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ORIGINAL ARTICLE



Inhibitory activity of narirutin on RBL-2H3 cells degranulation

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ABSTRACT

Context: It is an efficient strategy to apply inhibition of mast cell degranulation for evaluating anti-allergic effects of compounds. Previous works confirmed that narirutin had anti-allergic activity in OVA induced allergic asthma murine model. However, the mechanism is not clear.

Objective: Here, inhibitory mechanism of narirutin on RBL-2H3 cells degranulation was investigated.

Materials and methods: Cell viability was analyzed by CCK-8 kits, cell degranulation was analyzed by ELISA methods, morphology and ultrastructure of cells was observed by atomic force microscopy, intracellular Ca^{2+} concentration was measured by fluorescence microscope, mRNA expression were measured by PCR, and signaling pathways were measured by WB.

Results: The results showed that narirutin have no direct effects on mRNA expression of FcεRI subunit. However, it inhibited Ca^{2+} influx by suppressing the phosphorylation of Syk, LAT and PLCγ1 signaling pathway transduction. Subsequently, the inhibition of Ca^{2+} influx directly leads to NF-κB signaling pathway transduction decreased. Narirutin can also suppress the phosphorylation of MAPK signaling pathways by decreasing the expression of P-p38, P-ERK and P-JNK, inhibit the synergistic effect for Ca^{2+} influx, and then reduce the release of IL-4, TNF-α, histamine and β-HEX.

Conclusion: Our study suggested that the inhibitory mechanism of narirutin on RBL-2H3 cells degranulation could be related to regulate MAPK, NF-κB and Tyrosine kinase signaling pathway.

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KEYWORDS

Allergy; narirutin; degranulation; mechanism; RBL-2H3 cell

Introduction

Allergy is a crucial public health problem in the world [1]. World Allergy Organization (WAO)–IAACI defines allergy as specific immunologic mechanism initiating inflammatory, causes symptoms and signs of allergic disease, such as asthma, rhinitis, conjunctivitis, and dermatitis [2]. IgE and mast cells play interdependent and independent roles in the complex immunol responses [3]. IgE sensitizes mast cells by adsorbing on their surface, then binds with allergen and causes mast cell activation. After activation, mast cells may immediately degranulate and release substances as β-hex, histamine, IL-4 and TNF-α, which induce immediate hyper-sensitive reaction and lead to Calcium influx [4]. What is more, these inflammatory mediators may elevate levels of antibodies, then result in a sequence of acute allergic reactions, for instance, increase in vascular permeability, induction of bronchial smooth-muscle contraction, and recruitment of inflammatory cells [5]. Apparently, mast cells are almost existing in all tissues of body. As critical mediators in the stage of sensitization and effect, they may be activated by a number of stimulating factors [6]. Therefore, mast cell degranulation is a critical link in the pathogenesis of allergy, in other words, the degranulation suppressing is a good way to treat and prevent allergy [7–9].

Narirutin is the main component in *Citri Reciculatae Pericarpium* (CRP), which is one of traditional Chinese

medicine. We found it had anti-allergic activity which may be related with mast cell degranulation [10]. However, the exact mechanism of narirutin on mast cell degranulation is still unclear. The purpose of this work is to systematically discuss the potential mechanism of narirutin on RBL-2H3 cells, considerable efforts have been made on the effects of narirutin on RBL-2H3 cell degranulation by a series of experiments, such as release of β-Hex, histamine, IL-4, TNF-α and Ca^{2+} influx, FcεRI mRNA expression of high affinity receptor, as well as cellular signaling pathways.

Materials and methods

Chemicals and reagents

4-Nitrophenyl N-acetyl-β-D-glucosaminide, anti-DNP monoclonal mouse IgE, DNP-BSA were obtained from Sigma-Aldrich (St. Louis, MO, USA); Narirutin (Purity: 99.20%, HPLC) were provided by Chengdu Manster Biotechnology co., LTD. (Chengdu, China); DMEM, TRYPsin 0.25% EDTA were purchased from Gibco (Beijing, China); FBS was provided by Clark (Richmond, USA), Enhanced CCK8 assay kit, Fluo 3-AM were provided by Dojindo (Kyushu, Japan); Rat Histamine ELISA kit was purchased by MEIMIAN, China (Jiangsu, China); IL-4 ELISA was purchased by Bioss (Beijing, China); TNF alpha RAT ELISA Kit, Dynabeads® mRNA DIRECT™ Micro Kit assay kit

were obtained by Invitrogen life technology (California, USA); ambion® Primer Script™ 1st strand cDNA Synthesis kit were purchased from TaKaRa & Clontech (Shiga, Japan); NovoStart® SYBR qPCR SuperMix Plus assay kit were purchased from Novoprotein Scientific Inc (Beijing, China); Albumin Bovine V were purchased from Becton, Dickinson and Company (New Jersey, USA); Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb; p38 MAPK (D13E1) XP® Rabbit mAb (HRP Conjugate); Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb; p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (HRP Conjugate); Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb; SAPK/JNK Rabbit mAb; Phospho-Src Family (Tyr416) (D49G4); Lyn (C13F9) Rabbit mAb, Phospho-Syk (Tyr525/526) (C87C1) Rabbit mAb; Syk (D3Z1E) XP® Rabbit mAb; LAT antibody; Phospho-NF-κB p65(Ser536) Rabbit mAb; Phospho-IκBα/β (Ser176/180) (16A6) Rabbit mAb; Phospho-IκBα (Ser32) (14D4) Rabbit mAb and Anti-rabbit IgG, HRP-linked Antibody were provided by Cell Signaling Technology (Massachusetts, USA); IκBα + IκBβ Antibody were provided by Signalway antibody, USA; LAT (phospho Tyr191) Polyclonal Antibody; PLCγ1 Polyclonal Antibody and PLCγ1 (phospho Tyr771) Polyclonal Antibody were provided by Immunoway, American; P65, RELA Rabbit Polyclonal antibody and IκBα Rabbit Polyclonal antibody were provided by Proteintech (Rosemont, USA).

Cell culture and treatment

Rat basophilic leukemia (RBL-2H3) cells were purchased from the cell bank of Chinese academy of sciences (Shanghai, China). According to RBL-2H3 cells culture guidelines, cells were cultured in DMEM containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS, at the incubator with 37 °C and 5% CO₂.

Cell viability assay

Cell viability was analyzed by CCK-8 kits. RBL-2H3 cells were cultured in 96-well plates about 1.0×10^4 cells/well. After 24 h, discarded the medium and treated with different concentrations of narirutin (0~200 μM) for 20 h.

