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Research Article

Analysis of allergens in tubeimu saponin extracts by using rat basophilic leukemia 2H3 cell-based affinity chromatography coupled to liquid chromatography and mass spectrometry

An affinity two-dimensional chromatography method was developed for the recognition, separation, and identification of allergic components from tubeimu saponin extracts, a preparation often injected to treat various conditions as indicated by traditional Chinese medicine. Rat basophilic leukemia-2H3 cell membranes were used as the stationary phase of a membrane affinity chromatography column to capture components with affinity for mast cells that could be involved in a degranulation reaction. The retained components were enriched and analyzed by membrane affinity chromatography with liquid chromatography and mass spectrometry via a port switch valve. Suitability and reliability of the method was investigated using appropriate standards, and then, the method was applied to identify components retained from tubeimu saponin extracts. Tubeimoside A was identified in this way as a potential allergen, and degranulation assays confirmed that tubeimoside A induces RBL-2H3 cell degranulation in a dose-dependent manner. An increase in Ca^{2+} influx indicated that degranulation induced by tubeimoside A is likely Ca^{2+} dependent. Coupled with the degranulation assay, RBL-2H3 cell-based affinity chromatography coupled with liquid chromatography and mass spectrometry is an effective method for screening and identifying allergic components from tubeimu saponin extracts.

Keywords: Cell membrane chromatography / Degranulation / High-performance liquid chromatography with mass spectrometry / Mast cells / Tubeimoside A
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1 Introduction

Allergies are classified into four types, with type I being the most common allergic reaction. The type I allergic reaction, which is associated with asthma and allergic dermatitis, is provoked by binding of an allergenic antigen, such as cedar pollen, to immunoglobulin E (IgE). Antigen-bound IgE is subsequently recognized by the high-affinity IgE receptor ($\text{Fc}\epsilon\text{RI}$) expressed on the surface of mast cells and basophiles, following which chemical mediators such as histamine, 5-HT, and inflammatory cytokines are released from intracellular granules in a process called degranulation [1–4].

Nonallergic drug hypersensitivities, also referred to as anaphylactoid reactions, frequently occur when anesthetics and contrast agents are used in routine clinical applications

[5]. Chemotherapeutic drugs also frequently induce nonallergic drug hypersensitivities, and can induce mast cells to release chemical mediators in an IgE-independent manner [6]. The released mediators nonetheless induce acute allergic responses, including abnormal smooth muscle contractions, vasodilation, and increased vascular permeability. Thus, basophilic mast cells play a crucial role in initiating the allergic reaction. The rat basophilic leukemia (RBL) cell line was developed in 1973 from rats in which leukemia was induced with the chemical carcinogen β -chloroethylamine [7]. A subclone of the RBL-IV (HR+) line led to the isolation of the histamine-secreting RBL-2H3 clone in 1976 [8]. Because their granule content is similar to mast cells, RBL-2H3 cells are commonly employed as a convenient model to study mast cell degranulation, although in some aspects they are more similar to basophiles than other histamine-releasing cell types [9].

Traditional Chinese medicine has been widely used in clinical practice since ancient times in China, and modern clinicians continue to apply many of these ancient remedies. In the 1950s, injections based on traditional Chinese medicine were developed as new formulations to treat diseases, such as traditional Chinese medicine injections

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Abbreviations: BV, bed volumes; CMC, cell membrane chromatography; IgE, immunoglobulin E; RBL, rat basophilic leukemia

Colour Online: See the article online to view Fig. 1 in colour.

