



Fisetin inhibits IL-31 production in stimulated human mast cells: Possibilities of fisetin being exploited to treat histamine-independent pruritus

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ABSTRACT

Interleukin-31 (IL-31) is a recently discovered cytokine that is tightly linked to the pathogenesis of pruritus seen in atopic dermatitis. Flavonoids, like fisetin, are naturally occurring molecules with antioxidant, cytoprotective, and anti-inflammatory actions. Aim: the present study sought to investigate whether fisetin modulates IL-31 and histamine release in human mast cells (HMC-1). Main methods: HMC-1 cells were pretreated with fisetin at various doses and stimulated with phorbol-12-myristate 13-acetate and calcium ionophore A23187 (PI) for different time intervals. We evaluated IL-31 production and histamine release and signaling mechanism of the action of fisetin on IL-31 production. We also investigated the effects of fisetin on scratching behaviors in mice. Key findings: Fisetin decreased PI-stimulated mRNA expression and production of IL-31 in HMC-1 cells. Fisetin inhibited PI-induced phosphorylation of mitogen-activated protein kinases that further suppressed nuclear factor (NF- κ B) activation and translocation to the nucleus through the inhibition of I κ B- α phosphorylation. Fisetin also prevented mast cell release of histamine in HMC-1 cells. Mice *in-vivo* studies show that fisetin reduced scratching behaviors in mice. Significance: These pharmacological actions of fisetin provide new suggestions that fisetin can be of potential use for the treatment of pruritus that cannot be treated with histamine receptor blockers alone.

1. Introduction

IL-31 has recently been discovered to be a helical cytokine belonging to the gp130/IL-6 cytokine family that includes other cytokines like IL-6, IL-11, and IL-27 [1]. IL-31 has been directly linked to play a key role in the development of AD in mice and humans. For instance, transgenic mice over-expressing IL-31 or wild type mice administered with recombinant IL-31 protein developed characteristic skin phenotypes that closely mimic that of patients with atopic dermatitis [2]. Higher levels of IL-31 expression have been seen in biopsy specimens taken from patients with atopic dermatitis than those from healthy individuals [3]. Aside the role it plays in AD, IL-31 like IL-2, 4, 6 also plays a key role in mediating the itch seen in AD both in mice and human studies. For example, IL-31 mRNA was found to be expressed in NC/Nga mice AD model experiencing pruritus than in mice AD model without pruritus [4]. Prurigo nodularis and pruritic AD patients notably expressed IL-31 mRNA [5]. These data imply an important role for IL-

31 in the pathogenesis of atopic dermatitis particularly pruritus. The involvement of IL-31 in pruritus is further confirmed as IL-31 receptors have been shown to be expressed on cutaneous nerve fibers located in the skin closed to IL-31 producing cells like mast cells and T-cells [6]. This shows that IL-31 could also be a key factor that mediate non-histamine dependent pruritus in AD.

Mast cells are one of the major effector cells in the immune response system that secrete pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-13 and inflammatory mediators, including histamine, leukotrienes, serotonin, and prostaglandin (PG)E₂ [7]. Mast cell plays a key role in the mediation of itch in AD by releasing chemical mediators such as histamine [7], cytokines like IL-6, IL-22, and IL-31 [6].

Although various treatments have been employed to relieve pruritus and scratching in patients, chronic pruritus is still difficult to treat as current treatment options are frequently ineffective, thereby necessitating new therapeutic approaches. In addition, the most common

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conventional therapy-antihistamine used for the treatment of pruritus often fails as histamine is not the only mediator of pruritus [8]. This has led to the development of new approaches in antipruritic therapy. Phytochemicals have recently gained the attention to be exploited as possible replacement for the treatment of several diseases. Of this phytochemicals, flavonoids have shown anti-inflammatory, antioxidant, antibacterial and anti-allergic properties [9–11]. Fisetin, present in vegetables and fruits such as onions, strawberries, apples and the Japanese fruit Kaki [12], have been reported to suppress the production of cytokines like TNF α , IL-1 β , IL-4, IL-6 IL-8, and IL-13 [13]. However the ability of fisetin to suppress IL-31 and histamine production and pruritus in mice model of pruritus has not yet been described. This present study was designed to investigate if fisetin inhibits histamine and IL-31 production *in vitro* in HMC-1; determine the mechanism of inhibition and also to investigate the effects of fisetin on scratching behavior *in vivo* in mice.

2. Material and methods

2.1. Chemicals used in this study

Phorbol-12-myristate 13-acetate, calcium ionophore A23187, and fisetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against I κ B α , phospho-I κ B α , extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, p38 and phospho-p38, JNK, phospho-JNK, NF- κ B, phospho-NF- κ B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against IL-31 were purchased from Thermo Scientific (Rockford, IL, USA) and R&D Systems (MN, USA). Antibodies against actin came from Biosciences (San Diego, CA, USA). Western blot reagent and Western blot blotting buffer came from BIO-RAD (CA, USA) and thermos Scientific (Rockford, IL, USA). ELISA kits for IL-31, IL-6, IL-1 β , and histamine were purchased from Biolegend (San Diego, CA, USA), R&D Systems (Minneapolis, MN, USA) and Enzo (Farmingdale, NY) respectively. Inhibitors for ERK1/2, JNK, and NF- κ B were purchased from Sigma-Aldrich (St. Louis, MO, USA) while inhibitors for p-38 was purchased from Selleckchem (Houston, TX, USA). All other chemicals used in this study are of reagent grade and have been listed within the text.

