



Short Communication

A high expression Mas-related G protein coupled receptor X2 cell membrane chromatography coupled with liquid chromatography and mass spectrometry method for screening potential anaphylactoid components in kudiezi injection

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ABSTRACT

Kudiezi injection is a traditional Chinese medicine injection used widely in China to alleviate blood stasis and to stimulate blood circulation. Its use has been associated with a high rate of adverse effects including anaphylactoid reactions. In the present study, a two-dimensional system comprised of high expression Mas-related G protein-coupled receptor X2 cell membrane chromatography coupled online with HPLC-ESI-IT-TOF-MS was established, and used to screen and identify anaphylactoid components in kudiezi injection. Luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, luteoloside and luteolin were identified as the potential anaphylactoid components. The release of β -hexosaminidase and histamine from Laboratory of Allergic Disease 2 cells enabled evaluation of the anaphylactoid activities of these compounds *in vitro*. Both β -hexosaminidase and histamine release were enhanced markedly with increasing concentrations of the anaphylactoid components. The molecular docking assay showed excellent interactions between the potential anaphylactoid constituents and MRGPRX2. In general, the two-dimensional system developed in this study is effective in screening for the anaphylactoid components in Kudiezi injection.

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1. Introduction

Kudiezi injection (KI) is comprised of an extract of the raw herb *Ilexeris sonchifolia* Hance. It is used widely in the treatment of cardio- and cerebrovascular diseases including coronary heart disease, angina and cerebral infarction [1]. Despite excellent therapeutic effects, adverse reactions to KI have been reported frequently. These include severe allergic reactions such as anaphylactic shock and neurological damage [2–4]. Anaphylactoid reactions to KI are one of the more concerning adverse reactions. However, neither the specific constituents of KI that produce these reactions nor their biological targets have been identified. At present, methods for screening for allergens in traditional Chinese medicine injections focus primarily upon screening almost all of the components and the binding of haptens to bull serum albumin [5–7]. These methods have their shortcomings including being costly, time-consuming and inefficient, as well as having poor specificity. Therefore, a rapid and efficient solution is urgently needed.

Affinity chromatography is a good choice for complex samples. Cell membrane chromatography (CMC) is a type of biological affinity chromatography that combines the advantages of biomaterials and chromatography. It is an effective and powerful tool to identify target compounds from complicated samples [8–10]. CMC has also been used successfully to investigate the interaction between drugs and membrane receptors [11,12]. High expression cells make screening more precise and effective. As a multi-dimensional type of chromatography, CMC coupled with HPLC-MS or GC-MS has proven to be a rapid and effective solution for target constituents in complex samples [13,14]. Therefore, we elected to employ high expression CMC combined with HPLC-ESI-IT-TOF-MS in this study.

Mas-related G protein-coupled receptor X2 (MRGPRX2) is a G protein-coupled 7-transmembrane protein, belonging to a large family of G-protein-coupled receptors. In humans, it is located mainly in dorsal root ganglia, neurohypophysis, blood vessels, skin mast cells, cord blood-derived mast cells and CD34+ cell-derived mast cells. MRGPRX2 is also expressed in the human mast cell line, Laboratory of Allergic Disease 2 (LAD2) [15,16], where it is involved in allergy, inflammation and itch [17–19]. Ligands of MRGPRX2 that induce itch include substance P, which is increased in atopic skin, as well as bovine adrenal medulla peptide and compound 48/80 (C48/80). These ligands stimulate MRGPRX2 directly

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and without the need for an intermediary such as IgE. In recent years, exogenous ligands such as opioid compounds, neuromuscular relaxants, antifungal drugs, and aminoglycoside, sulfonamide and quinolone antibacterial agents have been discovered. These can stimulate MRGPRX2 on mast cells to induce β -hexosaminidase and histamine release [20–23]. Thus, we chose MRGPRX2 as the receptor in our CMC model to screen for the components of KI that incite anaphylactoid reactions.

We used MRGPRX2 highly expressed human embryonic kidney 293 (MRGPRX2-HEK293) cell membrane chromatography to troll for potential anaphylactoid constituents in KI. This was coupled online with HPLC-ESI-IT-TOF-MS to analyze and identify the retained constituents. We then investigated the release of β -hexosaminidase and histamine from LAD2 cells to determine the sensitizing effects of the anaphylactoid components.

2. Materials and methods

2.1. Drugs and reagents

C48/80, histamine (HA) and ciprofloxacin (CF) were obtained from Sigma-Aldrich (MO, USA). Losartan (LA), erlotinib (EB) and ribavirin (RN) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Luteolin-7-*O*-glucuronide (LNG), apigenin-7-*O*-glucuronide (ANG), luteoloside (LE) and luteolin (LN) were obtained from Chengdu Pufeide Biotech Co., Ltd. (Chengdu, China). All the aforementioned compounds had purity $\geq 98\%$. KI (lot#: 140403) was obtained from Shenyang Shuangding Pharmaceutical Co., Ltd. (Shenyang, China). Silica gel (ZEX-II, 5 μ m, 200 Å) was supplied by Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). HPLC grade methanol, acetonitrile and formic acid were purchased from Merck KGaA (Darmstadt, Germany). A MK-459 Millipore Milli-Q Plus ultrapure water system (Darmstadt, Germany) was employed to produce water for this study.

2.2. Preparation of standard and sample solutions

LA, EB, RN, CF, LNG, ANG, LE and LN standard solutions were prepared in methanol, and stored in the dark at -20°C . The mixed standard solutions of LNG, ANG, LE and LN were prepared before analysis. All the samples for chromatographic analysis were diluted to suitable concentrations using the mobile phase and filtered through a 0.45 μ m filter.

2.3. Cell line

MRGPRX2-HEK293 cells were a gift from the collaborating laboratory in Johns Hopkins University [21] and grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies, New York, USA) supplemented with fetal bovine serum (10%, Clontech, Mountain View, CA, USA) and penicillin-streptomycin (100 U/mL, Invitrogen, Scotland, UK), and maintained at 37°C under 5% CO_2 . The LAD2 cells were provided by A. Kirshenbaum and D. Metcalfe (National Institutes of Health, USA). They were maintained in a StemPro-34 medium supplemented with human stem cell factor (SCF; 100 ng/mL), StemPro-Nutrient supplement (10 mL/L), L-glutamine (2 mM) and penicillin-streptomycin (1:100), and grown at 37°C under 5% CO_2 .