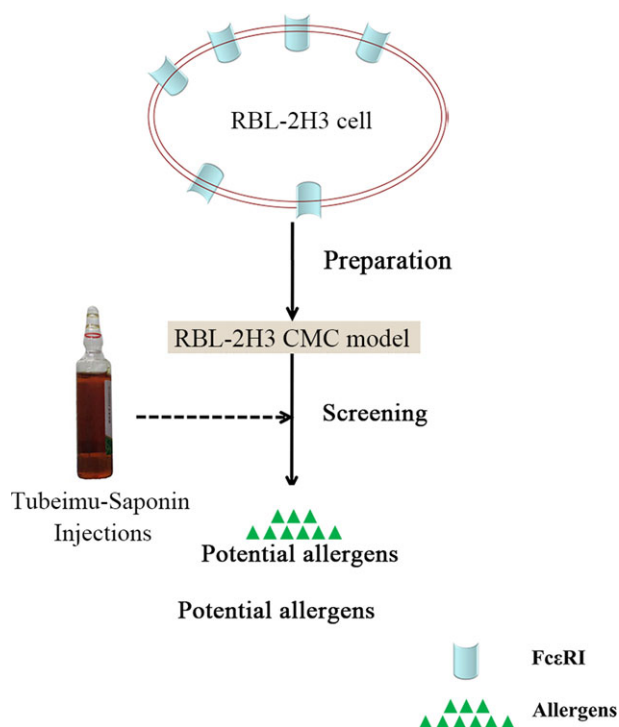


Figure 1. Scheme for screening potential allergenic components with the RBL-2H3-CMC model.

[10–12]. Despite positive therapeutic effects, the intravenous injection of compounds extracted from traditional Chinese medicine materials has in some cases led to acute allergic reactions, including anaphylactoid reactions [13]. Many researchers have put effort into detecting the components of these treatments that cause mast cell degranulation without IgE participation, with some studies suggesting that compounds such as chlorogenic acid, taxanes, radiocontrast agents, or vancomycin may be responsible. [14]. Since IgE is not involved, skin testing has not been a reliable predictor of these allergic reactions and therefore the development of alternative methods for allergy screening is required. The herbal medicine injections that are widely used in China and other countries [15] often have a highly complex mixture of components, presenting a significant problem for QC. As a result, adverse drug reactions resulting from herbal injections have occurred frequently [16, 17].

Bolbostemma paniculatum is a medicinal herb distributed in Hebei, Henan, Shanxi, and Shandong province in China. According to “Bencao Gangmu Shiyi,” a Qing Dynasty text written by Xuemin Zhao, *B. paniculatum* has many pharmacological effects, such as antitumor and antiviral activities. With the continued development of traditional Chinese medicine, researchers have found that total saponin extracts from tubers of *B. paniculatum* contain many components (Fig. 1). Among these, Tubeimoside A, B, and C have been identified as active components [18]. The injectable preparation, referred to as tubeimu saponin, consists of total saponin extracts of *B. paniculatum* that have been dissolved

with distilled water and filtered with activated charcoal. This traditional Chinese medicine preparation is reported to be effective for antipyretic, antidotal, anticondyloma, and anti-tumor [19] therapies. It was awarded a new drug certificate (Z20026653) by the China Food and Drug Administration in 2002 for condyloma therapy. It was reported that tubeimu saponin injection increases efficacy up to 90% compared with other preparations [20]. However, some side effects have also been reported in association with the clinical application of tubeimu saponin preparations, including toxicity, noninflammatory swelling, and hemolysis [21–23].

In this study, membrane extracted from RBL-2H3 cells was employed as the stationary phase for cell membrane chromatography (CMC), a type of biological affinity chromatography, to screen for allergens that could be contributing to anaphylactoid reactions. CMC coupled with LC–MS/MS has been shown to be an effective method for screening, analyzing, and identifying target components from complex systems [24–27]. Membrane-binding components of tubeimu saponin injections were captured using the RBL-2H3-CMC method and subsequently identified by LC–MS/MS (Fig. 1). A cell degranulation assay was then performed in order to determine the allergenic effects of the identified components [28, 29].

2 Materials and methods

2.1 Materials and reagents

Silica gel (ZEX-II, 5 μm , 200Å) was supplied by Qingdao Meigao Chemical (Qingdao, China). Quercetin ($\geq 98\%$, Lot number: MUST-12072505) and tubeimoside A ($\geq 98\%$, Lot number: MUST-12091704) was obtained from Chengdu MUST Bio-technology. *B. paniculatum* was purchased from a medical materials market and authenticated by Professor Niu Xiaofeng of the Department of Pharmacognosy at Xi'an Jiaotong University (Xi'an, China). DMSO, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), and trypsin were obtained from Sigma. HPLC-grade methanol was obtained from SK Chemicals (Ulsan, Korea).

2.2 Instruments and conditions

The CMC screening system consisted of three units, an LC-20AD pump, an SIL-20A autosampler, and an SPD-20A UV/Vis detector (Shimadzu Corporation, Kyoto, Japan). The RBL-2H3-CMC columns (10.0 mm $\times$ 2.0 mm I.D., 5 μm) used in this system were packed using an RPL-10ZD column-loading machine (Dalian Replete Science and Technology, Dalian, China). Briefly, the cell membrane stationary phase was dissolved in saline and placed into a stainless-steel slurry tube with a length of 31 cm and a diameter of 1.8 cm. The phase was packed on the column under 10 MPa pressure.