2.2. Treatment of cells with fisetin

HMC-1 cells were cultured in Iscove's modified Dulbecco's medium (Gibco, Grand Island NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. The cells were maintained at 37 °C in 5% CO $_2$ in a humidified incubator. The cells (5 $\times$ 10 ^{5} cells/mL) were seeded in 6-well dishes for 16 h and treated with fisetin ($\geq$ 98% purity) at indicated concentrations dissolved in DMSO for 1 h and stimulated with phorbol-12-myristate 13-acetate (50 nM) plus calcium ionophore A23187 (PI) for indicated time periods. The concentration of DMSO was below toxicity level of < 0.1%.

2.3. Reverse transcription (RT)-PCR analysis

HMC-1 cells, pre-treated with or without fisetin for 1 h were stimulated with 50 nM PI for 3 h. Total RNA was extracted and purified using Qiagen RNeasy Mini extraction kit (Hilden, Germany) according to the manufacturer's specifications. The concentration of total RNA was determined by spectrophotometry. One microgram of each sample was reverse transcribed to cDNA at 43.8 °C for 1 h using Bioneer cDNA synthesis kit (Daejeon, Korea) and T100™ Thermal Cycler, Bio-Rad. The primers and PCR conditions are shown in Table 1. PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with GelRed Nucleic acid stain. The gels were photographed using BIO-RAD Molecular Imager, and relative band intensity was analyzed using the machine's software.

Table 1

Primers and PCR conditions for analysis of gene expression in HMC-1 cells.

Cytokine	Primer sequence	Annealing temperature (°C)	No. of cycles	Product size (bp)
IL-31	5'tgtgccaacagacacccatg3' 3'tgttgggtccagaggtcaa5'	60	40	108
GADPH	5'cactctccacaccttgacgc3' 3'tccaccacctgttctgta5'	60	30	107

2.4. Cytoplasmic and nuclear protein extraction

Nuclear and cytoplasmic extracts were done using reagents from Thermo Scientific (Rockford, IL, USA). In brief, HMC-1 cells were pre-treated with or without fisetin for 1 h and stimulated with PI for 30 min. Cells were washed with ice-cold PBS, centrifuged at 500 $\times$ g for 2 min at 4 °C and resuspended in 0.1 mL of ice-cold cytoplasmic extraction reagent I. The tubes were vigorously vortexed and incubated on ice for 10 min, lysed gently with 5.5 μ L of cytoplasmic extraction reagent II. After vortexing and centrifuging at 16000 $\times$ g for 5 min at 4 °C, the supernatant was collected as cytoplasmic fraction. The fractions were stored at –70 °C. The pellets were subsequently resuspended in 50 μ L of nuclear extraction reagent and lysed vigorously for 40 min on ice to obtain the nuclear fractions which were stored at –70 °C until used. The protein concentration in the cytoplasmic and nuclear extract was evaluated by using a BCA protein assay (Pierce, Rockford, IL).

2.5. Western blotting

Twenty micrograms of cytoplasmic or nuclear proteins were subjected to Western blot analysis. The proteins were denatured in 5 $\times$ SDS-PAGE loading buffer (Biosesang, Gyeonggi-do, Korea) for 5 min at 95 °C and separated by 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon PVDF; Millipore, Bedford, MA, USA). The membrane was then blocked with 5% non-fat dry milk in TBST (0.05% Tween-20 in TBS, pH 7.4) for 1 h at room temperature and further incubated overnight with various antibodies. The blots were washed five times with TBST and incubated with respective secondary antibodies for 1 h 30 min. After washing, the antibody-specific proteins were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA). To ensure equal protein loading, the membranes were stripped and reprobed with anti β -actin antibodies using the protocol described above. The density of each band in an immunoblot was analyzed using the ImageJ gel analysis software (developed by the National Institutes of Health, Maryland, USA).

2.6. ELISA

The secretion of IL-31 and Histamine were measured by an enzyme-linked immunosorbent assay (ELISA). HMC-1 cells were pre-treated with or without fisetin and potent inhibitors of NF- κ B [P8765 (50 μ M)], JNK [SP600125 (10 μ M)], ERK [PD98059 (100 μ M)], p38 MAPK [SB203580 (100 μ M)] for 1 h and stimulated with PI for 12 h (IL-31). After centrifugation, cell culture media supernatant was used to perform the ELISA following each of the Kit's accompanying manual. For the standard curve, recombinant IL-31 and Histamine standards were run alongside the samples and used to calculate the various concentrations of IL-31 and histamine. All steps were performed at room temperature, and all standards and samples were assayed in triplicate.

2.7. Animals

The 5-week-old male ICR mice used in this study were obtained from Orient Bio Inc. (Iksan, Korea). Mice were randomly divided into four groups of five mice per group and acclimatized for 1 week before

the start of the experiment. The mice were fed with commercial standard laboratory diet and water *ad libitum*, maintained in an air-conditioned room with temperature $22 \pm 2^\circ\text{C}$, humidity 50–60%, and 12/12 h light-dark cycle. The animal protocols were performed following Jeonju University Institutional Animal Care and Used Committee guidelines.

