Chinese Medicine, 2015, 6, 147-157
Published Online June 2015 in SciRes. http://www.scirp.org/journal/cm
http://dx.doi.org/10.4236/cm.2015.62016

Specificity Screening of Potential Active Components from Moutan Cortex for Rat Mesangial Cells HBZY-1 by Cell Membrane Immobilized Chromatography

Junfei Gu[1,2,3], Minghua Zhang[1], Jiarui Yuan[1,3], Bingjie Zhao[1,3], Liang Feng[1*], Xiaobin Jia[1], Li Zhang[1], Yuesheng Wang[4], Luqi Huang[2*]

[1]Key Laboratory of New Drug Delivery System of Chinese Materia Medica, Jiangsu Provincial Academy of Chinese Medicine, Jiangsu Branch of China Academy of Chinese Medical Sciences, Nanjing, China
[2]State Key Laboratory Breeding Base of Dao-Di Herbs, China Academy of Chinese Medical Sciences, Beijing, China
[3]College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China
[4]Institute of Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing, China
Email: [*]wenmoxiushi@163.com, [*]huangluqi@263.net

Received 5 May 2015; accepted 20 June 2015; published 23 June 2015

Abstract

Moutan Cortex (MC) has been demonstrated to have an inhibitive effect on inflammation and oxidative stress responses in mesangial cells in our previous study. However, little is known about the components of MC contributing to this benefit. In the present study, cell membrane immobilized chromatography (CMC), a fast and useful method, was presented for screening potential active components of MC. HBZY-1 cells were incubated with MC (200 μg/mL) at the optimal incubation time (90 min). HPLC-DAD analysis and LC/ESI/MS/MS were performed to distinguish the active components and identify its structural ion fragments. The results showed that eight components binding to HBZY-1 cells were mudanoside B, paeoniflorin sulfonate, paeoniflorin, tetragalloyl glucose (isomeride), hexagalloyl glucose, mudanopiside A, and paeonol. In conclusion, our established CMC might be a useful method for screening potential active components in complicated traditional Chinese medicines. These components might be associated with the efficacy of MC on prevention and treatment of diabetic nephropathy.

Keywords

Cell Membrane Immobilized Chromatography, Moutan Cortex, HBZY-1 Cell, Screening

[*]Corresponding authors.

How to cite this paper: Gu, J.F., *et al*. (2015) Specificity Screening of Potential Active Components from Moutan Cortex for Rat Mesangial Cells HBZY-1 by Cell Membrane Immobilized Chromatography. *Chinese Medicine*, **6**, 147-157.
http://dx.doi.org/10.4236/cm.2015.62016

Components, Diabetic Nephropathy

1. Background

Diabetic nephropathy (DN), one of microvascular complications in diabetic patients as the end-stage renal disease, is the leading cause of high morbidity and mortality of diabetes mellitus (DM) throughout the word [1]. The optimal treatment for DN continues to evolve as newer therapies for decreasing DN progression through a multifaceted target [2]. Recent studies show that the beneficial effects of traditional Chinese medicines (TCMs) on the treatment and prevention of DN have attracted the increasing interest of researchers [3].

As a well-known traditional herbal medicine, Moutan Cortex (MC), the root bark of *Paeonia suffruticosa* Andr., has been used for DN treatment for a long time in China and other Asian countries [4]. It is worth noting that MC has an inhibitive effect on inflammation and oxidative stress responses in DN [5] [6]. Phytochemical study has shown that MC contains lots of chemical compounds such as paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, apiopaeonoside, benzoylpaeoniflorin, paeonol, sugars, etc. [7]. Actually, as we know, not all of components in TCMs are responsible for the disease treatment [8]. Of all the components in MC, paeonol has been investigated to have the anti-diabetic effect [9]-[11]. In addition, paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin and paeonol could inhibit the aggregation of platelet which might exacerbate the response of DN [12]. It has also been investigated that paeoniflorin and oxypaeoniflorin have the antioxidant and anti-inflammation activities on AGEs-induced mesangial cell damage in our present study [13]. However, little is known about the mutil-components of MC contributing to the prevention and treatment of DN.

Recently, more and more methods have been developed for the screening of active components in TCMs, such as high throughput screening, and DNA sequencing [14]-[16]. Pharmacology researches have demonstrated that the action of drugs may be derived from the interaction of bioactive component molecules with some receptors or channels on cell membrane. Cell membrane immobilized chromatography (CMC), one of cell membrane affinity chromatography, is composed of the biologic membrane immobilized part and the chromatographic analysis part. Specifically, the cell membrane of organism is used as a solid phase for targeting effective components in TCMs. Then, the potential active components can be analyzed by chromatographic techniques timely. The effective components in TCMs can be screened quickly and efficiently [17]. Hence, the use of CMC by biomembrane of organism extraction together with chromatography analysis may be a powerful tool for screening potential active components in TCMs.