The analytical system consisted of a DGU-20A₃ degasser, two LC-20AD pumps, an SPD-M20A diode array detector, an

LC/MS-2010EV mass spectrometer, and an LC/MS Solution work station (Shimadzu Corporation, Kyoto, Japan). A Shimadzu Shim-pack VP-ODS column (150 mm $\times$ 2.0 mm I.D., 5 μ m, Kyoto, Japan) was used as the second-dimension column. The conditions of the system setup were as follows: mobile phase A, composed of water with acetic acid (0.5%, v/v) and triethylamine (0.1%, v/v), and mobile phase B (methanol) were combined in an ODS column (150 mm $\times$ 2.0 mm I.D., 5 μ m) with a gradient of 40% mobile phase A. We performed gradient elution using a flow rate of 1.0 mL/min with two different mobile phases. Because of the fast flow rate of the second dimension mobile phase (1.0 mL/min) a P470 high pressure graduated microsplitter valve (Upchurch Scientific, Washington, USA) was used to split the mobile phase so that the mobile phase flow rate would be compatible with the LCMS-2010EV mass spectrometer (0.2 mL/min). The column temperature was maintained at 37°C and a DAD detector was used. The conditions of the mass spectrometer were as follows: ionization mode ESI, nebulizer gas N₂ (purity, 99.999%), flow rate 1.5 L min/min, drying gas N₂ (purity 99.999%), pressure 0.1 MPa, interface temperature 230°C, heat block temperature 200°C, curved desolvation line temperature 250°C, detector voltage 1.5 kV, curved desolvation line voltage 10 V, positive and negative ionization mode, scanning from m/z 50–1500.

The two systems were combined with a VICI_{AG} 10G-0911V ten-port two-position valve (Valco Instrument, Houston, USA), and two Shimpack VP-ODS precolumns (10 mm $\times$ 2.0 mm I.D., 5 μ m, Shimadzu Corporation, Kyoto, Japan) were used as the enrichment columns.

2.3 Sample preparation

The tubeimu saponin injection was prepared with a series of extraction, filtration, and concentration steps. Briefly, an extract of 20 g of *Rhizoma Bolbostemmatidis* powder was refluxed at 65°C in 400 mL 70% ethanol for 1 h and the filtrates were re-extracted three additional times under the same conditions. The total ethanol extract was filtered on a macroporous resin (NKA-9) column that was then washed with 7 BV (bed volumes) of distilled water. Subsequently, total saponins were eluted from the macroporous resin with 3 BV of 95% ethanol. Then the filtrates were concentrated and dried by distillation under reduced pressure [30, 31]. A 0.1 g of the tubeimu saponin extract was dissolved in 2 mL of methanol as a working solution. For storage, concentrated solutions of tubeimu saponin (1 mg/mL each) were kept in the dark at 4°C.

Tubeimoside A and quercetin standard solutions were separately prepared in methanol (1 mg/mL). The solutions were freshly prepared and stored in the dark at –20°C. When these standard solutions were used, they were diluted to suitable concentrations using fresh mobile phase solutions.

2.4 Preparation of RBL-2H3-CMC column

Rat basophilic leukemia-2H3 mast cells were originally acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai), and were cultured in Dulbecco's modified Eagles' Minimal Essential Medium containing 4.5 g/L glucose and supplemented with 10% fetal calf serum from Gibco-BRL (Rockville, USA). Cells were cultured in CF EasyFill-2 culture vessels (Nunc EasyFill™, Nunc) in a humidified atmosphere with 5% CO₂ at 37°C. When cell confluence reached about 80–90%, RBL-2H3 cells were harvested and washed with physiological saline (pH 7.4). The RBL-2H3-CMC columns were prepared as described previously [32]. Briefly, a cell membrane suspension prepared from RBL-2H3 cells (7×10^6) was mixed with 0.05 g heat-activated silica (105°C for 30 min). To pack the columns, the RBL-2H3 cell membrane stationary phase (20 mL of 2.5 mg/mL) dissolved in physiological saline was pumped into a stainless-steel slurry tube (length 31 cm and diameter 1.8 cm) using a packing pump (RPL-10ZD, Replete, Dalian, China). Physiological saline with a 2 mL/min flow rate was injected into the slurry tube under pressure (<10 MPa). Finally, the cell membrane stationary phase was packed into the CMC column (10 mm $\times$ 2.0 mm I.D.). The mobile phase (water) had a flow rate of 0.2 mL/min and the columns were kept at 37°C. UV detection was performed at 254 nm for Quercetin and at 210 nm for tubeimoside A.

