

RESEARCH ARTICLE

Licochalcone A inhibits MAS-related GPR family member X2-induced pseudo-allergic reaction by suppressing nuclear migration of nuclear factor- κ B

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Licochalcone A (Lico A) is a natural flavonoid belonging to the class of substituted chalcone that has various biological effects. Mast cells (MCs) are innate immune cells that mediate hypersensitivity and pseudo-allergic reactions. MAS-related GPR family member X2 (MRGPRX2) on MCs has been recognized as the main receptor for pseudo-allergic reactions. In this study, we investigated the anti-pseudo-allergy effect of Lico A and its underlying mechanism. Substance P (SP), as an MC activator, was used to establish an in vitro and in vivo model of pseudo-allergy. The in vivo effect of Lico A was investigated using passive cutaneous anaphylaxis (PCA) and active systemic allergy, along with degranulation, Ca^{2+} influx in vitro. SP-induced laboratory of allergic disease 2 (LAD2) cell mRNA expression was explored using RNA-seq, and Lico A inhibited LAD2 cell activation by reverse transcription polymerase chain reaction (RT-PCR), western blotting, and immunofluorescence staining. Lico A showed an inhibitory effect on SP-induced MC activation and pseudo-allergy both in vitro and in vivo. The nuclear factor (NF)- κ B pathway is involved in MRGPRX2 induced MC activation, which is inhibited by Lico A. In conclusion, Lico A inhibited the pseudo-allergic reaction mediated by MRGPRX2 by blocking NF- κ B nuclear migration.

KEYWORDS

Licochalcone A, mast cells, MRGPRX2, NF- κ B, pseudo-allergy

1 | INTRODUCTION

Pseudo-allergy is a hypersensitivity reaction mediated by a non-IgE-dependent mechanism and, thus, does not require prior exposure to the antigenic sensitization and causes mast cell (MC) degranulation directly (Babina et al., 2018; Chen et al., 2017; Lin et al., 2019; Pichler, 2019; Pichler & Hausmann, 2016). MCs are key mediators of allergic reactions that participate in the occurrence of allergic reactions. MCs are innate immune cells found in tissues throughout organisms and are particularly abundant at sites exposed to the environment, such as the skin and mucosal surfaces (Gonzalez-de-Olano & Alvarez-Twose, 2018)

(Kubo, 2018; Subramanian et al., 2016). MCs secrete a variety of inflammatory mediators, such as histamine, interleukin, and tumor necrosis factor, which eventually lead to allergic reactions (Benyon et al., 1989; Mukai et al., 2018; Velez et al., 2018).

Generally, after the body is exposed to the allergen, it produces specific IgE that binds to the Fc fragment of IgE receptor I (Fc ϵ RI) of MCs. When the allergen enters the body again, it binds to the specific IgE and activates Fc ϵ RI, which can cause MC degranulation and induce allergic reactions (Berings et al., 2018; Finkelman et al., 2016). Increasing evidence shows that MCs can be directly activated by exogenous or endogenous mediators to produce allergic reactions without IgE (Babina et al., 2018). MRGPRX2, a G protein-coupled receptor (GPCR) expressed on MCs, mediates IgE-independent allergy

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(Che et al., 2018; Hamamura-Yasuno et al., 2020). Presently, the US Food and Drug Administration (FDA) has approved developed IgE antibody reagents for the targeted treatment of allergic reactions. However, there are no relevant reports on drugs for IgE-independent allergic diseases.

Licochalcone A (Lico A), a natural flavonoid compound derived from the root of *Glycyrrhiza* spp. plants, is known as licorice (Gan-Cao in Chinese) (de Freitas et al., 2020). Structurally, it belongs to the class of substituted chalcone, and it has a characteristic α , β -unsaturated carbonyl group (Zhang et al., 2018). The chalcone nucleus of Lico A is replaced by two phenolic hydroxyl groups, a methoxyl group, and an isoprene side chain (Chen et al., 1993). Recent studies have suggested that Lico A has various pharmacological activities, including anti-inflammatory, antioxidant, antitumor, antiviral, and antimicrobial (Chen et al., 2017; Chu et al., 2012; Wang et al., 2015). However, to the best of our knowledge, there is still no research on the anti-allergic effects of this compound.

In this study, we used substance P (SP), an MC activator, to establish in vitro and in vivo models of pseudo-allergy. The potential anti-pseudo-allergic effect of Lico A was investigated, and the effect of SP on the mRNA of MCs was analyzed using RNA-seq technology. We also tested the effect of Lico A on key genes involved in MC activation. The present study was conducted to explore the effect of Lico A on pseudo-allergy, as well as the mechanisms of the activity.

2 | MATERIALS AND METHODS

2.1 | Drug and reagents

Licochalcone A purity >98% was from Baoji Chenguang Biotechnology Co., Ltd. p-Nitrophenyl N-acetyl- β -D-glucosamide, FITC-avidin, Evans blue, and substance P (SP) were provided by Sigma-Aldrich (St. Louis, MO). Fluo-3, AM was bought from Thermo Fisher Scientific (Waltham, MA). Chloral hydrate was obtained from HUSHI (Shanghai, China). Triton X-100 was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Mouse TNF- α , CCL2 ELISA kits were purchased from Sino Biological Inc. (Beijing, China).

2.2 | Cell lines

LAD2 human MCs were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, Bethesda, MD). Cells were cultured in StemPro-34 medium supplemented with 100 U penicillin-streptomycin, 2 mM L-glutamine, and 100 ng/ml human stem cell factor in a 37°C incubator with 5% CO₂. The HEK293 expressed MRGPRX2 cells were established and cultured in DMEM supplemented with 10% FBS, 100 U penicillin-streptomycin (HyClone, UT).

2.3 | Animals and ethical statement

Adult male C57 mice (6–8 weeks old) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Mice

were housed in individual cages in a temperature-controlled room, fed a standard dry chow twice a day, with free access to water. The breeding environment was on a 12 hr light/dark cycle. Room temperature was 20–25°C, with a relative humidity of 40%. Animals were randomly assigned to groups.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. All mice experimental protocol was approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-713). All animals were performed under chloral hydrate anesthesia.

