Anti-pseudo-allergic Components in Licorice Extract Inhibit Mast Cell Degranulation and Calcium Influx

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Research

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Abstract

Background

Pseudo-allergic reactions (PARs) widely occur upon the application of drugs or functional foods. Anti-pseudo-allergic ingredients from natural products have attracted much attention. This study aimed to investigate anti-pseudo-allergic compounds in licorice.

Methods

The anti-pseudo-allergic effect of licorice extract was evaluated in rat basophilic leukemia 2H3 (RBL-2H3) cells. Anti-pseudo-allergic compounds were screened by using RBL-2H3 cell extraction and the effects of target components were verified in RBL-2H3 cells, mouse peritoneal mast cells (MPMCs) and mice. Molecular docking was performed to determine the potential ligands of MAS-related G protein-coupled receptor-X2 (MRGPRX2), a pivotal target of PARs.

Results

Glycyrrhizic acid (GA) and Licochalcone A (LA) were screened and shown to inhibit Compound48/80-induced degranulation and calcium influx in RBL-2H3 cells. GA and LA also inhibited degranulation in MPMCs and increase of histamine and TNF-α in mice. LA could bind to MRGPRX2, as determined by molecular docking.

Conclusions

Our study provides a strong rationale for using GA and LA as novel treatment options for PARs. LA is a potential ligand of MRGPRX2.

Background

Pseudo-allergic reactions (PARs), are caused by non-immunoglobulin E-based release of mediators from mast cells and are acute life-threatening hypersensitivity reactions with severe clinical manifestations that resemble those of generalized anaphylaxis, *e.g.* urticaria, pruritus, angioedema, gastrointestinal symptoms or bronchospasm. PARs usually occur upon the application of drugs, functional foods or food additives, which has been a serious public health concern [1, 2]. MAS-related G protein-coupled receptor-X2 (MRGPRX2) has been found to mediate PARs [3–5]. The activation of mast cell receptors by certain drugs can promote Ca^{2+} mobilization [6] and induce the release of cytokines and chemokines, such as β-hexosaminidase, histamine, tumor necrosis factor (TNF-α).

At present, the treatment of allergic-like diseases mainly includes recovery from immune disorders and attenuated allergy-related inflammatory release. Anti-allergy drugs mainly include anti-histamines, mast cell stabilizers, and hormone drugs [7], which can only help relieve allergic symptoms and reduce the pain caused by allergic reactions. Therefore, it is important to find more effective candidate drugs to suppress mast cell activation,especially during MRGPRX2-dependent PARs.

Glycyrrhiza uralensis Fisch. (Gan-Cao), commonly called “licorice”, is one of the most frequently used ingredients in traditional Chinese medicine (TCM). Licorice products are also most often consumed as flavoring and sweetening agents in food products in Western countries [8]. Licorice is promoted as an herb that can be used to treat peptic ulcers, eczema, skin infections, cold sores, menopausal symptoms, liver disease, respiratory ailments, inflammatory problems, chronic fatigue syndrome, acquired immune deficiency syndrome, and even cancer [9, 10]. However, there are few studies on anti-pseudo-allergic effect of licorice.

In this report, the anti-pseudo-allergic effect of licorice extract (LE) was preliminarily evaluated in rat basophilic leukemia 2H3 (RBL-2H3) cells. Potential anti-pseudo-allergic compounds in licorice were screened using RBL-2H3 cell extraction and the effects of target components were verified in mouse peritoneal mast cells (MPMCs) *in vitro* and mice *in vivo*. Finally, molecular docking was performed to preliminarily determine the potential antagonists of MRGPRX2.

Methods

Reagents and materials

Modified Eagle’s medium (MEM) was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from ScienCell (Carlsbad, CA). Compound 48/80 (C48/80), *p*-nitrophenyl-N-acetyl-β-D-glucosami- nide and poly-D-lysine hydrobromide (PDL) were purchased from Sigma Aldrich (Saint Louis, MO). Triton X-100 was obtained from Amresco (Solon, OH). Penicillin and streptomycin were purchased from Beyotime Biotechnology (Shanghai, China). Reference substances were purchased from Duan Li Bio-technology (Nanjing, China). Fluo-4 AM was purchased from Beyotime Biotechnology. ELISA kits for histamine and TNF-α were purchased from Jiangsu Meimian Industrial Co., Ltd (Yancheng, China). The Annexin V-FITC fluos staining kit was purchased from KeyGEN BioTECH (Nanjing, China).

Animals

ICR male mice (18–22 g) were obtained from Yangzhou University (Yangzhou, China). Animals were housed under a 12-h light–dark cycle at 22 °C and relative humidity of 55 ± 5%, and had free access to food and water. Protocols involving animals were carried out according to the guidelines set by the Institutional Animal Care and Use Committee of Jiangsu Province Academy (Jiangsu, China).

Cells

RBL-2H3 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in MEM supplemented with 15% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.11 g/L sodium pyruvate in a humidified atmosphere of 5% CO_2 at 37 °C.

Isolation of MPMCs

Adult male mice were killed. A total of 10 mL ice-cold phosphate-buffered saline (PBS) was used to perform two sequential peritoneal lavages, which were combined and centrifuged at 800 *g* for 10 min at 4 °C. The supernatant was decanted to leave a small mast cell pellet (≈ 2 mL), and 2 mL of 30% Percoll and 80% Percoll were added successively to form an interface (with a final volume ratio: 1:1:1). After centrifugation at 600 *g* for 15 min at 4 °C, cells at the junction interface were collected and washed with PBS twice (200 *g*, 10 min, 4 °C). MPMCs were resuspended in DMEM with 10% FBS. Purity and viability were determined by toluidine blue staining and trypan blue exclusion.

Preparation of LE

Licorice was ultrasonically extracted for 30 min, using 70% ethanol as the solvate. The filtrate was evaporated under reduced pressure at 50 °C, and the residue was suspended in H_2O and freeze-dried to obtain LE.

RBL-2H3 cell extraction

LE was dissolved in MEM without FBS and filtered through a membrane (pore size, 0.22 μm) to create the sample solution. After incubation of RBL-2H3 cells with the filtrate at 37 °C and 5% CO_2 for 1 h, the supernatant was discarded. The deposited cells were washed six times with 3 mL of PBS each time. The eluates were discarded except the last one, which was collected and used as a control for UPLC-DAD-Q-TOF-MS/MS analysis. Then, the deposited cells were denatured and extracted with 3 mL of 80% methanol. After centrifugation at 9000 *g* for 10 min, the supernatant was collected and dried. The residue was dissolved in methanol again and filtered through a membrane (pore size, 0.45 μm) for UPLC-DAD-Q-TOF-MS/MS analyses [11]. Cells incubated with MEM without FBS were prepared as the control sample using the same procedures described above.