β-Hex measurement

According to Itoh Tand's and Ohguchi K's research [11], degranulation of RBL-2H3 cells came to peak when the anti-DNP-IgE dose was 0.5 μg/mL, DNP-BSA dose was 0.5 μg/mL. RBL-2H3 cell degranulation was described by the release of β-hex on the base of a previous approach. RBL-2H3 cells (1×10^5 cells/well) were sensitized with 0.5 μg/mL of anti-DNP monoclonal mouse IgE overnight treated with different concentrations of narirutin (0~200 μM) for 2.5 h, then stimulated with DNP-BSA (0.5 μg/mL) for 1 h, subsequently stopped with the ice about 10 min. Supernatants were collected, centrifuged (4 °C, 14,800 rpm, 10 min) and transferred to a 96-well plates and incubated with 50 μL 4-Nitrophenyl N-acetyl-β-D-glucosaminide (1 mM) for 1.5 h at 37 °C. The

reactions were stopped by 200 μL/well stop solution (0.1 mM Na₂CO₃/NaHCO₃). Finally, the OD values at 405 nm were measured using a microplate reader.

The inhibition of the release of β-hex as follows [12]:

$$\text{Inhibition} = [1 - (A_S - A_{BS}) / (A_M - A_B)] \times 100\%$$

A is the OD value at 405 nm.

A_S: RBL-2H3 cells (+), Anti-DNP-IgE (+), DNP-BSA (+), narirutin (+);

A_{BS}: RBL-2H3 cells (−), Anti-DNP-IgE (−), DNP-BSA (−), narirutin (+);

A_B: RBL-2H3 cells (−), Anti-DNP-IgE (−), DNP-BSA (−), narirutin (−);

A_M: RBL-2H3 cells (+), Anti-DNP-IgE (+), DNP-BSA (+), narirutin (−)

Cytokine measurements

RBL-2H3 cells (5×10^5 cells/well) were cultured in 6-well plates. Anti-IgE-sensitized (0.5 μg/mL) RBL-2H3 cells were incubated with different concentrations of narirutin (0~100 μM) for 2.5 h, subsequently interleukin IL-4, Rat Histamine and tumor necrosis factor (TNF-α) were released under DNP-BSA (0.5 μg/mL) stimulation for 1 h. The reactions were stopped by ice-bath. Cell secretory components were collected by sterile tube. Centrifuge at 4 °C for 2500 rpm, 20 min, carefully collect the supernatant. Then, cytokine in cell supernatants was measured by ELISA kits.

AFM imaging

According to the research of Pi J, Li T, and Liu J, place cover slides on 6-well plates. RBL-2H3 cells (1×10^5 cells/well) were cultured in 6-well plates and were sensitized with 0.5 μg/mL of anti-DNP monoclonal mouse IgE for 12 h. After cells were washed triple with PBS, cells were treated with narirutin (100.00 μM) for 2.5 h. Then, it was stimulated with DNP-BSA (0.5 μg/mL). Subsequently, cells were washed triple with PBS, fixed with 4% paraformaldehyde solution for 10 min, washed triple with distilled water and dried in air. Finally, morphology and ultrastructure of RBL-2H3 cells were observed by Atomic force microscopy (Agilent 5500 SPM, USA) [13].

Intracellular Ca²⁺ concentration measurement

RBL-2H3 cells (1×10^5 cells/well) were cultured in 96-well black plates and were sensitized with 0.5 μg/mL of anti-DNP monoclonal mouse IgE for 12 h. After cells were washed three times with D-PBS, cells were incubated for 40 min with Fluo 3-AM (5 μM), and then treated with different concentrations of narirutin (0.00, 40.00, 60.00, 80.00 and 100.00 μM) for 2.5 h. Finally, it was stimulated with DNP-BSA (0.5 μg/mL). Fluorescence intensity was immediately conducted under an inverted fluorescence microscope (Nikon ECLIPSE Ts2, Japan). Mean fluorescence intensity was calculated by using Image J [14–16].

MRNA expression measurement

RBL-2H3 cells (1×10^4 cells/plate) were cultured in plates. RBL-2H3 cells were treated with different concentrations of narirutin (0.00, 40.00, 60.00, 80.00 and 100.00 μ M). The mRNA was isolated from the RBL-2H3 cells using Dynabeads[®] mRNA DIRECT[™] Micro Kit assay and reverse transcribed using PrimerScript[™] 1st strand cDNA Synthesis kit. The polymerase chains reaction was performed with NovoStart[®] SYBR qPCR SuperMix Plus assay kit.

The following primer pairs were synthesized (Invitrogen Custom Primers)

Fc ϵ RI α , 5'-CATTGTGAGTGCCACCATTC-3', 5'-TTCTTCCAGCTACGGCATCT-3';

Fc ϵ RI β , 5'-TTGTCTGCTCCACACTCCAG-3', 5'-AGGCTGCCTCTCACCAGATA-3';

Fc ϵ RI γ , 5'-CACCTGTTCTCAGCTGCATT-3', 5'-GGATCAGGGAA GGAAGAGG-3';

GAPDH, 5'-AGACAGCCGCATCTTCTTGT-3', 5'-CTTGCCGTGG GTAGAGTCAT-3'

Three-step polymerase chains reaction amplification procedure was adopted: 95 °C for 1 min, then, 95 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s and 40 cycles, the PCR reaction was performed with Gene amplification instrument (Dlin, China); The amplification products of the PCR reactions were analyzed by 1.5% agarose gel electrophoresis. DNA bands were visualized with Gel Imager (F1-F2 Fuses Type BIO-RAD, USA) [7].

Immunoblot analysis

RBL-2H3 cells (1×10^6 cells/plate) were cultured in plates and treated as before. After stimulated with DNP-BSA, interest proteins were extracted from cells lysis products with RIPA lysis. Lysates were centrifuged at 14,800 rpm for 10 min at 4 °C for purification by Refrigerated centrifuge table high speed (H2300R-2, cence[®], China). The concentrations of protein were measured by Beyotime protein quantitative kit. 50 μ g loading quantity of supernatant was analyzed by SDS-PAGE and transferred to PVDF membrane. Special membrane was closed with skim milk powder or Albumin Bovine V for 2~4 h and incubated with primary antibodies at 4 °C overnight. The membrane was probed with secondary antibodies for 1 h at room temperature. After washing, immunoblotting detection was performed by Chemiluminescent gel imager (c300 Azure Biosystems, USA). The protein bands were visualized with Ultra-sensitive ECL chemiluminescence detection reagents [17].

