
The human mast cell line-1 cell membrane chromatography coupled with HPLC-ESI-MS/MS method for screening potential anaphylactic components from chuanxinlian injection

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Abstract

Chuanxinlian injection is a traditional Chinese medicine injection widely used in China to treat sore throat, cough, and dysentery. While, the high occurrence of severe adverse reactions had been reported in clinical practice in recent years. In the present study, a human mast cell line-1 cell membrane chromatography coupled with HPLC-ESI-MS/MS method was established to screen and identify potential anaphylactic components in chuanxinlian injection, and the dehydroandrographolide was identified as a potential anaphylactic component. *In vitro* anaphylactic assay showed that intracellular Ca^{2+} concentration clearly increased under dehydroandrographolide (100 μM) treatment, β -Hexosaminidase and histamine release in human mast cell line-1 cells were both markedly enhanced with increased concentrations of dehydroandrographolide, confirming the anaphylactic activity of dehydroandrographolide. The application for chuanxinlian injection in this study suggested that the developed human mast cell line-1 cell membrane chromatography coupled with HPLC-ESI-MS/MS system may be effective and rapid for screening the potential anaphylactic components from complex samples.

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1. Introduction

As one of the major utility formulations of traditional Chinese medicines (TCMs) in China, Traditional Chinese medicine injections (TCMIs) were widely used due to their speedy action and favorable bioavailability (Patwardhan et al., 2005; Yu et al., 2006; Yi et al., 2015; Sun et al., 2016). With the extensive application, adverse drug reactions (ADRs) of TCMIs had been reported frequently in recent decades, including damage on cardiovascular system, digestive system, skin and mucous membrane, nervous system even death (Zhang et al., 2012; Cheng et al., 2009). Based on the reports, ADRs occurred in 5% of patients treated with TCMIs, which accounted for almost 70% of ADRs of TCMs. And the incidence of ADRs induced by certain TCMI was even about 12% (Xu et al., 2015; Tang et al., 2015; Zhao et al., 2009; Sun et al., 2015). Therefore, ADRs had become the most critical problem in the development of TCMIs and the modernization of TCMs. Chuanxinlian injection (CXLI) is made of extracts of *Andrographis paniculata*, and is clinically used to treat sore throat, cough, and heat dysentery. Nevertheless, the ADRs, including fever, chills, anaphylactic reactions, difficult breathing and anaphylactic shock, had been reported to occur within minutes or half an hour after treatment (Hu et al., 2015). Therefore, it is significant to establish an effective and rapid method for screening and identification of potential anaphylactic components in CXLI.

Study showed that anaphylactoid reaction makes up over 70% ADRs of TCMIs, which occurred in the patient firstly treated and led to more dangerous damage (Xu et al., 2015). Many low-molecular-weight compound (LMWC) could cause anaphylactoid reaction, including antibiotic, anesthetic, muscle relaxants, non-steroidal anti-inflammatory drugs and iodinated contrast media. After drugs recognized by the receptor, mast cell degranulation and release of chemical mediators will appear, leading to rapid anaphylactic responses, including vasodilation, smooth muscle contraction and increased vascular permeability (Mcneil et al., 2015). The resulted secretion can be measured experimentally, which makes mast cell as a nice *in vitro* cellular model for studying anaphylactoid reaction. Human mast cell line-1 (HMC-1), a cell line established from the peripheral blood of a patient with mast cell leukemia, shows many characteristics of immature mast cells. Due to high affinity IgE

receptor FcεRIα and FcεRIγ distributed on the cell membrane surface, it was frequently employed in anaphylactoid reaction study (Guhl et al., 2010).

To evaluate the safety of TCMIs, the Chinese Pharmacopeia stipulates to detect tannin, protein, resin and pyrogen, without regarding the LMWC. And anaphylactic LMWCs were ordinarily evaluated by screening most components in TCMIs, which was obviously time-consuming and inefficient (Hu et al., 2015). As one of the bionic biological affinity chromatography, cell membrane chromatography (CMC) presented a simple and convenient way to screen active components from complicated samples without any pretreatment. And many target components have been successfully screened from complicated TCMIs by different membrane receptors (Wang et al., 2010; Li et al., 2015). In addition, cell membrane chromatography could also propose a comparable but much safer method for studying the interactions between ligands and membrane receptors comparing with the classical radioligand binding assay (Wang et al., 2006; Zeng et al., 2009). And mast cell membrane chromatography model has been reported to be successfully used to screen potential anaphylactic components (Han et al., 2016; Han et al., 2014; Zhang et al., 2014). With the development of technology, CMC coupled with GC-MS, HPLC-MS, or HPLC-MS/MS have shown to be an effective strategy for rapid screening, analyzing, and identifying potential anaphylactic components from complex systems (He et al., 1996; He et al., 2001; Wang et al., 2010; Hou et al., 2009; Li et al., 2015; Han et al., 2016).