UPLC-DAD-Q-TOF-MS/MS analysis for identification of components combined with RBL-2H3 cells

UPLC-DAD-Q-TOF-MS/MS analysis was carried out using 1290 Infinity UPLC instrument (Agilent Technologies, Santa Clara, CA) coupled with a 6538 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a dual electrospray ionization (ESI) source. The mobile phase consisted of water-0.05% formic acid (A) and acetonitrile (B). Gradient elution conditions were as follows: 0–2 min, 10%–25% B; 2–4 min, 25%–35% B; 4–14 min, 35%–70% B; 14-22 min, 70%-82%; 22-24 min, 82%-95%, followed by a return to the initial condition. The flow rate was 0.3 mL/min. Chromatographic separation was carried out at 30 °C on an Ultimate UHPLC LP-C_{18} (1.8 μm, 2.1×100 mm). Samples were detected at 237 nm. Parameters used for MS detection were optimized as follows: drying gas temperature, 350 °C;

flow rate of drying gas (N_2), 8.0 L/min; fragmentor voltage, 100 V; nebulizer, 40 psi; capillary, 3500 V; skimmer, 65 V; Oct RFV, 750 V. The sample collision energy was set at 35 V. All data acquisitions and analyses were controlled by Mass Hunter software (Agilent Technologies). Mass spectra were recorded in the range *m/z* 100–2000 with accurate mass measurement of all peaks. Each sample was analyzed in negative mode.

Measurement of cell viability

Cell viability was determined by the MTT assay. RBL-2H3 cells were seeded in a 96-well plate at 1×10^4 cells/well. After 24 h of incubation at 37 °C in an atmosphere of 5% CO_2, cells were treated with different concentrations of test compounds for 30 min. Then, the supernatant was discarded and cells were incubated with MEM containing 0.5 mg/mL MTT at 37 °C and 5% CO_2. After 3 h, the medium was removed and 150 μL dimethyl sulfoxide added to each well. Absorbance was measured at 570 and 650 nm.

MPMCs were seeded in a 96-well plate at 3×10^4 cells/well. After 12 h of incubation at 37 °C in an atmosphere of 5% CO_2, cells were treated with different concentrations of test compounds for 30 min. Then the method was the same as above.

Measurement of β-hexosaminidase release

β-Hexosaminidase release from RBL-2H3 cells was examined as described previously with some modifications [12].

RBL-2H3 cells (1×10^4 cells/well) or MPMCs (3×10^4 cells/well) were cultured for 24 h in a 96-well plate, washed with MEM/ DMEM without FBS, and incubated with MEM/DMEM containing different concentrations of the sample solutions for 30 min at 37 °C in an atmosphere of 5% CO_2. Cells in the blank control and positive control were treated with MEM/DMEM alone under the same incubation conditions. Then, the cells in sample solutions and positive control were stimulated with C48/80 (30 μg/mL) for 30 min at 37 °C in an atmosphere of 5% CO_2. Cells in the blank control were incubated with MEM/DMEM without FBS.

β-Hexosaminidase released into the supernatant and cell lysate was quantified by hydrolysis of *p*-nitrophenyl-N-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) for 60 min at 37 °C. The absorbance of each well was measured at 405 nm. Percentage of release of β-hexosaminidase was calculated as a percentage of the total content, using the following formula:

$$\beta - \text{hexosaminidase}(\%) = \frac{\text{OD}(\text{supernatant})}{\text{OD}(\text{supernatant}) + \text{OD}(\text{lysate})} \times 100$$

Measurement of histamine and TNF-α release

RBL-2H3 cells (5×10^4 cells /well) were cultured for 24 h in a 24-well plate, and washed with MEM without FBS. The administration method was the same as used for the measurement of β-hexosaminidase release. The supernatants were collected and centrifuged at 845 *g* for 10 min. Histamine content in the supernatant was determined using an ELISA histamine kit according to the manufacturer's instructions.

Mice were randomly divided into eight groups (n = 8), including the blank control group, positive control C48/80 group and treatment groups. The treatment groups were orally administered different doses of the target components. The blank control group and positive control group were administered saline. After 30 min, the positive control group and treatment groups were challenged via an intravenous injection with C48/80 (2.5 mg/kg). The blank control group was intravenously injected with saline. Blood was collected after 10 min, and serum was obtained through centrifugation (825 *g*, 10 min, room temperature). Histamine content and TNF-α in the serum were determined using ELISA histamine kits according to the manufacturer's instructions.

Fluorescence microscopy

According to the instructions provided with the Annexin V-FITC detection kit, RBL-2H3 cells were incubated with different sample solutions for 30 min and treated with C48/80 (30 μg/mL). After 30 min, the cells were washed twice with cold PBS and then binding buffer containing Annexin V-FITC (5 μL) was added. After 5 min in the dark, cells were examined and photographed at 630× magnification under a laser scanning confocal microscope (Zeiss LSM700, Jena, Germany).

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using the fluorescence indicator Fluo-4 AM. RBL-2H3 cells were plated onto a 96-well plate coated with 50 μg/mL PDL and balanced at 37 °C in an atmosphere of 5% CO_2. After 24 h, the cells were treated with different sample solutions for 30 min, and loaded with Fluo-4 AM for 40 min at 37 °C in an atmosphere of 5% CO_2. Then the cells were washed five times with Locke's buffer (pH 7.4, containing 2.144 g/L HEPES, 0.417 g/L potassium chloride, 9 g/L sodium chloride, 1.009 g/L glucose, 0.096 g/L magnesium chloride, 0.256 g/L calcium chloride and 500 μL 0.1 mM glycine), to leave a final volume of 150 μL in each well. Then, the plate was transferred to Varioskan Multifunctional microplate reader 3001 (Thermofisher Scientific, Germany). Cells were excited at 488 nm, and emission detected at 520 nm. Fluorescence was continuously scanned for 1 min to establish the baseline. Then, 50 μL of C48/80 (120 μg/mL□4×) was added to the positive well and different sample wells, respectively, yielding a final volume of 200 μL/ well. Locke's buffer was added to the control well. Fluorescence was continuously scanned for 5 min.

Molecular docking

To investigate the interactions between receptors and ligands, molecular docking studies were conducted using Auto Dock 4.2 (The Scripps Research Institute, USA). The docking model of MRGPRX2 used in this

study was based on the homology model of MRGPRX2 reported by Dr. Bryan L Roth and Protein Data Bank [13].