2.4 | Cell viability assay

The viability of LAD2 cells treated with Lico A was assessed using the cell counting kit-8 (sigma). Briefly, the cells were plated at a density of 2.5×10^4 cells/well in a 96-well plate and incubated overnight at 37°C. The cells were then treated with different concentrations of Lico A (12.5, 25, 50, 100 μ M) for 24 hr. Subsequently, CCK-8 colorimetric solution was added to each well and the plate was incubated for another 2 hr at 37°C under 5% CO₂. Absorbance at 490 nm was measured by a model 550 microplate reader (Bio-Rad, Carlsbad, CA) and the viability of the cells was compared with the negative control.

2.5 | β -Hexosaminidase release assay

LAD2 cells were incubated with indicated concentrations of Lico A for 30 min and then with SP (4 μ g/ml) mixture for 30 min at 37°C. The cell lysates and supernatants were collected. Degranulation was measured by detection of β -hexosaminidase release as described previously (Wang et al., 2016). Briefly, 2.5×10^4 cells in 100 μ l per condition were seeded into a 96-well plate and incubated overnight in an environment at 37°C under 5% CO₂. The 96-well plate was then centrifuged. The culture medium was removed, and 50 μ l Lico A at different concentrations (12.5, 25, 50 μ M) were added to each well. After 30 min incubation at 37°C under 5% CO₂, cells in each well were stimulated with 50 μ l SP at a final concentration of 4 μ g/ml for another 30 min at 37°C. Hereafter, the plate were centrifuged at 400g for 5 min, and 50 μ l supernatant was transferred into other wells. Remaining cells were lysed with 100 μ l triton 1% X-100 (v/v), and 50 μ l supernatant was removed into new wells. Hexosaminidase activity was determined by incubating the supernatant and cell lysate with 1 mM p-Nitrophenyl N-acetyl- β -D-glucosamide for 90 min at 37°C. Reactions were stopped with 0.1 mM Na₂CO₃/NaHCO₃ and the absorbance was measured at 405 nm. IC₅₀ value was calculated by the inhibition ratio of β -hexosaminidase release, which was caused by different concentration of Lico A.

2.6 | Intracellular Ca²⁺ mobilization assay

LAD2 cells and HEK293 expressed MRGPRX2 cells were seeded in a 96-well plate (1×10^4 cells/well) and incubated for 24 hr at

37°C. The cells were then washed twice with calcium imaging buffer (CIB; 125 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO₃, 20 mM sucrose, pH 7.4), and loaded with the incubation buffer (3.5 mM Fluo-3 a.m., 0.1% [w/v] F-127, diluted with CIB at different concentrations of Lico A) at 37°C in the dark. An incubation buffer without Lico A was regarded as the negative control. After 30 min of incubation, the cells were then again washed twice with CIB and used immediately for imaging. For activation, SP at a final concentration of 4 µg/ml was added to each well, and we captured 200 graphs of the cells under blue light (1 s per picture) using an inverted fluorescent microscope for 120 s (Nikon, Ti-U, Japan).

2.7 | Paw swell in SP-induced mice

Adult male mice (6–8 weeks old) were randomly divided into the following groups (three mice per group): vehicle, SP group, SP + Lico A (20, 40, 80 mg/kg) groups. The mice of SP + Lico A groups were orally administrated with different concentrations of Lico A (20, 40, 80 mg/kg), respectively. Each mouse was anesthetized with an intraperitoneal injection of 0.2 ml 4% chloral hydrate half an hour after gavage. Fifteen minutes after the induction of anesthesia, all the mice were treated with intravenous injection of 0.2 ml 0.4% of Evans blue solution. The thickness of the paw was measured using a Vernier caliper. Five minutes later, 5 µl of 30 µg/ml SP was injected into the left paw and saline alone at same volume was injected into the right paw as a negative control. Paw thickness was measured again 15 min after the injection. Mice were then all sacrificed. The photo of mice was recorded and paw tissues were removed into 1.5 ml epoxy tubes, dried at 50°C for 24 hr, and weighed individually to calculate normalization. The extravasation of Evans blue was analyzed by addition of 500 µl acetone-saline (7:3) mixture into tubes and cutting paw tissues into pieces. Then, the tubes were centrifuged at 1,000g for 15 min, and the 200 µl supernatant in each tube was transferred into 96-wells plate. The absorbance was measured at 620 nm with a spectrophotometer (Bio-rad, CA) and normalized against tissue weight.

2.8 | Body temperature

All the mice (6–8 weeks old) were randomly divided into following groups (six mice per group): vehicle, SP group, SP + Lico A (20, 40, 80 mg/kg) group. The mice of SP + Lico A groups were orally administrated with different concentrations (20, 40, 80 mg/kg) of Lico A, respectively. Exactly 0.2 ml of 30 µg/ml SP or same volume of saline as a negative control was injected through tail vein 30 min after gavage. Immediately, a biological function experimental system was used to measure body temperature of each mice every 3 min for 30 min by inserting a probe into the anus of the mice. The change in body temperature was recorded.

2.9 | Measurement of cytokines release in mice

Exactly 8 hr after the experiment of body temperature, the blood of each mice was extracted through eyeballs and collected into 1.5 ml epoxy tubes in an ice-cold condition. Hereafter, the tubes were centrifuged at 4,000g for 15 min, the supernatant, namely serum, was transferred into other tubes and stored at −80°C. TNF-α and CCL2 are the cytokines that have been recognized as signals of MC activation and pseudo-allergic reaction (Komi et al., 2020). The levels of inflammatory cytokines, TNF-α and CCL2, in the serum were detected with Mouse TNF-α, Mouse CCL2 ELISA kit (Sino Biological Inc, Beijing, China) according to provided instructions.

2.10 | RNA-seq technology

Total RNA was extracted from LAD2 cells using Trizol reagent, and the RNA concentration was measured with a microreader at 260/280 nm. The eligible total RNA was purified, amplified, labeled, hybridized, washed, and scanned. We conducted quality control on the raw data, and selected the data through quality control to carry out various analyses based on the gene expression level such as principal component, correlation, differential gene screening, etc.