2.5 Verifying the RBL-2H3-CMC–HPLC–MS/MS system

To validate the RBL-2H3-CMC–HPLC–MS/MS approach, the capabilities of the RBL-2H3-CMC column was investigated. The mixed standard solution of atenolol (β -receptor antagonist), nicotine (nicotinic acetylcholine receptor agonist), nimodipine (calcium channel antagonist), and quercetin (membrane binding kinase inhibitor) [33] was analyzed using RBL-2H3-CMC columns.

The RBL-2H3-CMC–HPLC–MS/MS system was connected by a two-position, ten-port switch valve. Quercetin, a kinase inhibitor that binds RBL-2H3 cell membranes [33], was used in this analysis to determine the suitability of the RBL-2H3-CMC–HPLC–MS/MS system. The retained fraction was enriched and switched into the HPLC–MS/MS system for analysis and identification of compounds. The reproducibility of the enrichment was investigated by injecting quercetin three times, and the consistency of the peak area of enriched quercetin on the VP-ODS column was used as an indicator of reproducibility. By utilizing the peak area of the enriched quercetin on the VP-ODS column compared with the peak area of the direct HPLC–MS/MS analysis of quercetin, the ratio of enrichment was determined.

2.6 Screening retained components using RBL-2H3-CMC-HPLC-MS/MS

The tubeimu saponin samples were analyzed by the RBL-2H3-CMC-HPLC-MS/MS system. The components that were retained on the RBL-2H3-CMC columns were analyzed and identified by HPLC-MS/MS. In order to confirm the screening results, standard solutions of potential allergenic components were also analyzed by the method.

2.7 β -Hexosaminidase degranulation assays

RBL-2H3 cells were grown in 24-well plates to 70–80% confluence for the degranulation experiment. For degranulation assays, cells were treated with various concentrations of tubeimoside A for 30 min. Samples of culture supernatant (50 μ L) were then incubated for 1.5 h at 37°C with 100 μ L of *p*-nitrophenyl-*N*-acetyl- β -glucosaminide (5 mmol/L, Sigma) in 0.1 mol/L sodium citrate buffer (pH 4.5). Then, the reaction was terminated by adding 200 μ L of 0.2 mol/L sodium carbonate buffer (pH 10.5). Release of *p*-nitrophenolate in the medium was measured spectrophotometrically in a 96-well plate reader at a wavelength of 405 nm. The amount of β -hexosaminidase released from the cells was expressed as a percentage of the total β -hexosaminidase, which was determined from the cells lysed in 0.1% Triton X-100. The data are presented as the mean $\pm$ S.D. The amount of histamine was assayed using a histamine ELISA kit (KYM, Beijing) according to the manufacturer's instructions.

2.8 Intracellular Ca^{2+} measurement

Measurement of the levels of intracellular Ca^{2+} was performed with a calcium-reactive fluorescence probe called Fluo-3 (Biotium). Briefly, RBL-2H3 cells were incubated with tyrode buffer containing 4 μ mol/L Fluo-3-AM (Biotium) for 30 min at 37°C. The Fluo-3-loaded RBL-2H3 cells were washed twice and stimulated with serial concentrations of tubeimoside A in tyrode buffer. The change in fluorescence of Fluo-3 was measured at an excitation wavelength of 488 nm and an emission wavelength of 515 nm for 90 min.

3 Results and discussion

3.1 Suitability of the RBL-2H3-CMC-HPLC-MS/MS method

The selectivity of the RBL-2H3-CMC was tested using quercetin as the positive control and atenolol, nicotine, and nimodipine as the negative controls. Among these four test compounds, only quercetin was retained on the RBL-2H3 membranes (Fig. 2). The reproducibility of results from a single RBL-2H3-CMC column was investigated by injecting quercetin five times, and the RSD of the retention time of