Statistical analyses

All analyses were performed using GraphPad Prism v5.01 (GraphPad Software, La Jolla, CA). Data are presented as the mean ± SEM. One-way analysis of variance followed by Dunnett's test was used for multiple comparisons. $p < 0.05$ was considered significant. Each experiment was repeated at least three times.

Results

LE inhibited C48/80-induced degranulation and calcium influx in RBL-2H3 cells

Little cytotoxicity was observed even when administering a high dose of 100 μg/mL LE (Fig. 1A). Degranulation was monitored by β-hexosaminidase and histamine release. LE significantly inhibited C48/80-induced β-hexosaminidase release (Fig. 1B) and histamine release (Fig. 1C) from RBL-2H3 cells. An increase in cytosolic calcium is essential both for degranulation and the release of de novo synthesized mediators in mast cells [14]. Therefore, we investigated the effect of LE on Ca^{2+} mobilization in RBL-2H3 cells. Pretreatment with LE resulted in a dose-dependent decrease in C48/80-induced calcium influx (Fig. 1D and E), indicating that LE reduced C48/80-induced degranulation via the calcium signaling pathway.

UPLC-Q-TOF-MS/MS analyses of the RBL-2H3-binding components of LE

RBL-2H3 cell extraction was used to screen the components binding to RBL-2H3 cells. Compared with the total ion chromatograms of LE (Fig. 2A), glycyrrhizic acid (GA, peak 1) and licochalcone A (LA, peak 2) were screened (Fig. 2B) in the extracted ion chromatogram mode and the mass spectra were compared with that of the reference substances solution (Fig. 2C). No components were detected in the control sample (Fig. 2D) and final-wash eluate (Fig. 2E).

GA and LA inhibited C48/80-induced degranulation and calcium influx in RBL-2H3 cells

The anti-pseudo-allergic effect of the screened components was preliminarily verified in RBL-2H3 cells. GA had no effect on cell viability (Fig. 3A) and could dose-dependently inhibited C48/80-induced β-hexosaminidase release (Fig. 3B) and histamine release (Fig. 3C) from RBL-2H3 cells. GA also decreased calcium influx triggered by C48/80 in a concentration-dependent manner (Fig. 3D and E). LA also possessed little cytotoxicity (Fig. 4A) and significantly reduced β-hexosaminidase release (Fig. 4B) and

histamine release (Fig. 4C). Similarly, LA resulted in a dose-dependent decrease in calcium influx (Fig. 4D and E).

GA and LA inhibited C48/80-induced phosphatidylserine translocation in RBL2H3 cells

It has been reported that the membrane phospholipid phosphatidylserine translocated from the inner to outer leaflets of cytomembranes during MC degranulation [15, 16]. Annexin V-FITC can bind to cells exposed to phosphatidylserine, causing the cells to appear green. Compared with that in the control group (Fig. 5A) and C48/80 group (Fig. 5B), pretreatment with GA (Fig. 5C) and LA (Fig. 5D) reduced phosphatidylserine translocation in RBL-2H3 cells.

GA and LA inhibited C48/80-induced degranulation in MPMCs

To further verify the reliability of the results obtained in RBL-2H3 cells, the same indicators in MPMCs were determined. Similarly, GA (Fig. 6A) and LA (Fig. 6B) showed little cytotoxicity against MPMCs. GA (Fig. 6C) and LA (Fig. 6D) inhibited the release of β- hexosaminidase in MPMCs, which was consistent with the results observed in RBL-2H3 cells.

GA and LA inhibited the C48/80-induced increases in histamine and TNF-α in mouse serum

To validate whether GA and LA could inhibit PARs *in vivo*, mice were used to evaluate the effect on the histamine and TNF-α levels in serum. GA (Fig. 7A and B) and LA (Fig. 7C and D) both significantly attenuated C48/80-induced increases in histamine and TNF-α levels.

LA is a potential ligand of MRGPRX2

As shown in Fig. 8A and B, the docking results showed that only LA could bind to MRGPRX2 via TYR 320 and CYS 210 in a sphere space field model, indicating that LA may inhibit PARs mediated by MRGPRX2.

Discussion

Licorice is often used as a sweetener as well as in TCM and has anti-inflammatory activity. However, a systematic study of the anti-pseudo-allergic effect of LE and its ingredients is lacking. In the current study, we found that LE could significantly inhibit C48/80-induced degranulation and calcium influx in RBL-2H3 cells. GA and LA were screened by RBL-2H3 cell extraction and identified by EIC mode. We demonstrated that GA and LA were potential anti-pseudo-allergic components in licorice by performing further verification in RBL-2H3 cells, MPMCs and mice. Meanwhile, we found that LA was a potential MRGPRX2 ligand by performing molecular docking. This is the first systematic evaluation of the anti-

pseudo-allergic effect of LE and the first screen for potential effective constituents in LE. GA and LA are reported to inhibit C48/80-induced PARs for the first time in our study.

The screening and analysis of specific bioactive components in TCMs are still troublesome issues. Pharmacologic studies have shown that most drugs play a role first by combing with receptors or channels on cell membranes. Therefore, cell membrane chromatography and cell membrane extraction have been used in many studies and have some advantages over conventional methods (which are time-consuming and arduous) for the preliminary investigation of potential bioactive components of TCMs. Based on our previous study [17], GA and LA were screened and identified by RBL-2H3 cell extraction, which supplied a feasible method for preliminarily identifying potential anti-pseudo-allergic components in TCMs.

GA and LA could both inhibit mast cell degranulation and calcium influx, but only LA bound to MRGPRX2 according to molecular docking. As shown in Fig. 3C and 4C, the mode of change of the $[Ca^{2+}]_i$ seemed to be different between GA and LA, and the minimum effective concentration of LA was significantly lower than that of GA, which suggested that their upstream mechanisms of inhibiting mast cell activation might not be identical. In addition, isoliquiritigenin has been reported to inhibit IgE-independent allergies via the MRGPRX2 pathway [18]. LA and isoliquiritigenin are both flavonoids, while GA is a triterpenoid saponin, indicating that flavonoids in licorice possess the potential to inhibit MRGPRX2-mediated PARs. The structure-effect relationship of flavonoids in licorice should be investigated further to discover more effective natural antagonists of MRGPRX2 and predict their derivatives.