Statistical analysis

Dates were expressed as the means $\pm$ standard deviation (SD). Student's *t*-test or One-way ANOVA was used to assess significance between control and narirutin groups. Statistical analysis was performed using Graph Pad Prism 7.00, IBM

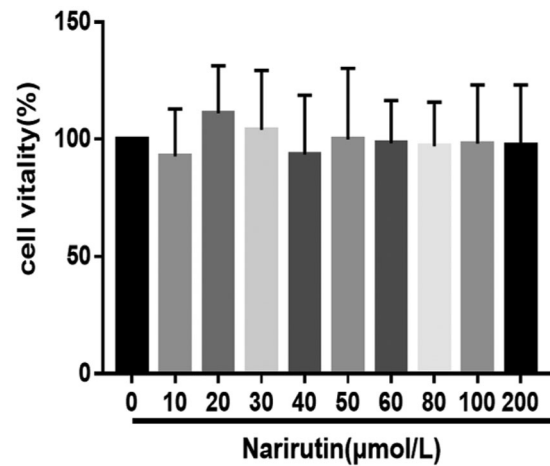


Figure 1. Effect of narirutin on the vitality of RBL-2H3 cells. RBL-2H3 cells were treated with different concentrations of narirutin for 20 h, and the OD value was measured after adding CCK8 for 3 h. Mean $\pm$ SD was used to represent the data.

SPSS Statistics 21 and origin 8.0. $p < .05$ was considered to be significant with at least three groups.

Results

Measurement of RBL-2H3 cells vitality

It is widely proposed that the effects of narirutin on RBL-2H3 cells vitality can be presented by CCK8 assay. According to the instructions, relative cell vitality of RBL-2H3 cells was treated with narirutin for 20 h, as the results shown (Figure 1), narirutin do not have any effects on the vitality of cells.

Release of β -hex, histamine, IL-4 and TNF- α

Generally, RBL-2H3 cells degranulation was described by release of β -hex on the base of a previous approach [8,9]. After sensitization and stimulation, degranulation level was defined as 100% in RBL-2H3 cells of untreated group. As shown in Figure 2(A), β -hex release were $68.04 \pm 2.02\%$, $54.29 \pm 0.98\%$, $42.91 \pm 1.11\%$, $41.87 \pm 1.58\%$, $36.5 \pm 0.93\%$, $30.74 \pm 1.67\%$, $29.32 \pm 0.54\%$, and $46.92 \pm 1.24\%$ after treated with different concentrations of narirutin. It can be easily found when the concentrations of narirutin at 25.00, 50.00 and 100.00 μ M, the β -hex release were lower than 40%.

In addition, histamine is also one of inflammatory factors as indicator to detect RBL-2H3 cells degranulation. After stimulation, stored histamine was released from IgE-sensitized cells. The releasing process is occurring consistently with β -hex release [18]. From Figure 2(B), in comparison with sensitized group, the amount of histamine release in narirutin treated groups were significantly reduced to 53.80 ± 0.77 , 52.53 ± 2.27 , 48.09 ± 2.72 , 48.80 ± 3.19 , and 48.37 ± 2.12 ng/mL at the concentrations of 6.25, 12.50, 25.00, 50.00 and 100.00 μ M.

Contents of pro-inflammatory cytokines such as IL-4 and TNF- α were also detected in supernatant. As shown in Figure 2(C), Comparing narirutin groups on sensitized group, when the concentrations of narirutin were 12.50, 25.00, 50.00 and