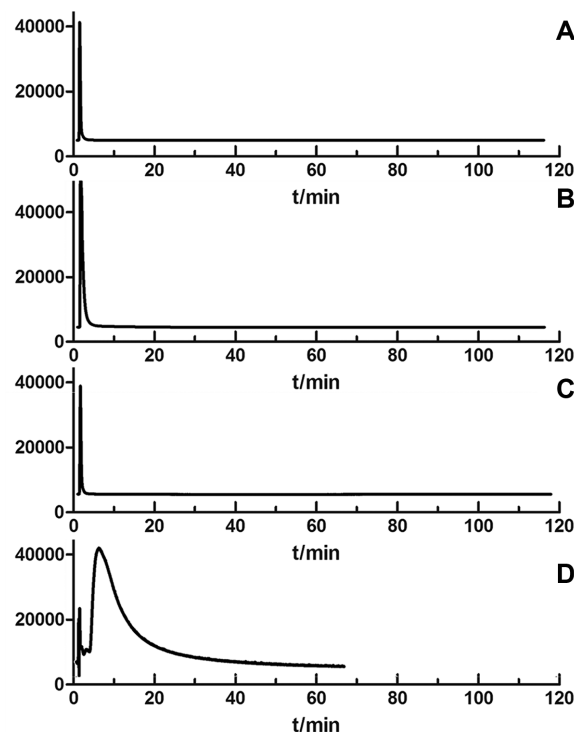


Figure 2. Chromatography of four samples using RBL-2H3-CMC. (A) 1 mg/mL atenolol; (B) 1 mg/mL nitrendipine; (C) 1 mg/mL captopril; (D) 1 mg/mL quercetin.

quercetin on the RBL-2H3-CMC column was 0.69% ($n = 5$). The reproducibility between RBL-2H3-CMC columns was investigated by injecting quercetin into three different columns, and the RSD of the retention time was calculated at 4.65% ($n = 3$). Suitability testing of the RBL-2H3-CMC method was investigated in the present study. To determine the lifetime of the RBL-2H3-CMC column, quercetin was repeatedly injected over a 48 h period. Quercetin had an obvious retention on the RBL-2H3-CMC column after 48 h. Therefore, it could be concluded that RBL-2H3-CMC column could be used for at least 48 h, meeting the requirement of the planned screening experiments.

As shown in Fig. 3A, the retention time of quercetin on the affinity column was about 3.9 min. There is a tailing peak showing R_1 retention. Based on previous studies, the mechanism of retention in the CMC affinity chromatography method is presumed to be a ligand–receptor interaction [34]. Therefore, the retained fraction R_1 (between the two dotted lines) was enriched with an enrichment column, and then it was switched to an analytical column for chromatographic separation and MS identification (Fig. 3C). Data from the HPLC-MS/MS analysis confirmed that the retained fraction was quercetin (see chromatogram in Fig. 3B). Therefore, the RBL-2H3-CMC-HPLC-MS/MS system was deemed suitable for the recognition, analysis, and identification of components retained by RBL-2H3 cells.

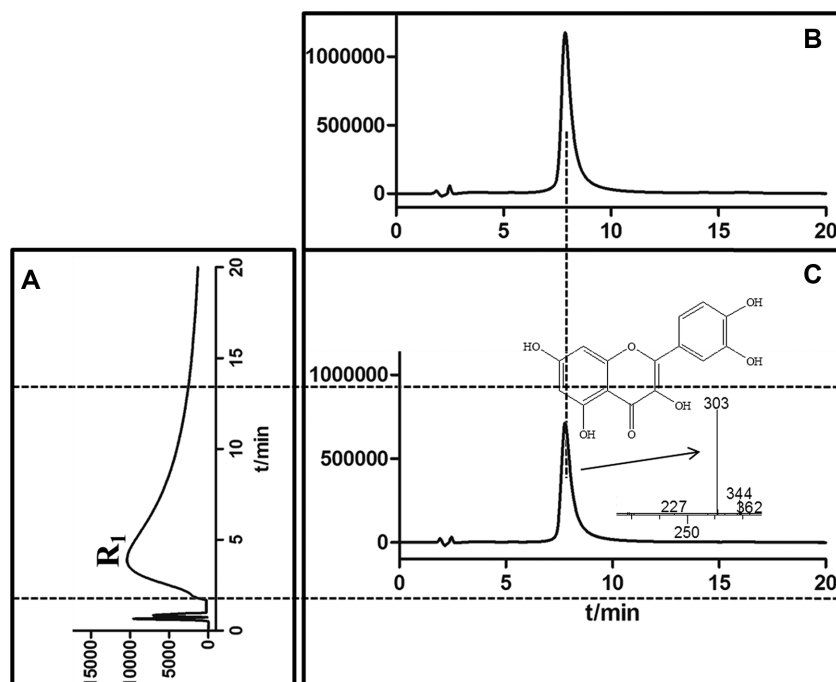


Figure 3. Results of quercetin analyzed by the RBL-2H3-CMC-HPLC-MS/MS method. (A) shows quercetin retained on the RBL-2H3-CMC column, (B) shows the direct HPLC-MS/MS chromatograms of the quercetin standard solution, and (C) shows the HPLC-MS/MS chromatograms of the retained fraction.