2.8. Compound 48/80-induced itch

Compound 48/80 was used to stimulate an immediate-type allergic reaction mediated by itch as previously described [14]. Briefly, the mice ($n = 5/\text{group}$) were intraperitoneally administered 50, and 100 mg/kg body weight of fisetin, 1 h later, the mast cell degranulation compound, compound 48/80 (50 μg) was injected into the subcutaneous layer of the left shoulder of the mice. After that, according to Mihara et al.'s method [15], scratching behavior was monitored with micro-cameras (ONCCTV, Seoul, Korea) for 1 h and the number of scratches on the site of the compound 48/80 injections was counted in a double-blinded manner.

2.9. Histology and immunohistochemistry

At the end of the counting, the mice were sacrificed, and the compound 48/80-injected sites ($1 \times 1 \text{ cm}$) were excised and 5 μm frozen sections were immediately obtained and stained with hematoxylin and eosin (H&E) and toluidine blue for histological analysis. The numbers of mast cells were counted using the light microscope (Leica, Wetzlar, Germany).

Immunohistochemistry method, using Ready-to-use IHC/ICC kit one-step HRP Polymer anti-Mouse, Rat & Rabbit IgG with DAB (Biovision, Milpitas, CA, USA) was used to analyze mast cell degranulation at the compound 48/80-injected sites. Skin tissues that had been stored at -80°C were frozen sectioned, fixed with acetone, peroxidase and protein blocked before being incubated with antibody against mast cell tryptase (at antibody concentration of 0.1 $\mu\text{g}/\text{mL}$). Evaluation of skin sections was performed by counting the number of tryptase-positive mast cells degranulation sites under the Leica light microscope at $100\times$ oil immersion magnification in 30 high power fields. Histologic analysis was carried out by 2 independent observers in a blinded fashion.

2.10. Statistical analysis

Student's *t*-test determined statistically significant differences among each group. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Fisetin suppresses the expression of IL-31 mRNA expression

To examine whether fisetin modulates gene expression of IL-31 in PI-stimulated HMC-1, cells were pre-treated with fisetin at concentrations of 2.5, 5, and 10 μM for 1 h and then stimulated with PI for 3 h. As shown in Fig. 1, pre-treatment of fisetin dose-dependently inhibited PI-induced gene expression of IL-31. Significant differences at $P < 0.01$ and 0.001 were obtained in cells treated with 5 and 10 μM of fisetin respectively, compared with cells PI-stimulated cells.

HMC-1 (5×10^5 cells/mL) was pre-treated with fisetin (2.5, 5, and 10 μM) for 1 h and stimulated with PMA (50 nM) + A23187 (1 μM) for 3 h. RT-PCR determined the expression level of mRNA. Each bar represents the mean $\pm$ SD of three measurements. Statistically significant difference, $^{**}P < 0.01$ and $^{***}P < 0.001$ between the treated cells and the positive control cells.

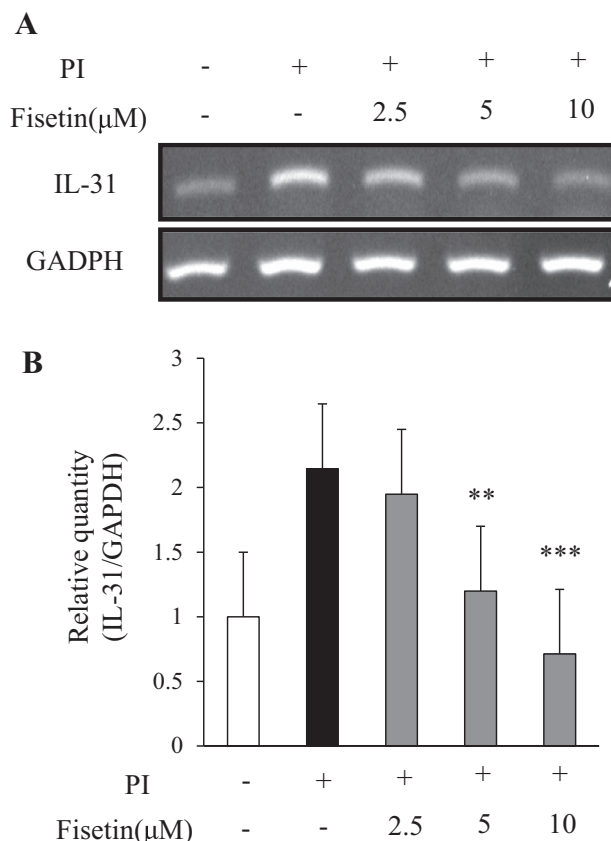


Fig. 1. Effect of fisetin on the expression IL-31 mRNA in stimulated HMC-1 cells.

3.2. Fisetin suppresses the expression of IL-31 protein expression

Protein expression of the IL-31 protein was examined in HMC-1 cells pre-treated with fisetin at concentrations of 2.5, 5, and 10 μM for 1 h and then stimulated with PI. Protein expressions were detected 5 h after stimulation. As shown in Fig. 2, pre-treatment of PI-stimulated cells with fisetin also dose-dependently suppressed IL-31 protein expression in HMC-1. Significant differences at $P < 0.05$ and 0.01 were obtained in cells treated with 5 and 10 μM of fisetin respectively, compared with cells PI-stimulated cells.

Cells (5×10^5 cells/mL) were pre-treated with fisetin (2.5, 5, and 10 μM) for 1 h prior to PMA (50 nM) + A23187 (1 μM) stimulation for 5 h. Western blotting analyzed expression of IL-31 cytokine protein. Band intensity for each protein expressions was normalized against actin. Each bar represents the mean $\pm$ SD of three measurements. Statistically significant difference, $^{*}P < 0.05$ and $^{**}P < 0.01$ between the treated cells and the positive control cells.