In the present study, a HMC-1/CMC-HPLC-ESI-MS/MS method was established to screen and analyze potential anaphylactic components in CXLI. In addition, intracellular Ca²⁺ mobilization assay, β-hexosaminidase release, and histamine release were performed to verify the anaphylactic effects of the retained components *in vitro*.

2. Materials and methods

2.1. Materials and reagents

Silica gel (ZEX-II, 5 μm, 200Å) was obtained from Qingdao Meigao Chemical (Qingdao, China). The HILIC column (2.1 mm×150 mm, 3 μm) was purchased from Agela Technologies (Tianjin, China). Quercetin (purity ≥98%, lot#: 100081-201408) was supplied

by the national institutes for food and drug control, and dehydroandrographolide (DGE) (purity $\geq 98\%$, lot#: 150414) was purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). CXLI (lot#:12091201) was obtained from Henan kanghua Pharmaceutical Co., Ltd. (HeNan, China). The 1640 medium was purchased from Gibco (Life Technologies, USA), and the fetal bovine serum was obtained from Clontech (Mountain View, CA, USA), penicillin and streptomycin was purchased from Invitrogen (Scotland, UK). DMSO was obtained from Sigma (Massachusetts, USA). Fluo-3 and Pluronic F-127 were procured from Biotium (California, USA). Histamine (HA) was purchased from Sigma. HA-d₄ (purity $\geq 98\%$) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). HPLC grade acetonitrile and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Water was produced by a MK-459 Millipore Milli-Q Plus ultra-pure water system (Darmstadt, Germany). All analytes and solutions were kept at 4 °C and filtered using a 0.45 μ m nylon membrane before use.

2.2. Instruments

A ten-port valve (Valco Instrument Co. Inc., Houston, TX, USA) and one Merck RP-18e pre-column (10 mm $\times$ 4.6 mm I.D., Merck KGaA, Darmstadt, Germany) were used to couple HMC-1/CMC with the HPLC-ESI-MS/MS system. A HPLC system (LC-2010AHT) was used in HMC-1/CMC system. The conditions of the HMC-1/CMC system were as follows: mobile phase of water with a flow rate 0.2 mL/min, UV detection at 254 nm. Two Nexera LC-20ADXR pumps, a SPD-20A UV/VIS detector, a CBM-20A communication bus module, an LCMS-8040 triple quadrupole mass spectrometer, and a Lab Solutions work station (Shimadzu Corporation, Kyoto, Japan) were used in the HPLC-ESI-MS/MS system. Both the HMC-1/CMC column and the RP-18e column (100 mm $\times$ 4.6 mm I.D., Merck KGaA, Darmstadt, Germany) were kept at 37 °C. The conditions for the HPLC-ESI-MS/MS system were as follows: the mobile phase was acetonitrile (A) - 0.1% formic acid water (B), and the gradient program was: 0-7 min, 95%-70% B; 7-15 min, 70%-67% B; 15-20 min, 67%-10% B. with a 1.5 mL/min flow rate, UV detection at 254 nm. The effluent from the UV detector

was split with a splitter valve to provide a flow rate of 0.3 mL/min into the mass spectrometer. MS/MS conditions were: nebulizer gas (N₂; purity > 99.999%), flow rate 3.0 L/min; drying gas (N₂; purity > 99.999%), flow rate 15.0 L/min; interface, ESI source: desolvation line (DL) temperature 250 °C, heat block temperature 400 °C; interface voltage 4.5 kV, interface current 4.7 A; detector voltage 1.72 kV; CID gas (He; purity > 99.999%), pressure 230 kPa; positive- and negative- ionization scan mode (Q1 and Q3), scanning from m/z 50-1000.

2.3. Standard solutions and sample preparation

CXLI, DGE and quercetin standard solutions (1 mg/mL) were stored at 4 °C in the dark. All samples for column chromatography analysis were diluted using the mobile phase to suitable concentrations right before analysis, and the injection volume was 10 µL