2.4. Preparation of MRGPRX2-HEK293/CMC column

Approximately 7×10^6 MRGPRX2-HEK293 cells in exponential phase were washed three times with phosphate-buffered saline (pH 7.4) and centrifuged ($110 \times g$) at 4°C for 5 min. The washed cells were added to Tris-HCl (50 mM, pH 7.4) before ultrasonication for 30 min and homogenization for 3 min in an ice-bath. Crude

cell membrane was obtained after centrifuging ($1000 \times g$) at 4°C for 10 min, and further centrifuging ($12,000 \times g$) of the supernatant at 4°C for 20 min. Purified cell membrane was acquired following additional centrifuging ($12,000 \times g$) at 4°C for 20 min. For the cell membrane stationary phase, the cell membrane suspension, in 5 mL phosphate-buffered saline, was adsorbed on 45 mg silica which activated at 105°C for 30 min, under vacuum, agitation and ice-bath conditions. The obtained mixture was stirred for 30 min and then allowed to stand for 14 h. The MRGPRX2-HEK293/CMC column (10 mm $\times$ 2.0 mm I.D.) was obtained by packing the cell membrane stationary phase with the wet packing procedure.

2.5. MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS

A two-dimensional method of MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS was developed. In this method, LC-2010AHT was applied in the MRGPRX2-HEK293/CMC system (the first dimension, ^1D) with the following conditions: ultrapure water mobile phase with a flow rate of 0.2 mL/min, UV detection (254 nm), and column kept at 37°C . A ten-port valve (Valco, TX, USA) with an RP-18e pre-column (used as the trap column, 10 mm $\times$ 4.6 mm I.D., Merck KGaA, Darmstadt, Germany) was used to couple ^1D with the HPLC-ESI-IT-TOF-MS system (the second dimension, ^2D), which comprised: two pumps (LC-20AD), an auto-sampler (SIL-20A), a degasser (DGU-20A3), a diode array detector (SPD-M20A), a communication bus module (CBM-20A), an ion trap-time of flight mass spectrometer (LCMS-IT-TOF-MS) and an LCMS solutions work station (Shimadzu Corporation, Kyoto, Japan). The conditions for ^2D were: 0.1% formic acid with 20 mM ammonium acetate (A) and acetonitrile (B) as the mobile phase with a gradient elution (0–18 min, 2–18% B; 18–25 min, 18–30% B; 25–35 min, 30–90% B), a RP-18e column (100 mm $\times$ 4.6 mm I.D., Merck KGaA, Darmstadt, Germany) with a 1.0 mL/min flow rate and kept at 37°C , and UV detection (254 nm). The effluent was split by a splitter valve to provide a flow rate of 0.3 mL/min into the mass spectrometer (Shimadzu, Kyoto, Japan). The optimal mass spectrometer parameters were: N_2 nebulizing gas (1.5 L/min) and drying gas (109 kPa), temperature of desolvation line and heat block set at 200°C , CID gas was Ar, voltage of the interface and detector set at 4.5 and 1.57 kV, respectively. All gases had purity $> 99.999\%$. The mass range was set at m/z 100–1000 in positive and negative ionization scan mode.

2.6. Validation of the two-dimensional system

For the MRGPRX2-HEK293/CMC column model, selectivity was verified by receptor-specific standard solutions of LA (angiotensin II receptor antagonist), EB (epidermal growth factor receptor antagonist), RN (broad-spectrum nucleoside antiviral agent) and CF (antibacterial ligand of MRGPRX2). For the two-dimensional system, CF was used as a positive-control to verify suitability. CF standard solution was injected into the ^1D system, with CF recognized and retained by the MRGPRX2 receptor on the MRGPRX2-HEK293/CMC column. The retained fraction was then enriched on the trap column and finally switched to the ^2D system for further analysis and identification.

2.7. Practical application of the two-dimensional system

KI was analyzed using the two-dimensional system established in this study. For further verification, standard solutions of the retained components screened from KI were also assayed using this system.

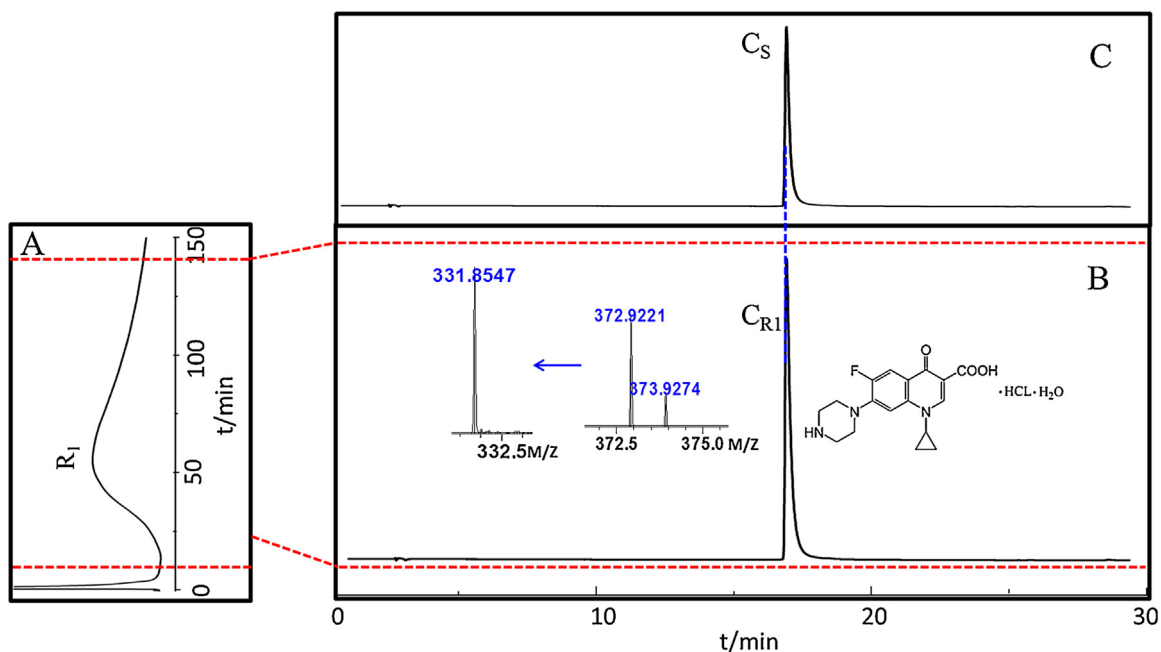


Fig. 1. Chromatograms of CF analyzed by the MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS method.