3.3. Fisetin suppresses the production of IL-31 cytokine

To confirm the effect of fisetin on the gene and protein expression of IL-31, culture supernatants were assayed to check the concentrations of the various cytokines by ELISA methods. HMC-1 cells were pre-treated with fisetin (2.5, 5, and 10 μM) before stimulation with PI for 12 h, and culture supernatants were collected, stored at -70°C and analyzed using ELISA. As shown in Fig. 3A, PI-induced increased in the concentrations of IL-31, was significantly ($P < 0.001$ with fisetin 10 μM pre-treatment) and dose-dependently decreased when stimulated cells were pre-treated with fisetin.

Cells (5×10^5 cells/mL) were pre-treated with fisetin at indicated

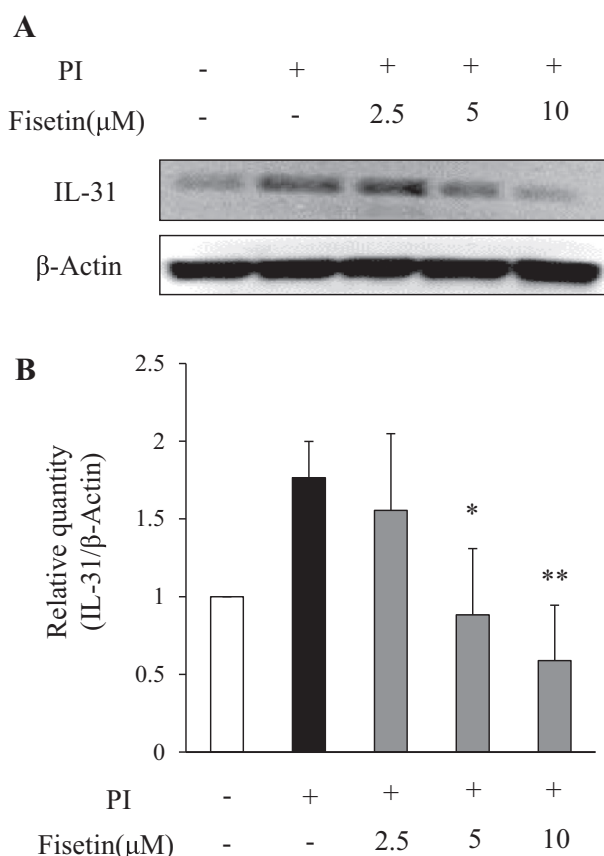


Fig. 2. Effect of fisetin on IL-31 cytokine protein expression in stimulated HMC-1 cells.

concentrations for 1 h prior to PMA (50 nM) + A23187 (1 μ M) stimulation for 12 h. ELISA analyzed the production of the IL-31 cytokine (A) and histamine (B). Each bar represents the mean $\pm$ SD of three measurements. Statistically significant difference, ** P < 0.01 and *** P < 0.001 between the treated cells and the positive control cells.

3.4. Fisetin inhibits histamine production

To also verify the effect of fisetin on histamine release by HMC-1 cells, cells were pre-treated with fisetin (0.5, 1, and 2 μ M) before stimulation with PI for 12 h, and culture supernatants were collected,

stored at -70°C and later analyzed for histamine concentrations. PI stimulated the release of histamine in HMC-1 cells. Histamine release was dose-dependently suppressed with a significant difference at P < 0.01 for fisetin 1 μ M and P < 0.001 for fisetin 2 μ M when the stimulated cells were pre-treated with fisetin (Fig. 3B).

3.5. Fisetin suppresses the activation of mitogen-activated protein kinases (MAPKs) and transcription activities of NF- κ B

To elucidate the mechanism of action of fisetin in suppressing IL-31 production, the phosphorylation of MAP kinases (p38, ERK, and JNK) and the activation and translocation of NF- κ B/p65 protein to the nucleus in PI-stimulated cells were examined. In HMC-1 cells, phosphorylations were detected 30 min after stimulation with PI. PI stimulation led to phosphorylation of MAPKs and also phosphorylation and translocation of NF- κ B/p65 protein from the cytosol to the nucleus. However, Fisetin significantly and dose-dependently suppressed phosphorylation of p38, ERK, and JNK in stimulated HMC-1 cells (Fig. 4A, B, C, D). Next, to confirm if the activations of MAP kinases and NF- κ B actually mediates production of IL-31 in HMC-1, the effects of JNK (SP600125), ERK1/2 (PD98059), p-38 (SB203580), and NF- κ B (P8765)-specific inhibitors on PI-stimulated HMC-1 were investigated. In line with the effects of fisetin, pretreatment with these potent inhibitors at nontoxic concentrations (cytotoxicity data not shown) blocked IL-31 production (Fig. 4E). In addition, Fisetin significantly and dose-dependently inhibited nuclear translocation of NF- κ B. The activated forms of cytoplasmic I κ B- α (p-I κ B- α) and cytoplasmic and nuclear NF- κ B (p-NF- κ B) was also significantly and dose-dependently decreased when cells were pre-treated with fisetin (Fig. 5).

Cells (5×10^5 cells/mL) were pre-treated with fisetin (2.5, 5, and 10 μ M) and inhibitors (for ERK1/2, JNK, p-38, and NF- κ B) for 1 h prior to PMA (50 nM) + A23187 (1 μ M) stimulation for 30 min and 12 h respectively. Western blotting analyzed activations of ERK1/2, JNK, p-38, and NF- κ B (A) and band quantification (B, C, D) was done using ImageJ software. ELISA was used to measure IL-31 after treatment of cells with various inhibitor. Each bar represents the mean $\pm$ SD of three measurements. The statistically significant difference, * P < 0.05 and *** P < 0.001 between the treated cells and the positive control cells.