2.11 | Real-time quantitative PCR

LAD2 cells were pretreated with 50 µM Lico A for 8 hr, then stimulated with SP for 2 hr. Total cellular RNA of LAD2 cells was extracted with a Trizol RNA isolation kit according to the manufacturer's instrument. Subsequently, reverse transcription was performed to obtain complementary DNA (cDNA) by treatment with gDNA wiper mix (Bio-Rad) for 2 min and HiScript II qRT SuperMix II (Bio-Rad) for 15 min at 45°C. The reaction was then stopped by incubating the solution at 85°C for 5 s, after which 2 µl cDNA was prepared for further analysis. Real-time quantitative PCR reactions were performed with 2 µl cDNA and 18 µl master mix, containing iQ SYBR Green Supermix (Bio-Rad), corresponding gene-specific forward primer, and reverse primer in a CFX384 real-time PCR detection system (Bio-Rad) in triple tests: initial denaturation at 90°C followed by 40 cycles of 10 s at 95°C and 30 s at 55°C. The genes' mRNA level was expressed as cycle threshold (Ct) values and the relative expression level of genes were calculated using the formula $2^{-\Delta\Delta Ct}$ with GAPDH as the reference gene. Specific primer sets used are as follows (5' to 3'): human NF-κB p65, 5'-TTGAGGTGTATTTCACGGGACC-3' (forward) and 5'-GCACATCAGCTTGCGAAAAGG-3' (reverse); human GAPDH, 5'-ATGGGGAAGGTGAAGGTCG-3' (forward), and 5'-GGGGTCATTGATGGCAACAATA-3' (reverse).

2.12 | Western blot analysis

Exactly 4 µg/ml SP was added to LAD2 cells and incubated for 2 hr at 37°C, then total proteins of the cells with 4 µg/ml SP were extracted

using RIPA lysis buffer containing 10% protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics) for 30 min on ice. Subsequently, insoluble protein lysate was obtained by centrifugation at 13,500g for 10 min at 4°C. The protein concentration of the samples was examined by a BCA protein quantification kit with bovine serum albumin as a standard, following the manufacturer's instructions. After the extracted lysates were mixed in 5× loading buffer from Thermo Fisher Scientific, Inc. (Waltham, MA) and then boiled for 5 min, the protein in the cell lysates was denatured, and equivalent amounts of protein were loaded and run on a 10% gel using SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd., Xi'an, China). The separated proteins were transferred onto polyvinylidene fluoride membranes (PVDF: Hangzhou Microna Membrane Technology Co., Ltd, Hangzhou, China) and blocked by constant stirring with 5% non-fat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd., Xi'an, China) for 2 hr at room temperature. The membranes were then incubated with the following primary antibodies: NF-κB p65 (1:10,000), phospho-NF-κB p65 (1:1,000) · IκB-α (1:2,000) overnight at 4°C. After being incubated, the membranes were washed four times with TBST for every 10 min and then incubated with the secondary antibodies (1:20,000 dilution in TBST) for 30 min at room temperature. The membranes were then washed four times with TBST for 10 min each and developed with enhanced chemiluminescence (ECL) kit. A Lane 1DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to capture the images of the developed blots, and Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD) was used to quantify the protein levels.

2.13 | Immunofluorescence staining assay

The LAD2 cell suspension was dropped on the glass supports pre-coated with poly-lysine. After dried under 56°C, the samples were fixed with cold acetone for 10 min. Then, the slides were washed three times with PBS at room temperature for 3 min each time, followed by permeation treatment with 0.5% Triton X-100 solution for 10 min. After cleaning with PBS again, the remaining water was sucked up with absorbency paper. Subsequently, the slides were incubated for 15 min at room temperature with 100 μl biotin-labeled goat anti-rabbit IgG, and the serum was poured out. Then, 100 μl of the prepared NF-κB primary antibody solution (1:100) (Abcam, Cambridge, MA) was added to the cells and incubated at 37°C. After 1 hr, the excess primary antibody was removed, slides were washed with PBS three times again, and the samples were incubated with 100 μl fluorescent secondary antibody (Abcam, Cambridge, MA) for 2 hr at room temperature. After washing the cells with PBS, slides were mounted using a drop of anti-fluorescence quenching sealant containing DAPI (Xi'an Yifei Biotechnology Co., Ltd., Xi'an, China) to visualize the nuclei. The outcomes were evaluated by confocal microscopy.

2.14 | Statistical analysis

All experiments were performed with randomization, and data are presented as the mean ± standard error of mean (SD) from at least

three independent experiments. One-way analysis of variance (ANOVA) was used to determine statistical differences between multiple groups. Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

3 | RESULTS

3.1 | Lico A inhibits SP-induced LAD2 cell activation

To explore the effect of Lico A on cell viability at different concentrations, the cell counting kit-8 assay was performed. The results showed that at 50 μM, Lico A had almost no effect on the cell viability, which showed a rate of >80% even at a concentration of 200 μM (Figure 1A). To determine whether Lico A affects degranulation of LAD2 cells induced by SP, we monitored β-hexosaminidase release in LAD2 cells treated with different concentrations of Lico A.

As shown in Figure 1B, 47.89% ± 9.987% of β-hexosaminidase was released from the cells after SP stimulation, whereas treatment with Lico A dose-dependently inhibited the release of β-hexosaminidase in these cells. Compared with the vehicle group ($n = 3$, 47.89% ± 4.48%), the inhibitory effect was maximum at 50 μM, with a β-hexosaminidase release rate of 27.80% ± 4.478% (Figure 1B). The half-maximal inhibitory concentration (IC₅₀) was also determined to be 16.15 ± 3.38 μM by the release of β-hexosaminidase following exposure to a series of increasing concentrations of Lico A (Figure 1B,C).

Once MCs are activated, Ca²⁺ flux is significantly increased (Holowka et al., 2016). LAD2 and MRGPRX2-expressing HEK293 cells were pretreated with different concentrations (12.5, 25, and 50 μM) of Lico A and incubated in CIB with Fluo-3 a.m. for 30 min. As shown in Figure 1D, compared with the vehicle group LAD2 cell, the inhibition of calcium mobilization in the Lico A-treated group increased with increasing concentrations. Similar results were observed in MRGPRX2-expressing HEK293 cells, where Lico A also reduced calcium mobilization in a dose-dependent manner. At a concentration of 50 μM, the intracellular Ca²⁺ concentration showed no obvious change (Figure 1E), which demonstrated the inhibitory effect of Lico A on MRGPRX2-induced cell activation.

