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Nevadensin relieves food allergic responses and passive cutaneous anaphylaxis in mice through inhibiting the expression of c-Kit receptors†

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Nevadensin (NEV), a natural flavonoid compound derived from *Lysionotus pauciflorus* Maxim, has numerous biological activities. However, few researchers have examined its potential impact on alleviating allergies. In the present study, NEV was found to upregulate rectal temperature, suppress the development of diarrhea, and decrease the levels of serum specific immunoglobulin E, histamine and mouse MC protease-1 in ovalbumin-allergic mice. Moreover, NEV also alleviated passive cutaneous anaphylaxis reactions and inhibited the release of β -hexosaminidase and histamine in bone marrow-derived mast cells. Furthermore, we provide the first demonstration that NEV decreases the expression of c-Kit and suppresses the proliferation of bone marrow-derived mast cells and accelerates their apoptosis. These findings indicated that *L. pauciflorus*-derived NEV might have the potential to alleviate food hypersensitivity.

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Introduction

Food allergy is a potentially life-threatening allergic reaction affecting a substantial proportion of the population, with a prevalence of nearly 8% among children and 5% among adults worldwide.¹ Egg allergies have a prevalence of 1.2%–2.0% in children, who experience a marked reduction in quality of life.² To date, the management of food allergies relies largely on strict avoidance of food allergens in the diet and using emergency medications. However, eggs add indispensable nutrients to children's diets. Meanwhile, accidental exposure to allergens occurs frequently and consistently in young children and adults.³ Additionally, curative anti-allergic drugs have some undesirable adverse effects. Thus, there is a

clear need to develop and use safe and natural anti-allergic active substances.

L. pauciflorus, a species of Gesneriaceae in the genus *Lysionotus*, is widespread in Southern China and is used in the treatment of lymph node tuberculosis, cough with tachypnoea and rheumatic pains.⁴ This plant has attracted great attention because of its flavonoids, which have distinctive effects including anti-allergy, anti-thrombosis, oxygen-free-radical scavenging and anti-oxidation.^{5,6} In our previous studies, dihydromyricetin from *L. pauciflorus* has been demonstrated to attenuate mouse allergic responses by modulating the activation of mast cells (MCs) in both the sensitization stage and effector stage.⁷ NEV, as the main and most abundant flavonoid in *L. pauciflorus*, was reported to possess anti-inflammatory, antibacterial, asthma relieving and antitussive activities.⁸ Brightling *et al.* have shown that asthma is associated with MC degranulation and inflammation, which often occur in allergies.⁹ However, few studies have focused on the anti-allergic activities and potential application value of NEV in the field of food allergy. Thus, exploring the anti-allergic activities of NEV should be of value.

Food allergy can be classified into immunoglobulin (Ig) E-mediated, non-IgE-mediated and mixed responses.¹⁰ Chicken egg yolk and white are responsible for causing an IgE-mediated response, the most common in food allergy.⁵ Moreover, ovalbumin, the most abundant (58%) protein in egg white, has been reported to elicit allergic reactions more frequently than egg yolk.¹¹ Thus, a mouse allergy model induced by ovalbumin (OVA) is often used to evaluate the anti-allergic activity of various materials.^{12,13} An imbalance in T helper

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cells (Th) 1/Th2 is responsible for initiating the IgE-mediated food allergy reactions by producing different types of cytokines.¹⁴ Interleukin (IL)-4, IL-5 and IL-13 released from Th2 cells induce the class switching of B cells, thus leading to the production of specific IgE antibodies.¹⁴ Subsequently, IgE binds high-affinity IgE receptors (FcεRI) on the surfaces of MCs, which then release abundant allergic mediators within minutes of re-exposure to allergens. Studies have demonstrated that targeting MCs may be an effective strategy for the treatment of allergic disorders.¹⁵

MCs, which are characterized by the receptors FcεRI and c-Kit, are widely recognized as the major effector cells in IgE-mediated MC response.¹⁶ The interaction between FcεRI and IgE is characterized by a slow dissociation rate ($k_{\text{off}} < 10^{-5} \text{ s}^{-1}$). These cells are permanently sensitized with IgE and thus are ready to respond immediately upon allergen challenge.^{15,17} FcεRI aggregation leads to a cascade of intracellular signaling transduction including the phosphorylation of tyrosine kinases. C-Kit (a receptor tyrosine kinase) regulates the activation, differentiation, survival and apoptosis of MCs.^{15,18} When stem cell factor (SCF, the ligand of c-Kit) binds its receptor, c-Kit dimerizes, autophosphorylates and consequentially activates the downstream signaling events.¹⁵ c-Kit potentiates FcεRI-mediated degranulation and cytokine production through reinforcement or potentiation of FcεRI signals, although it cannot induce degranulation on its own.^{18,19} Yamaki *et al.* have concluded that sunitinib (a tyrosine kinase inhibitor) relieves oral antigen-induced anaphylaxis viapreventing MC activation and have indicated that sunitinib and its related kinase inhibitors might be potential drugs for the treatment of food allergies.²⁰ However, most studies have focused on FcεRI-mediated responses, whereas the potential effects of natural active substances on c-Kit-mediated responses are poorly understood.^{21,22}

In this study, the cytotoxicity and the anti-allergic activity of NEV were first detected in RBL-2H3 cell assays. Moreover, an OVA-induced mouse model and PCA model were established to further verify its anti-food allergy activities. Furthermore, bone marrow-derived MCs (BMMCs) were generated to further investigate the potential underlying the anti-food allergic mechanism.