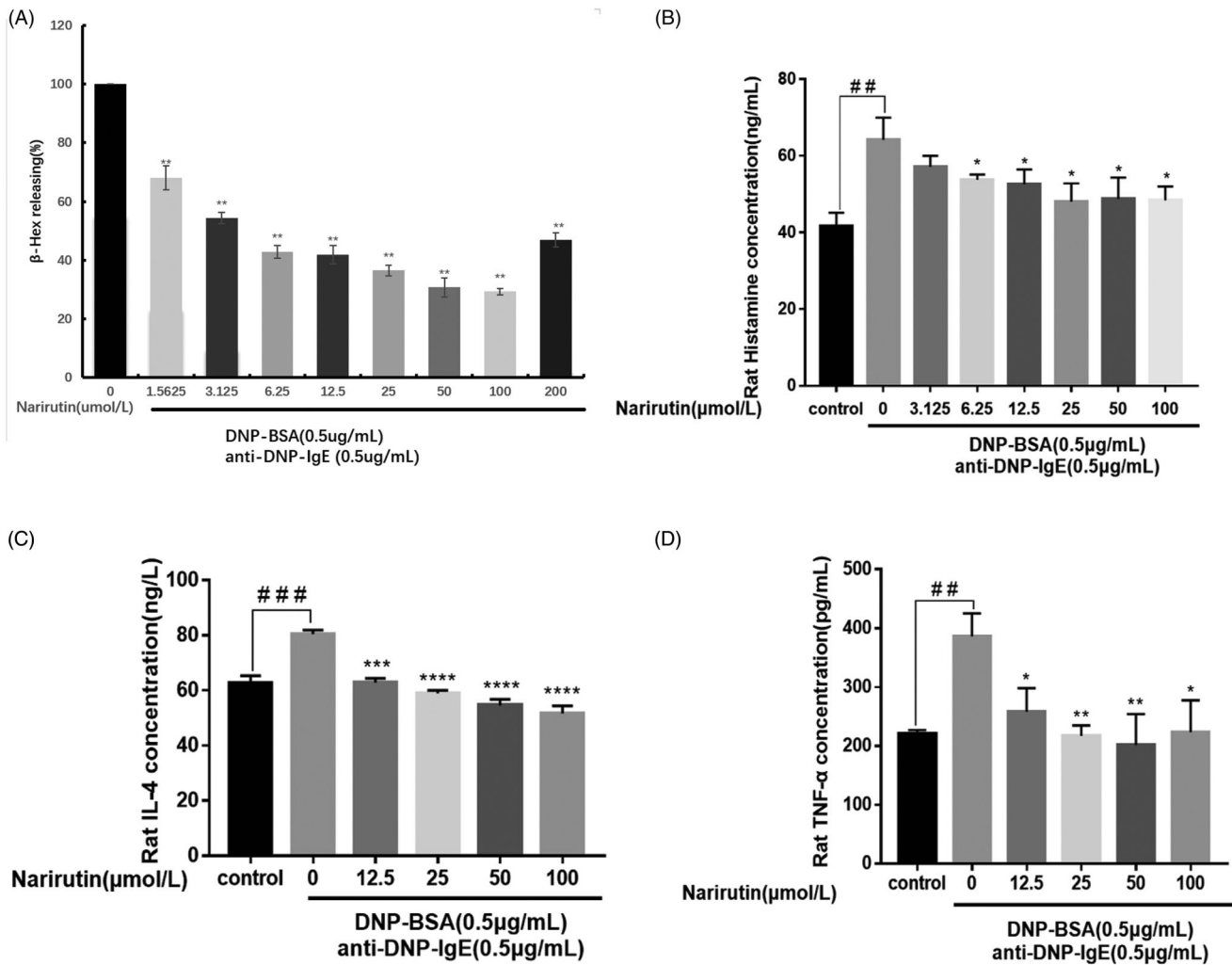


Figure 2. Effect of narirutin on β -hex, histamine, IL-4, TNF- α releasing. The sensitized RBL-2H3 cells were pretreated with different concentrations of narirutin for 2.5 h, with that it was stimulated by DNP-BSA, and the OD value was measured after the reaction is terminated. Mean $\pm$ SD was used to represent the data, # $p < .05$, ## $p < .01$, control cells group compared with sensitized RBL-2H3 cells group; * $p < .05$, ** $p < .01$, narirutin treated RBL-2H3 cells group compared with sensitized RBL-2H3 cells group.

100.00 μ M, production of IL-4 significantly decreased to 62.76 ± 0.91 , 58.93 ± 0.66 , 54.55 ± 1.28 , 51.63 ± 1.58 ng/mL. Similarly, TNF- α release were significantly decreased to 258.1 ± 23.26 , 217.1 ± 10.15 , 201.1 ± 30.51 , 223.1 ± 31.43 pg/mL at concentration of 12.50, 25.00, 50.00 and 100.00 μ M (Figure 2(D)).

In conclusion, the drop-off in β -hex, histamine, IL-4 and TNF- α release between narirutin groups and sensitized group suggests that narirutin could inhibit RBL-2H3 cell degranulation especially at concentration of 12.50, 25.00, 50.00 and 100.00 μ M.

AFM imaging of RBL-2H3 cells

In an attempt to clarify morphological information of RBL-2H3 cell degranulation at micrometer scale, some researchers used AFM to study cell degranulation [19,20]. AFM has high resolution, we could investigate morphological changes of RBL-2H3 cells induced by DNP-BSA stimulation at micrometer scale.

As shown in Figure 3, single cell topography looked like a shuttle in A1 and A3, but that of A2 looked like a sphere and it was brighter than A1. C2 had a much stronger stereo feeling than C1 and C3 in their 3D topographical image. These results demonstrated that narirutin had a positive effect on stabilizing morphology of activated cells. Small holes can be observed on cell membrane of B1 and B3 obviously, in contrast, the membrane projection and holes approximately disappearing were observed in B2 cells. Muchmore, B2 cells were surrounded by more prominent unknown vesicles and granular material. That is just the fact that cell degranulation may be a process of exocytosis. In other words, there are many particles stored in cells that would be released when cells were stimulated, these vesicles from the cytoplasm to outside the membrane. More importantly, as shown in the curve, vesicles of sensitized RBL-2H3 cells had a higher height as comparing with other groups. These results showed that DNP-BSA can promote sensitized RBL-2H3 cells transfer more vesicles to the edge and supernatant of RBL-2H3 cells, and lead to cell more granules release. However, narirutin could suppress this phenomenon in a certain extent.

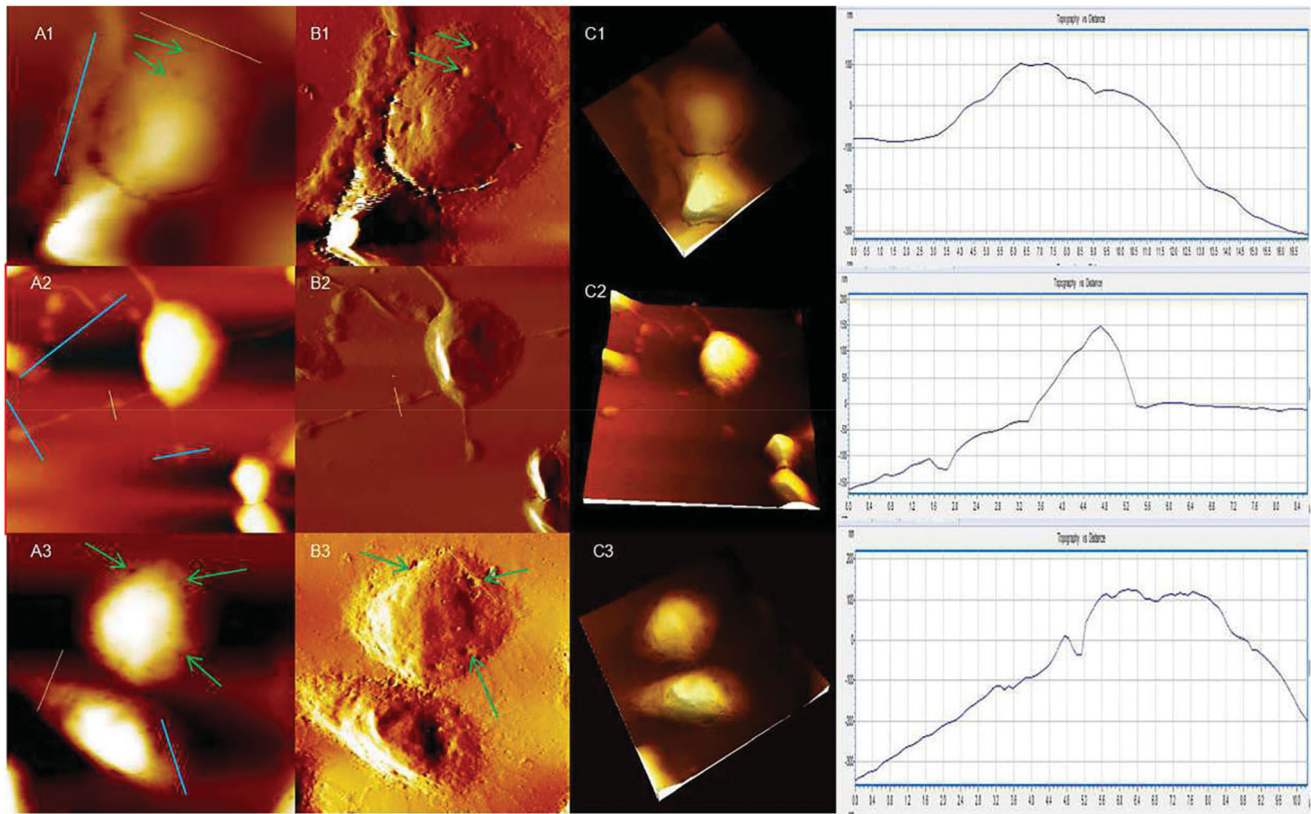


Figure 3. Morphology and ultrastructure RBL-2H3 cells induced by DNP-BSA stimulation. Scale bar in 90 μm , A1-C1, A2-C2 and A3-C3 respectively is morphology and ultrastructure, topography and the corresponding 3 D images of RBL-2H3 cells. A1-C1, A2-C2 and A3-C3 respectively is control RBL-2H3 cells, sensitized RBL-2H3 cells and narinutin treated RBL-2H3 cells. The green arrows indicate holes on the plasma membrane, the blue lines indicate the size of vesicles.