2.4. Cell culture and preparation of HMC-1/CMC column

The HMC-1 cells were cultured in 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin. in an atmosphere containing 5% CO₂ at 37 °C. The culture medium was replaced every two days with fresh medium, and the cells were maintained at a density of 2×10⁶ cells/mL. The harvested HMC-1 cells (7×10⁶) at 80-90% confluency was washed three times with physiological saline by centrifugation at 110×g, 4 °C for 5 min. Tris-HCl (50 mM, pH 7.4) was added to resuspend the HMC-1 cells, and then ruptured by ultrasonication for 30 min, homogenization for 3 min. The resulting homogenate was centrifuged at 1000×g, 4 °C for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000×g, 4 °C for 20 min. The crude membrane precipitation was resuspended in 10 mL Tris-HCl (50 mM, pH 7.4) and centrifuged again at 12,000 × g, 4 °C for 20 min. The precipitate was resuspended by 5 mL physiological saline and used for preparation of cell membrane stationary phase (CMSP). Silica gel (45 mg) was activated at 105 °C for 30 min, and cell membrane suspension (5 mL) was adsorpted on the activated silica under vacuum and vortex at 4 °C. The mixture was subsequently stirred for 30 min and

left for 14 h. Eventually, the HMC-1/CMC column was manufactured by packing the stationary phase prepared as above into a column (10 mm×2.0 mm I.D.) with a wet packing procedure on a column loading machine.

2.5. Selectivity and suitability of HMC-1/CMC-HPLC-ESI-MS/MS method

The standard solution of quercetin (confirmed as a positive drug in the previous study (Johannes et al., 1999)), gefitinib (selective epidermal growth factor receptor inhibitor), metoprolol (β_1 -receptor blocker), and captopril (angiotensin converting enzyme inhibitor) were used to examine the specific selectivity of the HMC-1/CMC column. The positive drug, quercetin, was also used to verify the suitability of this method. The standard solution was injected into HMC-1/CMC and the retained fraction was enriched and switched into the HPLC-ESI-MS/MS system for further analysis and identification.

2.6. Screening retained components using HMC-1/CMC-HPLC-ESI-MS/MS method

The CXLI was analyzed by the HMC-1/CMC-HPLC-ESI-MS/MS method. The components retained on the HMC-1/CMC column were analyzed and identified by HPLC-MS/MS. For further verification, the standard solutions of allergenic component screened from CXLI was also assayed using this method.

2.7. *In vitro* pharmacological effects

2.7.1 Intracellular Ca^{2+} mobilization assay

HMC-1 cells were plated into an EP tube and washed twice with CIB (NaCl 125 mM, KCl 3 mM, CaCl_2 2.5 mM, MgCl_2 0.6 mM, HEPES 10 mM, glucose 20 mM, NaHCO_3 1.2 mM, sucrose 20 mM, brought to pH 7.4 with NaOH). Then, the cells were loaded with the incubation buffer (1 μL of Fluo-3, 1 μL of Pluronic F-127 and 998 μL of CIB) for 30 min at 37 °C under 5% CO_2 . After that, mast cells were washed twice with CIB and plated at 1×10^4 cells per well in 96-well plates in 100 μL CIB, after which, the cells were used immediately for imaging. The calcium imaging conditions were magnification of the cells for 200 times,

with one photograph being taken per second under blue light. The cells were identified as responsive if intracellular calcium concentrations rose by at least 50% after injection of the drugs.

2.7.2 β -hexosaminidase release

HMC-1 cells were incubated in 96-well plate at 3000 cells/well overnight at 37 °C under 5% CO₂. After centrifugating at 200×*g* for 5 min, the culture medium was removed, and DGE (90 μ L per well) was added at the indicated concentrations (200 μ M, 100 μ M, 50 μ M, 25 μ M, 12.5 μ M), followed by incubation for 30 min at 37 °C under 5% CO₂. Cells with TM buffer was treated as negative control, and the A+P (5 μ M A23187 and 50 nM PMA in TM buffer) was used as a positive control. The 96-well plate was then centrifuged at 200×*g* for 5 min, the supernatant (50 μ L per well) was taken out and placed in a new well, and the β -hexosamine (50 μ L per well) was added to the well. Cells were lysed with 0.1% Triton X-100 to determine the total β -hexosaminidase. The β -hexosaminidase in the supernatant and cell lysates was quantified through hydrolysis of p-nitrophenyl N-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was stopped by adding buffer (0.1 M sodium carbonate/sodium bicarbonate pH 11.0). The percentage of β -hexosaminidase release was assessed by measuring the samples at 405 nm using a microplate spectrophotometer and calculated as follows:

$$\beta - \text{hexosaminidase release rate \%} = \frac{A_s}{A_t} \times 100\%$$

Where A_s stands for the absorbance of the culture supernatant at 405 nm and A_t stands for the sum of the absorbance of the cell lysate and supernatant at 405 nm.