Materials and methods

Materials

RPMI 1640, Eagle's Minimum Essential Medium, fetal bovine serum, trypsin solution and penicillin-streptomycin solution were obtained from Hyclone (Logan, UT, USA). Anti-dinitrophenyl (DNP)-IgE, 4-methylumbelliferyl-*N*-acetyl-β-D glucosaminide, OVA and Evans blue were purchased from Sigma-Aldrich (St Louis, MO, USA). DNP-BSA was purchased from Biosearch (Petaluma, CA, USA) and Imject alum was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). ELISA kits for anti-OVA-IgE, histamine and mouse MC proteinase (mMCP)-1 were obtained from Cayman (Ann Arbor, MI, USA), IBL (Hamburg, GER) and R&D Systems (Minneapolis, MN, USA), respectively. Goat anti-mouse IgG1 and IgG2a antibodies

were purchased from Abcam (Cambridge, UK). Recombinant mouse IL-3 protein and mouse SCF were obtained from Peprotech (Rocky Hill, NJ, USA). Carboxyfluorescein succinimidyl amino ester (CFSE) and propidium iodide (PI)/RNase Staining Buffer were purchased from BD Pharmingen (San Diego, CA, USA) and eBioscience (San Diego, CA, USA), respectively. NEV from *L. pauciflorus* was obtained as a gift from Guizhou Medicinal University (Guiyang, China). The purity of NEV was greater than 98%.

Female BALB/c mice (6–8 weeks) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). In the mouse model, the mice were randomly divided into five groups with five mice each. They were raised in an SPF environment with a relative humidity of $55 \pm 10\%$ and stable temperature ($22 \pm 1^\circ\text{C}$). This experiment was performed in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. All protocols were approved by the Animal Research Ethics Board of Jimei University (Xiamen, China, no. SCXK 2016-0006).

Obtention of NEV

In this study, NEV was obtained as a gift from Guizhou Medicinal University (Guiyang, China). A dried plant of *L. pauciflorus* was extracted using EtOH. After removing the organic solvent, a series of separation steps were used to separate NEV from the resulting extract, including the liquid-liquid partition and a number of reversed-phase and normal chromatographic techniques.²³ NEV was dissolved in DMSO to 100 mg mL^{-1} , and then the solution was diluted with PBS to the working concentration.

RBL-2H3 assay

RBL-2H3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in an incubator with $5\% \text{ CO}_2$ at 37°C . The effect of NEV on RBL-2H3 cell viability was determined *via* MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide] assays. The levels of histamine and β-hexosaminidase from RBL-2H3 cells treated with or without NEV were measured as previously described.²⁴

Food allergy model protocol and allergic symptoms

BALB/c mice were housed 1 week in advance of the experimentation. On days 0 and 14, the mice were sensitized through intraperitoneal (i.p.) injection of $100 \mu\text{g}$ of OVA and 2 mg of alum in $200 \mu\text{L}$ of phosphate-buffered saline (PBS). From day 28, the mice were orally challenged with 50 mg of OVA in $200 \mu\text{L}$ of PBS every 3 days, for a total of five challenge events. The OVA-sensitized and OVA-challenged mice were treated without (OVA group) or with NEV (NEV groups) at different doses ($1, 5$ or 10 mg kg^{-1}) by gavage daily from day 27 to day 40. Mice sensitized with 2 mg of alum in $200 \mu\text{L}$ of PBS and challenged with PBS served as the controls (PBS group). As the indices of food allergy symptoms, rectal temperature, anaphylaxis and diarrhea were measured in accordance with a previous study; the mice were placed alone in cages and their feces were counted to calculate the proportion of loose stools in the total stools.²⁵

Determination of specific antibodies, allergic mediators and cytokines in serum

One hour after the fifth challenge with OVA, serum was obtained *via* the tail vein for the determination of histamine and mMCP-1. On day 41, the mice were sacrificed and the serum was collected for the detection of specific antibodies and cytokines. Specific IgG1 and IgG2a were determined with ELISA as previously reported.²⁵ Serum anti-OVA-IgE, IL-4, IFN- γ , histamine, mMCP-1 and IL-10 were measured with commercial ELISA kits in accordance with the manufacturer's directions.

PCA model

An IgE-mediated PCA model was used according to a previous study.¹² Anti-DNP-IgE (0.1 μg) were injected subcutaneously into the right ear of each mouse. After 24 h, to evaluate the potential effects of NEV on MCs, NEV (1, 5 or 10 mg kg^{-1}) was administered to the mice by oral gavage 1 h before the antigen challenge (intravenously with 0.2 mg of DNP-BSA in 100 μL of PBS containing 10 mg mL^{-1} Evans blue). After 30 min, the mice were photographed and sacrificed. The right ears were collected and mixed in 250 μL of 3 M KOH at 37 °C overnight. Phosphoric acid and acetone (5 : 13, 225 μL) were mixed and added, after which the samples were centrifuged (3000 rpm, 15 min). The absorbance of the supernatants was measured at 620 nm.

Generation of BMMCs and β -hexosaminidase and histamine release measurement in BMMCs

BMMCs were generated from bone marrow cells as previously reported.²⁶ After a 4-week stimulation with mouse IL-3 and mouse SCF, the cells were identified by flow cytometric analysis of the cell surface expression of c-Kit and Fc ϵ RI α . Data were acquired using a Guava easyCyte 6-2L system and analyzed using the ACS Paragon Plus Environment Guava Soft 3.1.1 software (Millipore, MA, USA).

The effect of NEV on the levels of β -hexosaminidase and histamine released from mature BMMCs were determined according to a previous study.²⁴

The maturation of BMMCs

To verify the effects of NEV on the maturation of BMMCs, bone marrow cells were cultured under stimulation with mouse IL-3 and mouse SCF to induce the generation of BMMCs, and treated without or with NEV (1, 5 or 10 $\mu\text{g mL}^{-1}$) during the generation for 4 weeks (the medium was changed every 2 days) and the cells were identified by flow cytometric analysis every week.

The proliferation and cell cycle of BMMCs

Mature BMMCs were first labeled with CFSE according to the manufacturer's instructions and then treated with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days. Three days later, the cells were collected and the proliferation of BMMCs was determined by flow cytometry. Mature BMMCs were treated with different doses of

NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days and then the cells were washed, fixed with 70% ethanol at 4 °C and resuspended in PI/RNase Staining Buffer for 15 min at room temperature. The proliferation and cell cycle of BMMCs were analyzed using the FlowJo V10 software.