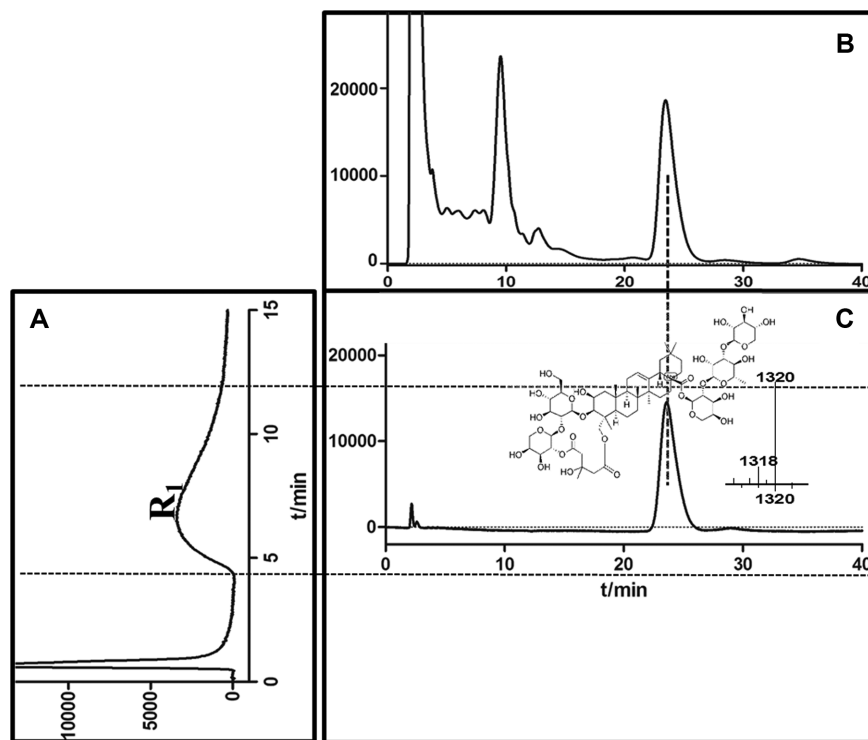


Figure 4. Results of tubeimu saponin injection analyzed by the RBL-2H3-CMC on-line HPLC-MS/MS method. (A) shows one retained fraction, R₁ (between two dotted lines), on the RBL-2H3-CMC column, (B) shows the direct HPLC-MS/MS chromatograms of tubeimu saponin injections, and (C) shows the HPLC-MS/MS chromatograms of the retained fraction, and it was identified as tubeimoside A.

3.2 Application of the RBL-2H3-CMC-HPLC-MS/MS method

Tubeimu saponin injections were subsequently analyzed with the RBL-2H3-CMC-HPLC-MS/MS method (Fig. 4). One significant fraction (R₁, retention time, ~5.8 min) was retained on the RBL-2H3-CMC column (Fig. 4A). The retained fraction was enriched on the enrichment column, and via the

column switching device the fraction was directly injected into the HPLC-MS/MS system. The HPLC-MS/MS chromatogram of the tubeimu saponin injection (Fig. 4B) was compared to the chromatogram of the retained fraction (R₁). On the basis of this comparison, the retained fraction was identified as tubeimoside A. In order to verify the recognition, analysis, and identification using the RBL-2H3-CMC-HPLC-MS/MS method, a standard solution of tubeimoside A was