2.7.3 Histamine release

As β -hexosaminidase release, HMC-1 cells were also grown in 96-well plate at 3000 cells/well overnight at 37 °C under 5% CO₂ atmosphere. Then the 96-well plate was centrifugated at 200×*g* for 5 min and the growth medium was removed. DGE (100 μ L per well) dissolved with TM buffer was added at the indicated concentrations (200 μ M, 100 μ M,

50 μ M, 25 μ M, 12.5 μ M), cells treated with TM buffer and A+P (5 μ M A23187 and 50 nM PMA in TM buffer) were performed as negative control and positive control, respectively. After incubation for 30 min at 37 °C under 5% CO₂, the 96-well plate was centrifuged at 200 $\times g$ for 5 min, and the supernatant (50 μ L per well) was collected and placed on ice for 10 min. 100 μ L of internal standard solution (d₄-histamine, 5 ng/mL dissolved in acetonitrile) was added into each sample collected above. After vortex and centrifugation at 12,000 $\times g$, 4 °C for 10 min, the supernatant was analyzed by an optimized UHPLC-ESI-MS/MS method based on previous report (Koyama et al., 2011). In our method, histamine was analyzed with a HILIC column, and isocratic elution, a mixed solution of acetonitrile-water with 0.1% formic acid and 20 mM ammonium formate (80:20, v/v), was conducted at a flow rate of 0.3 mL/min.

3. Results and discussion

3.1 Selectivity and suitability of HMC-1/CMC-HPLC-ESI-MS/MS method

To examine the specific selectivity of the HMC-1/CMC model, the standard solutions of quercetin, gefitinib, metoprolol, and captopril were injected into this model, respectively. The chromatograms of the standard solutions were displayed in Fig. 1. Only quercetin was retained on the HMC-1/CMC model, indicated that the HMC-1/CMC column has specific selectivity for ligands of Fc ϵ RI receptor.

To verify the suitability of HMC-1/CMC-HPLC-ESI-MS/MS method, quercetin solution was also injected into. The retained fraction (R₁) on the HMC-1/CMC column (Fig. 2A) was enriched and switched into the HPLC-ESI-MS/MS system and was considered to be quercetin (Fig. 2B). To further confirm the above result, quercetin was directly analyzed by HPLC-ESI-MS/MS system (Fig. 2C). MS spectra of R₁ and comparison of retention time between R₁ (Fig. 2B) and quercetin (Fig. 2C) identified R₁ as quercetin. All the results indicated that this method could be served for further study.

3.2 Screening potential anaphylactic components in CXLI

The verified HMC-1/CMC-HPLC-ESI-MS/MS method was further used to screen potential anaphylactic components in CXLI. Chromatograms of CXLI obtained by the HMC-1/CMC-HPLC-ESI-MS/MS method were shown in Fig. 3. The total analysis time, including the CMC and the HPLC-ESI-MS/MS section, was ≤ 40 min. The retained fraction (R_2) of the CXLI on the HMC-1/CMC column was displayed in Fig. 3A, the HPLC-ESI-MS/MS chromatograms of R_2 and CXLI were listed in Fig. 3B and Fig. 3C, respectively. The mass spectrum and the proposed fragmentation pathway of R_2 were presented in Fig. 4. The molecular ion of R_2 and its daughter ions were 333.15, 315.20, 297.15, 274.30, and 257.15, respectively (Fig. 4A). The fragment ion at m/z 315.20 was formed by the loss of 18 (H_2O) from the m/z 333.15. The loss of 18 (H_2O) from the m/z 315.20 produced the ion at 297.15. The loss of 42 (propylene, C_3H_6) and an electron from the m/z 315.20 produced the ion at 274.30 (Fig. 4B). The further loss of 17 (hydroxyl) produced the ion at 257.15 (Li et al., 2008; Li et al., 2011). In summary, R_2 was characterized as DGE.

To further verify the screening result, the DGE standard solution was analyzed with the same method. As shown in Fig. 5, DGE possessed the same retention behaviour and MS spectra on the HMC-1/CMC-HPLC-ESI-MS/MS system compared with the retained fraction of CXLI, indicating the retention of DGE in CXLI.

3.3 *In vitro* pharmacological effects of potential anaphylactic component

Intracellular Ca^{2+} mobilization assay, histamine release and β -hexosaminidase release tests in HMC-1 cells were used to investigate the anaphylactic activities of DGE *in vitro*. As shown in Fig. 6, the intracellular calcium concentrations in HMC-1 cells clearly increased by DGE at 100 μM (Fig. 6A). In addition, DGE could increase β -hexosaminidase and histamine release in a dose-dependent manner from 12.5 to 100 μM , the β -hexosaminidase release rate and histamine release were 13-29% and 45-59 ng/mL, respectively (Fig. 6B and Fig. 6C). All the results confirmed the anaphylactic effects of DGE.