3.2 | Lico A inhibits PCA induced by SP in vivo

A PCA mouse model was used to investigate the anti-pseudo-allergic effect of Lico A in vivo. The degree of paw swelling and Evans blue exudation of the mouse paw were recorded to assess the severity of local allergic reactions. Images of the mice were subsequently captured (Figure 2A).

Compared with the vehicle group, which exhibited a 30.02% ± 3.876% increase in paw swelling, this effect was obviously inhibited by Lico A in a dose-dependent manner. At a maximum concentration of 80 mg/kg, the rate of increase was less than half of that in the vehicle group (Figure 2B). Similarly, the absorption value shown below also

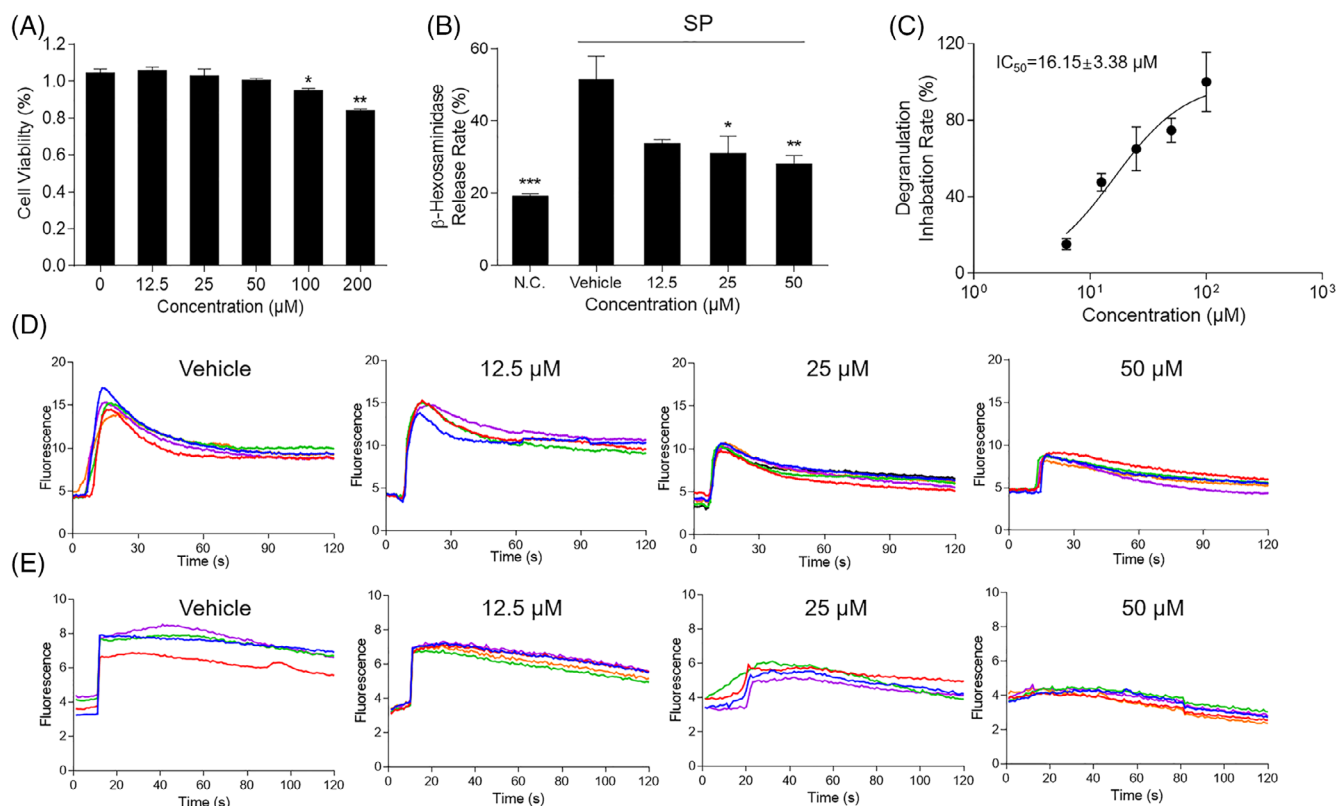


FIGURE 1 Licochalcone A (Lico A) inhibits substance P (SP)-induced laboratory of allergic disease 2 (LAD2) cell activation. (A) Cells were treated with different concentrations of Lico A (0–200 μM) for 12 hr. Cell viability was determined using cell counting kit (CCK)-8 assay. (B) LAD2 cells were pretreated with different concentrations of Lico A for 30 min, followed by activation with 4 μg/ml SP. Degranulation was revealed by the release of β-hexosaminidase. (C) Half-maximal inhibitory concentration (IC_{50}) of Lico A was calculated based on inhibition of β-hexosaminidase release in Lico A-treated LAD2 cells. (D) Lico A inhibited calcium mobilization in LAD2 induced by SP in a dose-dependent manner. (E) Lico A inhibited calcium mobilization in MRGPRX2-expressing HEK293 cells induced by SP. Data are means $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

suggested that Lico A had a dose-dependent inhibitory effect on Evans blue exudation in the mouse paw (Figure 2C).

3.3 | Lico A inhibits systemic anaphylaxis induced by SP in mice

Hypothermia is an important feature of systemic anaphylaxis (Hong et al., 2020) and can reflect the severity of anaphylaxis induced by SP. Initially, the mice were orally administered different concentrations (20, 40, and 80 mg/kg) of Lico A. After 30 min, SP (30 μg/ml) or an equal volume of saline was injected IV, and the body temperature was immediately measured and recorded every 3 min for 30 min. The results demonstrated that Lico A (40 mg/kg) significantly improved body temperature compared with that of mice treated with the vehicle (Figure 3A).

Cytokines, one of the most important allergic mediators released from activated MCs, can be detected in serum to evaluate the anti-pseudo-allergic activity of Lico A in vivo. In our study, the blood serum of all the mice was collected. The serum levels of tumor necrosis factor (TNF)-α were determined after SP challenge and reached 56.71 pg/ml, whereas pretreatment with 40 mg/kg Lico reduced the

levels to 33.52 ± 23.19 pg/ml (Figure 3B). In addition, determination of C-C motif chemokine ligand 2 (CCL2) showed high CCL2 levels of 56.71 ± 3.33 pg/ml (Figure 3C), which were also increased after SP treatment. However, the levels were reduced significantly to 41.02 ± 17.19 pg/ml following treatment with Lico A at up to 40 mg/kg. These results suggest that Lico A has a potent effect on SP-induced systemic anaphylaxis.