The apoptosis of BMMCs

The apoptosis of normal BMMCs after stimulation with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days was measured with flow cytometric analysis as described previously.²⁷ To determine the effects of NEV on the apoptosis of BMMCs after the removal of SCF, we treated mature BMMCs with NEV for 3 days, resuspended the cells without NEV and SCF, and continued cultivation for 16 h. Then, the apoptosis of cells was analyzed. To verify the effects of NEV on the apoptosis of activated BMMCs, mature BMMCs were treated as follows: (1) sensitized with anti-DNP-IgE (0.5 $\mu\text{g mL}^{-1}$) overnight; (2) treated with or without NEV (2, 10, or 20 $\mu\text{g mL}^{-1}$) for 1 h; (3) stimulated with DNP-BSA (1 $\mu\text{g mL}^{-1}$) and then the apoptosis was determined with flow cytometric analysis.

Statistical analysis

Experimental results are expressed as the mean $\pm$ standard deviation. Each experiment was performed at least three times. The differences between the groups were analyzed by ANOVA. Significant differences between the NEV and OVA groups were assessed with Duncan's multiple range tests. The *P* value threshold for significance was set at 0.05 or 0.01.

Results

NEV suppressed the release of β -hexosaminidase and histamine in RBL-2H3 cells

In this study, NEV was obtained from *L. pauciflorus* plant material, and its chemical structure is presented in Fig. 1A. MTT assays were performed to determine the effect of NEV on the viability of RBL-2H3 cells. As shown in Fig. 1B, NEV (0–20 $\mu\text{g mL}^{-1}$) did not result in any cytotoxicity toward RBL-2H3 cells. As shown in Fig. 1C and D, NEV suppressed the release of β -hexosaminidase and histamine in RBL-2H3 cells in a concentration-dependent manner.

NEV attenuated OVA-induced mouse allergic symptoms

An OVA-induced food allergy model was used to verify the anti-allergic activities of NEV (protocol in Fig. 2A). As expected, hypothermia, diarrhea and a high allergic score were observed in OVA-sensitized and OVA-challenged mice (Fig. 2B–D). Moreover, treatment with NEV significantly attenuated oral OVA-challenge-induced food allergy, as assessed on the basis of hypothermia (Fig. 2B), diarrhea (Fig. 2C) and anaphylaxis score (Fig. 2D).

NEV decreased the levels of OVA-specific IgE, histamine and mMCP-1

Compared with that in the PBS group, the level of OVA-specific IgE in the OVA group sharply increased (Fig. 3A). Daily treat-

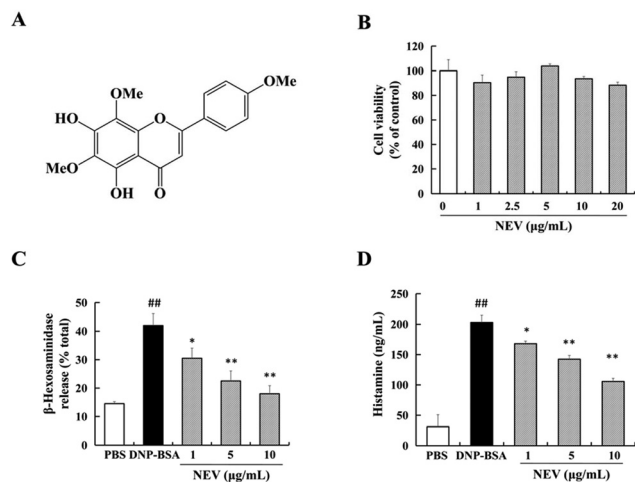


Fig. 1 Chemical structure of NEV and its inhibitory effects on the release of β -hexosaminidase and histamine in RBL-2H3 cells. (A) The chemical structure of NEV from *L. pauciflorus*. (B) The cytotoxicity of NEV toward RBL-2H3 cells was measured by MTT assays. RBL-2H3 cells were incubated with NEV at concentrations of 1–20 $\mu\text{g mL}^{-1}$. (C) The effect of NEV on the level of β -hexosaminidase in RBL-2H3 cells. RBL-2H3 cells were successively sensitized overnight with anti-DNP-IgE (0.1 $\mu\text{g mL}^{-1}$), treated with NEV (1, 5 or 10 $\mu\text{g mL}^{-1}$) for 1 h and stimulated with 0.5 $\mu\text{g mL}^{-1}$ DNP-BSA for 1 h in 48-well plates. Then the quantities of β -hexosaminidase in the cell supernatant and lysate were measured. (D) The effect of NEV on the level of histamine in RBL-2H3 cells. Cells were sensitized and treated as in C, except for stimulation with DNP-BSA for 15 min. The level of histamine in the cell supernatant was evaluated by ELISA. * $P < 0.05$; ** $P < 0.01$ for all NEV groups compared with the DNP-BSA group. # $P < 0.05$; ## $P < 0.01$ for the PBS group compared with the DNP-BSA group. The data represent the mean $\pm$ SD of three independent experiments.

ment with NEV (1, 5 or 10 mg kg^{-1}) led to significantly decreased levels of OVA-specific IgE in the serum (3495.88, 3630.67 and 3498.71 ng mL^{-1} , respectively). Nevertheless, no significant differences were observed in the levels of OVA-specific IgG1 and IgG2a between the NEV and OVA groups (ESI Fig. S1A†). The levels of IL-4 (Th2-dependent), IFN- γ (Th1-dependent) and IL-10 (regulatory T-dependent) in the serum were determined. However, NEV exhibited no marked effects on the levels of these cytokines (ESI Fig. S1B–S1D†). To monitor MC degranulation, we measured the levels of histamine and mMCP-1 in the serum. As presented in Fig. 3B, administering NEV (1, 5 or 10 mg kg^{-1}) significantly decreased the histamine levels (946.81, 759.73 and 706.26 ng mL^{-1} , respectively), which is lower than that of the OVA group (1471.99 ng mL^{-1}). In addition, NEV markedly decreased the level of mMCP-1, but this effect was not concentration dependent (Fig. 3C).