(A) Chromatogram of CF retained on the MRGPRX2-HEK293/CMC column.

(B) Chromatograms of the retained fraction (R_1) by HPLC-ESI-IT-TOF-MS.

(C) Chromatogram of CF by HPLC-ESI-IT-TOF-MS.

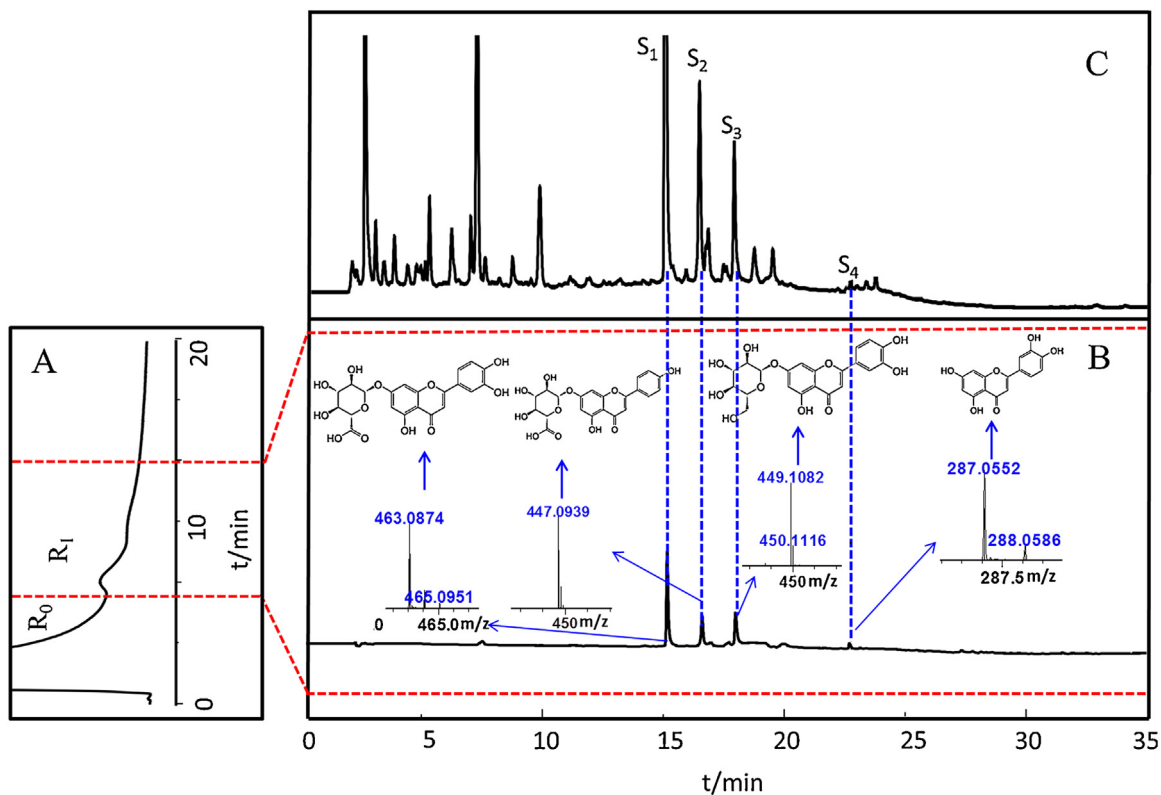


Fig. 2. Chromatograms of KI analyzed by the MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS method.

(A) Chromatogram of KI on the MRGPRX2-HEK293/CMC column.

(B) Chromatograms of the retained fraction (R_2) by HPLC-ESI-IT-TOF-MS.

(C) Chromatogram of KI by HPLC-ESI-IT-TOF-MS.

2.8. In vitro pharmacological effects

2.8.1. β -hexosaminidase release

Briefly, LAD2 cells were cultivated overnight in a 96-well plate under a 5% CO_2 atmosphere at 37 °C. They were then centrifuged

(200 × g) for 5 min to gather the cells at the bottom before removing the supernatant. Drugs at different concentrations (90 μ L per well, n = 3) were added to each well and further incubated for 30 min. TM buffer and C48/80 (30 μ g/mL) were added separately as neg-

ative and positive controls ($n=3$), respectively. After centrifuging ($200 \times g$) for 5 min, the supernatant ($50 \mu\text{L}$ per well) was moved to a new well and β -hexosamine ($50 \mu\text{L}$ per well) was added. The released β -hexosaminidase was quantified, as the absorbance at 405 nm, after reacting at 37°C for 90 min, with the reaction terminated using the stop buffer. To determine total β -hexosaminidase, the cells were lysed using 0.1% Triton X-100.

2.8.2. Histamine release

The histamine release assay was conducted using LAD2 cells according to our previous work and in triplicate [24]. Details are provided in the Supplementary material.

2.9. Molecular docking assay

For further verification of the combination of potential anaphylactoid components with MRGPRX2, a molecular docking assay was performed using Surflex-DockMode of the SYBYL-X 2.0 program package (Tripos, MO, USA). The MRGPRX2 structure was calculated using Modeller and expanded upon using elastic network modeling to increase the amount of sampled backbone conformations [20]. The sphere space field and ribbon models were chosen for this project.

2.10. Statistical analysis

The data were analyzed using SPSS software, and presented as mean $\pm$ standard error of the mean (S.E.M.). A two-tailed non-paired Student's *t*-test was used for independent sample variance analysis, with differences considered statistically significant at $*P<0.05$, $**P<0.01$, and $***P<0.001$.

3. Results and discussion

3.1. Validation of the two-dimensional system

For MRGPRX2-HEK293/CMC column selectivity, LA, EB, RN and CF were analyzed using the ^1D system, with resultant chromatograms shown in Supplementary Fig. 1. None of the three negative drugs (LA, EB and RN) were retained on the MRGPRX2-HEK293/CMC column, while CF was retained obviously. This showed that only the specific ligand, CF, was recognized by the MRGPRX2 receptor and retained on the column. This suggested that the MRGPRX2-HEK293/CMC column could screen the target compounds selectively.