3.4 | SP upregulates mRNA expression in LAD2 cells

To explore the effect of Lico A on gene expression, RNA-seq was performed to measure the mRNA levels extracted from LAD2 cells. Two experimental control groups, the positive control (PC), which was stimulated with 4 μg/ml SP for 8 hr, and the negative control (NC), were set up. Subsequently, the differential genes between the NC and PC groups were detected and immune-related genes in the gene ontology (GO) classification were selected and screened step-by-step through a heat map analysis. The obtained heat map of the upregulated genes in the PC group showed 24 upregulated genes following SP treatment (Figure 4A).

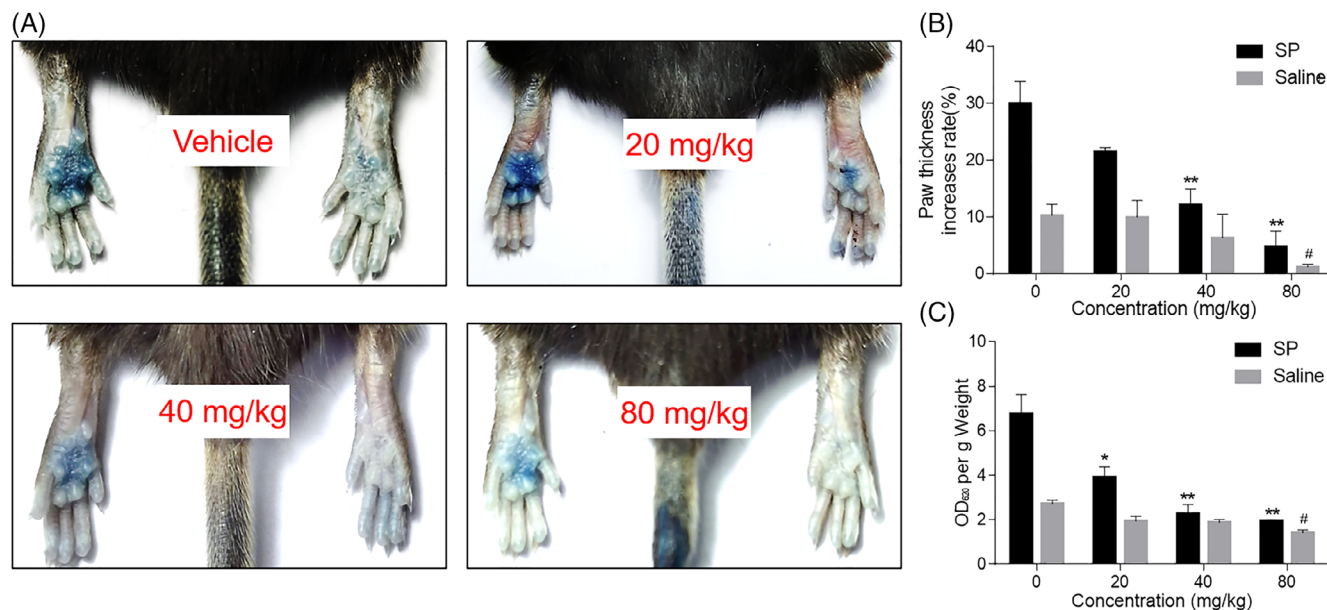


FIGURE 2 Lico A inhibited passive cutaneous anaphylaxis (PCA) induced with substance P (SP) in mice. (A) Representative images of Evans blue extravasation. Quantification of (B) increase in paw thickness and (C) Evans blue extravasation in the paw 15 min after injection of SP. Data are means $\pm$ SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. # means a significant difference between right paws [Colour figure can be viewed at wileyonlinelibrary.com]

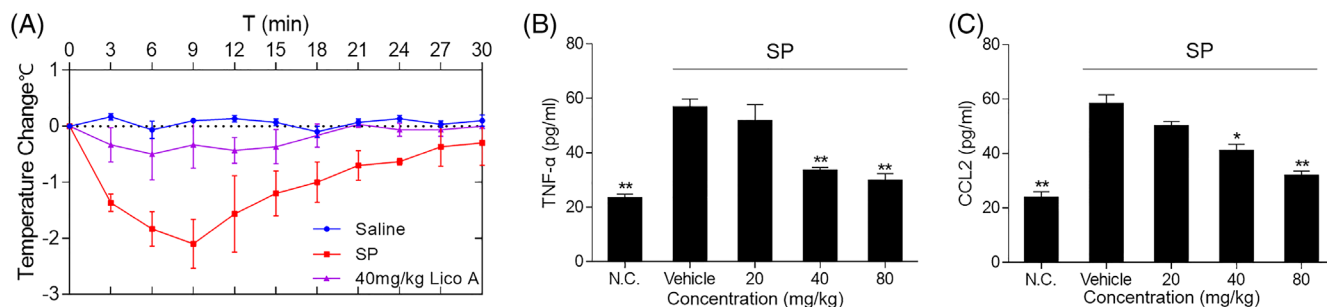


FIGURE 3 Lico A inhibits systemic anaphylaxis induced by substance P (SP) in mice. (A) Lico A (40 mg/kg) was administered by gavage, and 30 min later, 30 μ g/ml SP was injected IV to induce systemic anaphylaxis, or saline as a negative control. Hypothermia was detected in 30 min. Lico A inhibited (B) tumor necrosis factor (TNF)- α and (C) CCL2 release in serum of mice. Data are means $\pm$ SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

The fragments per kilobase of transcript per million mapped reads (FPKM) values of the 24 genes upregulated by SP in the NC and PC groups were measured and reflected the expression levels of each gene (Figure 4B). The NC group showed no gene expression, whereas the PC group showed a higher gene expression, which further confirmed that SP upregulated the 24 genes. The network relationships of the 24 upregulated genes were explored through a protein interaction map, which identified 18 closely related genes (Figure 4C).