NEV alleviated mouse PCA reactions and suppressed the release of β -hexosaminidase and histamine in BMMCs

To further explore the inhibitory effects on MCs, we established a mouse PCA model. Evans blue accumulated in the ears of the DNP-BSA-stimulated group mice, as compared to the PBS group mice (Fig. 3D). The content of Evans blue in the

DNP-BSA group increased from 0.30 (PBS group) to 0.91 (Fig. 3E). The administration of NEV caused a marked decrease in Evans blue content (Fig. 3D and E). Additionally, the content of Evans blue in the NEV 10 mg kg^{-1} group was close to that in the PBS group (0.31 vs. 0.30).

To further verify the effects of NEV on MCs, we determined the levels of β -hexosaminidase and histamine in BMMCs. As presented in Fig. 3F and G, NEV suppressed the release of β -hexosaminidase and histamine in mature BMMCs in a dose-dependent manner.

NEV suppressed the expression of c-Kit on the surfaces of BMMCs

As shown in Fig. 4, NEV markedly decreased the number of mature BMMCs from the first week to the third week. However, in the fourth week, NEV had a slight effect on the number of mature BMMCs, thus suggesting that NEV did not change the inevitable trend of bone marrow cell differentiation into BMMCs under the induction of mouse IL-3 and mouse SCF. Unexpectedly, NEV significantly inhibited the expression of c-Kit on the surfaces of BMMCs from the second week to the fourth week (Fig. 5A). To further explore the possible effects of NEV on mature BMMCs after the suppression of c-Kit expression, we aimed to shorten the processing time of NEV on BMMCs cells by increasing the dose of NEV (2, 10 and 20 $\mu\text{g mL}^{-1}$). As expected, treatment with NEV (10 or 20 $\mu\text{g mL}^{-1}$) for 3 days led to a significant decrease in c-Kit expression (Fig. 5B).

NEV inhibited the proliferation of BMMCs

Because the c-Kit receptor regulates the proliferation of cells,²⁸ BMMCs were labeled with CFSE for the exploration of the effects of NEV on cell proliferation. The CFSE fluorescence intensity of BMMCs after a 3-day cultivation in the NEV groups was significantly enhanced, suggesting that NEV inhibited the proliferation of BMMCs in a concentration-dependent manner (Fig. 6A). We further explored whether NEV inhibited the proliferation of BMMCs by regulating the cell cycle using PI dye. After treatment with NEV (20 $\mu\text{g mL}^{-1}$), the percentage of cells in the G0–G1 phase increased from 83.2% (Control group) to 86.9% (Fig. 6B and C), which indicated that NEV had a weak arrest effect on BMMCs in G0–G1 phase.

NEV accelerated the apoptosis of BMMCs

Bioactive substances have been reported to alleviate mouse food allergic responses by inducing the apoptosis of MCs.^{27,38} Moreover, the c-Kit receptor was reported to be able to regulate apoptosis; thus we labeled BMMCs with PI and Annexin V to detect their apoptosis.²⁸ Viable cells with intact membrane exclude PI, and the membranes of damaged or dead cells are permeable to PI. Cells stained negative for PI and positive for Annexin V are undergoing apoptosis (early apoptosis), and double-positive cells are in the final stages of apoptosis or already dead (late apoptosis). Non-stained cells did not undergo measurable apoptosis.²⁹ First, the effect of NEV on the apoptosis of normal BMMCs was detected by flow cytome-

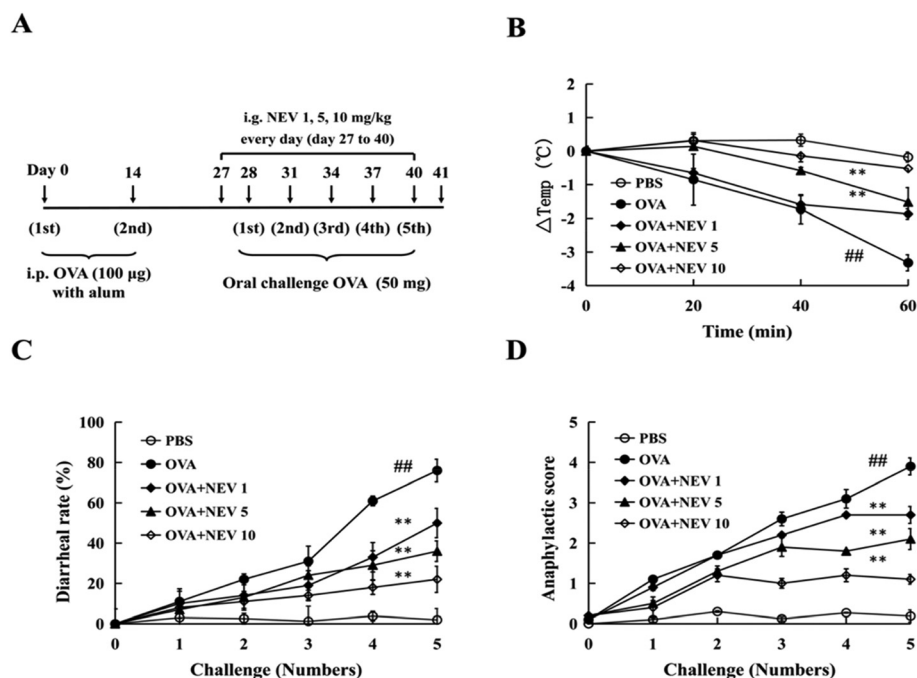


Fig. 2 NEV attenuated OVA-induced mouse allergic symptoms. (A) Food allergy model. Groups of mice ($n = 5$ per group) were sensitized with 100 μg of OVA and 2 mg of alum in 200 μL of PBS by intraperitoneal injection and orally administered with NEV (1, 5 or 10 mg kg^{-1}). (B) The rectal temperature of mice was measured (1 h after the fifth OVA challenge). (C) The situation of diarrhea in mice was assessed (1 h after each OVA challenge, by calculating the proportion of loose and total stools in the stool). (D) The anaphylactic scores of mice were evaluated (1 h after each OVA challenge). * $P < 0.05$; ** $P < 0.01$ for all NEV groups compared with the OVA group. # $P < 0.05$, ## $P < 0.01$ for the PBS group compared with the OVA group. The data represent the mean $\pm$ SD of three independent experiments.

try. However, no significant effects of NEV on the apoptosis of normal BMMCs (Fig. 7A), dendritic cells and L-O2 human liver cells (not showed) were observed. Because SCF is thought to play a role in enhancing c-Kit-mediated responses, whereas NEV has an opposite effect, we sought to verify whether apoptosis might be accelerated in mature BMMCs after the removal of SCF. As expected, NEV induced the apoptosis of BMMCs after SCF removal (Fig. 7B). The effect of NEV on the apoptosis of BMMCs activated by the Fc ϵ RI-IgE complex was further explored. There were no significant differences in the apoptosis of BMMCs between the control group and DNP-BSA group (Fig. 7C). Interestingly, NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) induced early apoptosis in BMMCs in response to allergens, with apoptosis rates of 15.67%, 17.57% and 18.53%, respectively (Fig. 7C).