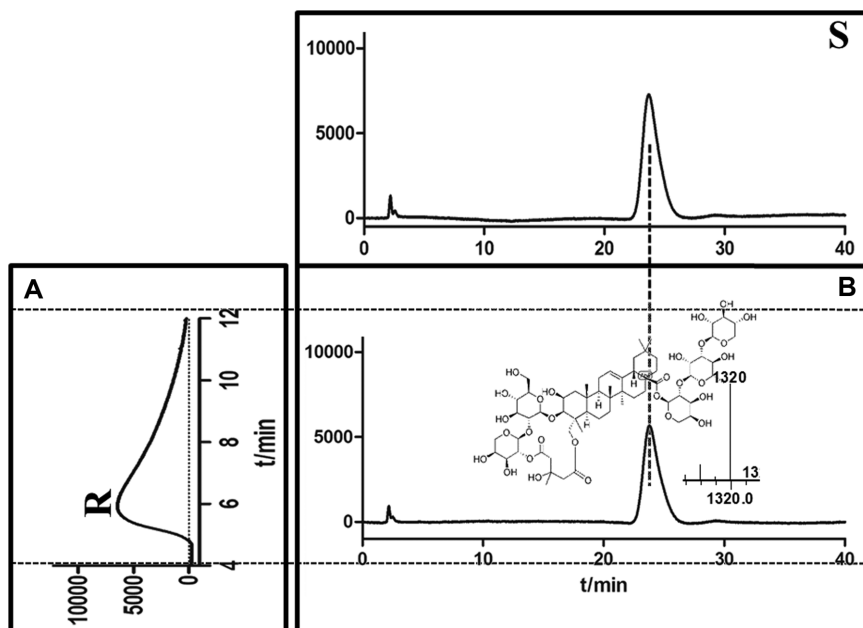


Figure 5. Results of tubeimoside A standard solution analyzed by RBL-2H3-CMC online HPLC–MS/MS method. (A) shows the RBL-2H3-CMC chromatogram of tubeimoside A (between two dotted lines), (B) shows the HPLC–MS/MS chromatograms of the retained fraction and it was identified as tubeimoside A, and (S) shows the direct HPLC–MS/MS chromatograms of the tubeimoside A standard solution.

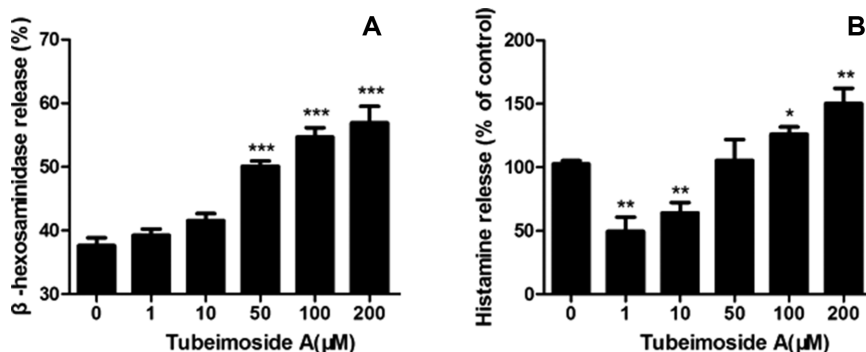


Figure 6. Effect of tubeimoside A on degranulation. (A) β -Hexosaminidase release from RBL-2H3 cell treated with 0, 1, 10, 50, 100, 200 μ M tubeimoside A, and (B) histamine release from RBL-2H3 cell treated with 0, 1, 10, 50, 100, 200 μ M tubeimoside A. Each value represents the mean $\pm$ SD, $n=3$. ** $p < 0.01$, *** $p < 0.005$ in comparison with the 0 μ M treated group.

also analyzed. This analysis showed that tubeimoside A was retained on the RBL-2H3-CMC column (R, retention time ~ 5.8 min; Fig. 5A). The retained fraction was analyzed by HPLC–MS/MS and was identified as tubeimoside A (Fig. 5C). The chromatogram of the tubeimoside A standard (Fig. 5) exhibited the same retention peak as the fraction retained by RBL-2H3-CMC from the tubeimu saponin sample (Fig. 4C). Based on the results (Figs. 4 and 5) of the RBL-2H3-CMC column analysis, tubeimoside A was recognized, analyzed, and identified as the likely allergenic component.

3.3 Effect of tubeimoside A on degranulation of mast cells

It is not known how tubeimoside A acts on RBL-2H3 cells, and therefore it was determined whether tubeimoside A could induce degranulation. Based on cell viability results from an MTT assay (a colorimetric assay for assessing cell toxicity of component), it was determined that a tubeimoside concentration below 200 μ M would maintain cell variability. Therefore, RBL-2H3 cells were treated with

serial concentrations of tubeimoside A below the calculated IC_{50} . The cells treated with tubeimoside A degranulated, and it should be noted that they also appeared round and transparent.