Therefore, the present study was conducted to establish an appropriate CMC method for screening the potential active components in MC which interacted with mesangial cells. Furthermore, these active components, which were related to the protective effect of MC on DN, were revealed for the first time in our study.

2. Materials and Methods

2.1. Materials and Reagents

MC, the dried roots of *Paeonia suffruticosa* Andr. (Batch No. 20120415), was from Anhui Huqiao Chinese Medicine Technology Co., Ltd. (Tongling, Anhui Province). The pharmaceutical botany of the medicinal material was identified by Prof. Dekang Wu from Nanjing University of Chinese Medicine. Standard substances of paeoniflorin and paeonol, each content was more than 98% were offered by National Instisutes for Food and Drug Control. Mudanoside B, tetragalloyl glucose, hexagalloyl glucose, mudanopiside A, which the content were exceeded 98%, were supplied by BOC Sciences (NY, USA). D-glucose was purchased from Sigma (St. Louis, MO, USA). Basal DMEM medium and fetal bovine serum (FBS) was provided by Gibco (BRL, USA). All other reagents used in this study were from commercial sources.

2.2. Sample Preparation

Crude slices (50 g) of MC were weighted and extracted in 250 mL 80% (v/v) ethanol for 2 h in a reflux system (two times). The two extracts were merged and filtered through 0.45 μm membrane filter (PALL, USA). The solvent was removed in a rotary evaporator at 55˚C. The concentrated extract was then diluted to 0.5 g/mL and

stored at 4˚C for further use. The chromatograms of MC extract were analyzed by HPLC-DAD at 254 nm (**Figure 1**).

2.3. Preparation of AGEs

Advanced glycation end products (AGEs) were prepared according to previous method [18] [19]. In brief, 5 g bovine serum albumin (BSA) and 9 g D-glucose were dissolved adequately in 100 mL phosphate buffer saline (PBS, 0.2 M, pH = 7.4). After being passed through 0.22 μm microporous membrane filter, the solution was then incubated in 5% CO_2/95% air at 37˚C under sterile conditions. The reaction mixture was dialyzed in 0.2 M PBS overnight to remove unincorporated glucose molecules after the incubation of 3 months. The content of AGEs was measured by human AGEs ELISA kit, the final concentration was 858 mg/mL, and then stored at 4˚C until use.

2.4. Cell Culture

Rat mesangial cells line HBZY-1 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cells were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 80 units/mL of penicillin/streptomycin. Cells were incubated in 25 cm^2 plastic cell culture dishes in 5% CO_2/95% O_2 at 37˚C. All medium was renewed every 2 days to provide sufficient nutrition. After 80% - 90% confluent layer, cells were used for the further experiments.

2.5. Cell Membrane Extraction of Sample

HBZY-1 cells were incubated in a plastic dish at a generating of 80% - 90% confluent layer. AGEs of 1 mL was added into 9 mL PBS (pH = 7.4) to induce mesangial cell dysfunction. The MC extraction (50, 100, 200 μg/mL) was then incubated with live cells at 37˚C according to the previous description [7]. These cells were washed with 2 mL PBS to remove the unbound components for six times. The sixth washing eluate was retained for HPLC or LC/ESI/MS/MS analysis. Sequentially, 5 mL dissociation solution containing citric acid (pH 4.0) was added to dissociate the binding molecules at 37˚C for 60 min. The liquid supernatants of 5 mL were evaporated to dryness by high-purity nitrogen for 12 h at room temperature. Chromatographic methanol of 0.8 mL was added to each of them to dissolve the samples dryness. After vortex-mixing for 2 min, the supernatants of all the samples were taken and evaporated to dryness again for 5 h at room temperature. The residue was redissolved in 0.2 mL methanol followed by vortex-mixing for 2 min and centrifugation at 11,000 *g* for 10 min. Finally, the supernatants of all the samples were taken for HPLC and LC/ESI/MS/MS analysis.

2.6. HPLC Analysis

HPLC analysis system of Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD), a quaternary pump, a column heater-cooler and an autosampler was performed to separate and analyze these components of MC and eluates. The samples were separated on an Agilent TC-C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase gradient conditions consisted of acetonitrile (A) and 0.1% formic acid (B) was 0 - 20 min, 5% - 10% A; 20 - 30 min, 10% A; 30 - 80 min, 10% - 18% A; 80 - 120 min, 18% - 50% A. The flow rate was 0.8 mL/min and the column temperature was maintained at 25˚C with a 10 μL

