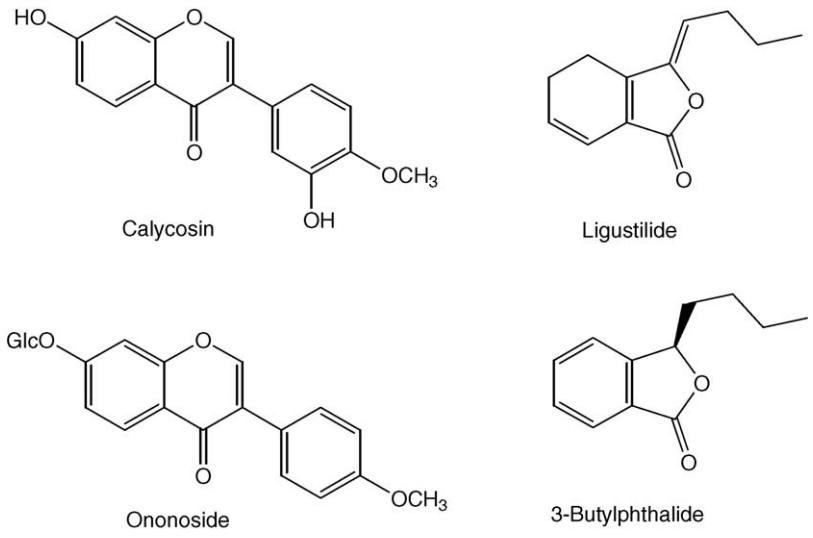

Fig. 8. Chemical structures of potential bioactive components found in Danggui Buxue decoction.

that this newly proposed strategy might be a useful approach for rapidly screening and analyzing potential bioactive candidates from TCM.

4. Conclusion

In present study, a novel strategy for screening and determining bioactive components of combined prescription of TCM using live cell extraction and HPLC–DAD–MS analysis was proposed and successfully applied for the investigation of potential bioactive candidates of Danggui Buxue decoction and its compositions, Radix Angelica Sinensis and Radix Astragli for endothelial cells. The results indicated that this newly proposed strategy might be a useful approach for predicting bioactive candidates in TCM with high probability.

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References

[1] G. Ye, Y.Z. Li, Y.Y. Li, H.Z. Guo, D.A. Guo, J. Pharm. Biomed. Anal. 33 (2003) 521–527.
[2] M. Homma, K. Oka, T. Yamada, T. Niitsuma, H. Ihto, N. Takahashi, Anal. Biochem. 202 (1992) 179–182.
[3] L.C. He, S.C. Wang, X.D. Geng, Chromatographia 54 (2001) 71–76.
[4] D.C. Schriemer, Anal. Chem. 76 (2004) 441A–448A.
[5] H.P. Luo, L.R. Chen, L.L. Zhu, Z. Ding, X.J. Xu, Anal. Chem. 75 (2003) 3994–3998.
[6] L.L. Zhu, L.R. Chen, H.P. Luo, X.J. Xu, Anal. Chem. 75 (2003) 6388–6393.
[7] R. Moaddel, L.L. Lu, M. Baynham, I.W. Wainer, J. Chromatogr. B 768 (2002) 41–53.
[8] R. Moaddel, I.W. Wainer, J. Pharm. Biomed. Anal. 30 (2003) 1715–1724.
[9] B.X. Yuan, L.C. He, G.D. Yang, Chin. J. Pharmacol. Toxicol. 17 (2003) 70–73.
[10] X.J. Zhao, G.C. Dang, G.D. Yang, L.C. He, Chin. J. Anal. Chem. 30 (2002) 195–197.
[11] K. Gao, L.C. He, G.D. Yang, Chin. Pharm. J. 38 (2003) 14–16.
[12] M.J. Liang, L.C. He, Chin. J. Anal. Chem. 32 (2004) 83–86.
[13] I. Gottschalk, C. Lagerquist, S.S. Zuo, A. Lundqvist, P. Lundahl, J. Chromatogr. B 768 (2002) 31–40.
[14] Z.B. Dong, S.P. Li, M. Hong, Q. Zhu, J. Pharm. Biomed. Anal. 38 (2005) 664–669.
[15] Z.B. Hu, L. Fan, Z.F. Han, D.Z. Wu, C.Q. Song, Chin. Tradit. Herbal Drugs 31 (2000) 756–758.
[16] Y. Wu, P.D. Zhu, J. West Chin. Univ. Med. Sci. 32 (2001) 593–595.
[17] C.H. Cho, Q.B. Mei, P. Shang, S.S. Lee, H.L. So, X. Guo, Y. Li, Planta Med. 66 (2000) 348–351.
[18] L.Z. Lin, X.G. He, M. Lindenmaier, G. Nolan, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordel, J. Chromatogr. A 876 (2000) 87–95.
[19] L.Z. Lin, X.G. He, L.Z. Lian, W. King, J. Elliott, J. Chromatogr. A 810 (1998) 71–79.
[20] Z.X. Wang, Q.F. Ma, Q. He, Chin. Tradit. Herbal Drugs 14 (1983) 97–99.
[21] W.H. Huang, C.Q. Song, Chin. J. Chin. Mater. Med. 26 (2001) 147–152.
[22] Z.H. Song, Z.N. Ji, C.K. Lo, T.T. Dong, K.J. Zhao, O.T. Li, C.J. Haines, S.D. Kung, K.W. Tsim, Planta Med. 70 (2004) 1222–1227.
[23] H.L. Xu, Y.P. Feng, Acta Pharmacol. Sin. 20 (1999) 929–933.
[24] Y. Fan, D.Z. Wu, Y.Q. Gong, J.Y. Zhou, Z.B. Hu, Eur. J. Pharmacol. 481 (2003) 33–40.