Cells were pre-treated with fisetin (2.5, 5, and 10 μ M) for 1 h prior to PMA (50 nM) + A23187 (1 μ M) stimulation for 30 min. NF- κ B activation and translocation were assayed by Western blot (C-NF- κ B, cytoplasmic NF- κ B; C-p-NF- κ B, cytoplasmic p-NF- κ B; C-I κ B- α , cytoplasmic I κ B- α ; C-p-I κ B- α , cytoplasmic p-I κ B- α) in the cytoplasm (A) and in the nucleus (B). Band quantification (C, D, E, F) was done using ImageJ software. Each bar represents the mean $\pm$ SD of three measurements.

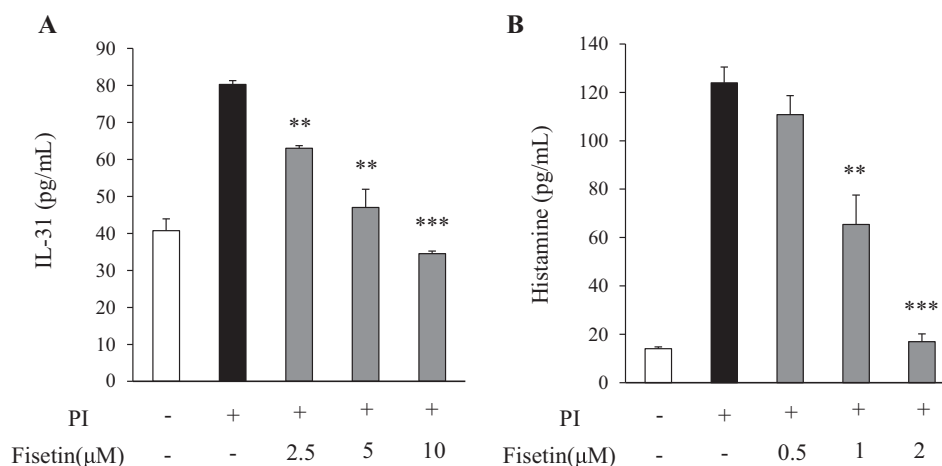


Fig. 3. Effect of fisetin on IL-31 and histamine production in stimulated HMC-1 cells.