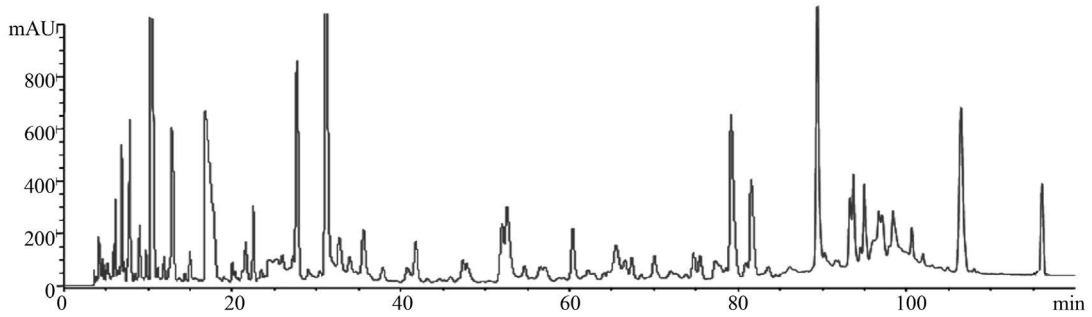

Figure 1. HPLC-DAD chromatograms was detected at 254 nm of MC extracted in 80% (v/v) ethanol.

injection volume at 254 nm.

2.7. LC/ESI/MS/MS Analysis for Identification of Compounds

In this study, LC/ESI/MS/MS analysis was used to identify the compounds which were dissociated from cell membrane of HBZY-1 cells. The analysis was performed under negative-ion mode by ThermoQuest mass spectrometer (San Jose, CA, USA). The optimal operating parameters were as follows: ion spray voltage, −4 kV; the nebulizer gas (N_2): 0.45 L/min; curtain gas (N_2): 0.2 L/min; declustering potential 1 (DP1): −20 V; focusing potential (FP): −80 V; declustering potential 2 (DP2): −20 V. The mass spectrometer was detected over a range of m/z 80 to 1000 in the full scan mode.

2.8. Statistical Analysis

All data were expressed as means ± standard deviation (SD). Statistical analysis was performed by GraphPad Prism TM 5.0 statistical package with analysis of variance. Comparison between groups was using t-test. The value of p less than 0.05 was considered to be a statistically significant difference.

3. Results and Discussion

3.1. The Optimization of Conditions in Cell Membrane Immobilization

The incubation concentration and incubation time are two important factors for CMC. Taken cells viability into consideration, different concentration extracts of MC including 50, 100, 200 μg/mL were examined to screen the optimal incubation concentration. As shown in **Figure 2(A)** and **Figure 2(B)**, after being incubated with three concentrations of MC, the most components of MC had a high peak area at the concentration of 200 μg/mL. It indicated that MC of 200 μg/mL had a better interaction with HBZY-1 cells. To make sure a better incubation for screening of components, 200 μg/mL MC was introduced for the cell membrane immobilization in our experiments. In addition, the selected concentration is consistent with that used in the evaluation of efficacy in our previous study [20].

Another important factor in CMC is the incubation time for the binding of components of MC to HBZY-1 cells. Different incubation time might affect the binding level of molecules to targets on cell membrane. Generally, the incubation time was 30 min as described by previous study in platelets or macrophages [17] [21]. In our present study, different time points including 15, 30, 60, 90, 120 min were examined for the most appropriate incubation time in HBZY-1 cells immobilized system. As shown in **Figure 3(A)** and **Figure 3(B)**, only 6 peaks were found in 15 min, whereas, 8 peaks were shown after 30 min. The peak areas of these 8 peaks were increased from 30 min to 90 min in a time-dependent manner. However, there were no more peaks and significant increase of peak area of 8 peaks in the chromatogram at 120 min. These data suggested that 90 min was the optimal time for the incubation.

Eventually, HBZY-1 cells were incubated with MC at the concentration of 200 μg/mL for 90 min. The optimal incubation time and concentration were used for the immobilization.

3.2. Cell Membrane Immobilization of Components in MC

Studies show that cell membrane immobilized chromatography (CMC) is a powerful tool for screening of active compounds in TCM extract relying on the living cells incubated in the culture flask associated with the pathogenesis through the relevant passageway. Hence, there are two important factors in potential active components screening from MC in the biologic membrane immobilized part: the style of cell membrane and the pathogenic passageway participating in DN.

It is well known that the targets related to pathological changes on the cell membrane are not identical in different types of cells. There is specific for the binding of bioactive components by the immobilization of different cell membranes. Reports have shown that mesangial cells, one of the main cell types of glomerulus, play important roles in the development of chronic kidney diseases including DN [22]. Hence, HBZY-1 cells were used for immobilizing potential active components of MC.