Due to specific bonding of the ligand-receptor, CF-MRGPRX2, the standard CF solution was injected into the two-dimensional system as a positive-control to validate suitability (Fig. 1) [21]. The LC work station recorded the CF chromatogram on the MRGPRX2-HEK293 column (Fig. 1A), with the sole retained fraction (R_1) enriched by the trap column and switched to the ^2D system. After analysis and identification of R_1 in the HPLC-ESI-IT-TOF-MS system, we obtained its chromatogram and mass spectrometry information (Fig. 1B), which identified R_1 as *cf*. For comparative purposes, the chromatogram of CF from the HPLC-ESI-IT-TOF-MS system is shown in Fig. 1C. Hence, we showed that the MRGPRX2-HEK293 coupled with HPLC-ESI-IT-TOF-MS system could be employed successfully to analyze the constituents that had MRGPRX2 receptor activity.

3.2. Practical application of the two-dimensional system

The developed two-dimensional system was then used to screen for the potential anaphylactoid components in KI (Fig. 2). When KI was analyzed using the ^1D system, the unconjugated compo-

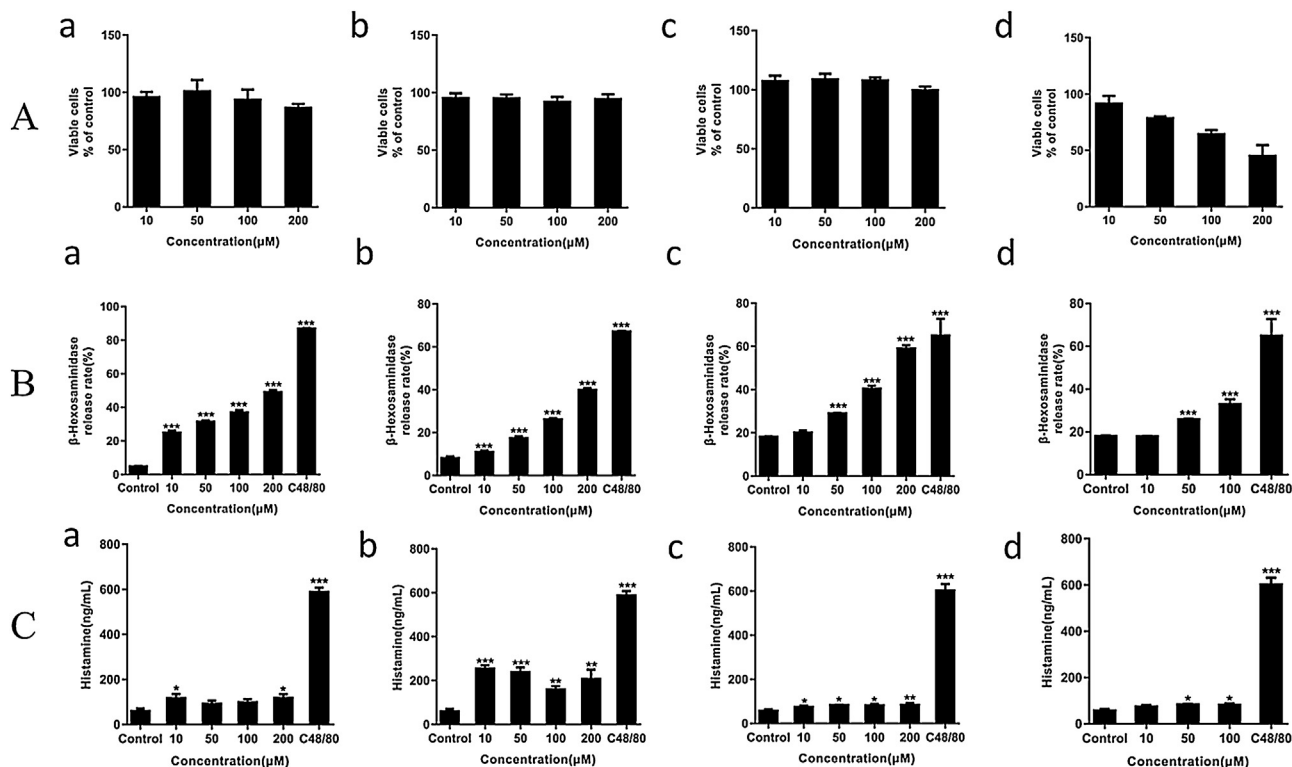


Fig. 3. *In vitro* pharmacological effects of LNG, ANG, LE and LN.

(A) Effect of LNG, ANG, LE and LN on LAD2 cell viability as measured by CCK-8 assay.

(B) β -hexosaminidase release by LAD2 cells treated with LNG, ANG, LE and LN.

(C) Histamine release by LAD2 cells treated with LNG, ANG, LE and LN.