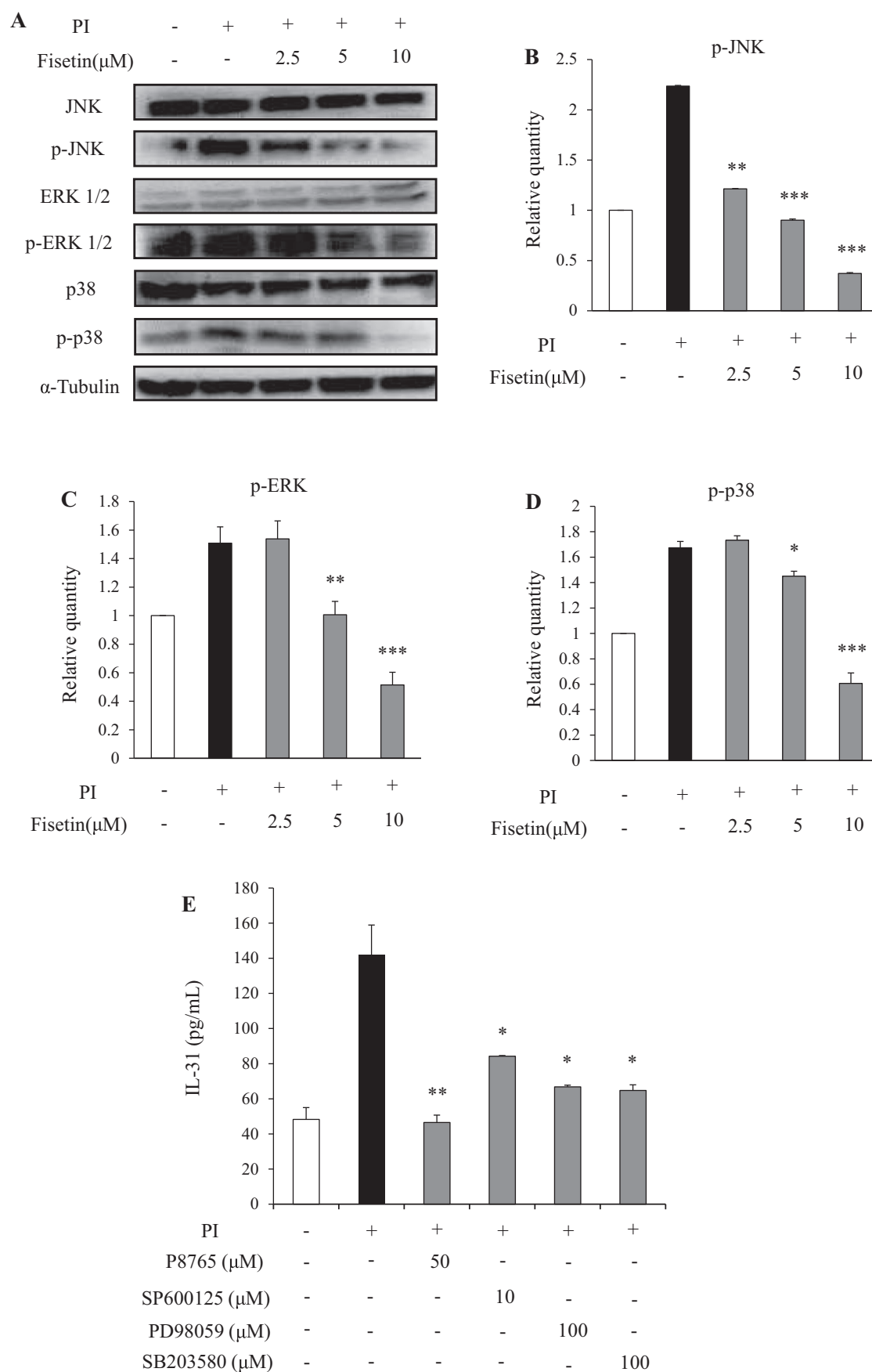


Fig. 4. Effect of fisetin on activation of MAP Kinase in stimulated HMC-1 cells.

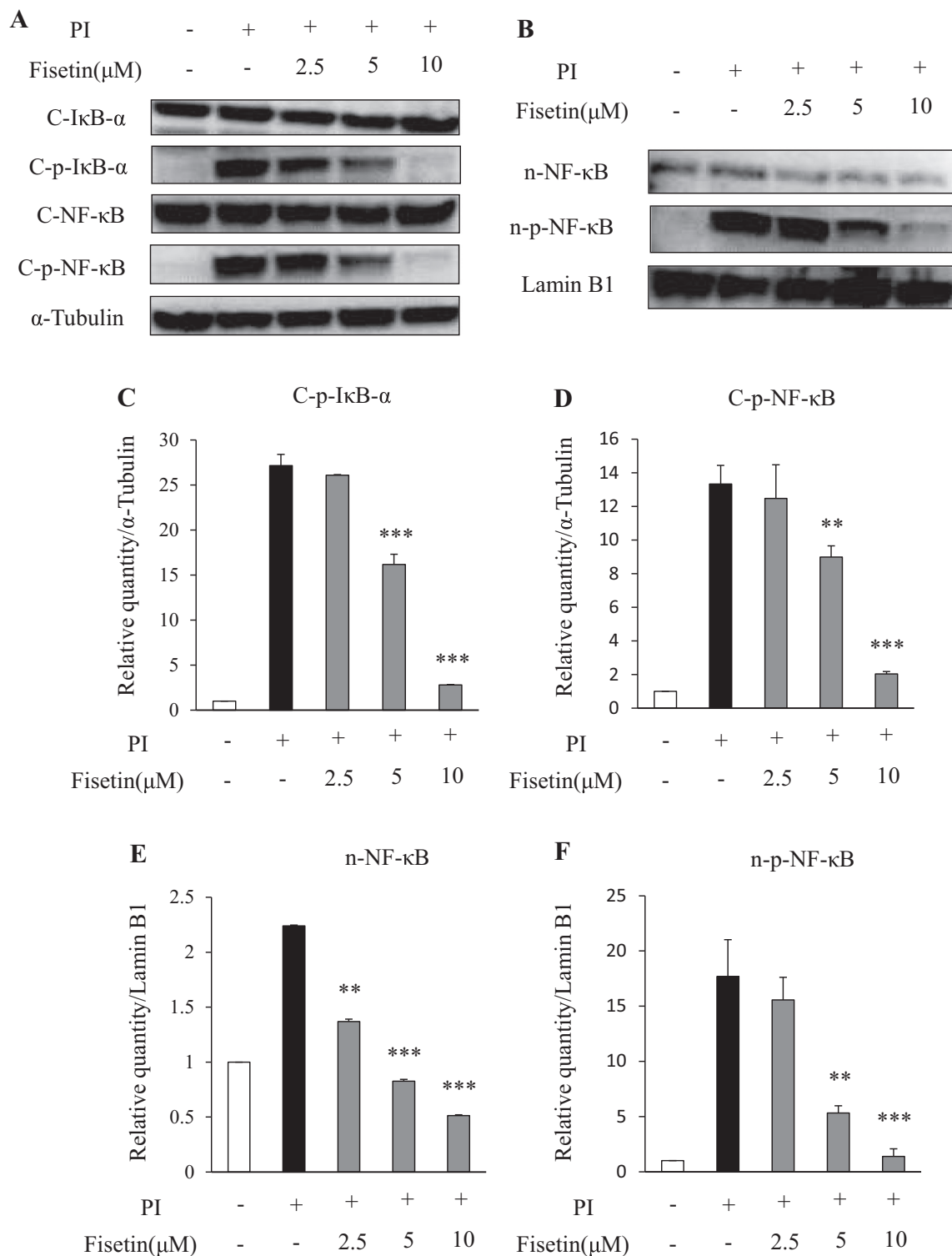


Fig. 5. Effect of fisetin on transcription activities of NF- κ B in stimulated HMC-1 cells.

The statistically significant difference, ** $P < 0.01$ and *** $P < 0.001$ between the treated cells and the positive control cells.