AGEs, derived from the non-enzymatic glycation reaction, have been found to accumulate in glomerular basement membrane and mesangial cells and are demonstrated to involve in the progression of DN [23]. The in-

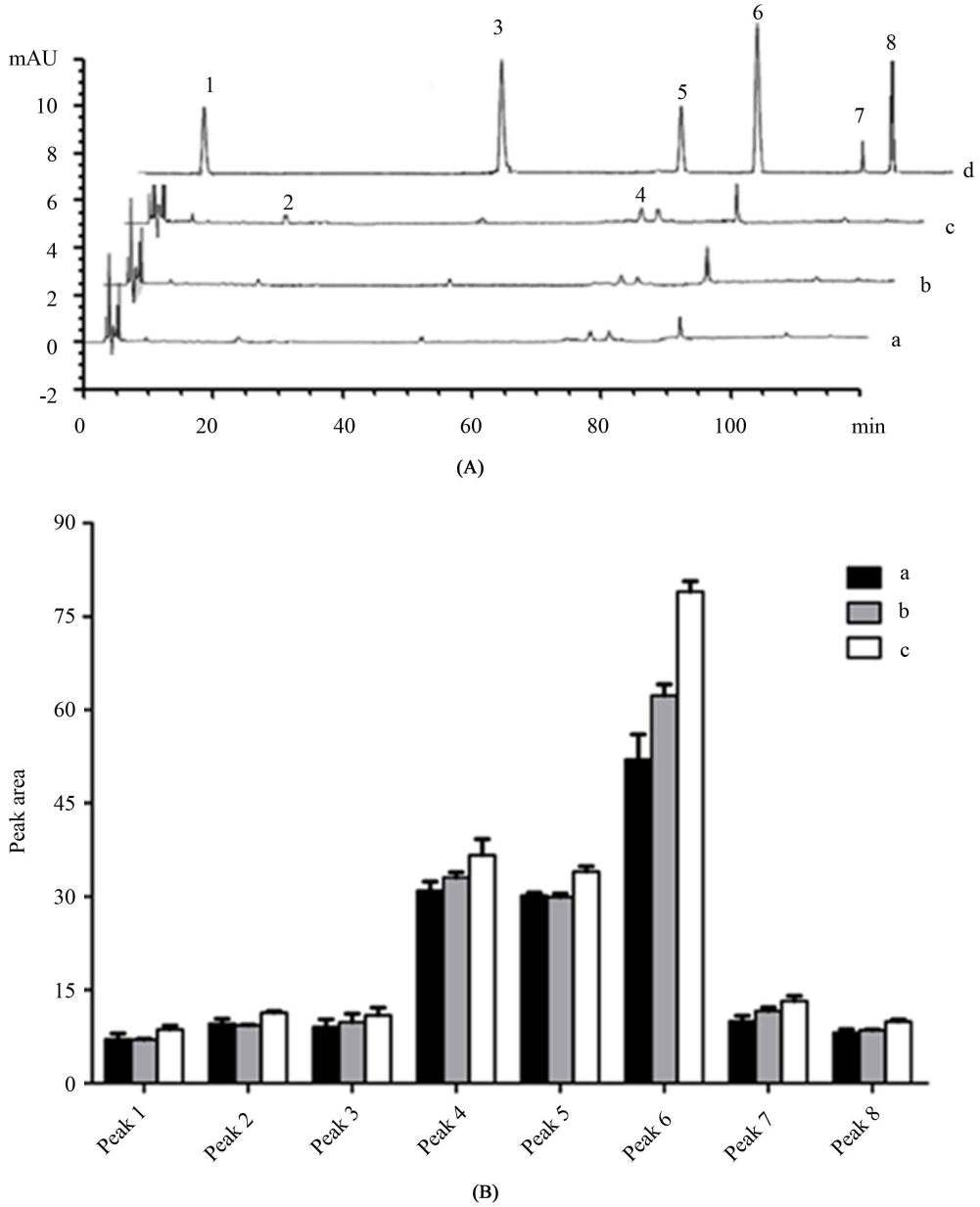

Figure 2. HPLC-DAD chromatograms was detected at 254 nm of MC after incubation with HBZY-1 cells in different concentrations (A). The peak area of 8 components of MC in different concentrations (B). (a) MC of 50, (b) MC of 100, (c) MC of 200 μg/mL at 37˚C, (d) the mixed standard of (1) mudanoside B; (3) paeoniflorin; (5) tetragalloyl glucose; (6) hexagalloyl glucose; (7) mudanopiside A; (8) paeonol.

teraction between AGEs and their specific receptors RAGE (AGEs-RAGE) on HBZY-1 cells membrane could stimulate the over-expressions of receptors and trigger signaling events contributing to the cells damage [24]. More importantly, the active components of MC play a protective role in the pathogenesis of mesangial cells under pathological conditions. Consequently, in the present study, AGEs were introduced to induce mesangial cells dysfunction for immobilizing these potential active components of MC which were binding to cell membrane.