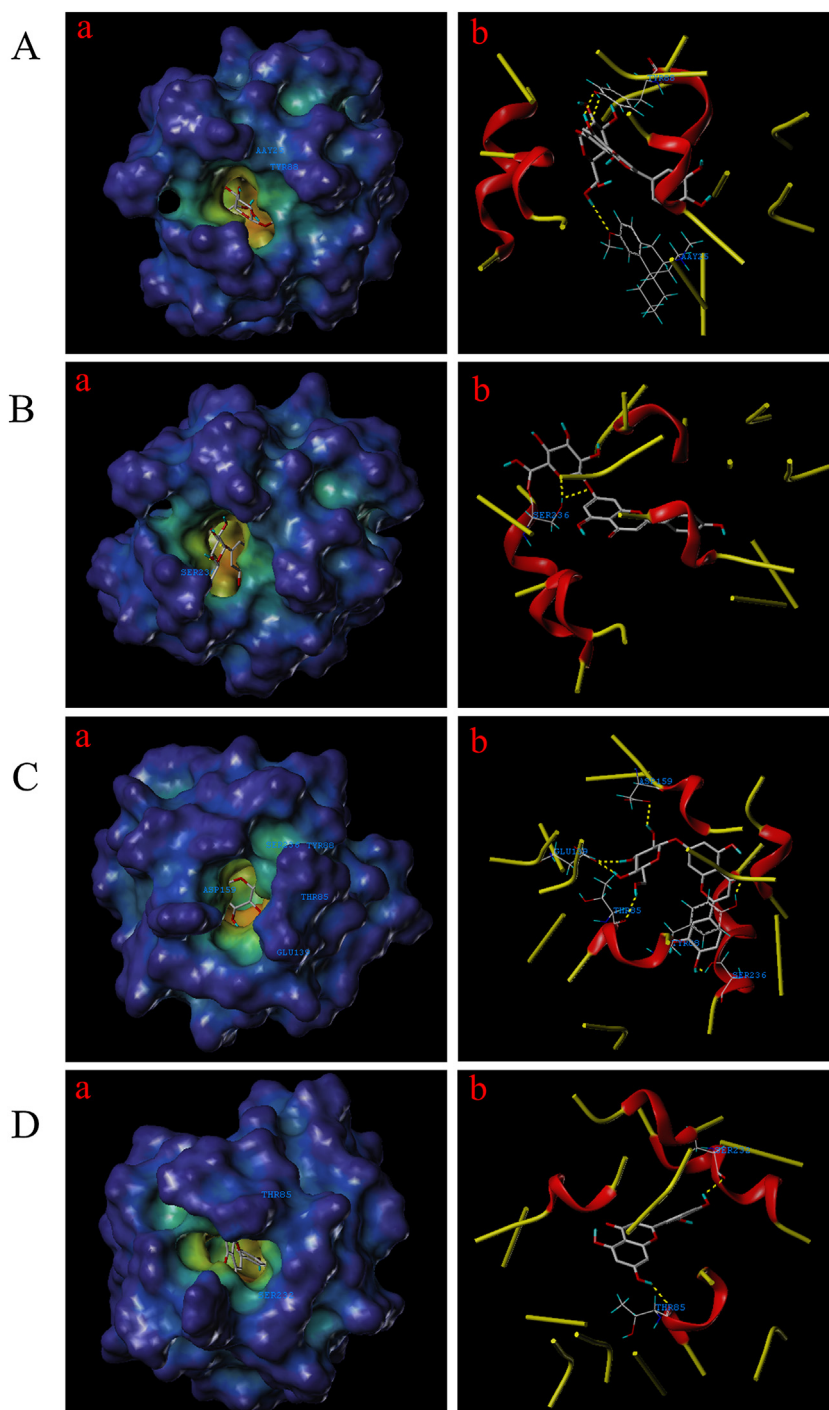


Fig. 4. Molecular docking modeling of LNG, ANG, LE and LN with MRGPRX2.

(A) Molecular docking modeling of LNG with MRGPRX2.

a. Ribbon model of LNG and MRGPRX2, b. Sphere space field model of LNG and MRGPRX2.

(B) Molecular docking modeling of ANG with MRGPRX2.

a. Ribbon model of ANG and MRGPRX2, b. Sphere space field model of ANG and MRGPRX2.

(C) Molecular docking modeling of LE with MRGPRX2.

a. Ribbon model of LE and MRGPRX2, b. Sphere space field model of LE and MRGPRX2.

(D) Molecular docking modeling of LN with MRGPRX2.

a. Ribbon model of LN and MRGPRX2, b. Sphere space field model of LN and MRGPRX2.

nents on the MRGPRX2-HEK293/CMC column flowed out first along with the solvent. With repetitive adsorption-elution, the conjunct components flowed out subsequently. Fig. 2A shows the MRGPRX2-HEK293/CMC chromatogram of KI, with one retained fraction (R_2 , retention time about 7 min) appearing on the MRGPRX2-

HEK293/CMC column. R_2 was enriched in the trap column, and after a switch of the ten-port valve, R_2 was further identified using the coupled HPLC-ESI-IT-TOF-MS system. The HPLC-ESI-IT-TOF-MS chromatograms of R_2 are shown in Fig. 2B. These were identified as LNG, ANG, LE and LN depending on retention time and the mass

spectrometry information from the ²D system. For comparison, the ²D chromatogram of KI is presented in Fig. 2C. The retention time was evaluated several times by continuous analysis of the positive drug and KI (Supplementary Figs. 2, 3), and showed excellent repeatability (Supplementary Tables 1, 2).

To confirm the veracity of the above results, the mixture of LNG, ANG, LE and LN was also analyzed using the same process and system (Supplementary Fig. 4). The same chromatograms and mass spectrometry fragments were demonstrated. Collectively, these results suggested that LNG, ANG, LE and LN were indeed the potential anaphylactoid constituents of KI.

3.3. In vitro pharmacological effects

LNG, ANG, LE and LN-induced anaphylactoid reactions involved the release of β -hexosaminidase and histamine, which are characteristic mediators for mast cells stimulated by allergens. Hence, cell viability under serial concentrations of LNG, ANG, LE and LN was tested to determine the concentration scope of these compounds for inducing β -hexosaminidase and histamine release. As depicted in Fig. 3A, LNG, LE and LN did not exhibit any LAD2 cell toxicity at concentrations of 10, 50, 100 and 200 μ M whereas ANG had no toxic effect at the three lower concentrations. The release of β -hexosaminidase and histamine from LAD2 cells treated with serial concentrations of LNG, ANG, LE and LN, negative control and positive control (C48/80, 30 μ g/mL) was 25–49% and 123–128 ng/mL for LNG, 11–40% and 159–254 ng/mL for ANG, 20–59% and 74–84 ng/mL for LE, 19–31% and 74–82 ng/mL for LN. There were significant differences in β -hexosaminidase and histamine release between all four treatments and the negative control (Fig. 3B and C). Furthermore, LNG and ANG produced effects that were clearly more pronounced than with LE and LN. From the results in Fig. 3, it can be seen that LNG, ANG, LE and LN from KI can induce β -hexosaminidase and histamine release in LAD2 cells.

3.4. Molecular docking

To investigate the interactions between the potential anaphylactoid constituents and MRGPRX2, molecular docking was performed using the Surflex-Dock mode of the SYBYL-X 2.0 program package. The docking results showed that LNG, ANG, LE and LN matched the active pocket of MRGPRX2 well in the sphere space field model (Fig. 4Aa, Ba, Ca, Da). Detailed analysis of the interaction between the potential anaphylactoid constituents and MRGPRX2 is shown in the ribbon model (Fig. 4Ab, Bb, Cb, Db). Based on the docking results, it was found that LNG formed three hydrogen bonds with TYR88 and AAY25, ANG formed one hydrogen bond with SER236, LE formed six hydrogen bonds with ASP159, GLU139, THR35, TYR88 and SER236, and LN formed two hydrogen bonds with THR85 and SER232 in MRGPRX2. These results suggested that the potential anaphylactoid constituents bind well to MRGPRX2.