4. Conclusions

In summary, the HMC-1/CMC-HPLC-ESI-MS/MS method was established and applied to screen, analyze, and identify potential anaphylactic components in CXLI, and DGE was identified as the potential anaphylactic component. Intracellular Ca^{2+} concentration, β -Hexosaminidase and histamine release further confirmed the anaphylactic activity of DGE. These findings suggested that the HMC-1/CMC-HPLC-ESI-MS/MS method can serve as an effective and rapid method for screening potential anaphylactic components from complex samples.

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Figure captions

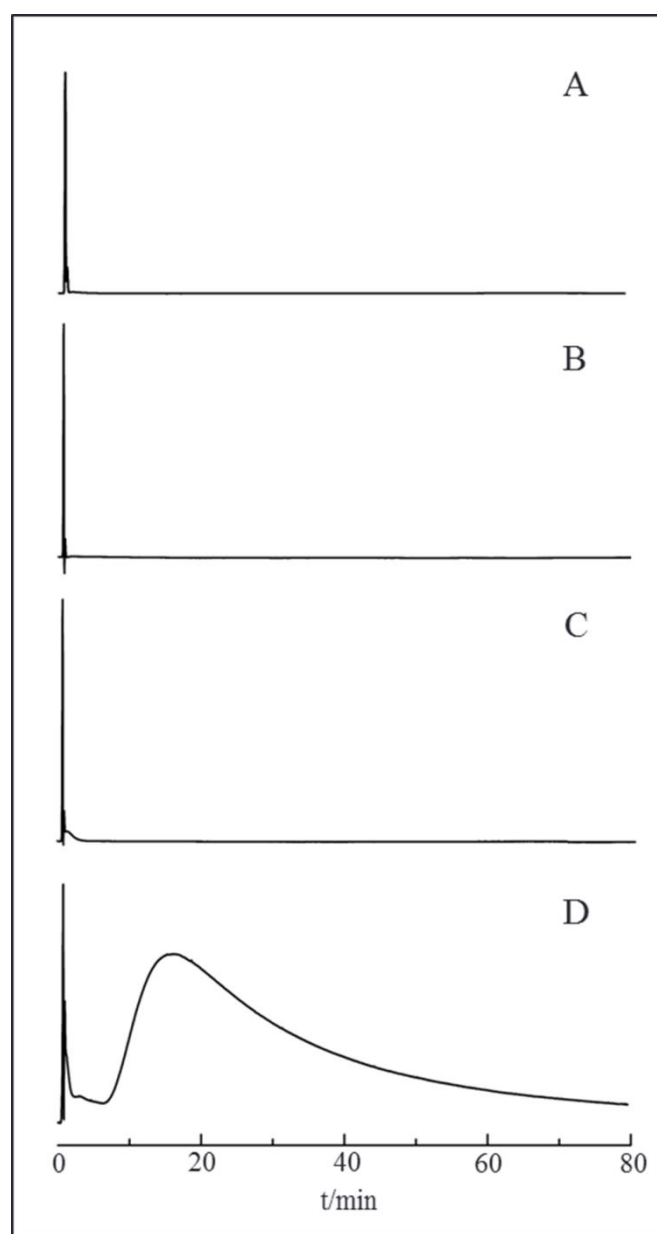


Figure 1 Chromatograms of gefitinib (A), metoprolol (B), captopril (C) and quercetin (D) on HMC-1/CMC column.