Discussion

As an important traditional Chinese herb, *L. pauciflorus* has been demonstrated to have a wide range of bioactive activities and to be highly safe.³⁰ Moreover, NEV is the main active compound in *L. pauciflorus* and it is necessary to study its physiological and pharmacological activities. And these research results will benefit the development and utilization of *L. pauciflorus*.⁸ In this study, NEV (12.5 g) was isolated from *L. pauciflorus* plant material (100 kg) and was found to sup-

press the release of β -hexosaminidase and histamine at doses of 1, 5 or 10 $\mu\text{g mL}^{-1}$ in RBL-2H3 cells. Moreover, NEV significantly alleviated OVA-induced mouse allergic symptoms (including hypothermia, diarrhea and high allergy scores) at concentrations of 1, 5 or 10 mg kg^{-1} .

To further assess the anti-allergic potential of NEV, we determined several serum indicators in mice. Th1-/Th2-related cytokines play an important role in the Th1/Th2 balance, which is a key mechanism of allergic diseases.³¹ Hsu *et al.* have demonstrated that dioscorins increase IgG2a and IFN- γ levels and decrease IL-5 levels, thereby suppressing OVA-induced allergic reactions, possibly through modulating imbalanced Th1/Th2 immune responses.¹⁴ Studies have confirmed that IL-10 exerts immunosuppressive effects, thus preventing excessive immune responses and body damage.^{32,33} However, NEV showed no clear inhibitory effects in suppressing the levels of OVA-specific IgG1, IgG2a, IL-4, IFN- γ and IL-10 at any concentration; therefore, NEV may not have significant effects in regulating the balance of Th1/Th2 or immunosuppressive effects. However, NEV significantly inhibited the release of OVA-specific IgE, while it had no inhibitory effect on IL-4. As reported, the population and differentiation of B cells affect the production of IgE.^{24,34} Thus, there is a possibility that NEV may inhibit the production of IgE by affecting B cells. Meanwhile, specific IgE antibody has been reported to be essential in initiating food allergy by participating in MC activation.³⁵ Furthermore, the levels of histamine and

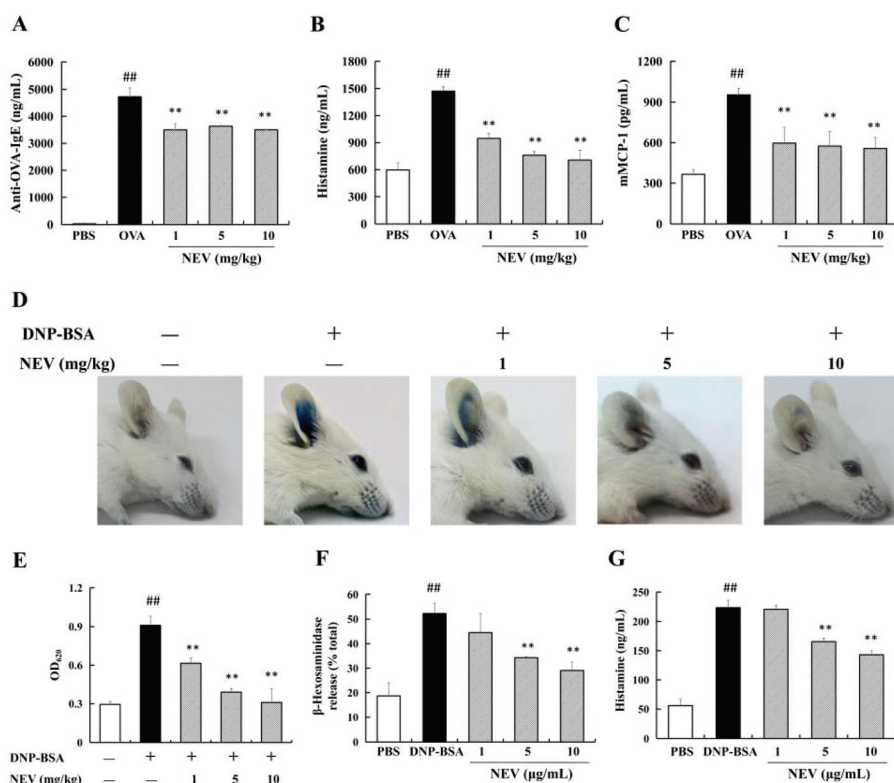


Fig. 3 NEV suppressed the levels of OVA-specific IgE, histamine and mMCP-1, alleviated PCA reactions, and inhibited the levels of β -hexosaminidase and histamine in BMMCs. (A) NEV decreased the level of OVA-specific IgE in the serum, which was collected from eyeballs on day 41. (B) NEV suppressed the level of histamine in the serum, which was collected from the tail veins of mice within 1 h after the last challenge. (C) NEV inhibited the level of mMCP-1 in the serum, which was collected from the tail veins of mice within 1 h after the last challenge. (D) NEV alleviated mouse PCA reactions. Groups of mice ($n = 5$ per group) were sensitized with anti-DNP-IgE after 24 h and mice were orally gavaged with NEV after 1 h and challenged with DNP-BSA via the tail vein. The ears of mice with absorbed Evans blue were photographed. (E) NEV alleviated the accumulation of Evans blue in mice ears. 3 M KOH and acetone and phosphoric acid (13 : 5) were used to extract the absorbed dye, and then the absorbance was measured at 620 nm with a microplate reader. (F) NEV decreased the level of β -hexosaminidase in BMMCs. Mature BMMCs (1×10^6 cells per mL) were successively sensitized overnight with anti-DNP-IgE ($0.5 \mu\text{g mL}^{-1}$), treated with NEV (1, 5 or $10 \mu\text{g mL}^{-1}$) for 1 h and stimulated with DNP-BSA ($1 \mu\text{g mL}^{-1}$) for 1 h. Then, the quantities of β -hexosaminidase in the cell supernatant and lysate were determined. (G) NEV decreased the level of histamine in BMMCs. Mature BMMCs were sensitized and treated as in (F), except for stimulation with DNP-BSA for 15 min, and the levels of histamine in the cell supernatant were measured by ELISA. (A–C) $^*P < 0.05$; $^{**}P < 0.01$ for all NEV groups compared with the OVA group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for the PBS group compared with the OVA group. (D–G): $^*P < 0.05$; $^{**}P < 0.01$ for all NEV groups compared with the DNP-BSA group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for the PBS group compared with the DNP-BSA group. The data represent the mean $\pm$ SD of three independent experiments.