PARs usually occur upon the application of drugs or functional foods. An increasing number of studies have reported the anti-pseudo-allergic compounds from natural products [19–22]. Licorice is widely considered to be an effective candidate because of its strong effect and low toxicity [23], and our study provides a strong rationale for the use of GA and LA as functional food components or novel treatment options for PARs.

Conclusions

In conclusion, GA and LA were identified as potential anti-pseudo-allergic components in LE. Our study provides a strong rationale for using GA and LA as novel treatment options for PARs. Above all, this study also lays the foundation for the further study of flavonoids in licorice as potential MRGPRX2 antagonists.

Abbreviations

PARs: Pseudo-allergic reactions; MEM: Modified Eagle's Medium; MPMC: mouse peritoneal mast cell; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide; PBS: phosphate-buffered saline; PDL: poly-D-lysine hydrobromide; Q-TOF MS/MS: quadrupole time-of-flight tandem mass spectrometry; LE: licorice extract.

Declarations

Ethics approval and consent to participate

All procedures and assessments were approved by Animal Ethics Committee of Jinling Hospital.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the study: LW, HS, JQ; performed the biological experiments: LW, GZH, YL, SJJ; statistical analysis: LW, DSK; wrote the paper: LW; revised the paper: ZMF, JQ, HS. All of the authors have read and approve the final manuscript.

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Figures

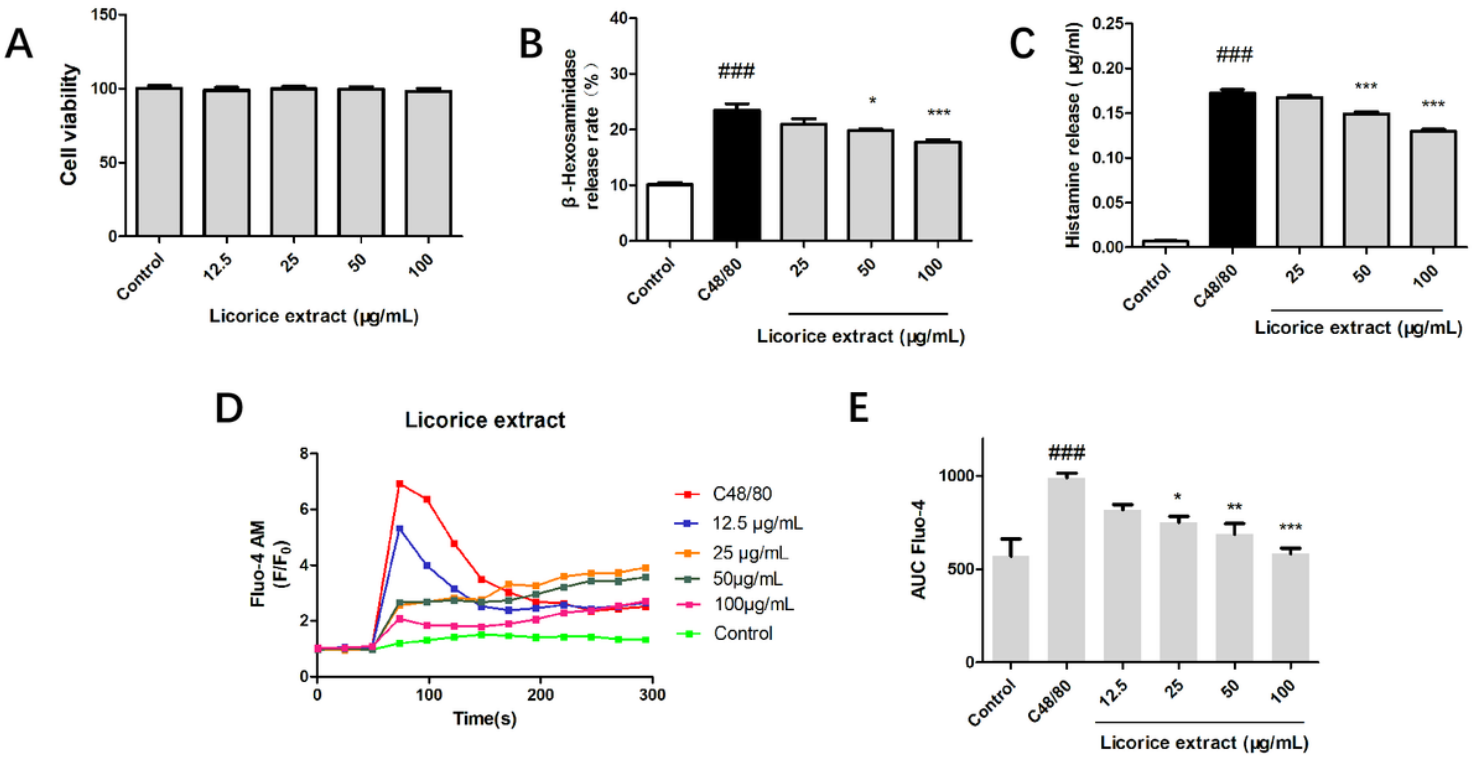

Figure 1

LE inhibited C48/80-induced degranulation and calcium influx in RBL-2H3 cells. (A) LE had no effect on cell viability. (B) LE inhibited C48/80-induced β-hexosaminidase release. (C) LE inhibited C48/80-induced histamine release. (D) LE reduced C48/80-induced calcium influx dose-dependently. (E) Quantification of con-centration-response relationship for LE. Data are presented as mean ± SEM. ###p < 0.001, C48/80 (30 μg/mL) group compared with control group. *p < 0.05, **p < 0.01, ***p < 0.001, compared with C48/80 group.