This result suggested the interaction strength of different proteins through the size and color depth of the circles. Furthermore, the 18 closely related genes in the protein interaction map were enriched using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and the resulting bubble chart is shown in Figure 4D. Eighteen genes were highly correlated with cytokine–cytokine receptor interaction, *Salmonella* infection, and toll-like receptor, cytokine, and NF- κ B signaling pathways.

A literature review identified several genes, such as *TNF*, granulocyte-macrophage colony-stimulating factor (*CSF2*), C-X-C motif chemokine ligand 8 (*CXCL8*), C-C motif chemokine ligand 3 (*CCL3*), *CCL4*, and *CCL7*, that play important roles in the protein interaction diagram, and are all regulated by NF- κ B (Mitchell & Carmody, 2018). Therefore, we speculated that NF- κ B is a key upstream gene of each gene upregulated by SP.

3.5 | Lico A inhibits upregulation of mRNA expression induced by SP in LAD2 cells

Based on the speculation that SP upregulates mRNA expression through the NF- κ B signaling pathway, RT-PCR was performed to determine the effect of Lico A on six genes (*TNF*, *CSF2*, *CXCL8*, *CCL3*, *CCL4*, and *CCL7*), which are regulated by NF- κ B. As shown in Figure 5,

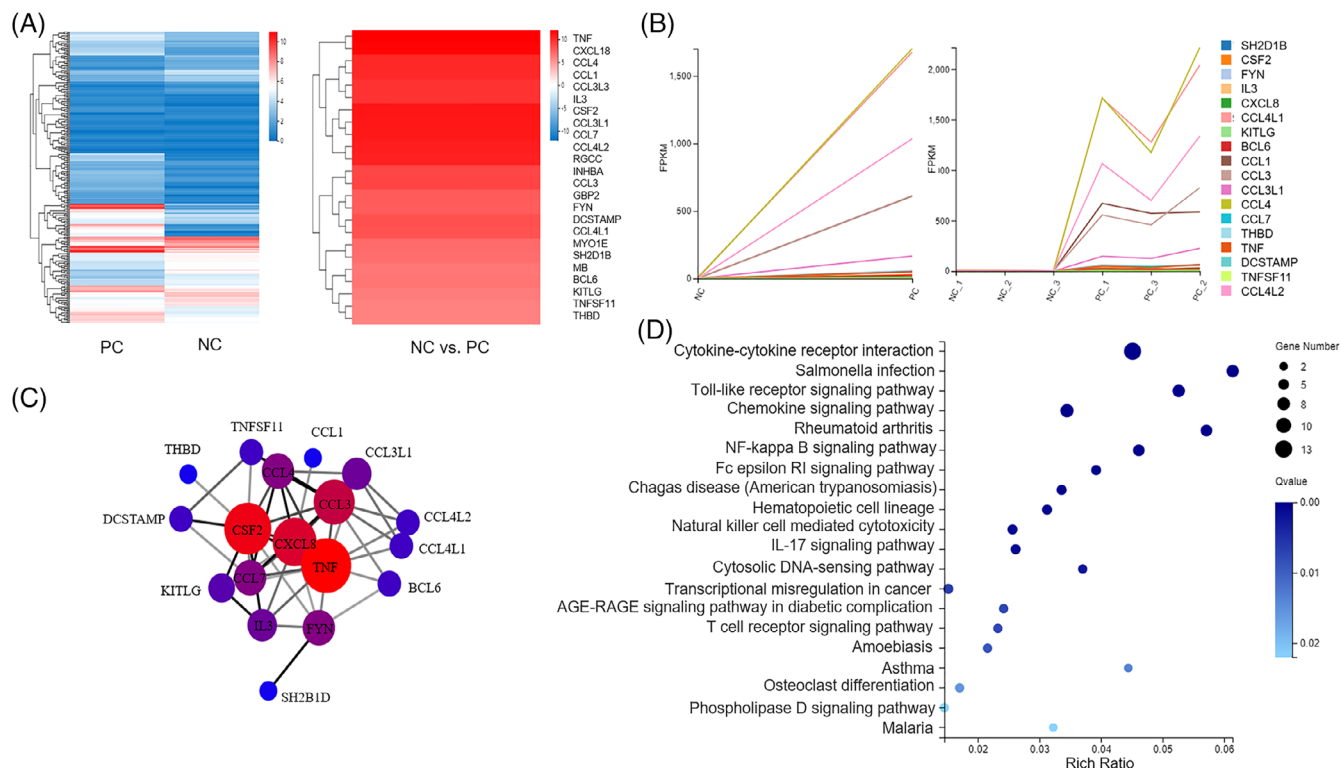
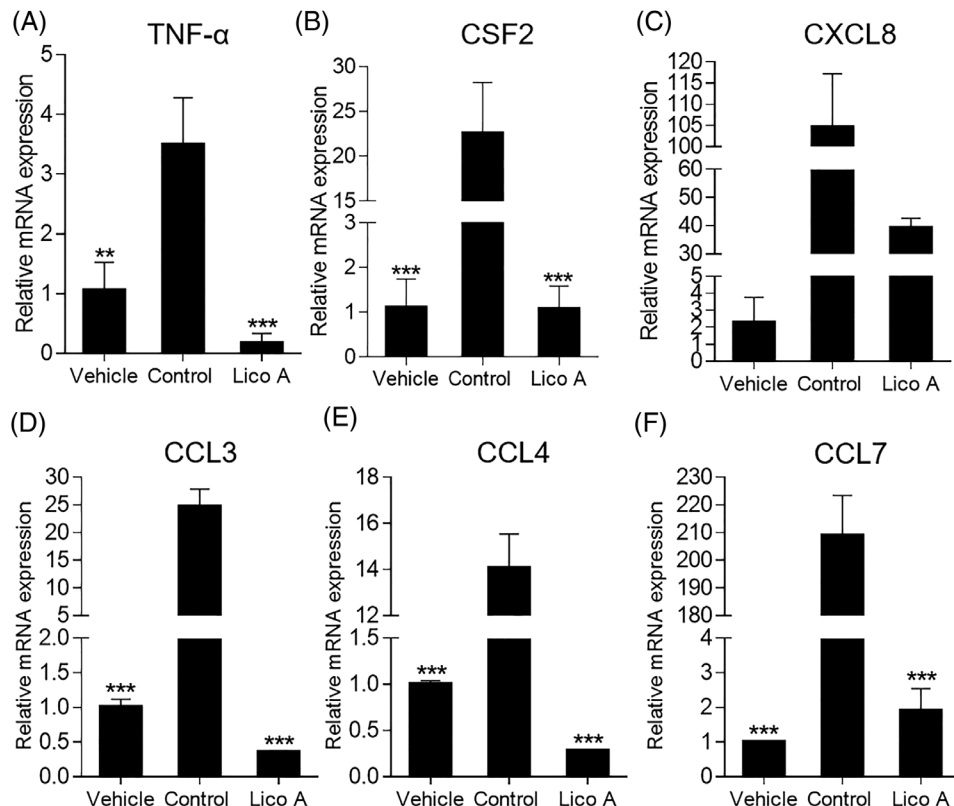