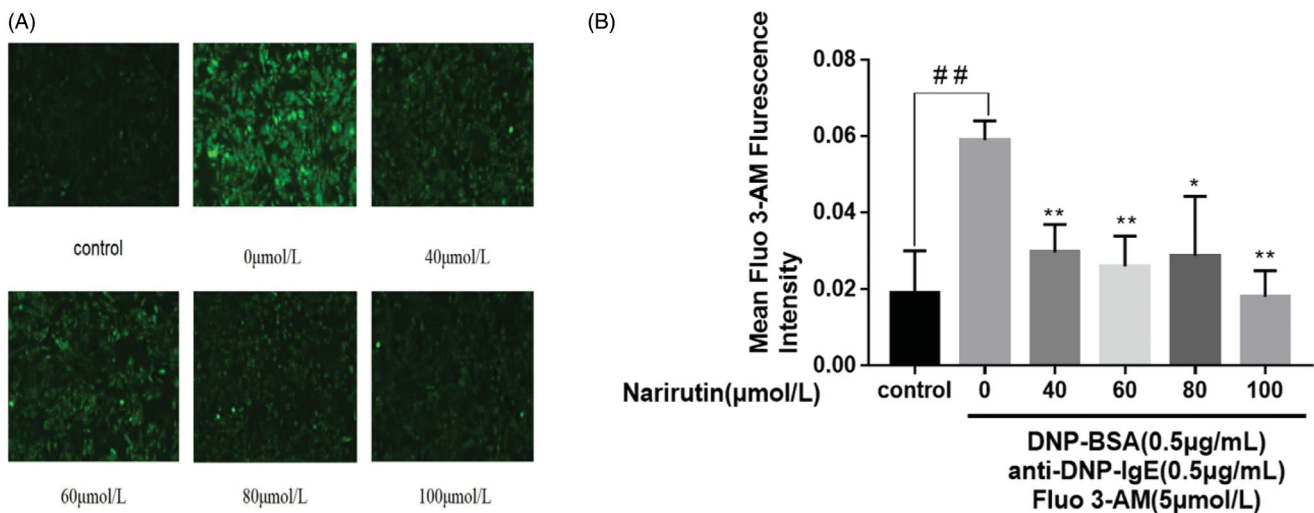


Figure 4. Effect of narinutin on Ca^{2+} influx in RBL-2H3 cells. (A) Intracellular fluorescence images of different concentrations narinutin treated RBL-2H3 cells; (B) The mean intracellular fluorescence intensity of different concentrations narinutin treated RBL-2H3 cells, Mean $\pm$ SD was used to represent the data, $^{\#}p < .05$, $^{\#\#}p < .01$, control cells group compared with sensitized RBL-2H3 cells group; $^*p < .05$, $^{**}p < .01$, narinutin treated RBL-2H3 cells group compared with sensitized RBL-2H3 cells group.

Effect on Ca^{2+} influx

In order to further confirm the inhibitory effects, Ca^{2+} influx were also evaluated by Fluo 3-AM. According to the results of Figure 4(A,B), when the concentration of narinutin was

from 25.00 to 100.00 μM , mean intracellular fluorescence intensity was significantly weaker than the sensitized group (0 μM). What is more, the intensity of 100.00 μM group was similar to control. The results indicated the influx of Ca^{2+} was inhibited by narinutin.

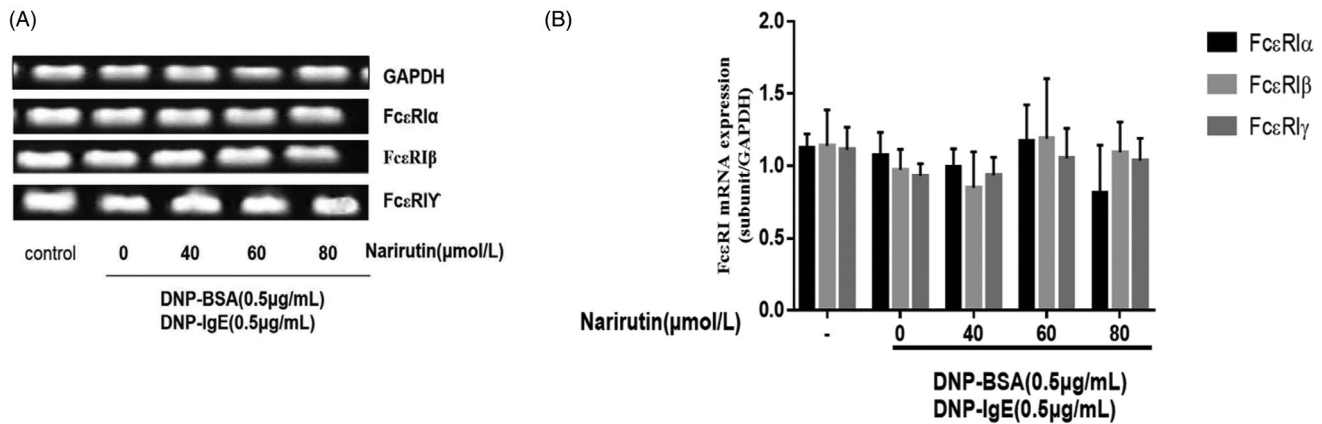


Figure 5. Effect of narirutin on the mRNA expression of FcεRI on RBL-2H3 cells. The images were taken using Gel Imager F1-F2 Fuses Type. FcεRI expression, band intensities of PCR products from agarose gel electrophoresis were analyzed and compared relative to GAPDH. The GAPDH expression pattern in RBL-2H3 cells was used as an internal control. The quantitative expression of mRNA on the sublineages of FcεRI and GAPDH was determined by Image J. Mean $\pm$ SD was used to represent the data, control cells group compared with sensitized RBL-2H3 cells group; narirutin treated RBL-2H3 cells group compared with the sensitized RBL-2H3 cells group.