After the analysis of HPLC in samples of the incubation buffer of MC, the sixth eluate and blank dissociation eluate, the result of immobilization product was shown in **Figure 4**. We could easily find that there were 8 peaks of MC in immobilized sample after being compared with MC extract, the sixth eluate and blank dissociation

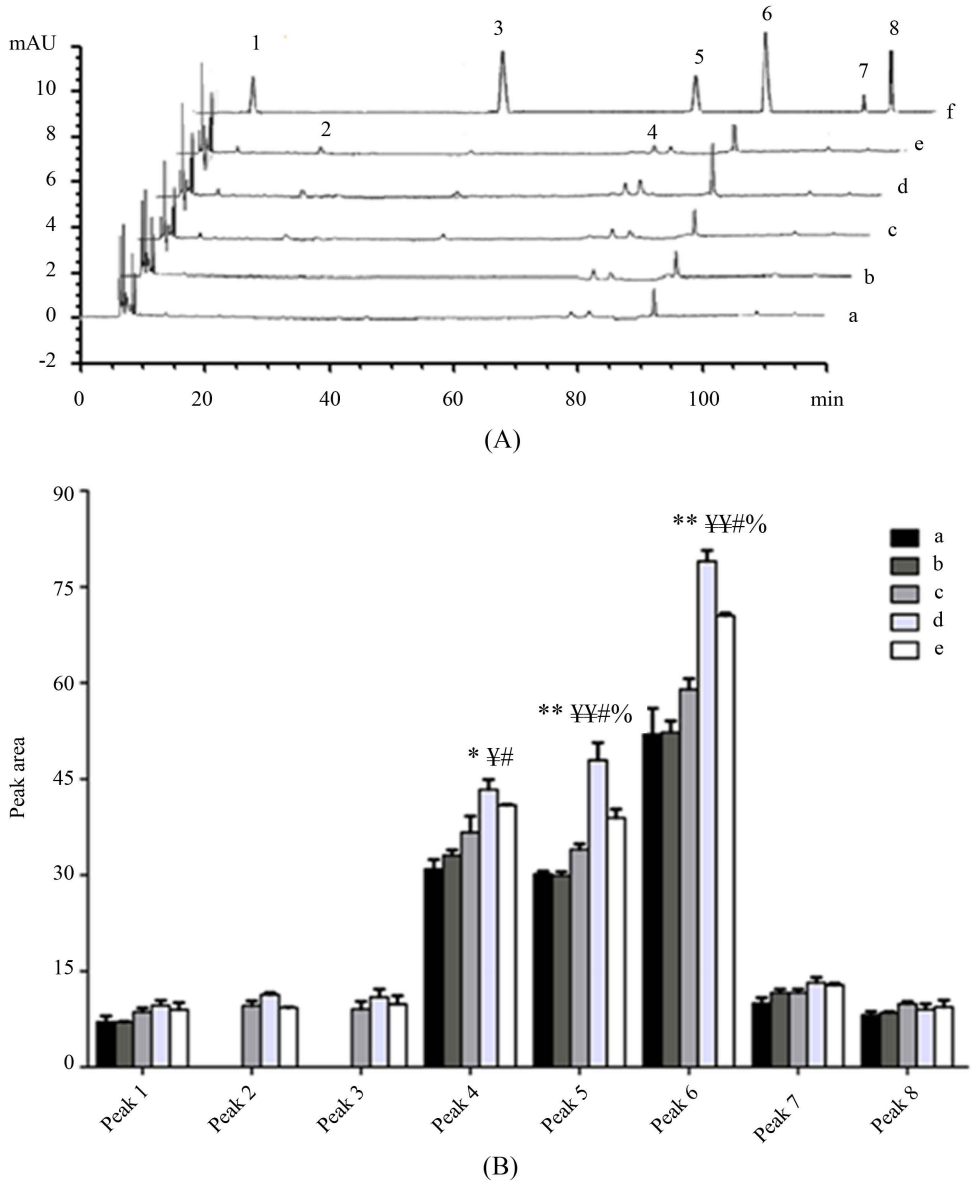

Figure 3. HPLC-DAD chromatograms detected at 254 nm of MC after incubation with HBZY-1 cells (A) and the peak area of 8 components of MC (B) at (a) 15 min, (b) 30 min, (c) 60 min, (d) 90 min, (e) 120 min, (f) the mixed standard of (1) mudanoside B; (3) paeoniflorin; (5) tetragalloyl glucose; (6) hexagalloyl glucose; (7) mudanopiside A; (8) paeonol. $^{*}p < 0.05$, (d) vs. (a); $^{**}p < 0.01$, (d) vs. (a); $^{¥}p < 0.05$, (d) vs. (b); $^{¥¥}p < 0.01$, (d) vs. (b); $^{\#}p < 0.05$, (d) vs. (c); % $p < 0.05$, (d) vs. (e).

eluate. The results indicated that peaks d1-d8 were the major components binding to cell membrane of HBZY-1. They might be the potential active components of MC for HBZY-1mesangial cells dysfunction.