FIGURE 4 Upregulation of mRNA expression by substance P (SP) in laboratory of allergic disease 2 (LAD2) cells. (A) Heat map of 24 genes upregulated by SP in LAD2 cells compared with negative control (NC) group after screening. (B) Fragments per kilobase of transcript per million mapped reads (FPKM) values of 24 genes upregulated by SP in NC and positive control (PC) groups. (C) Protein interaction map of 18 closely related genes. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map of 18 closely related genes suggested several highly related pathways. Data are means $\pm$ SD ($n = 3$) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 Inhibitory effect of Lico A on expression of (A) tumor necrosis factor (TNF), (B) granulocyte-macrophage colony-stimulating factor (CSF2), (C) C-X-C motif chemokine ligand 8 (CXCL8), (D) C-C motif chemokine ligand 3 (CCL3), (E) CCL4, and (F) CCL7 mRNA in substance P (SP)-stimulated laboratory of allergic disease 2 (LAD2) cells using reverse transcription polymerase chain reaction (RT-PCR). Data are means $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$



the mRNA expression of the six genes was increased in the control group compared with that in the vehicle group, which was 1.054 ± 0.33 , 1.098 ± 0.64 , 2.260 ± 1.52 , 1.003 ± 0.12 , 1.001 ± 0.04 , 1.000 ± 0.03 , respectively; however, it was significantly lower after treatment with Lico A than it was in the normal control. Specifically, the mRNA expression level of the Lico A-treated group was correspondingly reduced, which confirmed that all six downregulated genes, which are regulated by the NF- κ B pathway, are involved in the Lico A-induced inhibition of pseudo-allergic reactions.

3.6 | Lico A inhibits MC activation by blocking NF- κ B nuclear migration

To examine the underlying mechanisms of action of Lico A, we investigated its effects on SP-induced NF- κ B p65 upregulation in LAD2 cells using western blot analysis. Compared with the control group that was treated with SP alone, the levels of phosphorylated p65 were decreased in a dose-dependent manner in LAD2 cells co-treated with different concentrations of Lico A and 4 μ g/ml SP (Figure 6A). NF- κ B exists as an inactive form bound to the inhibitory protein I κ B- α in the cytoplasm, and degradation of I κ B- α must occur in order for NF- κ B to translocate to the nucleus (Chae et al., 2016). With the treatment of Lico A, the expression of the I κ B- α decreased, indicating that Lico A

has no effect on the degradation of I κ B- α , which further confirmed the inhibitory effect of Lico A on NF- κ B signaling pathway is related to the NF- κ B p65. We also investigated whether Lico A prevents translocation of NF- κ B (p65) from the cytosol to the nucleus using immunofluorescence staining.

In unstimulated LAD2 cells of the NC group, NF- κ B (p65) was mostly observed in the cytosol using confocal microscopy. Most of the intracellular NF- κ B (p65) entered the nucleus, and the fluorescence staining of both substances almost overlapped following SP treatment. In the Lico A + SP group, NF- κ B (p65) and nuclear fluorescence staining contour were clearly identified, as their positions were separate. This observation suggests that Lico A reduced the migration of NF- κ B (p65) significantly and the MC activation caused by SP (Figure 6B). Thus, NF- κ B is likely involved in the inhibition of pseudo-allergic reactions by Lico A.

4 | DISCUSSION

Pseudo-allergy is a global hypersensitivity reaction with a high incidence rate. Lico A is a flavonoid that is ubiquitous in a variety of plants and exhibits various pharmacological activities, whereas the effect on pseudo-allergy of Lico A has not been reported. In this study, we investigated the anti-pseudo-allergic effect and underlying