Effects on FcεRIα, FcεRIβ and FcεRIγ

It is well known that FcεRI receptor on the surface of mast cell plays a major role in degranulation, for it can connect with IgE and induce releasing. The effects of narirutin on expression of FcεRIα, FcεRIβ and FcεRIγ on RBL-2H3 cells were investigated by PCR. RBL-2H3 cells were sensitized with anti-DNP-IgE for 2.5 h and then stimulated by DNP-BSA to verify the effect of narirutin on the mRNA expression of FcεRI subunits. As shown in Figure 5(A,B), there was no significant difference of α, β and γ subunits mRNA expression among different groups, which means there was no effect of narirutin on the expression of FcεRI α, β and γ subunits expression.

Effect on MAPK, NF-κB and tyrosine kinase signaling pathway

In the following sections, effects of narirutin on signaling pathways were investigated. The anti-DNP-IgE stimulates the phosphorylation of MAPK signaling pathways including p38, JNK and ERK1/2, NF-κB signaling pathways including IκBα, IκKα/β and p65, as well as Tyrosine kinase signaling pathway including P-Lyn, P-Syk, P-LAT and P-PLCγ1.

The effects of narirutin on MAPK signaling pathways were shown in Figure 6(A1,A2). Narirutin suppressed phosphorylation of P-ERK1/2, P-JNK in dose-dependent manner at different concentrations significantly. For P-p38, the suppressing effect existed with dose-independent manner in the IgE-sensitized RBL-2H3 cells. Muchmore, expression of P-p38, P-ERK and P-JNK in the sensitized-RBL-2H3 cells were decreased by narirutin.

Activation of NF-κB requires phosphorylation of IκBα, leads to targeted degradation of IκBα proteins. Results of different groups treated with narirutin were shown in Figure 6(B1,B2). When compared with sensitized group, phosphorylation of IκBα was suppressed by narirutin significantly from concentration 40 to 100 μM with dose-dependent manner. For IκKα/β, there was also suppressing effect existing but with dose-independent manner. Expression of P-p65 was

significantly increased by Narirutin at concentration of 40.00, 60.00 and 80.00 μM, compared with sensitized group. In other words, narirutin suppressed the activities of allergic mediators *via* inhibition of NF-κB transcriptional enter the nucleus.

From Figure 6(C1,C2), effects of narirutin on tyrosine kinase signaling pathway were shown. The expression of P-Syk in sensitized group was higher than control group, and it was decreased significantly after treated with narirutin. In addition, expression of P-LAT and P-PLCγ1 was suppressed by narirutin. However, there was no significant difference on the expression of P-Lyn among different groups. Based on the above analysis, the suppression of narirutin on P-LAT, P-Syk and P-PLCγ1 lead to the inhibitory effect of activation of IgE on Lyn kinase signaling pathway, then subsequently regulated IgE-mediated degranulation and inflammatory cytokine secretion.

Discussion

In our previous work, we confirmed the anti-allergic activity of narirutin *in vivo*, but the mechanism was still unclear. IgE and mast cells play a major role in food allergy [21]. We found that narirutin has inhibitory activity on RBL-2H3 degranulation. Therefore, there are three aspects of this problem have to be addressed. The first aspect involves the inhibitory effects on RBL-2H3 cell degranulation and morphological information. The second aspect deals with effects of narirutin on FcεRI receptor on the surface of mast cell. The third aspect relates to signaling pathways on RBL-2H3 cells. 0–200 μM of narirutin were practicable for subsequent experiments, for there were no significant effects on vitality of cells. Based on the AFM results, it is clearly that narirutin could suppress the vesicles transfer to the edge and supernatant of RBL-2H3 cells promoted by DNP-BSA in sensitized RBL-2H3 cells. Furthermore, β-hex and histamine are commonly accepted as markers of mast cell activation degranulation, and they are also currently used indicators for the detection of degranulation of RBL-2H3 cells. The results