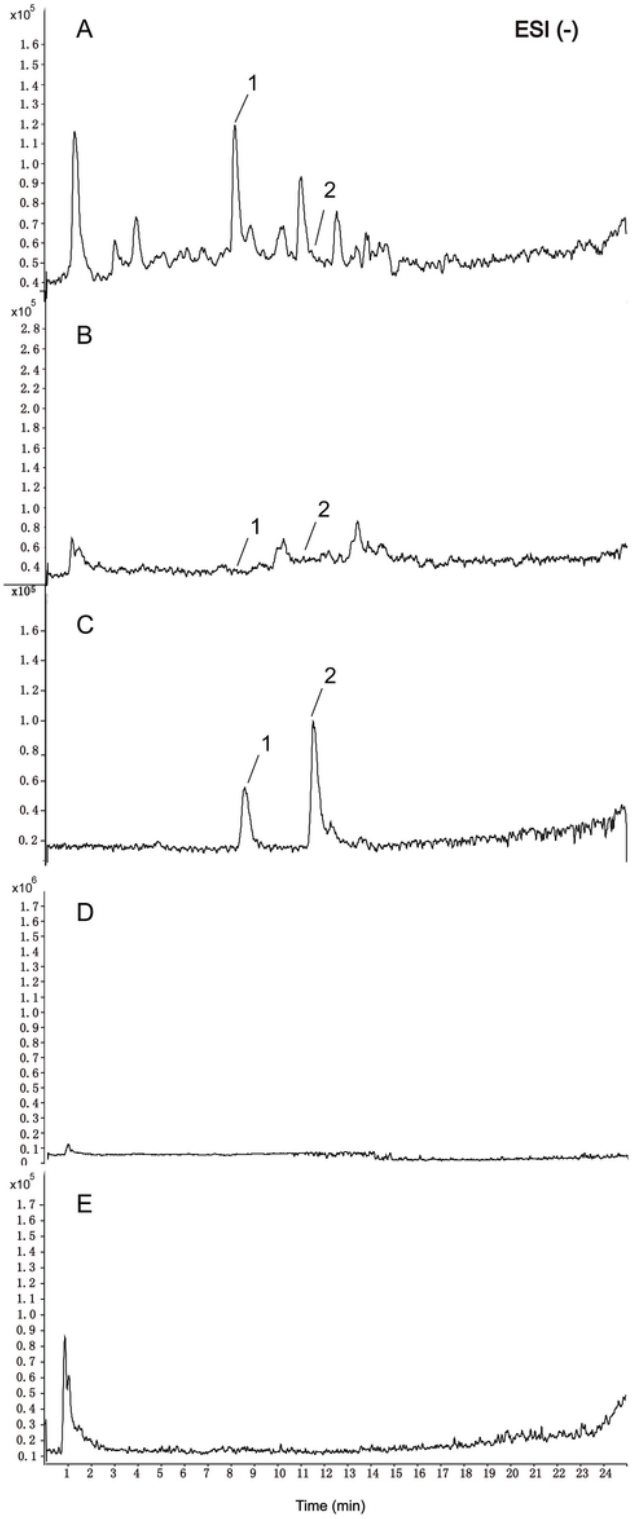

Figure 2

Total ion chromatograms of different samples in negative ion mode analyzed by UPLC–DAD-Q-TOF–MS/MS. (A) LE, (B) cell disruption, (C) reference substances, GA (peak 1) and LA (peak 2), (D) control sample, (E) final-wash eluate.

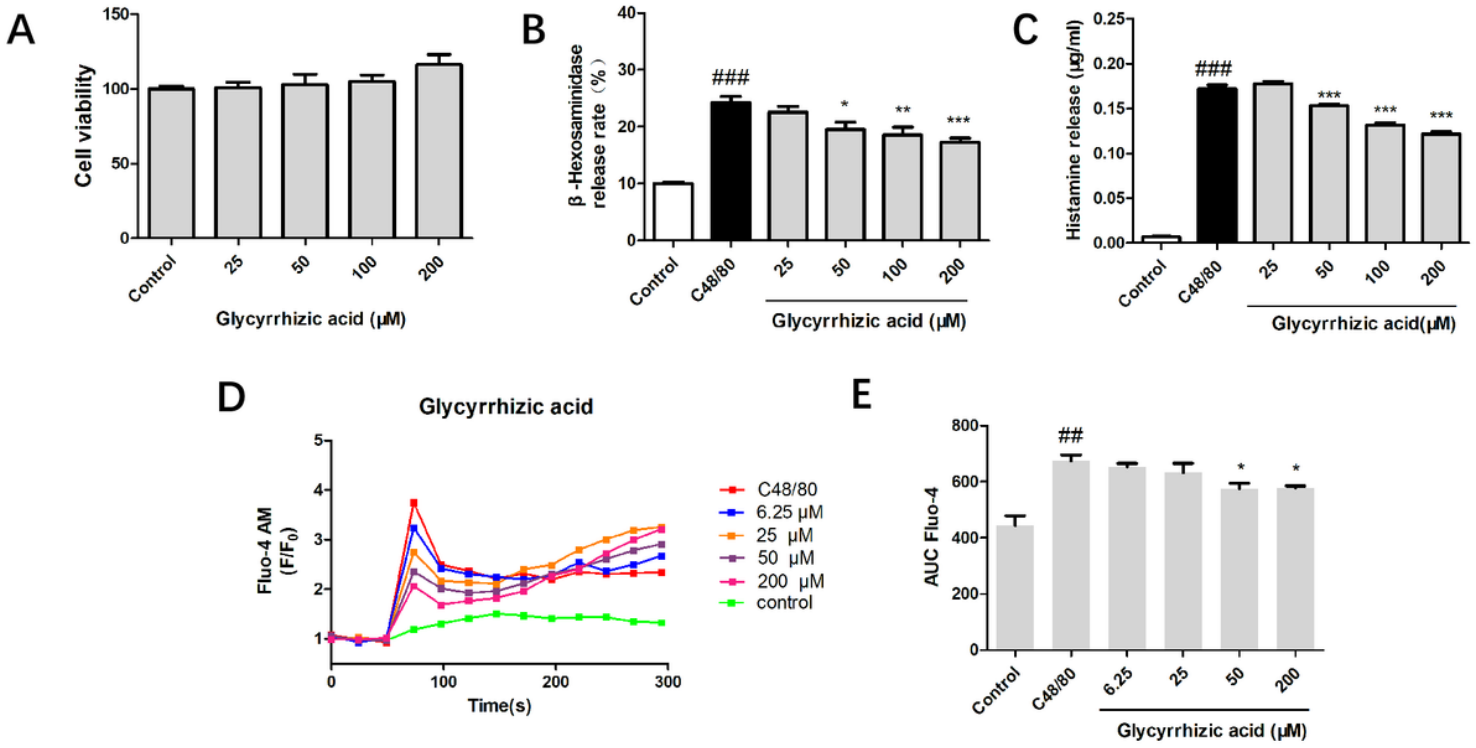

Figure 3

GA inhibited C48/80-induced degranulation and calcium influx in RBL-2H3 cells. (A) GA had no effect on cell viability. (B) GA inhibited C48/80-induced β-hexosaminidase release. (C) GA inhibited C48/80-induced histamine release. (D) GA reduced C48/80-induced calcium influx dose-dependently. (E) Quantification of concentration-response relationship for GA. Data are presented as mean ± SEM. ##p < 0.01, ###p < 0.001, C48/80 (30 μg/mL) group compared with control group. *p < 0.05, **p < 0.01, ***p < 0.001, compared with C48/80 group.