3.6. Fisetin reduces compound 48/80-induced itch in mice skin

The antipruritic effect of fisetin was also investigated in a compound 48/80-induced ICR mouse pruritus model. The control mice pretreated with saline exhibited limited scratching behavior, while subcutaneous

injection of compound 48/80 caused intense scratching in the ICR mice. Intraperitoneal pre-treatment with fisetin at dosages of 25 and 50 mg/kg inhibited compound 48/80-induced scratching. However, a statistically significant ($P < 0.01$) reduction of scratching behavior was seen only in mice pre-treated with 50 mg/kg of fisetin (Fig. 6A). Further histological examination of frozen skin tissues from the site of compound 48/80 injections reviewed a decrease in the infiltration of cells to the skin when mice were pretreated with Fisetin (Fig. 6B). Particularly,

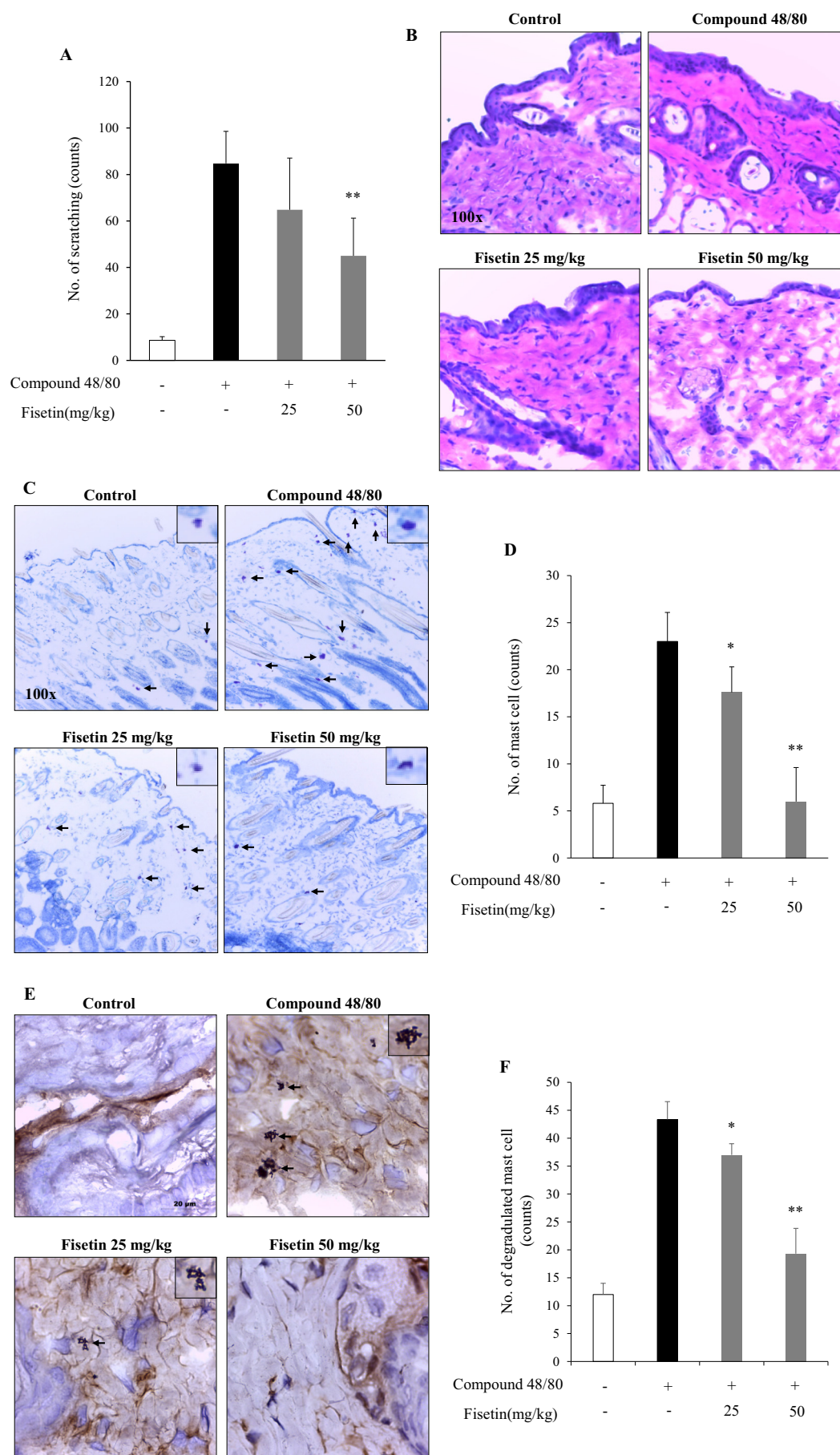


Fig. 6. Effects of fisetin on compound 48/80-pruritus in ICR mice.

Toluidine blue and immunohistochemistry staining of frozen tissue sections showed a statistically significant ($P < 0.05$) decreased in the infiltration and degranulation of mast cells in the mice skin administered with fisetin (Fig. 6C, D, E & F).

Five mice each were placed into four groups: compound 48/80 (–); compound 48/80 (+); compound 48/80 with fisetin 25 mg/kg; compound 48/80 with fisetin 50 mg/kg. Sixty minutes before injection of 50 µg/site (0.1 mL each) of compound 48/80 to subcutaneous layer of the mice skin, the mice were administered intraperitoneally with fisetin at the indicated concentration. (A) The number of scratches were monitored with micro-cameras for 60 min and counted in a double-blinded manner. (B) Skin tissue sections were stained with hematoxylin/eosin stains showing infiltration of cells. (C) Representative Skin tissue sections were stained with toluidine blue showing mast cells. (D) Mast cells were counted in 7 high power fields from each mice skin sections (E) Representative immunohistochemistry-stained skin tissue sections showing degranulated mast cells. (F) Degranulated mast cells were counted in 30 high power fields at 100× oil immersion magnification from each mice skin sections. Arrows indicate mast cells. Where applicable, data are presented as the mean ± SEM of five mice per group. The statistically significant difference at * $P < 0.05$, ** $P < 0.01$ between the treated groups and PI group.