mMCP-1, which are well-known products of MC activation that trigger both acute and chronic inflammation, were significantly decreased in mouse serum.¹² Thus, we hypothesized that NEV might attenuate OVA-induced mouse allergic reactions by suppressing the function of MCs. To test our hypothesis, we used a PCA model to monitor the inhibitory effect of NEV on MCs, as previously reported.^{36,37} As expected, NEV suppressed the content of Evans blue accumulation in mouse ears in a dose-dependent manner.

To further investigate the effect of NEV on MCs, we generated BMMCs. Some products including cytoplasmic particle-derived mediators, lipid-derived mediators and proinflammatory cytokines are released by activated MCs within minutes of antigen exposure.³⁸ In this study, NEV inhibited the release of β -hexosaminidase and histamine in BMMCs, thereby indicating that NEV suppressed Fc ϵ RI-mediated MC degranulation. Additionally, c-Kit has been suggested to be essential for the

development of MCs, and c-Kit-mediated signaling is critical for the differentiation and stimulation of MCs.^{16,39} Several small molecule inhibitors of c-Kit, such as imatinib and nilotinib, have been considered for the treatment of asthma, anaphylaxis or systemic mastocytosis, owing to the potential inhibition of c-Kit-mediated signaling.^{15,40} NEV has been demonstrated to diminish the expression of c-Kit in BMMCs within 3 days, and its effect time is shorter than that of coumarin, which has been reported to relieve mouse allergic reactions by decreasing c-Kit expression.⁴¹ According to reports, the number of MCs in local tissues and their homeostasis and function in the body largely depend on the degree of activation of c-Kit.¹⁸ Its activation in turn depends not only on the local concentration of SCF but also on the expression of c-Kit.¹⁸ NEV suppressed the proliferation of BMMCs and had a weak effect on arresting the cells in the G0–G1 phase, which may modulate the numbers of MCs *in vivo*. Studies have shown that

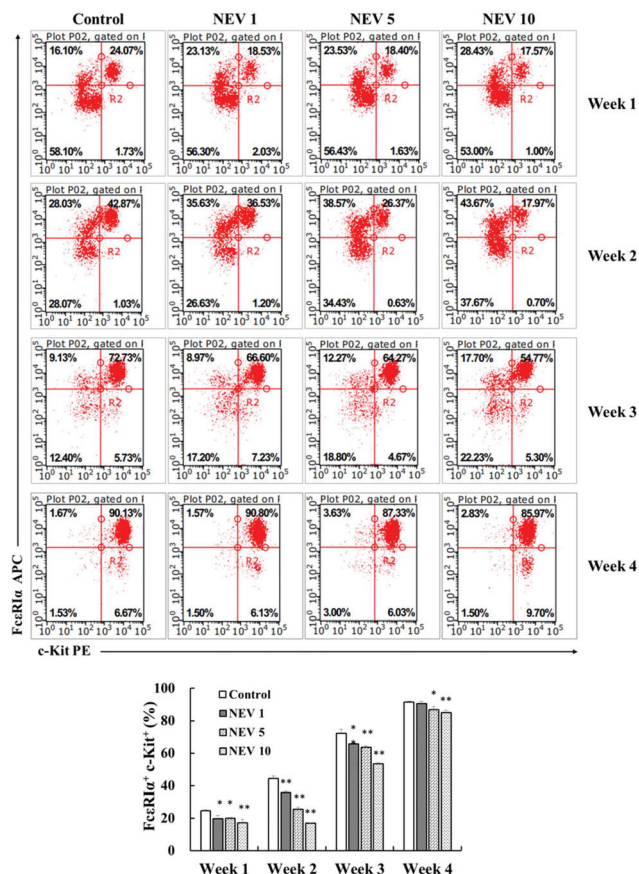


Fig. 4 NEV weakly regulated the maturation of BMMCs. Bone marrow cells were cultured under stimulation with mouse IL-3 and mouse SCF to induce the generation of BMMCs, and treated without or with NEV (1, 5 or 10 $\mu\text{g mL}^{-1}$) during the generation, and the medium was changed every 2 days. The expression of cell surface receptors c-Kit and FcεRIα was detected every week using flow cytometry. The data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$ for all NEV groups compared with the control group.

removal of mouse SCF can cause programmed cell death (Annexin V[−] PI⁺ cells) or induce cell apoptosis (Annexin V⁺ PI[−] cells or Annexin V⁺ PI⁺ cells), in agreement with our results.⁴² As reported, c-Kit can regulate the apoptosis of MCs by regulating signaling pathways such as PI3K-AKT.¹⁵ Meanwhile, SCF is related to the function and downstream signaling pathway of c-Kit.⁴³ The results showed that NEV can significantly inhibit the expression of c-Kit receptor in BMMCs. Meanwhile, NEV showed no significant effects on inducing the apoptosis of normal BMMCs, whereas NEV induced apoptosis in BMMCs after the removal of SCF. It indicated that NEV may accelerate the apoptosis of MCs through regulating the c-Kit-related signaling pathway, which is valuable and necessary for further exploration. As reported, allergen binding to IgE-FcεRI complex on the surface of MCs leads to FcεRI aggregation, thus inducing a cascade of intracellular signaling transductions.^{15,36} FcεRI aggregation would thus inhibit the apoptosis of MCs by upregulating the expression of antiapoptotic Bcl-2 family members, MCL-1 and BFL-1.⁴⁴ Thus, NEV