4. Conclusions

A MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS system was established and identified the potential anaphylactoid components of KI as LNG, ANG, LE and LN. MRGPRX2 target-based potential anaphylactoid components can be controlled and identified within one hour. *In vitro* pharmacological studies showed that all four compounds could precipitate release of β -hexosaminidase and histamine across a range of concentrations, and molecular docking found that LNG, ANG, LE and LN could bind well to MRGPRX2. These findings indicated that the two-dimensional system employed could efficiently complete the screening process by combining specific recognition via the

MRGPRX2-HEK293/CMC model with accurate identification via the HPLC-ESI-IT-TOF-MS online system. This method was demonstrated to be efficient and targeted. Overall, this system offers a useful screening method to identify target components in complex samples that act on MRGPRX2.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jpba.2018.07.027>.

References

- [1] Y.P. Chang, L. Li, Y.M. Xie, Y. Zhuang, Clinical outcomes research on parenterally administered Kudiezi in treatment of cerebral infarction, *China J. Chin. Mater. Med.* 38 (2013) 3155–3160.
- [2] Y. Shi, A.H. Yan, Adverse reaction summary of Kudiezi injection in 2008–2012, *J. Dis. Monit. Control* 6 (2012), 657–674.
- [3] J.B. Zhao, N. Huang, Q.U. Yi, 12 cases literature analysis of adverse reaction induced by Kudiezi injection, *Chin. J. Drug Eval.* 30 (2013), 111–112+116.
- [4] M. Lv, Z. Wang, D. Li, Literature analysis of adverse drug reactions induced by Kudiezi injection, *China Pharm.* 21 (2012) 38–39.
- [5] F. Wang, C.Y. Li, Y.F. Zheng, H.Y. Li, W. Xiao, G.P. Peng, Identification of the allergenic ingredients in Reduning injection by ultrafiltration and high-performance liquid chromatography, *Clin. Dev. Immunol.* 2 (2016) 1–7.
- [6] H. Wang, X.T. Zhang, D.D. Wang, H.Y. Sun, Y.X. Lan, Y.X. Lan, et al., An on-line analytical approach for detecting haptens in Shuang-huang-lian powder injection, *J. Chromatogr. A* 1513 (2017) 126–139.
- [7] X.G. Hu, Y. Wen, S.S. Liu, J.B. Luo, X.M. Tan, Z.H. Li, et al., Evaluation of the anaphylactoid potential of *Andrographis paniculata* extracts using the popliteal lymph node assay and P815 cell degranulation *in vitro*, *J. Transl. Med.* 13 (2015) 1–9.
- [8] L.C. He, X.D. Geng, Cell membrane receptors chromatography—a new method for drug and receptor interaction, *New Prog. Biomed. Chromatogr. Chin.* 3 (1996) 8–9.
- [9] M. Sun, X.F. Hou, Y.Y. Lin, J. Zhang, S.C. Wang, An improved cell membrane chromatography method for the simultaneous screening of two epidermal growth factor receptor antagonists from *radix scutellariae*, *J. Sep. Sci.* 38 (2015) 3145–3150.
- [10] L.C. He, S.C. Wang, X.D. Geng, Coating and fusing cell membranes onto a silica surface and their chromatographic characteristics, *Chromatographia* 54 (2001) 71–76.
- [11] W.N. Ma, L. Yang, Y.N. Lv, J. Fu, Y.M. Zhang, L.C. He, Determine equilibrium dissociation. Constant of drug-membrane receptor affinity using the cell membrane chromatography relative standard method, *J. Chromatogr. A* 1503 (2017) 12–20.
- [12] H. Du, J. Ren, S.C. Wang, L.C. He, Cell membrane chromatography competitive binding analysis for characterization of α (1A) adrenoreceptor binding interactions, *Anal. Bioanal. Chem.* 400 (2011) 3625–3633.
- [13] F. Wei, Q. Hu, J. Huang, S.L. Han, S.C. Wang, Screening active compounds from *Corydalis yanhusuo* by combining high expression VEGF receptor HEK293 cell membrane chromatography with HPLC-ESI-IT-TOF-MSn method, *J. Pharm. Biomed. Anal.* 136 (2017) 134–139.
- [14] S.C. Wang, M. Sun, Y.M. Zhang, H. Du, L.C. He, A new A431/cell membrane chromatography and online high performance liquid chromatography/mass spectrometry method for screening epidermal growth factor receptor antagonists from *Radix sophorae flavescentis*, *J. Chromatogr. A* 1217 (2010) 5246–5252.
- [15] H.H. Wu, M.Q. Zeng, E.Y. Cho, W.Q. Jiang, O. Sha, The origin, expression, function and future research focus of a G protein-coupled receptor, Mas-related Gene X2 (MrgX2), *Prog. Histochem. Cyto.* 50 (2015) 11–17.
- [16] S. Bulfone-Paus, G. Nilsson, P. Draber, U. Blank, F. Levi-Schaffer, Positive and negative signals in mast cell activation, *Trends Immunol.* 38 (2017) 657–667.
- [17] V.B. Reddy, T.A. Graham, E. Azimi, E.A. Lerner, A single amino acid in MRGPRX2 necessary for binding and activation by pruritogens, *J. Allergy Clin. Immunol.* 140 (2017) 1726–1728.