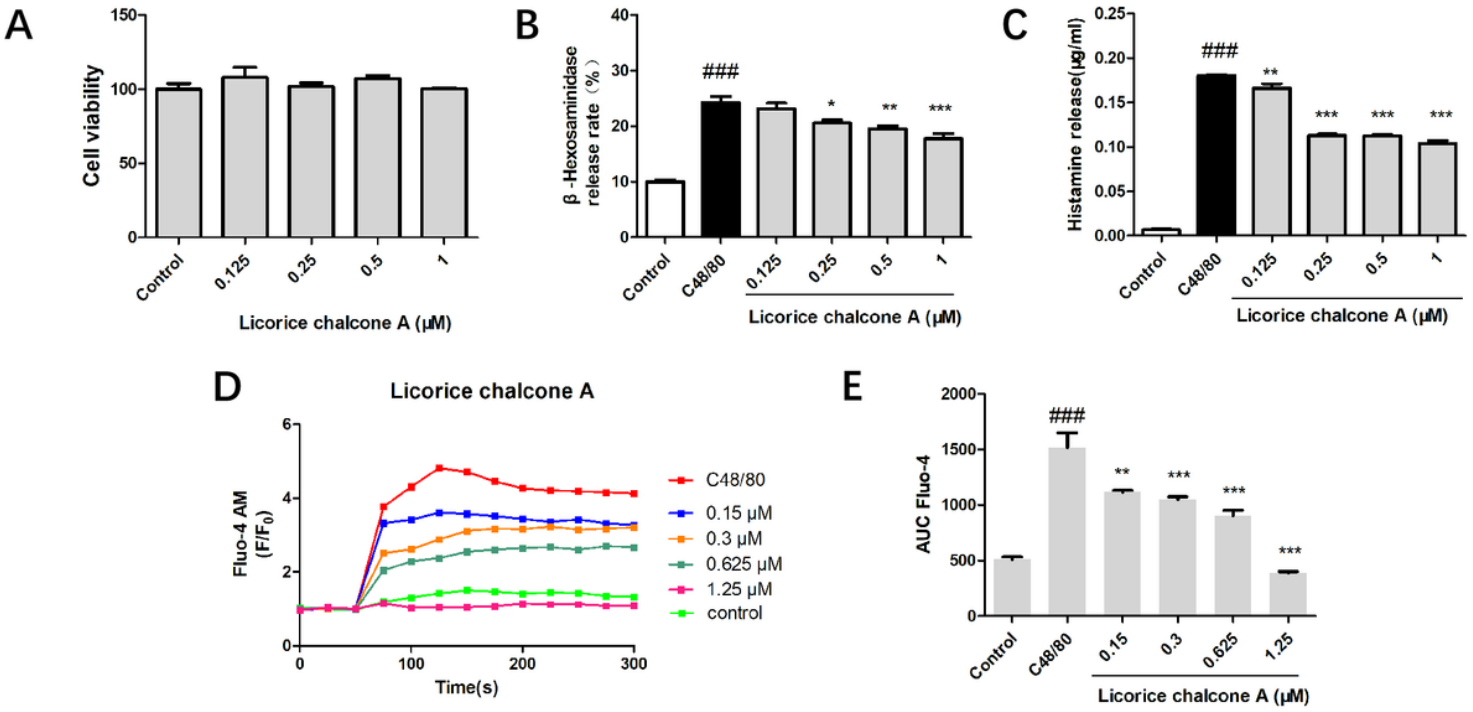

Figure 4

LA inhibited C48/80-induced degranulation and calcium influx in RBL-2H3 cells. (A) LA had no effect on cell viability. (B) LA inhibited C48/80-induced β-hexosaminidase release. (C) LA inhibited C48/80-induced histamine release. (D) LA reduced C48/80-induced calcium influx dose-dependently. (E) Quantification of con-centration-response relationship for LA. Data are presented as mean ± SEM. ###p < 0.001, C48/80 (30 μg/mL) group compared with control group. *p < 0.05, **p < 0.01, ***p < 0.001, compared with C48/80 group.