3.3. Identification of Detected Components

As a powerful technique for identification of molecular, LC/ESI/MS/MS was introduced for the identification of components in the present study. The components in immobilized sample could be directly identified using LC/ESI/MS/MS analysis. The MS spectra of d1-d8 were shown in **Figure 5** and the information was listed in **Table 1**. These components were identified as mudanoside B, paeoniflorin sulfonate, paeoniflorin, tetragalloyl glucose (isomeride), hexagalloyl glucose, mudanopiside A, paeonol according to the MS ion fragments (**Figure 6**).

By comparing the MS spectra, retention time and UV spectra with published literature data and standard sub-

Table 1. Characterization of 8 components in MC after incubation with HBZY-1 cells by LC/ESI/MS/MS.

No.	t_R/min	$[M-H]^-$	(−)ESI-MS^2 m/z (% base peak)	Compounds
1	11.0	463	443, 331, 169	mudanoside B
2	23.66	543	——	paeoniflorin sulfonate
3	52.44	479	449, 327, 165, 121	paeoniflorin
4	68.74	787	635, 617, 447, 277, 169, 125	tetragalloyl glucose
5	72.05	787	635, 617, 447, 277, 169, 125	tetragalloyl glucose
6	89.61	1091	939, 769, 169	hexagalloyl glucose
7	107.65	613	461, 431, 165, 151, 121	mudanopiside A
8	113.73	165	150, 122	paeonol

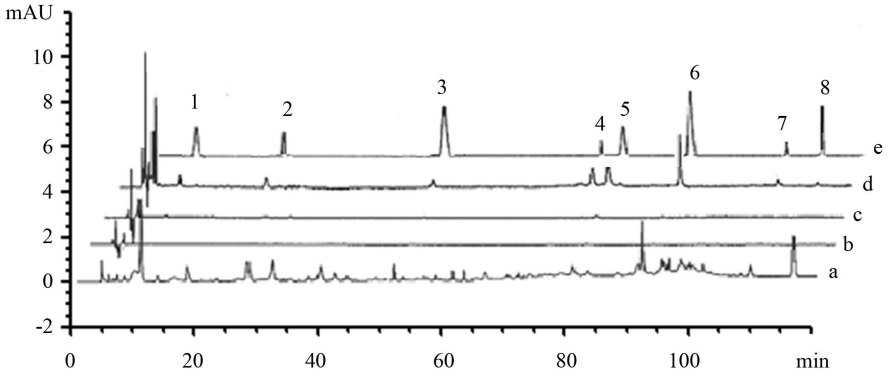

Figure 4. Chromatogram of the incubation of MC detected by HPLC-DAD (a), final washing eluate of biomembrane interacted with PBS (b), blank desorption eluate of biomembrane (c), desorption eluate of biomembrane interacted with MC (d), the mixed standard of (1) mudanoside B; (3) paeoniflorin; (5) tetragalloyl glucose; (6) hexagalloyl glucose; (7) mudanopiside A; (8) paeonol (e). Sample was detected at 254 nm.

stances [25], the components for peak d3 and d8 were identified as paeoniflorin and paeonol. Peak d1 exhibited an [M-H]-ion at m/z 463 was tentatively identified as mudanoside B in that their ion fragments at m/z 331 (a loss of a pentose (132 Da)) and m/z 169 (losses of a pentose (132 Da) according to previous references [25] [26].

It was well known that there was no components showing the [M-H]-ion at m/z 543 in MC, but d2 was identified as paeoniflorin sulfonate by comparison with the literature data previously [27]. Hence, it might be the product of the processing drug of MC which was changed from paeoniflorin.

It could also be easily seen that peak d4 and d5 had similar behavior displaying [M-H]-ions at m/z 787 in the ESI mass spectra, so it is very possible that they were isomeride. Their MS/MS spectra showed ion fragments at m/z 635, 617, 447, 277 169 and 125 with the losses of three gallic acids and a galloyl radical. Thus, they were proposed to be tetragalloyl glucose.

Peak d6 was identified as hexagalloyl glucose due to its [M-H]-ions at m/z 1091 in the full mass spectra. The greater 304 molecular weight of d6 compound than d4 and d5 was consistent with additional substitution of a galloyl group. The ion fragments of d6 at m/z 939 in that the losses of a gallic acid. For peak d8, it exhibited a [M-H]-ion at m/z 613. Its ion fragments at 151 and 121 attributed to [methoxybenzoic acid-H] and [benzoic acid-H]. The ion fragment at m/z 165 was reasonably identified as the pinane skeleton. Hence, compound d8 was assigned to be mudanpioside A.

MC is a well-known traditional herbal medicine that has been shown to hold a protective effect on DN. In the present study, the pharmacological activities of paeoniflorin and paeonol have been detected in anti-inflammation and anti-oxidation effect [13] [27] [28]. The pharmacological effect of other components will be investigated in our future study.