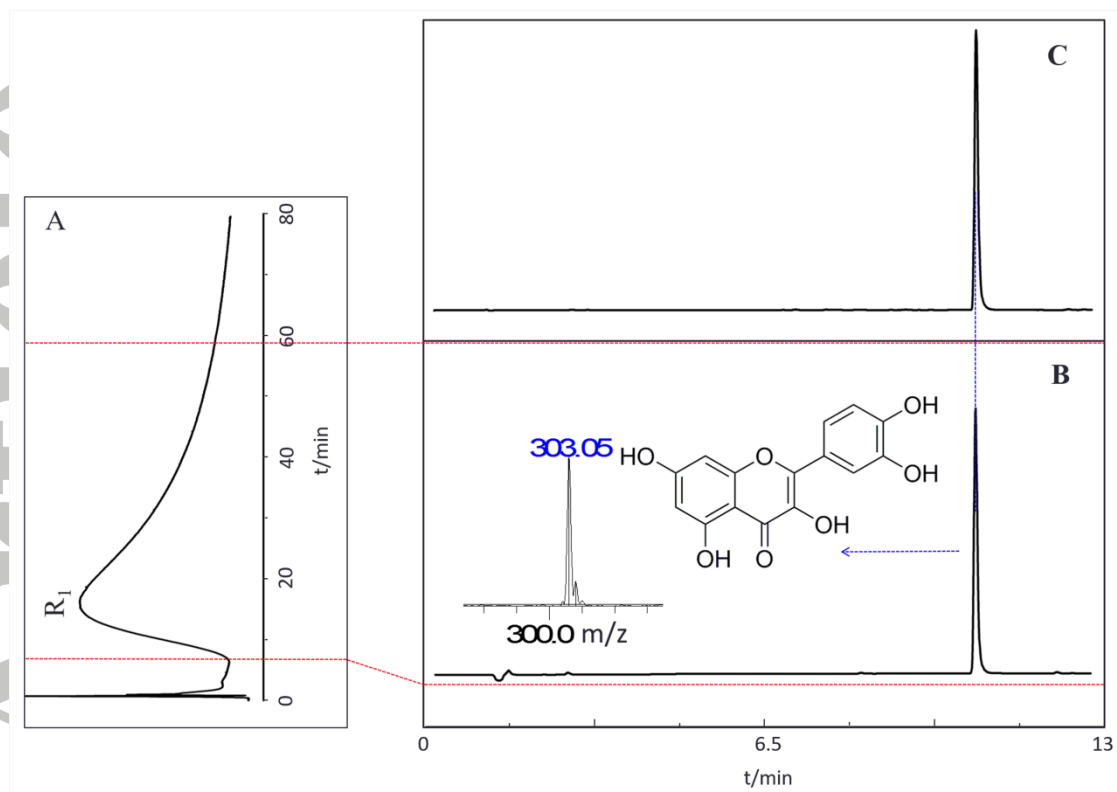


Figure 2 Chromatograms of quercetin analyzed by the HMC-1/CMC-HPLC-ESI-MS/MS method.

(A) Chromatogram of quercetin retained on the HMC-1/CMC column.

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

(C) Chromatogram of quercetin by HPLC-ESI-MS/MS.

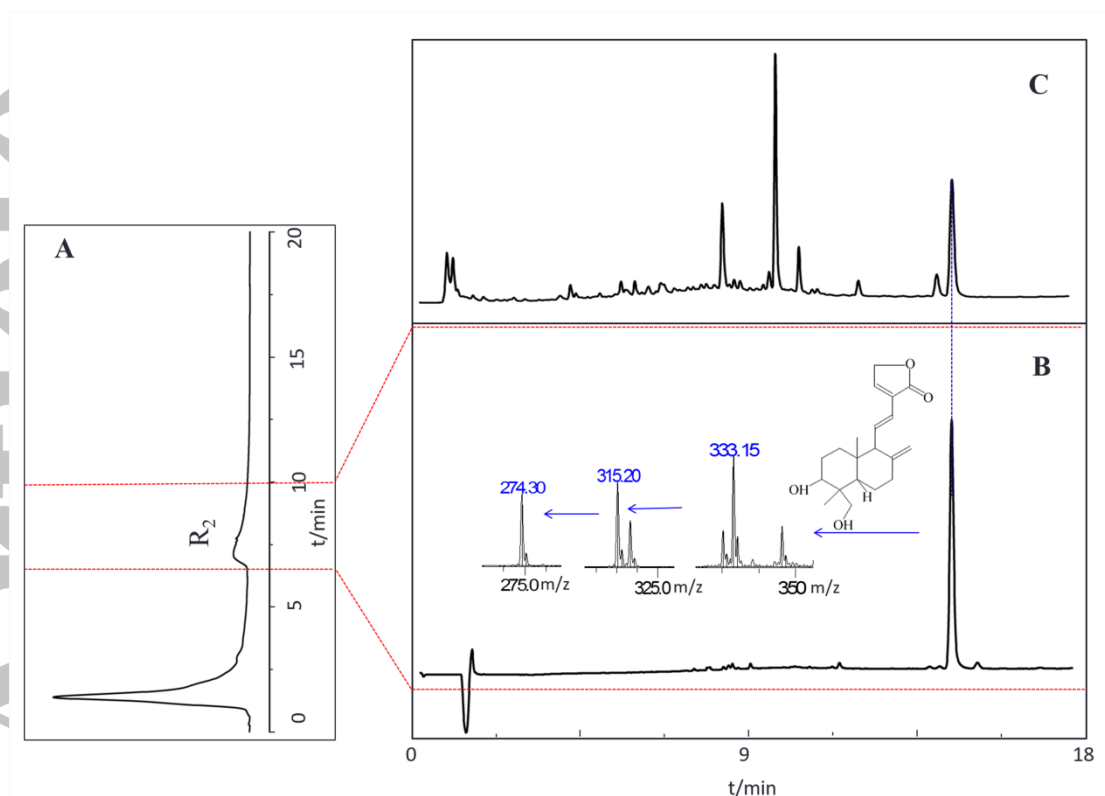


Figure 3 Chromatograms of CXLI analyzed by the HMC-1/CMC-HPLC-ESI-MS/MS method.