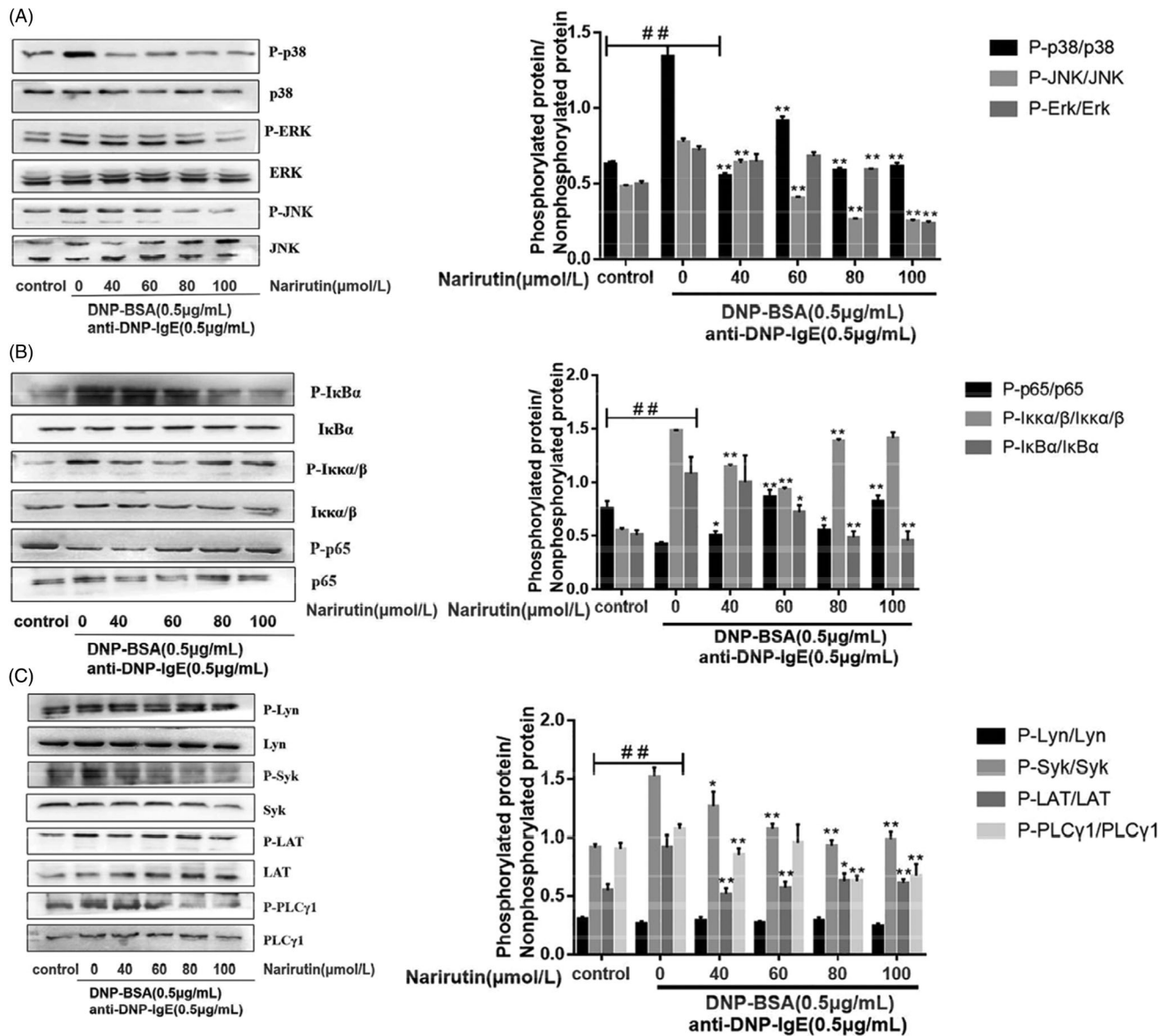


Figure 6. Effect of narirutin on the MAPK, NF- κ B and Tyrosine kinase signaling pathway on RBL-2H3 cells. The images were taken using Chemiluminescent gel imager c300 (Azure Biosystems). RBL-2H3 cells lysis products were used for protein immunoassay. The quantitative expressions of P-p38, P-Erk, P-JNK, P-p65, P-Lyn and P-Syk were analyzed by Image J. Mean $\pm$ SD was used to represent the data, $^{\#}p < .05$, $^{\#\#}p < .01$, control cells group compared with sensitized RBL-2H3 cells group; $^*p < .05$, $^{**}p < .01$, narirutin treated RBL-2H3 cells group compared with sensitized RBL-2H3 cells group.

manifested that narirutin clearly inhibited RBL-2H3 cells degranulation in a dose-dependent manner. In addition, references show that IL-4 induces B cells to produce IgE, and IgE induces T cells into helper T cells (Th2 cells). Th2 cells produce IL-4 and prevent other T cells from entering Th1 pathway. Narirutin could decrease the IL-4 release of RBL-2H3 cells. What is more, Tumor necrosis factor (TNF)- α is potent multifunctional cytokine that regulates inflammatory response and biological activity. Narirutin suppressed the releasing levels of TNF- α significantly. Consequently, we believe that narirutin had inhibitory effect on RBL-2H3 cell degranulation.

Concerning of the mechanism of activation RBL-2H3 cells, Fc ϵ RI receptor on the cell surface is important in the process of degranulation. As reported, the binding of IgE to mast cells was completely blocked in Fc ϵ RI knockout mice, and no degranulation was observed after anaphylaxis, which means

the expression of Fc ϵ RI have important effects on degranulation [22,23]. Fc ϵ RI expresses on RBL-2H3 cells surface. It is a tetrameric complex composed of immune multimeric receptors with one α subunit, one β subunit, and two γ -subunits. As a surface receptor for IgE, it only exists on mast cells and basophils [24]. When allergens bind to the specific IgE, consequent aggregation of receptors leads to a sequence of reactions. Then mast cells release β -hex and cytokines through the antigen-induced aggregation of Fc ϵ RI. The results indicated that there was no significant difference of α , β and γ subunits mRNA expression among different groups. It indicated that narirutin had no effect on the expression of Fc ϵ RI expression, which means the inhibitory effects of narirutin on degranulation have no direct relationship with the expression of Fc ϵ RI subunit.

Afterwards, we focused on signaling pathways. Ca^{2+} is the second messenger of intracellular signal transduction,

and the increasing of intracellular Ca^{2+} concentration is the core of degranulation [25–27]. We found that the intracellular fluorescence intensity of narirutin groups significantly decreased in contrast to the model group. This suggested that narirutin may stabilize RBL-2H3 cells by inhibiting extracellular Ca^{2+} influx. For MAPK signaling pathways, narirutin suppressed phosphorylation of MAPK signaling pathways by suppressed the expression of P-p38, P-ERK and P-JNK in sensitized-RBL-2H3 cells, which is agree with the results of Kouichi Sugaya and Xu J and Lv H [28,29]. NF- κ B plays a key part in inflammatory and immune reaction. It is present in cytosol in inactive state, sequestered in the cytoplasm by inhibitory proteins. NF- κ B p65 is activated under inducing of IgE, phosphor-I κ B α rapid degradation, with releasing of NF- κ B p65 enter into nucleus. Phosphor-I κ B α / β also regulates phosphor-NF- κ B p65, which can cause conformational changes and result in kinase activation. Narirutin decreased expression of phosphorylation of I κ B α and I κ B α / β and increased the phosphorylation of p65. The results implied narirutin inhibited NF- κ B transcriptional activation. Furthermore, Lyn activation triggers a cascade of signaling events. It can be mediated by Lyn phosphorylation of tyrosine residues within immune-receptor tyrosine-based activation motifs of the receptor proteins, subsequent recruitment and activation of other kinases including Syk [30]. Much more, activation of Syk leads to an increase in intracellular Ca^{2+} and the activation of MAPK. Thus, Lyn and Syk act as the early important intracellular signaling pathway knot of Fc ϵ RI mediated [31]. The results showed that narirutin inhibited P-LAT, P-Syk and P-PLC γ 1 but had no effect on Lyn, P-Lyn, LAT, Syk and PLC γ 1. It indicated that narirutin showed effects on Lyn activation. The LAT inhibition of narirutin leads to suppression of PLC γ 1. At last, narirutin inhibited IgE-mediated RBL-2H3 cell degranulation and inflammatory cytokine secretion.

Conclusions

In summary, we concentrated on the inhibitory mechanism of narirutin on RBL-2H3 cells by AFM, ELISA, PCR and Western-Blot. We confirmed the inhibitory effects of narirutin were applied by the following mechanisms. First, it inhibited Ca^{2+} influx by suppressing phosphorylation of Syk, LAT and plc γ 1 signaling pathway transduction. Subsequently, inhibition of Ca^{2+} influx directly lead to NF- κ B signaling pathway transduction decreased. Then, narirutin suppressed phosphorylation of MAPK signaling pathways by decreasing the expression of P-p38, P-ERK and P-JNK, inhibited the synergistic effect for Ca^{2+} influx, and reduced the release levels of IL-4 and TNF- α , at last, it inhibited degranulation of RBL-2H3 cells, and directly lead to the reduction of histamine and β -HEX release. In conclusion, the mechanism of narirutin on anti-allergy could be applied by regulating MAPK, NF- κ B and Tyrosine kinase signaling pathway.