- [18] T. Karhu, K. Akiyama, O. Vuolteenaho, U. Bergmann, T. Naito, K. Tatemoto, et al., Mast cell degranulation via MRGPRX2 by isolated human albumin fragments, *BBA-Gen. Subj.* 1861 (2017) 2530–2534.
- [19] M. Babina, S. Guhl, M. Artuc, T. Zuberbier, Allergic FcεRI-and pseudo-allergic MRGPRX2-triggered mast cell activation routes are independent and inversely regulated by SCF, *Allergy* 73 (2018) 256–260.
- [20] K. Lansu, J. Karpiak, J. Liu, X.P. Huang, J.D. McCorvy, W.K. Kroeze, et al., In silico design of novel probes for the atypical opioid receptor MRGPRX2, *Nat. Chem. Biol.* 13 (2017) 529–536.
- [21] B.D. McNeil, P. Pundir, S. Meeker, L. Han, B.J. Undem, M. Kulka, et al., Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions, *Nature* 519 (2015) 237–241.
- [22] T. Zhang, D.L. Che, R. Liu, S.L. Han, N. Wang, Y.Z. Zhan, et al., Typical antimicrobials induce mast cell degranulation and anaphylactoid reactions via MRGPRX2 and its murine homologue MRGPRB2, *Eur. J. Immunol.* 47 (2017) 1949–1958.
- [23] R. Liu, D.L. Che, T. Zhang, P. Pundir, J. Cao, Y.N. Lv, et al., MRGPRX2 is essential for sinomenine hydrochloride induced anaphylactoid reactions, *Biochem. Pharmacol.* 146 (2017) 214–223.
- [24] S.L. Han, Y.N. Lv, L.Y. Kong, D.L. Che, R. Liu, J. Fu, et al., Use of the relative release index for histamine in LAD2 cells to evaluate the potential anaphylactoid effects of drugs, *Sci. Rep.* 7 (2017).

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2. Materials and methods

2.2 Preparation of standard and sample solutions

Tyrode's modified solution (TM): 7.00 g NaCl, 0.35 g KCl, 0.37 g CaCl₂·2H₂O, 0.30 g MgSO₄·7H₂O, 0.16 g KH₂PO₄, 2.38 g HEPES, 1.31 g Sucrose and 1.00 g BSA in 1000 mL ultrapure water and sterilized.

Stop buffer used in β-hexosaminidase release assay: 0.1 mol/L Na₂CO₃/NaHCO₃.

2.8.2 Histamine release

To be more specific, about 30000 LAD2 cells per well in a 96-well plate were cultured overnight in the conditions of 37 °C in a 5% CO₂ atmosphere. After centrifugation (200 × *g*, 5 min), the culture supernatants were discarded, and the diluted drugs (in TM buffer), negative control (TM buffer) and positive control (30 μg/mL C48/80 in TM buffer) were added successively. After stimulation for 30 min at 37 °C in a 5% CO₂ atmosphere, centrifugation at 200 × *g* for 5 min was carried out again, and the supernatants (50 μL per well) were transferred immediately into the 1.5 mL tubes on ice, followed with 100 μL 5 ng/mL d4-HA acetonitrile solution. The mixtures were then centrifuged (12,000 × *g*, 20 min, 4 °C), and the supernatants were collected for analysis by HPLC-ESI-MS/MS. In the performed method of histamine release assay, a Venusil HILIC column (150 mm × 2.1 mm I.D., 3 μm, Agela Technologies, Tianjin, China) was employed, isocratic elution, the proportion of acetonitrile and water added with 0.1% formic acid and 20 mM ammonium formate was 82:18 (v/v), and the flow rate adopted was at 0.3 mL/min.

Supplementary Table 1 The retain times of CF on MRGPRX2-HEK293/CMC for 6 times and the RSD

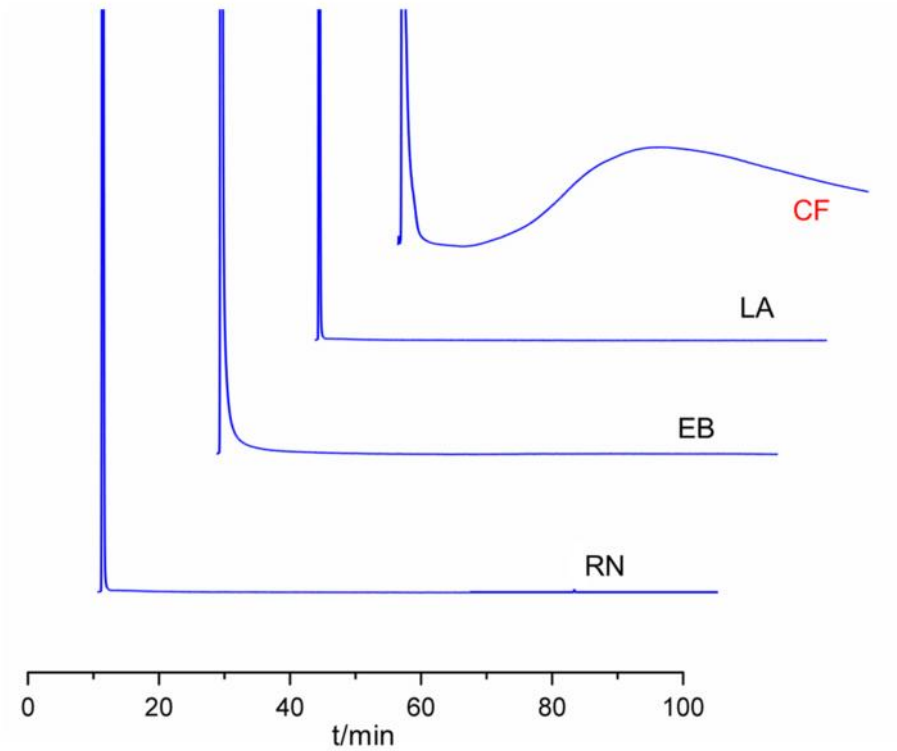
Retain time	RSD
54.001	0.89%
53.612	
53.458	
53.153	
52.843	
52.776	