(A) Chromatogram of CXLI on the HMC-1/CMC column.

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

(C) Chromatogram of CXLI by HPLC-ESI-MS/MS.

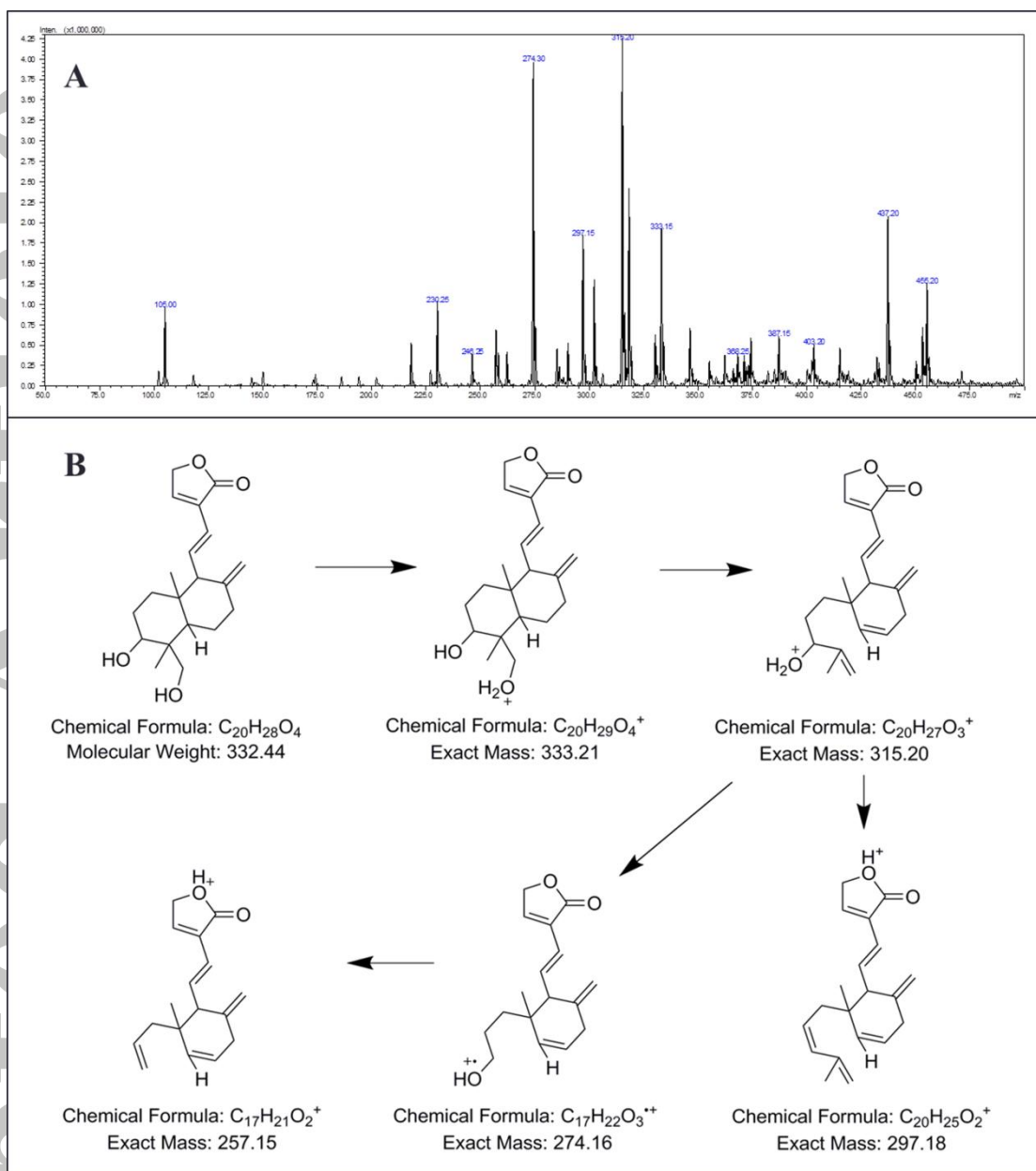


Figure 4 The mass spectrum and the proposed fragmentation pathway of R_2 .

(A) The mass spectrum of R_2 .

(B) The proposed fragmentation pathway of R_2 .