Author contributions

Liyan Niu complished experiments and wrote article; Xiaolei Shi designed and modified the article; Jihao Wei assisted in the completion of the experiment; Yongri Jin and Xuwen Li provided some important advices.

Disclosure statement

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References

- [1] Fenton M. Guidelines for the diagnosis and management of food allergy in the United States. *Clin Transl Allergy*. 2011;1:S10.
- [2] Johansson SGO, Bieber T, Dahl R, et al. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol*. 2004;113:832–836.
- [3] Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med*. 2012;18:693–704.
- [4] Vo TS, Kong CS, Kim SK. Inhibitory effects of chitoooligosaccharides on degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells. *Carbohydr Polym*. 2011;84:649–655.
- [5] Yunginger WJ, Sweeney KG, Sturner WQ, et al. Fatal food-induced anaphylaxis. *JAMA*. 1988;260:1450–1452.
- [6] Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev*. 1997; 77:1033–1079.
- [7] Lee T, Kwon J, Lee D, et al. Effects of *Cudrania tricuspidata* fruit extract and its active compound, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone, on the high-affinity IgE receptor-mediated activation of Syk in mast cells. *J Agric Food Chem*. 2015; 63:5459–5467.
- [8] Chung MJ, Sohng JK, Choi DJ, et al. Inhibitory effect of phloretin and biochanin A on IgE-mediated allergic responses in rat basophilic leukemia RBL-2H3 cells. *Life Sci*. 2013; 93:401–408.
- [9] Kim M, Lim SJ, Oidovsambuu S, et al. Gnetin H isolated from *Paenonia anomala* inhibits Fc ϵ RI-mediated mast cell signaling and degranulation. *J Ethnopharmacol*. 2014;154:798–806.
- [10] Anonymous. 2016. Key industry statistics for citrus growers. [retrieved 2017 Mar 15]. Available from: <https://www.citrusresourceswarehouse.org.za/home/document-home/information/cga-key-industry-statistics/3610-cga-key-industry-statistics-2016/file>
- [11] Itoh T, Ohguchi K, Nakajima C, et al. Inhibitory effects of flavonoid glycosides isolated from the peel of Japanese persimmon (*Diospyros kaki* Fuyu) on antigen-stimulated degranulation in rat basophilic leukaemia RBL-2H3 cells. *Food Chem*. 2011;126: 289–294.
- [12] Shi X, Niu L, Zhao L, et al. The antiallergic activity of flavonoids extracted from *Citri Reticulatae* Pericarpium. *J Food Process Preserv*. 2018;42:e13588.
- [13] Pi J, Li T, Liu J, et al. Detection of lipopolysaccharide induced inflammatory responses in RAW264.7 macrophages using atomic force microscope. *Micron*. 2014;65:1–9.
- [14] Zhao R, Wang S, Huang Z, et al. Lipopolysaccharide-induced serotonin transporter up-regulation involves PKG-I and p38MAPK

- activation partially through A3 adenosine receptor. *Biosci Trends*. 2015;9:367–376.
- [15] Chen W, Shen Y, Su H, et al. Hispidin derived from *Phellinus linteus* affords protection against acrylamide-induced oxidative stress in Caco-2 cells. *Chem Biol Interact*. 2014;219:83–89.
- [16] Kuba-Miyara M, Agarie K, Sakima R, et al. Inhibitory effects of an ellagic acid glucoside, okicamelliaside, on antigen-mediated degranulation in rat basophilic leukemia RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice. *Int Immunopharmacol*. 2012;12:675–681.
- [17] Xu J, Yuan C, Wang G, et al. Urolithins attenuate LPS-induced neuroinflammation in BV2 Microglia via MAPK, Akt, and NF- κ B signaling pathways. *J Agric Food Chem*. 2018;66:571–580.
- [18] Trivadila SS, Liu S, Sahid MNA, et al. Histamine uptake mediated by plasma membrane monoamine transporter and organic cation transporters in rat mast cell lines. *Eur J Pharmacol*. 2019;849:75–83.
- [19] Pi J, Huang L, Yang F, et al. Atomic force microscopy study of ionomycin-induced degranulation in RBL-2H3 cells. *Scanning*. 2016;38:525–534.
- [20] Deng Z, Zink T, Chen HY, et al. Impact of actin rearrangement and degranulation on the membrane structure of primary mast cells: a combined atomic force and laser scanning confocal microscopy investigation. *Biophys J*. 2009;96:1629–1639.
- [21] Siraganian RP, Castro ROD, Barbu EA, et al. Mast cell signaling: the role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. *FEBS Lett*. 2010;584:4933–4940.
- [22] Belostotsky R, Lorberboum-Galski H. Utilizing Fcepsilon-Bak chimeric protein for studying IgE-FcepsilonRI interactions. *Clin Immunol*. 2004;110:89–99.
- [23] Dombrowicz D, Flamand V, Brigman KK, et al. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. *Cell*. 1993;75:969–976.
- [24] Ra C, Jouvin MH, Kinet JP. Complete structure of the mouse mast cell receptor for IgE (Fc epsilon RI) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells. *J Biol Chem*. 1989;264:15323–15327.
- [25] Allen JD, Jaffer ZM, Park SJ, et al. p21-activated kinase regulates mast cell degranulation via effects on calcium mobilization and cytoskeletal dynamics. *Blood*. 2009;113:2695–2705.
- [26] Liao WC, Huang CC, Cheng HH, et al. Effect of calmidazolium on $[Ca^{2+}]_i$ and viability in human hepatoma cells. *Arch Toxicol*. 2009; 83:61–68.
- [27] Joo HM, Kang SJ, Nam SY, et al. The inhibitory effects of low-dose ionizing radiation in IgE-mediated allergic responses. *Plos One*. 2015;10:e0136394.
- [28] Kouichi S, Takehito T, Aika T, et al. Bisorbicillinol inhibits Lyn tyrosine kinase for allergic response on RBL-2H3 cells. *Bioorg Med Chem Lett*. 2019;29:832–835.
- [29] Lv H, Fan X, Wang L, et al. Daphnetin alleviates lipopolysaccharide/d-galactosamine-induced acute liver failure via the inhibition of NLRP3, MAPK and NF- κ B, and the induction of autophagy. *Int J Biol Macromol*. 2018;119:240–248.
- [30] Oliver JM, Burg DL, Wilson BS, et al. Inhibition of mast cell Fc ϵ RI-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J Biol Chem*. 1994;269:29697–29703.
- [31] Hinz M, Lemke P, Anagnostopoulos I, et al. Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. *J Exp Med*. 2002;196:605–617.