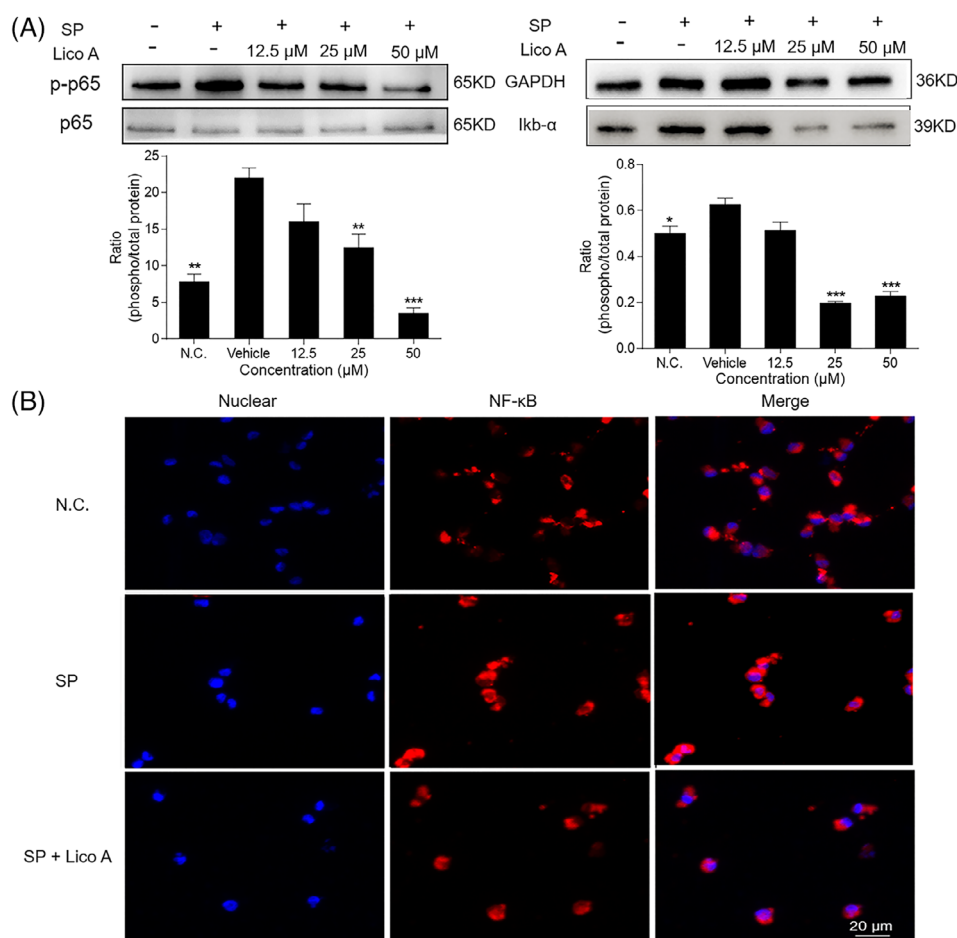


FIGURE 6 Lico A inhibits mast cell (MC) activation by blocking nuclear factor NF- κ B p65 phosphorylation and nuclear migration. (A) Effects of Lico A on phosphorylation of p65 and I κ B- α in substance P (SP)-stimulated laboratory of allergic disease 2 (LAD2) cells. (B) Immunofluorescence staining of NF- κ B (p65). Data are means $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

mechanism of action of Lico A for the potential in treatment of pseudo-allergy. The results showed that Lico A inhibited passive cutaneous anaphylaxis (PCA) and active systemic allergy in vivo, as well as degranulation, Ca^{2+} influx, and cytokine release in vitro, with the suppression of nuclear factor NF- κ B nuclear migration.

The MC MRGPRX2 is a key receptor for pseudo-allergy, and following its activation by ligands such as C48/80 and SP, intracellular Ca^{2+} flux has been reported to increase rapidly. This effect is accompanied by degranulation of MCs, immediate release of pre-synthetic substances such as β -hexosaminidase and histamine, and regulation of inflammatory factors such as TNF- α , IL-6, and CCL2, leading to pseudo-allergy (Sahid & Kiyoi, 2020). In this study, Lico A significantly inhibited degranulation and the rapid increase in intracellular Ca^{2+} in LAD2 cells induced by SP. Lico A showed an obvious inhibitory effect on MC activation, which was further verified using a pseudo-allergy PCA murine model and active systemic allergy in mice induced by SP.

As a responsive receptor of pseudo-allergy, the changes in downstream mRNA expression of MRGPRX2 after activation warrant further study. We comprehensively analyzed the mRNA level of SP-induced MRGPRX2 activation using RNA-seq technology, and found that 18 genes were highly correlated with cytokine-cytokine receptor interaction, *Salmonella infection*, and toll-like receptor, cytokine, and NF- κ B signaling pathways. Inflammatory factors such as TNF, CSF2, CXCL8, CCL4, CCL7, and CCL4L2, which play an important role in protein interaction, are all regulated by NF- κ B. Therefore, we speculated that NF- κ B plays a key role in MRGPRX2 activation by regulating many inflammatory factors. Furthermore, Lico A attenuated the expression levels of these six inflammatory factors (TNF, CSF2, CXCL8, CCL4, CCL7, and CCL4L2) regulated by NF- κ B, suggesting that Lico A possibly acts through the NF- κ B pathway. The p65 subunit of NF- κ B is an important protein that translocated from the cytosol into the nucleus after release from the inhibitor of NF- κ B α (I κ B α) (Cheon et al., 2018; Wang et al., 2018). Furthermore, it plays an essential role in the NF- κ B signaling pathway and regulates the production of inflammatory factors such as TNF- α and IL-8. We found that Lico A induced its biological effects by downregulating the phosphorylation of p65 using western blotting. Finally, we investigated the effects of Lico A on NF- κ B nuclear migration to confirm our inference and found that it occurred after stimulation of LAD2 cells with SP. Therefore, we confirmed that (Babina et al., 2018)NF- κ B participates in the MRGPRX2 downstream signals and that Lico A inhibited NF- κ B nuclear migration in LAD2 cells to inhibit the synthesis of cellular inflammatory factors.

There is a limitation in the study because of the lack of a PC on the pseudo allergic reaction. Dexamethasone is always used as a control drug to compare the effectiveness of different compounds on anaphylaxis in animal models (Kim et al., 2020). While it has serious side effects in clinical treatment as a kind of glucocorticoid, including diabetes and fatty liver (Cui et al., 2019), so we try to find a safe and effective new drug against the disease. Besides, the specific clinical reports on evaluation of pseudo-allergy are not clear, and the diagnostic criteria of the disease have not been unified yet, most studies are based on MC models (Falduto et al., 2021). As “the grandfather of

herbs”, licorice possesses potent pharmacological activities, and it is clinically applied as a whole medicine (Asl & Hosseinzadeh, 2008), there is no clinical report about Lico A acting as a single component isolated from licorice root (Liu et al., 2021), while the clinical data of the effect against acne of Lico A combined with other compounds are available (Dall'Oglio et al., 2019). For a more comprehensive study, toxicity data of Lico A are best investigated, which is another limitation of our study. There are no definite studies on the toxicology of Lico A in vivo. While it has been reported that Lico A has an inhibitory effect on inflammatory disease, such as colitis (Liu et al., 2018), at the series doses that we used in our study.

5 | CONCLUSIONS

Lico A inhibited pseudo-allergy mediated by MRGPRX2 both in vivo and in vitro, which was induced by suppression of NF- κ B nuclear migration. Presently, a variety of components have been reported to antagonize allergic reactions through MRGPRX2; however, they have been extracted from plants at a low content but high cost of effective drug concentration. Lico A is an effective constituent extracted from licorice, which is widely available worldwide. Moreover, Lico A belongs to the group of substituted chalcones that can also be chemically synthesized (Zhang et al., 2018). Thus, Lico A is an economical and effective anti-allergy reagent with considerable potential for treating pseudo-allergy-related diseases.

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CONFLICT OF INTEREST

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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