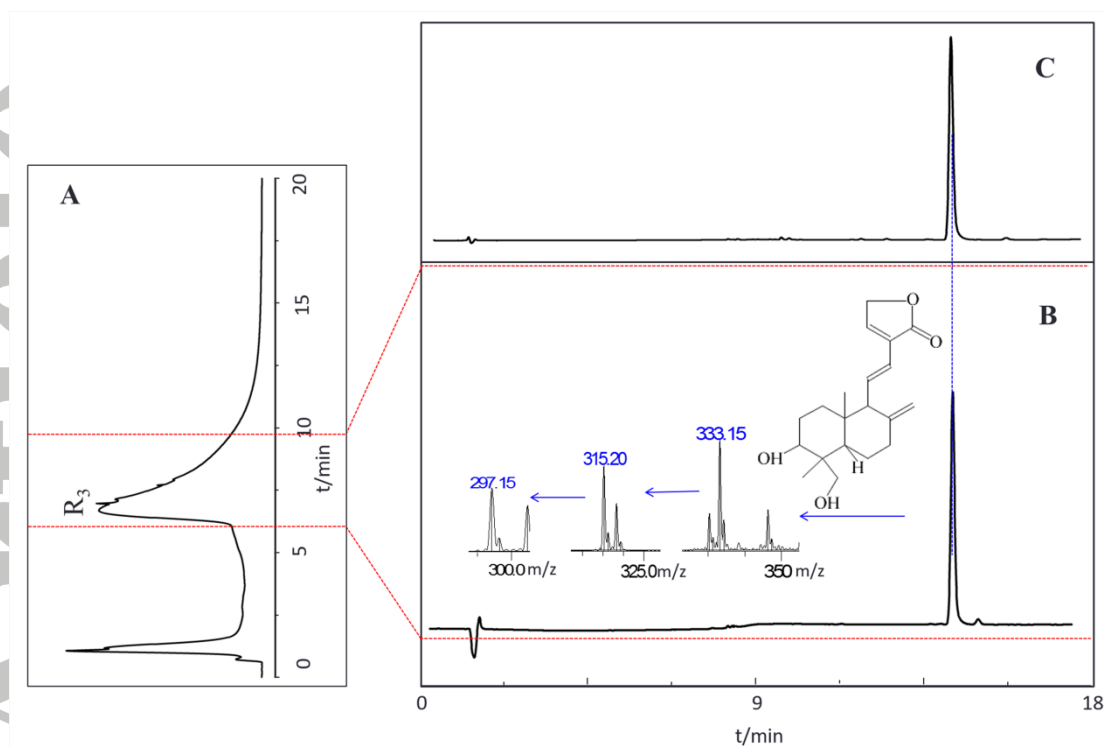


Figure 5 Chromatograms of DGE analyzed by the HMC-1/CMC-HPLC-ESI-MS/MS method.

(A) Chromatogram of DGE on the HMC-1/CMC column.

(B) Chromatograms of the retained fraction (R_3) by HPLC-ESI-MS/MS.

(C) Chromatogram of DGE by HPLC-ESI-MS/MS.

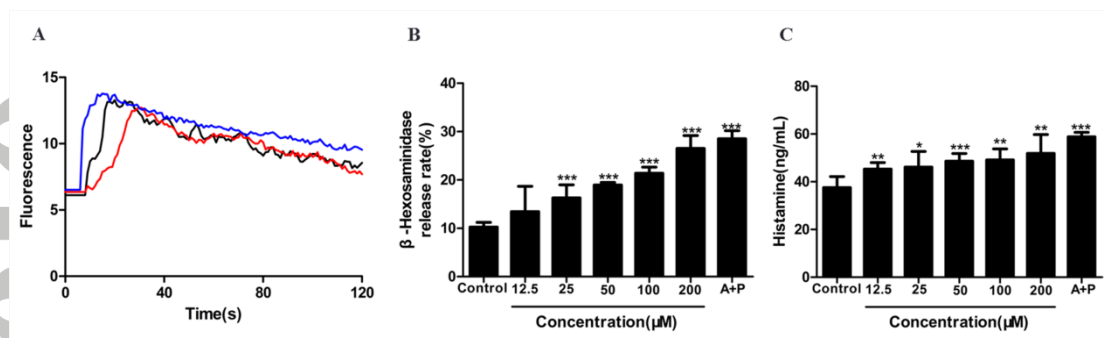


Figure 6 *In vitro* pharmacological effects of DGE

- (A) Representative Fluo-3 fluorescence incubation of HMC-1 showing changes in Ca^{2+} induced by DGE (100 μM).
- (B) β -hexosaminidase release of HMC-1 cells treated by DGE.
- (C) Histamine release of HMC-1 cells treated by DGE.