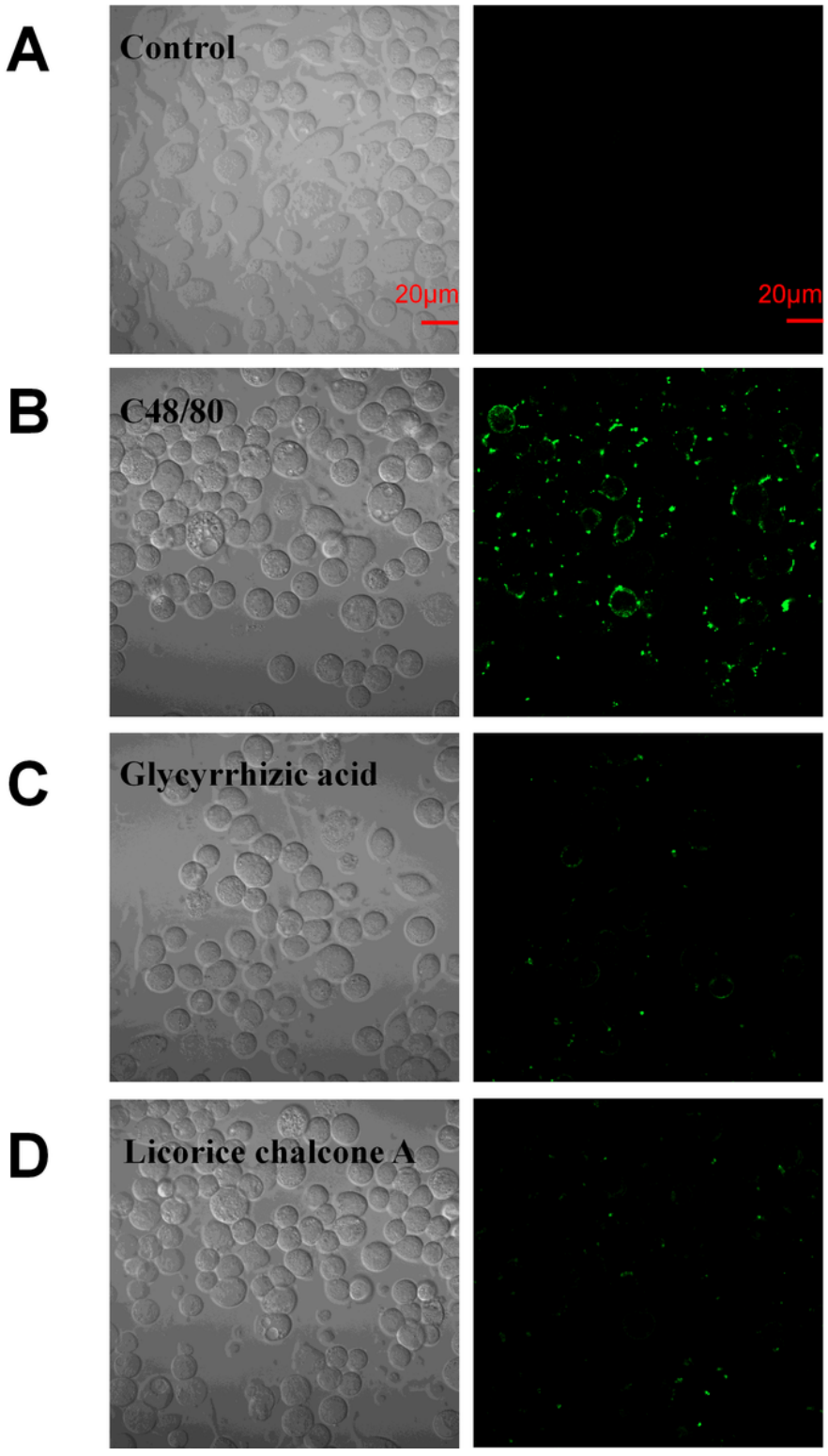

Figure 5

GA and LA inhibited C48/80-induced phosphatidylserine translocation in RBL 2H3 cells. (A) Control group, (B) C48/80 (30μg/ml) group, (C) GA (100 μM) group, (D) LA (0.50 μM) group.

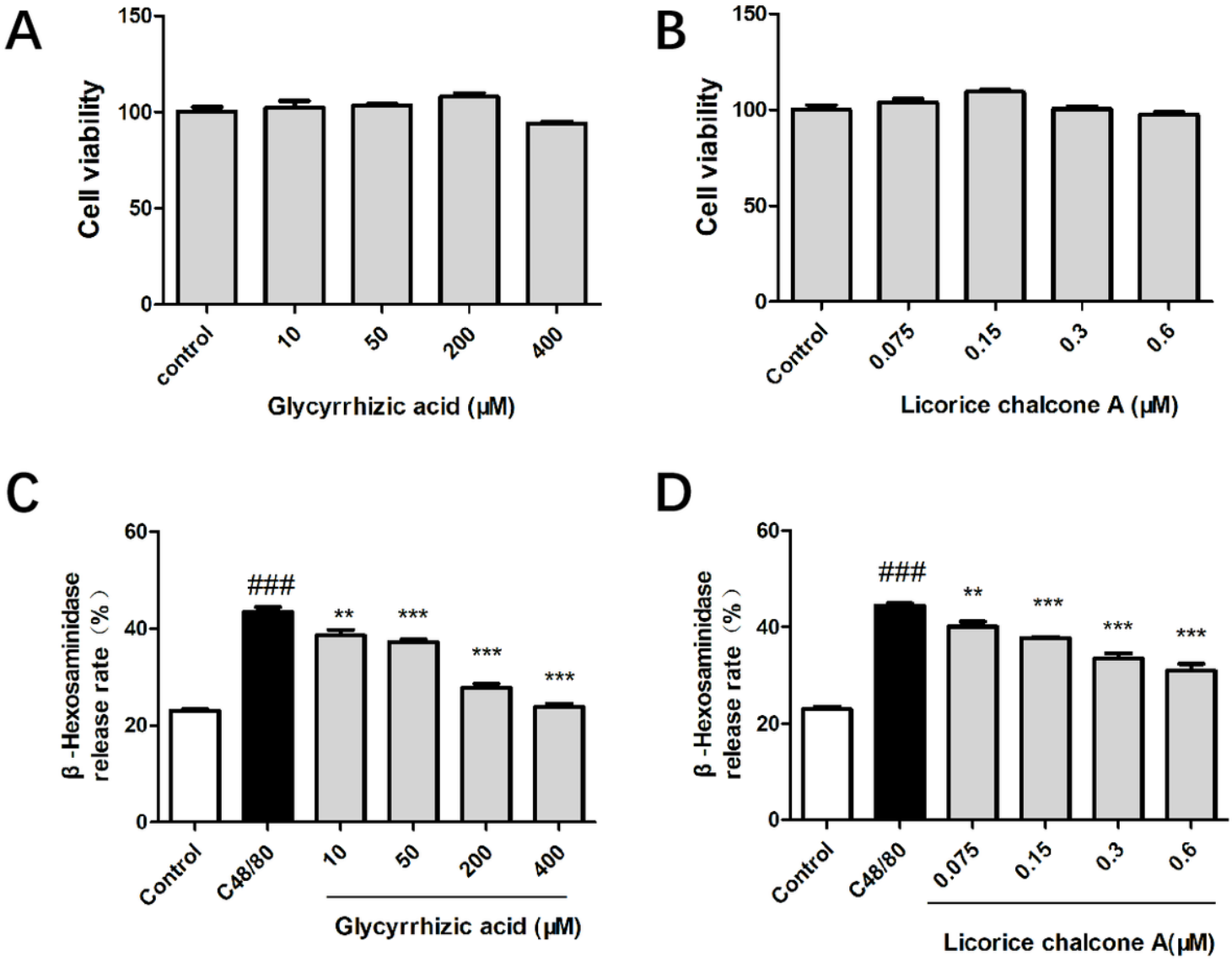

Figure 6

GA and LA inhibited C48/80-induced β-hexosaminidase release in MPMCs. (A) GA showed little cytotoxicity against MPMCs. (B) LA showed little cytotoxicity against MPMCs. (C) GA decreased dose-dependently C48/80-induced β-hexosaminidase release. (D) LA decreased dose-dependently C48/80-induced β-hexosaminidase release. Data are presented as mean ± SEM. ###p < 0.001, C48/80 (30 μg/mL) group compared with control group. **p < 0.01, ***p < 0.001, compared with C48/80 group.