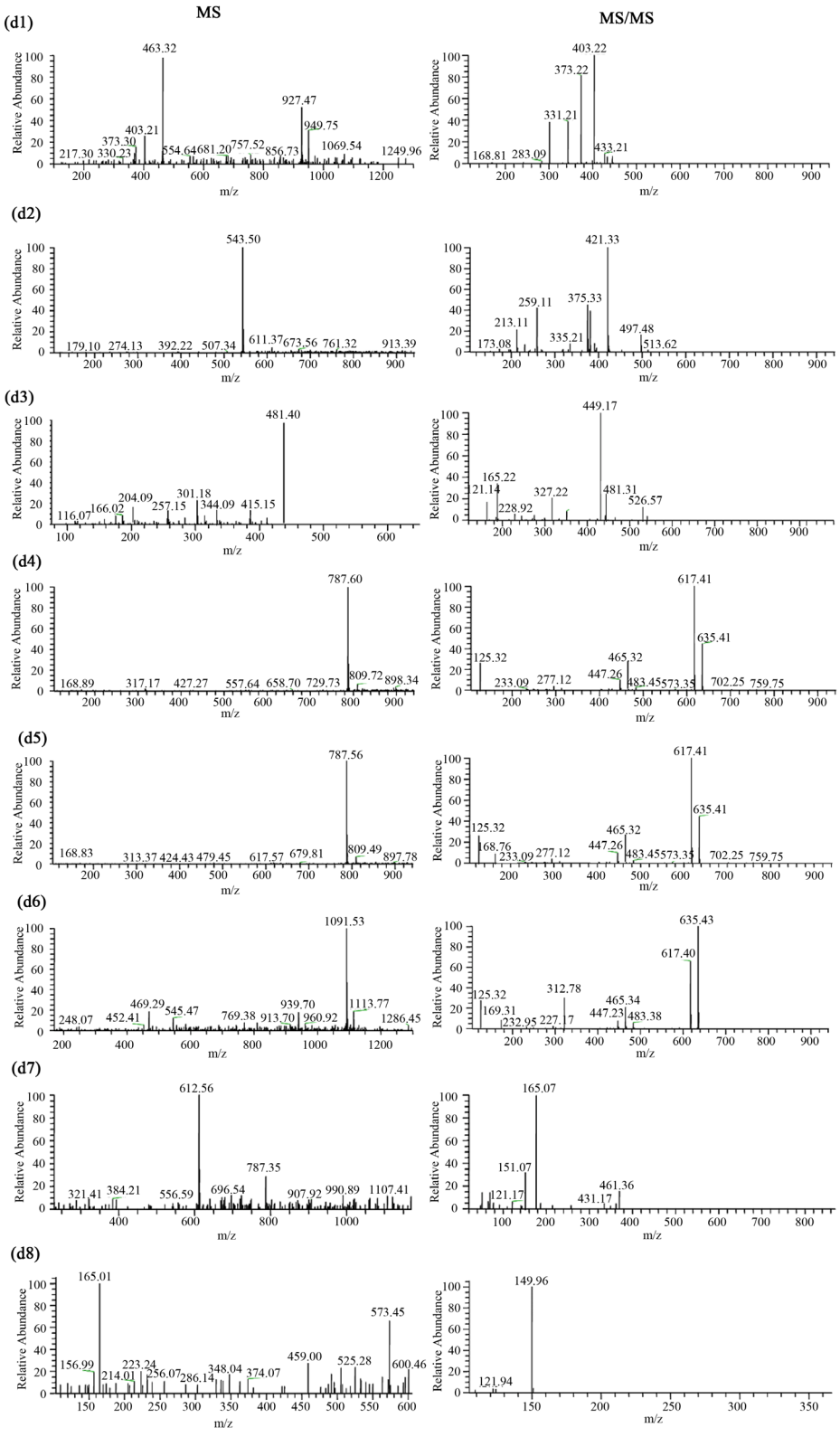

Figure 5. MS spectra of d1-d8 screened by cell membrane immobilized chromatography. (d1) mudanoside B; (d2) paeoniflorin sulfonate; (d3) paeoniflorin; (d4) tetragalloyl glucose; (d5) tetragalloyl glucose; (d6) hexagalloyl glucose; (d7) mudanopiside A; (d8) paeonol.