43 Supplementary Table 2 The retain times of KI on MRGPRX2-HEK293/CMC for 3 times and the RSD

Retain time	RSD
6.814, 10.005	
6.312, 9.707	4.04%, 1.56%
6.426, 9.792	

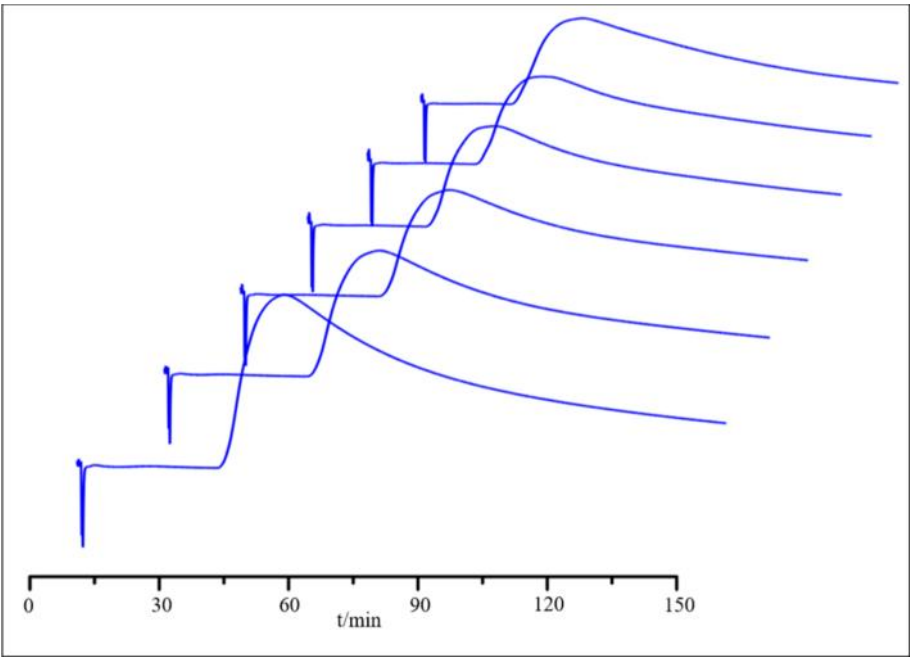
44

45 Supplementary Figure 1 Selectivity of the MRGPRX2-HEK293/CMC column

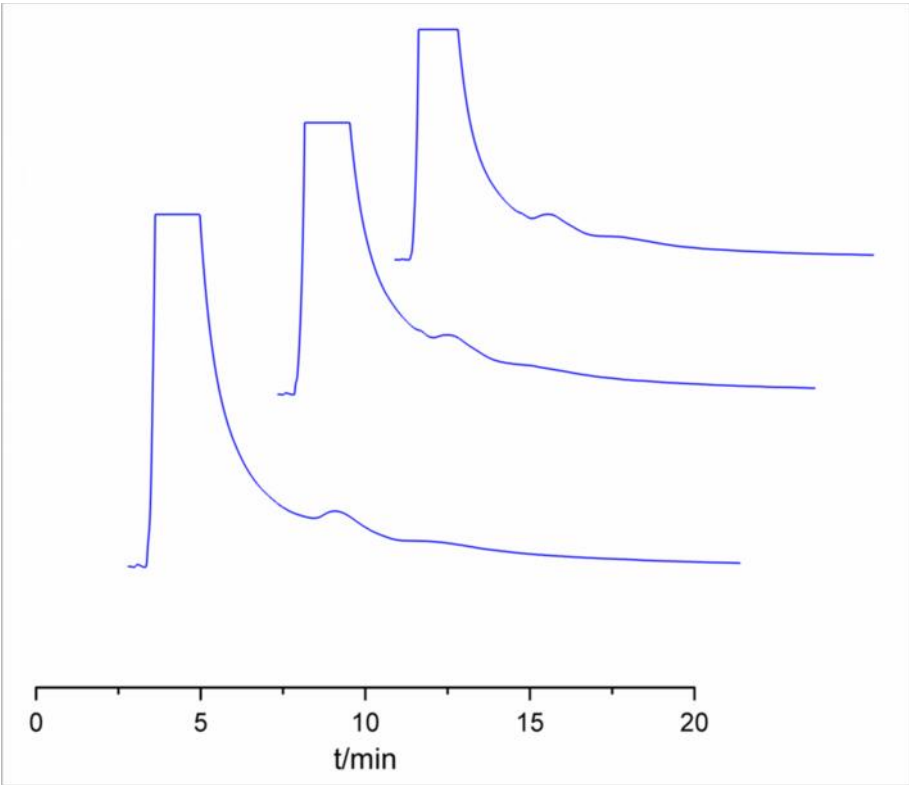


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47 Supplementary Figure 2 Chromatograms of CF on MRGPRX2-HEK293/CMC for 6 times

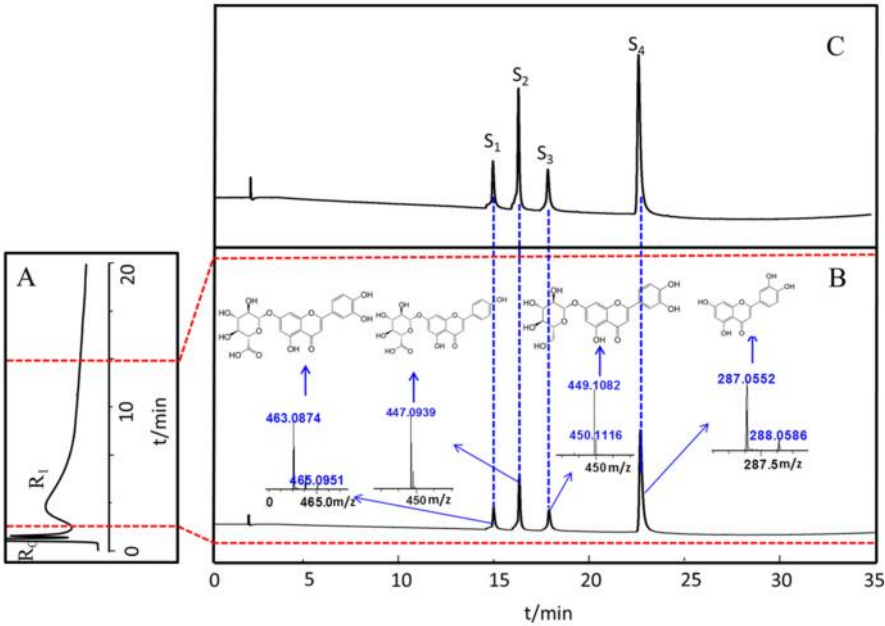


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51 Supplementary Figure 4 Chromatograms of the mixture of standard solutions of LNG, ANG, LE and LN
52 analyzed by the MRGPRX2-HEK293/CMC coupled online with HPLC-ESI-IT-TOF-MS method. (A)
53 Chromatogram of the mixture on the MRGPRX2-HEK293/CMC column. (B) Chromatograms of the retained
54 fraction (R3) by HPLC-ESI-IT-TOF-MS. (C) Chromatogram of the mixture by HPLC-ESI-IT-TOF-MS.



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