The granule-related enzyme β -hexosaminidase, which is an exoglycosidase expressed in mast cells, is released in parallel to histamine upon mast cell activation. β -Hexosaminidase release occurs in proportion to the degree of degranulation and is commonly used as a clinical marker for degranulation in mast cells [35]. RBL-2H3 cells were stimulated with increasing concentrations of tubeimoside A (Fig. 6A). Tubeimoside A concentrations exceeding 50 μ M significantly stimulated RBL-2H3 cell degranulation, with the maximum release of β -hexosaminidase occurring at a concentration of 200 μ M.

The release of chemical mediators such as histamine plays an important role in allergic reactions [36]. To further examine the effects of tubeimoside A on histamine release, the amount of histamine secreted from tubeimoside A-treated RBL-2H3 cells was determined by ELISA. Our data showed that tubeimoside A increased histamine secretion in a dose-dependent manner compared with the control (Fig. 6B).

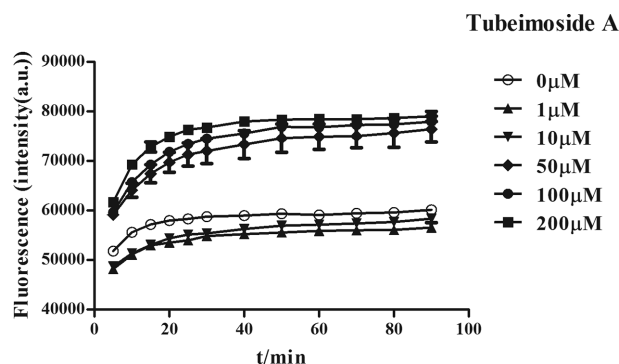


Figure 7. Effect of Tubeimoside A on Ca^{2+} influx. RBL-2H3 cell treated with 0, 1, 10, 50, 100, 200 μM tubeimoside A. The level of calcium fluorescence was recorded. Each data value represents the mean $\pm$ SD of three independent experiments.

3.4 Tubeimoside A induced $[\text{Ca}^{2+}]$ increase

Intracellular Ca^{2+} signaling can induce histamine release from mast cells without IgE binding to Fc ϵ RI receptors [37]. We tested whether tubeimoside A could trigger intracellular Ca^{2+} increase by loading RBL-2H3 cells with Fluro-3 AM dye and exposing them to 0, 1, 10, 50, 100, or 200 μM of tubeimoside A. The changes in cytosolic Ca^{2+} were measured by monitoring the intracellular fluorescence intensity. As shown in Fig. 7, tubeimoside A concentrations from 10 to 100 μM induced an increase of approximately onefold in fluorescence, while lower tubeimoside A concentrations ($< 10 \mu\text{M}$) caused a minimal elevation compared to untreated cells. This data demonstrated that tubeimoside A treatment resulted in a dose-dependent increase in Ca^{2+} concentration (Fig. 7). Histamine release is attributed to a high sustained Ca^{2+} by an external Ca^{2+} influx and a secondary Ca^{2+} release from intracellular Ca^{2+} stores (organelles). Interestingly, the release of histamine and the increase of Ca^{2+} followed the same trend. This phenomenon supported that Ca^{2+} signaling mediated the observed histamine release and subsequent degranulation in an IgE-independent manner.

4 Conclusion

Adverse drug reactions, including some acute allergic reactions, have been reported in some cases where tubeimu saponin injections were used. To avoid further adverse events, identification of the potential allergenic components from these preparations is urgently needed. The RBL-2H3 cell line is a histamine-releasing cell line commonly used in allergy and immunological research. RBL-2H3 cells may also degranulate in response to a variety of nonimmunological stimuli. Therefore, the RBL-2H3-CMC-HPLC-MS/MS method was applied to analyze and identify allergenic components from tubeimu saponin extracts in the present study. Tubeimoside A was readily identified by the rapid 2D screening method. The allergenic activity of tubeimoside A was further investigated by histamine and β -hexosaminidase release assays. In addition, it was shown that tubeimoside A treatment in-

creases intracellular Ca^{2+} in a dose-dependent manner. Based on the results using the RBL-2H3-CMC-HPLC-MS/MS analytical method, we conclude that tubeimoside A is likely to be at least one of the allergenic components present in tubeimu saponin injections.

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