4. Discussion

IL-31 has been directly implicated in pruritus found in patients with AD as it is expressed in mice AD model experiencing pruritus than in mice AD model without pruritus [4]. This is true as Rukwied et al., found that cetirizine, a histamine blocker could not abolish compound 48/80-induced pruritus in patients with AD [8] thus implying that other factors could be responsible for pruritus in AD. Fisetin, on the other hand, is known for its antidiabetic, anti angiogenic and anticarcinogenic properties by suppressing inflammatory processes that are associated with these diseases [16,17]. However, little or no study has been conducted to investigate the effect of fisetin in the regulation of IL-31, histamine and pruritus. Therefore, the present study sought to investigate the effect of fisetin on PI-induced HMC-1 activation vis-à-vis the production and release of IL-31 and histamine, and also to evaluate the effect fisetin has on compound 48/80-induced itch in ICR mice.

According to the present results, fisetin which showed no cytotoxicity to HMC-1 cells up to 80 µM (data not shown), suppressed the expression of IL-31 mRNA in stimulated HMC-1 cells. Further experiments showed that fisetin as well suppressed the expression of the IL-31 protein and the release of IL-31 cytokine. These results imply that the suppression mechanism of IL-31 by fisetin in HMC-1 at least begins with the regulation of IL-31 mRNA production. Fisetin had previously been reported to suppress several other cytokines like IL-1β/α, IL-4, IL-5, IL-6, IL-13, and TNFα [16–18]. MAPK cascade is the signaling pathway in the immune response that has an essential role in the intracellular signal network and regulates the expression of cytokines [19]. This makes MAPK a major target for the treatment of inflammatory and allergic diseases. Therefore, to further understand the molecular mechanism underlying the suppressive effects of fisetin on IL-31 production, the effect of fisetin on the activation of MAPKs in HMC-1 was confirmed as previous studies have shown the suppression of MAPKs in HMC-1 by fisetin (12, 13). To verify if MAPKs actually signals IL-31 production in PI-stimulated HMC-1 cells, we treated stimulated cells with potent inhibitors of ERK1/2, JNK, and p-38. All these inhibitors significantly blocked the secretion of IL-31 in HMC-1 cells thus implying the implications of these kinases in production of IL-31 in HMC-cells. The activation and translocation of NF-κB into the nucleus binds to DNA, coordinating the expression of inflammatory genes that encode cytokines, chemokines, enzymes and adhesion molecules involved in allergic diseases [20]. Activation of IκB-α is central to the activation of NF-κB though MAPK has also been implicated in NF-κB activation [21,22]. In this study, we also confirm that fisetin inhibited the

activation of IκB-α and NF-κB and further prevented the translocation and activation of NF-κB in the nucleus. Again, to show that activation of NF-κB can lead to production of IL-31 in HMC-1, we treated stimulated cells with potent inhibitors of NF-κB. NF-κB inhibitor also significantly blocked the secretion of IL-31 in HMC-1 cells. Thus it can be concluded that the molecular mechanism of fisetin in relation to the production and secretion IL-31 directly involves the suppression of MAPK and NF-κB activation. Interestingly, a novel NF-κB-binding element within the IL-31 promoter has been shown to mediate IL-31 expression in human Th2 cells [23].

HMC-1 cells are the main cells that are responsible for histamine synthesis. Since histamine also participates in the pathogenesis of itch, the effect of fisetin on histamine release by activated HMC-1 cells was investigated. Although cross linkage of IgE receptors on HMC-1 cells by IgE antibodies releases histamine, PI has also been previously shown to stimulate histamine release in HMC-1 cells [24]. In this study, we used PI to stimulate histamine release and showed that fisetin suppresses histamine release in HMC-1 cells. This means that fisetin can be further exploited as an alternative to histamine receptor blockers used in treating pruritus and hence can be used to target histamine dependent and histamine independent pruritus. Furthermore, fisetin suppressed compound 48/80-induced itch in mice *in-vivo* studies which may be due to its suppression of the infiltration and degranulation of inflammatory mast cell to the skin. Though further and detailed *in vivo* studies will be require to show that fisetin can also reduce IL-31 expression and histamine release in mice skin, one can speculate that the action of fisetin on HMC-1 cells vis-à-vis reduction of IL-31 and histamine release could play a role in the reduction of the itch in our *in vivo* studies. This could also be true as fisetin could reduce the infiltration and degranulation of mast cells into the skin.

5. Conclusion

In conclusion, this paper demonstrates for the first time that fisetin also suppresses the production of IL-31 through the suppression of MAPK and NF-κB activation in PI-activated HMC-1; inhibits histamine release in activated HMC-1 cells and reduced compound 48/80-induced Itch in ICR mice probably through the suppression of the infiltration and degranulation of inflammatory mast cells to the skin of mice. This study thus provides new evidence that fisetin can contribute importantly to the prevention or treatment of mast cell-mediated pruritus that cannot be abolished using histamine blockers alone. Further, *in vivo* studies will be needed to demonstrate precisely whether fisetin also prevents the expression of itching-related IL-31 cytokine and to understand its mechanism of action.

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