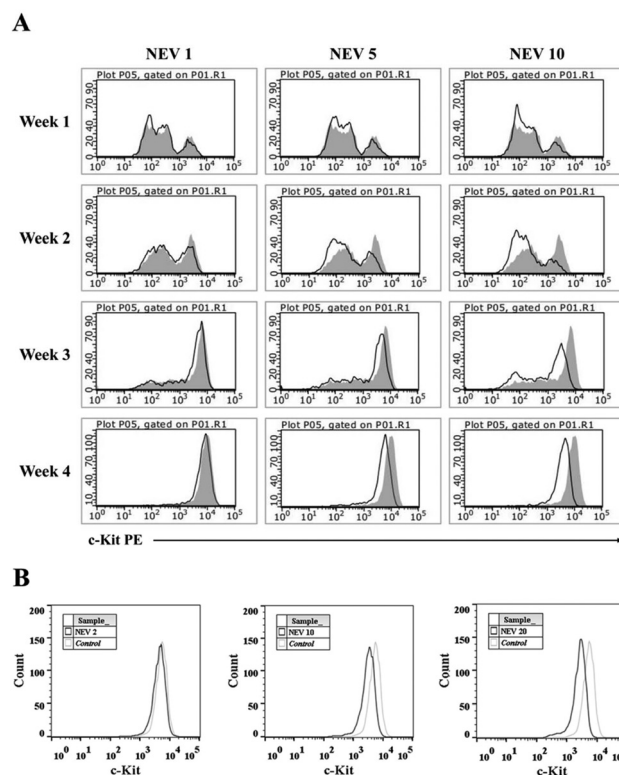


Fig. 5 NEV significantly suppressed the expression of c-Kit on the surfaces of BMMCs. (A) NEV significantly inhibited the expression of c-Kit during the maturation of BMMCs. The treatment of cells was as in Fig. 4. (B) NEV significantly suppressed the expression of c-Kit on the surfaces of mature BMMCs. Mature BMMCs were treated without or with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days, and the expression of c-Kit in mature BMMCs was determined using flow cytometry.

accelerated the apoptosis of allergen-activated BMMCs, which may result from its regulation of FcεRI-mediated signaling. And it is worth further exploring the regulation of the apoptosis of MCs using NEV. Taken together, NEV has the potential to alleviate OVA-induced food allergy by modulating the function of MCs, which may result from the inhibition of FcεRI and c-Kit/SCF signaling.

Our previous study has indicated that several flavonoids from *L. pauciflorus*, such as dihydromyricetin, myricetin and quercetin, can alleviate allergy.^{7,13,45} Dihydromyricetin attenuated OVA-induced mouse allergic reactions by blocking FcεRI-IgE interaction, thereby suppressing FcεRI-mediated MC reactions.⁷ This study provides the first demonstration that NEV has inhibitory effects on c-Kit-mediated MC reactions, in contrast to the above flavonoids in *L. pauciflorus*. These different effects may result from their chemical structures: NEV has three methoxy groups, whereas other flavonoids do not. As reported, the presence of multiple methoxides makes the compounds less polar and more planar, thereby facilitating the crossing of the cell membrane. Polymethoxylated flavones can also affect cell apoptosis and proliferation.^{46–48} Interestingly, the c-Kit receptors on the surface of MCs were attested to be associated with apoptosis and proliferation, which is in agree-

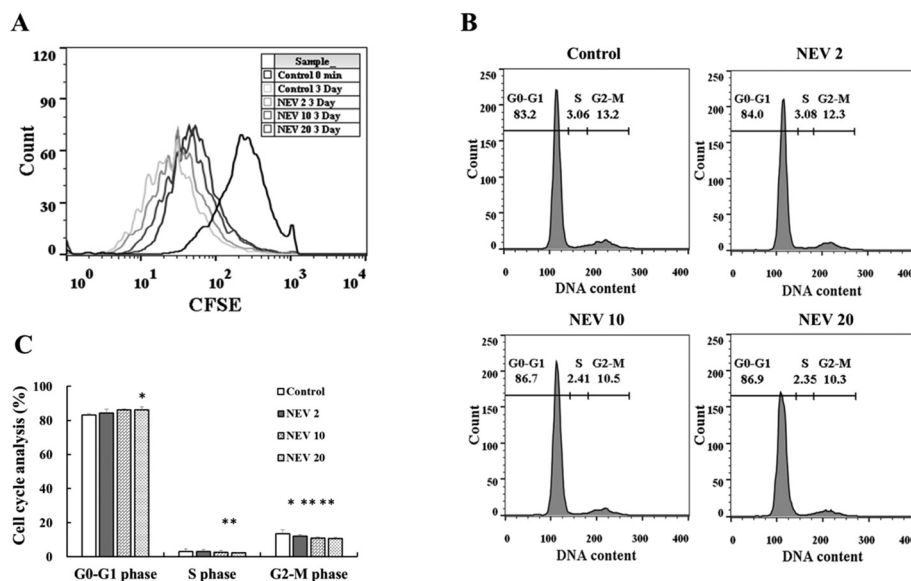


Fig. 6 NEV interfered with the proliferation of BMMCs and weakly blocked the cell cycle in BMMCs. (A) NEV interfered with the proliferation of mature BMMCs. Mature BMMCs were labeled with CFSE and treated without or with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days. Then, the fluorescence intensity of CFSE in BMMCs was detected using flow cytometry. (B) NEV weakly blocked the cell cycle in BMMCs. (C) Mature BMMCs were treated without or with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days. Then, the cells were stained with PI, and the cell cycle of BMMCs was measured using flow cytometry. $*P < 0.05$; $**P < 0.01$ for all NEV groups compared with the control group. The data represent the mean $\pm$ SD of three independent experiments.