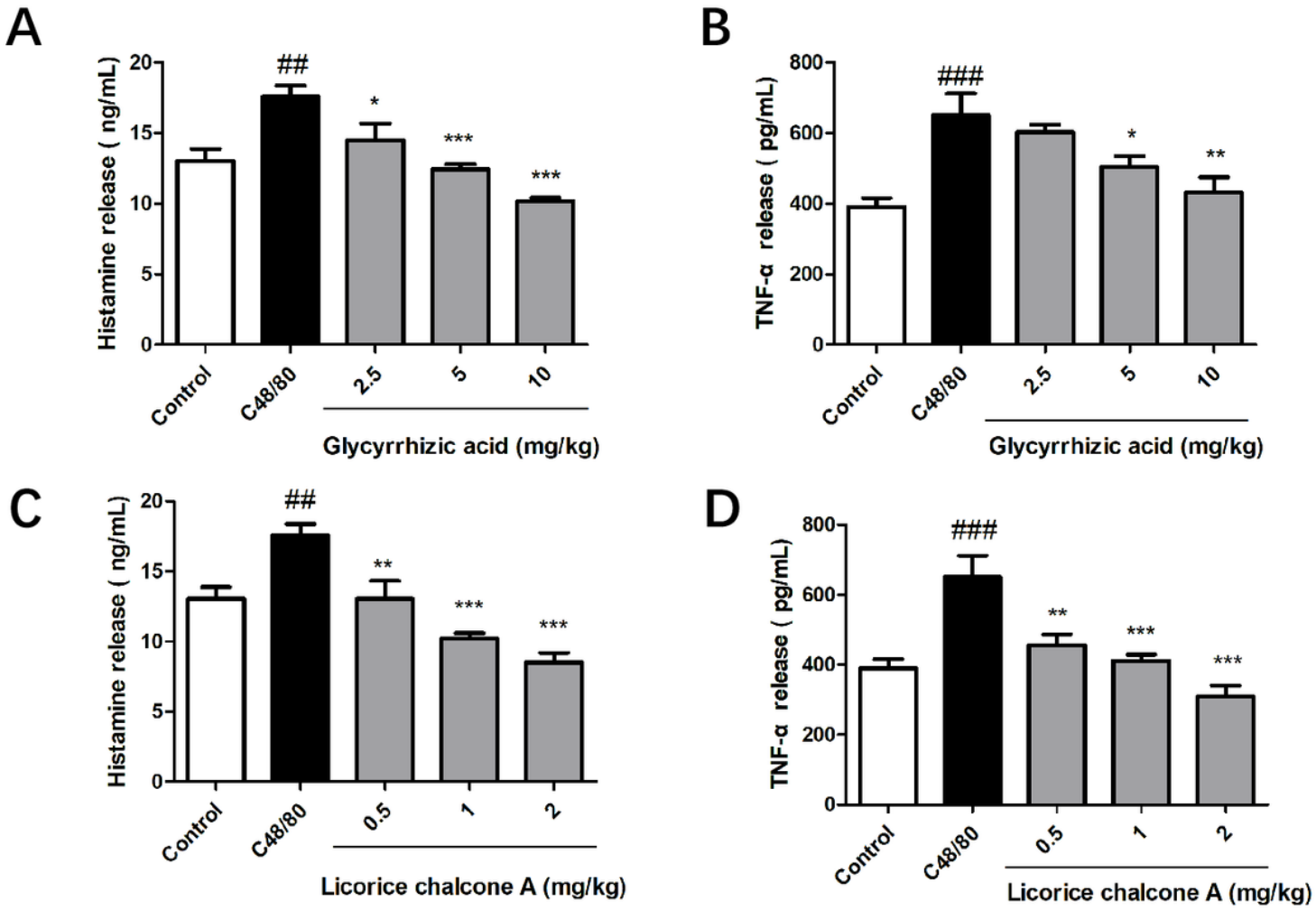

Figure 7

GA and LA inhibited C48/80-induced PARs in mice. GA inhibited C48/80-induced increase of histamine (A) and TNF-α (B). LA inhibited C48/80-induced increase of histamine (C) and TNF-α (D). Data are presented as mean ± SEM. ##p < 0.01, ###p < 0.001, C48/80 group compared with control group. *p < 0.05, **p < 0.01, ***p < 0.001, compared with C48/80 group.

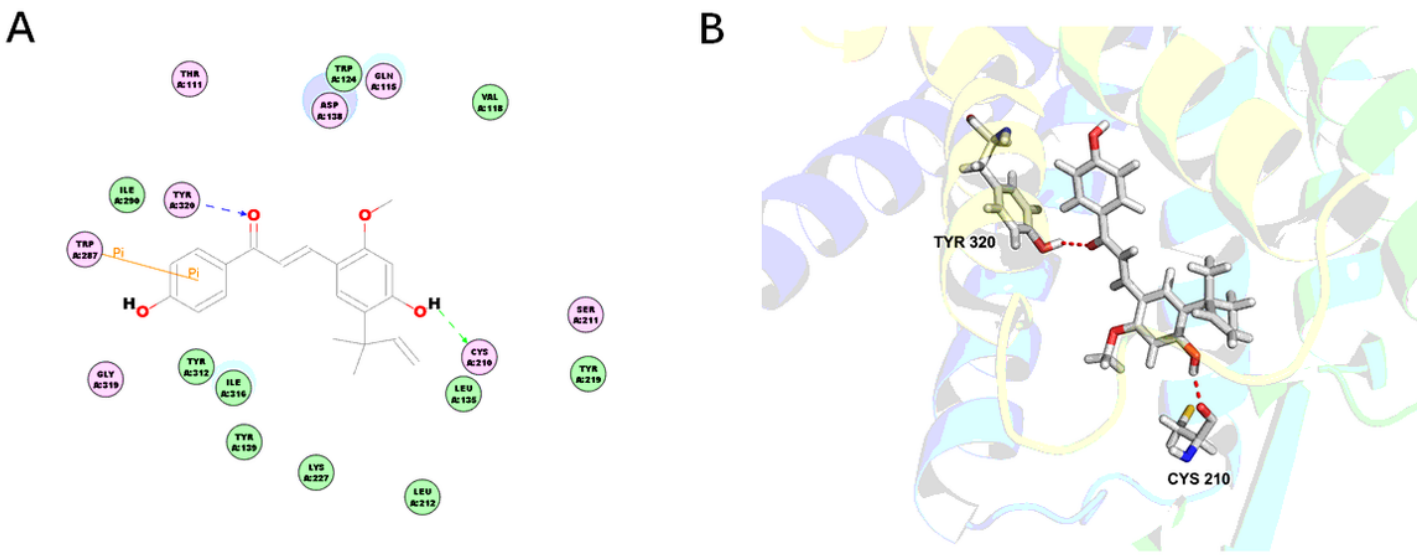

Figure 8

Molecular docking studies of LA and MRGPRX2. (A) 2D docking diagram. (B) 3D docking diagram