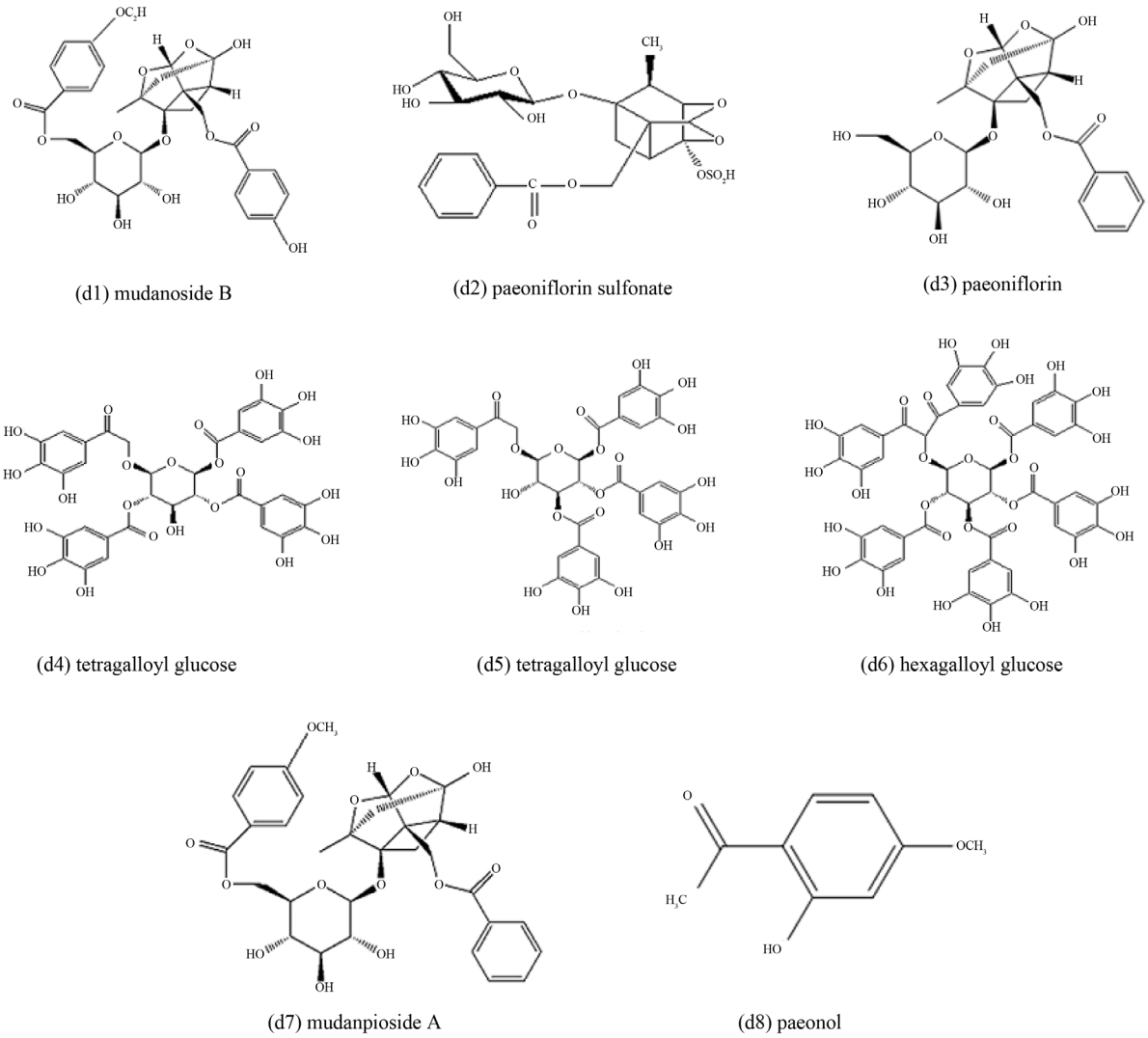

Figure 6. The structures of d1-d8 screened by cell membrane immobilized chromatography.

4. Conclusion

In the present study, CMC which relied on the living HBZY-1 cells incubated in the culture flask associated with the DN pathogenesis through the relevant passageway by biologic membrane immobilization part and LC/ESI/MS/MS part was presented for screening potential active components of MC. Finally, eight potential active components were obtained in MC binding to HBZY-1 cells. In conclusion, CMC might be a beneficial method in searching potential active components in complicated TCMs.

Acknowledgements

The authors gratefully acknowledge the financial support of the National Natural Science of China (81202906), Natural Science Foundation of Jiangsu (BK2012491), Major changes in the central level support projects (2060302) and National Natural Science Foundation of China (81130070).

Conflict of Interests

We declare that there is no conflict of interest. No author has any financial interest or conflict of interest involved with this study.

Authors' Contributions

JFG, MHZ, JRY, BJZ, LZ, XBJ and YSW contributed to the study design, analysis, and interpretation of data. LF and LQH participated in statistical analysis and drafted the manuscript. All authors approved the final manuscript.

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Abbreviations

AGEs: advanced glycation end products; **BSA**: bovine serum albumin; **CMC**: cell membrane immobilized chromatography; **DMEM**: Dulbecco's modified Eagle's medium; **DN**: diabetic nephropathy; **FBS**: fetal bovine serum; **MC**: Moutan Cortex; **TCMs**: traditional Chinese medicines.