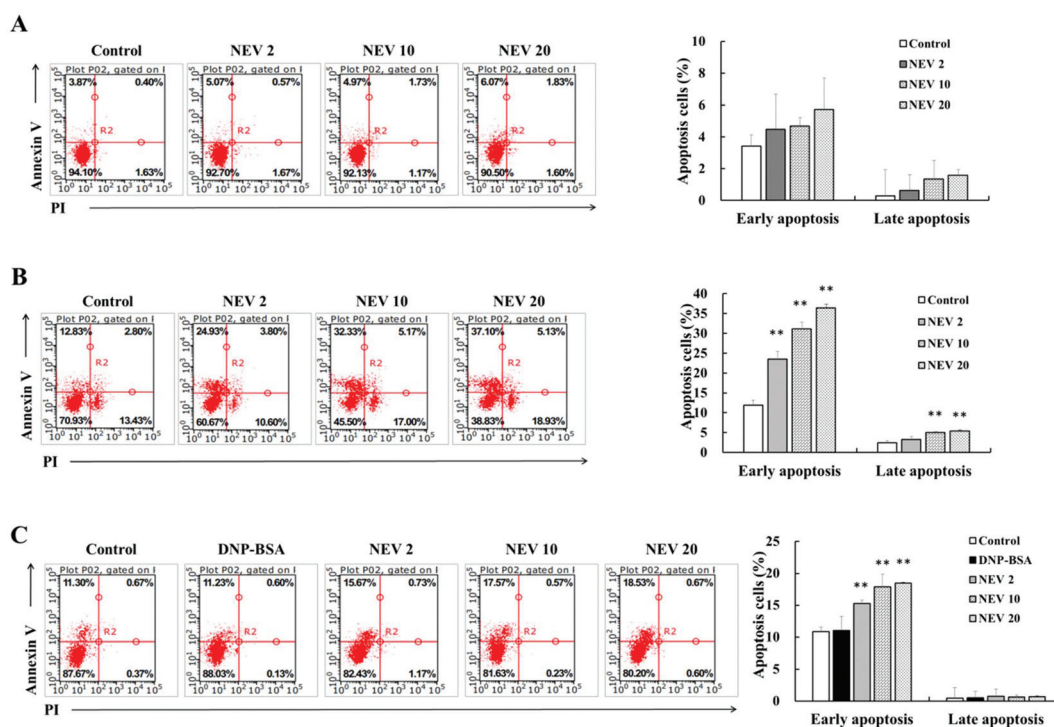


Fig. 7 Effects of NEV on the apoptosis of BMMCs. (A) NEV did not induce apoptosis in normal BMMCs. Mature BMMCs were treated without or with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days and then the apoptosis of BMMCs was detected using flow cytometry. (B) NEV treatment promoted apoptosis in BMMCs after the removal of mouse SCF. Mature BMMCs were treated without or with NEV (2, 10 or 20 $\mu\text{g mL}^{-1}$) for 3 days, and BMMCs were continuously cultured without stimulation with mouse SCF and NEV overnight. Then the apoptosis of BMMCs was determined. (C) NEV accelerated apoptosis in mature BMMCs activated by DNP-BSA. Mature BMMCs were successively sensitized overnight with anti-DNP-IgE (0.5 $\mu\text{g mL}^{-1}$), treated with NEV (-2, 10 or 20 $\mu\text{g mL}^{-1}$) for 1 h and stimulated with DNP-BSA (1 $\mu\text{g mL}^{-1}$) for 1 h. Then, the apoptosis of BMMCs was detected. (B) $*P < 0.05$; $**P < 0.01$ for all NEV groups compared with the control group. (C) $*P < 0.05$; $**P < 0.01$ for all NEV groups compared with the DNP-BSA group. The data represent the mean $\pm$ SD of three independent experiments.

ment with our experimental results. Wu *et al.* have demonstrated that nobilletin (a similar structure to NEV: they have the same parent structure and multiple methoxy groups) has the ability to inhibit airway inflammation in asthmatic mice.^{46,49} In addition, asthma is associated with mast-cell degranulation and inflammation and often occurs in allergies.⁹ In view of the inhibitory potential of NEV on food allergy, the effects of NEV on allergic asthma and other allergic models are worthy of exploration. Moreover, because of the presence of several flavonoids, extraction of *L. pauciflorus* may be suitable for applications in the field of anti-food allergy, but it must be verified and developed in the future.

Conclusion

In conclusion, NEV from *L. pauciflorus* attenuated OVA-induced allergic symptoms and decreased the levels of serum anti-OVA-IgE, histamine and mMCP-1. Moreover, NEV suppressed MC-mediated PCA reaction *in vivo* and inhibited the release of β -hexosaminidase and histamine in BMMCs. Furthermore, NEV diminished the expression of c-Kit, suppressed the proliferation of BMMCs and induced apoptosis in BMMCs. Therefore, NEV is expected to be a c-Kit receptor inhibitor that may provide new possibilities for the treatment of food allergen-induced allergic diseases.

Abbreviations

BMMC	Bone marrow-derived mast cell
CFSE	Carboxyfluorescein succinimidyl amino ester
DNP	Dinitrophenyl
Fc ϵ RI	High-affinity IgE receptor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MC	Mast cell
mMCP	Mouse mast cell proteinase
NEV	Nevadensin
OVA	Ovalbumin
PCA	Passive cutaneous anaphylaxis
RBL	Rat basophilic leukemia
SCF	Stem cell factor

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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Supplementary information

Figure legends

Figure S1. Effects of NEV on the levels of specific antibodies and cytokines.

A. The effect of daily treatment with NEV on serum OVA-specific IgG1 and IgG2a. Serum from eyeballs was collected on day 41.

B. The effect of daily treatment with NEV on serum IL-4. Serum from eyeballs was collected on day 41.

C. The effect of daily treatment with NEV on serum IFN- γ . Serum from eyeballs was collected on day 41.

D. The effect of daily treatment with NEV on serum IL-10. Serum from eyeballs was collected on day 41. [#] $P < 0.05$, ^{##} $P < 0.01$ for the PBS group compared with the OVA group. The data represent the mean $\pm$ SD.

Figure S1

