



Extracts of the leaves of *Pyrus ussuriensis* Maxim. Alleviate itch sensation via TSLP-dependent manner in mouse models of atopic dermatitis



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ABSTRACT

Pyrus ussuriensis Maxim. commonly known as “*Sandolbae*” in Korean is a pear tree widely distributed across East Asia. Recent studies indicate that *P. ussuriensis* Maxim. leaves (PUL) have antipruritic effects. This study aimed to determine the effects of PUL extract and its fractions in decreasing the itch sensation and skin lesions in two distinct animal models of atopic dermatitis (AD) induced by dinitrofluorobenzene (DNFB) or house dust mite (HDM). Our results showed that the total ethanol extract of PUL decreased the scratching behavior in mice with DNFB- and HDM-induced AD. Moreover, the ethyl acetate fraction of PUL significantly improved the overall condition of the mice with AD induced by HDM. Further, we used HEK293T cells that express receptors and ion channels for thymic stromal lymphopoietin (TSLP), a potent pruritogen for AD, to determine the mechanisms underlying the antipruritic effects of PUL extract/fractions. Specific subfractions of the PUL strongly inhibited the increase in calcium levels induced by TSLP. In addition, the specific subfraction of PUL inhibited the TSLP-induced increase in calcium levels in cultured mouse dorsal root ganglia neurons. Thus, our results showed that the PUL extract could be effective for alleviating pruritus, and the antipruritic effects were exerted probably via the inhibition of the TSLP pathway in peripheral sensory neurons governing the itch sensation in AD.

1. Introduction

Plants belonging to the genus *Pyrus*, commonly known as pear, are believed to be originated from the western mountainous parts of China. Pears are broadly categorized into two groups, namely Occidental and Oriental pears [1]. There are 12–15 distinct species of the Oriental or so-called “Asian” pears, and they are widely distributed across Eastern Asia, including China, Japan, and Korea. *Pyrus ussuriensis* Maxim., known as “*Sandolbae*” in Korean, is an abundantly found species in Korea. The fruit of this tree is unpalatable and has various beneficial effects in alleviating cough, gout, and rheumatic arthritis [2]. Moreover, an aqueous extract of *P. ussuriensis* fruits has acaricidal activities, which suggests their application as agents to prevent the growth of mites in stored food [3]. In addition, constituents derived from the stems and leaves of this pear have antimicrobial effects [4]. *P. ussuriensis* Maxim. has strong antioxidant [5] and anti-inflammatory effects [6]. To date, however, enough information is not available about the biological effects of the extracts of plants belonging to the genus *Pyrus*,

and thus, further studies are warranted to determine the biological effects of various species of this genus.

Itch is an unpleasant sensation that evokes scratching behavior. Historically, the discovery of an endogenous pruritogen histamine necessitated research on itch and thus, resulted in the development of antihistamine agents that are widely used for alleviation of various itch-related allergic symptoms. The histamine receptor and transient receptor potential cation channel subfamily V member (TRPV1) play a significant role in the initiation and transmission of histamine-induced itch in peripheral sensory neurons [7]. Itch is not completely alleviated by antihistamine agents in certain disease conditions such as atopic dermatitis (AD), which suggests that the itch sensation involves pathways independent of histamine. Recent studies indicate that numerous itch-inducing agents (pruritogens) activate various histamine-independent itch pathways. Agents such as cowhage, chloroquine, BAM8-22, β -alanine, and interleukin 31 (IL-31) are examples of pruritogens that activate their own specific receptors and/or ion channels that do not require the action of histamine receptors.

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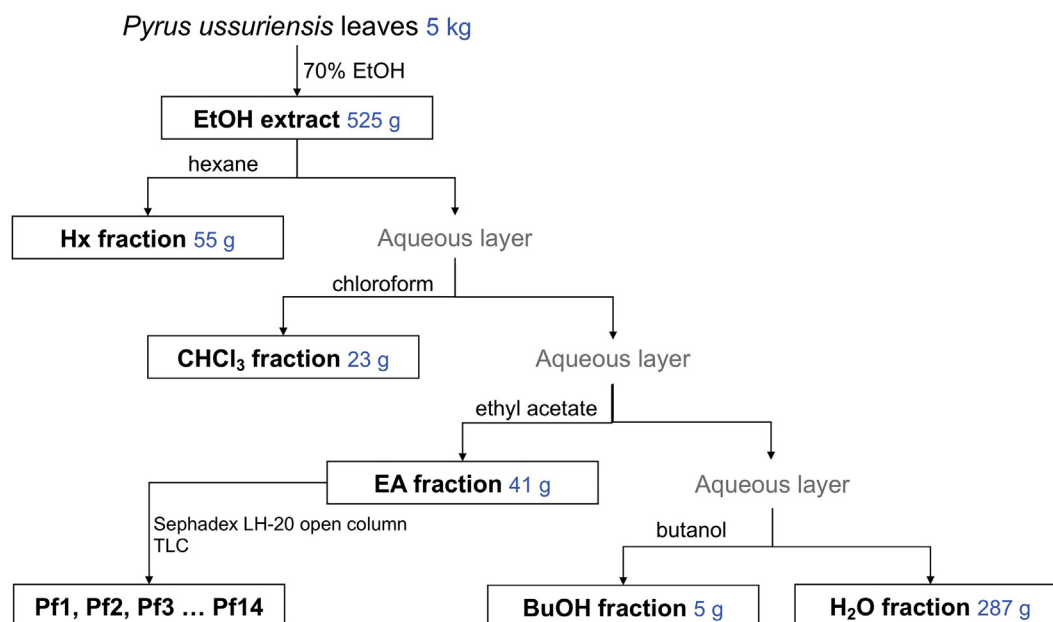


Fig 1. Schematic diagram of the preparation of *Pyrus ussuriensis* Maxim. leaves (PUL) extracts. Extract and fractions indicated in bold were used throughout the experiments.

The cytokine thymic stromal lymphopoietin (TSLP) is a histamine-independent pruritogen that has been suggested to play an imperative role in the pathogenesis of AD [8]. A recent study showed that TSLP acts directly on a subset of TRPA1-positive sensory neurons to trigger scratching behaviors in mice [9]. TSLP is mostly produced from keratinocytes, and subsequently stimulates Th2 helper cells, which can promote release of other Th2 cytokines. Under this view, it is plausible that TSLP could be the first signal in the pathogenesis of AD itch. Overall, these perspectives corroborate the importance of TSLP in the generation or transmission of AD itch.

An appropriate animal model is essential to understand the itch-scratch responses in AD. To date, various animal models of AD have been established [10], and animal models involving haptens such as dinitrofluorobenzene (DNFB) or 2,4-dinitrochlorobenzene (DNCB) for the induction of AD have been widely used. Although these mouse models have been widely used to induce AD-like symptoms, the haptens used to induce AD could be categorized as potent contact sensitizers, which evoke CD8⁺-T-cell-mediated skin inflammation [11]. Thus, DNFB-treated mice could be regarded as a model for “contact dermatitis” although both the diseases share various common AD-like symptoms such as elevated itch sensation, skin inflammation, increase in thickness of the skin, and an increase in TSLP in the skin [12].

Another animal model for AD involves induction of dermatitis by repetitive treatment of the skin of mice with debris of dead house dust mite (HDM). Allergens such as HDM are involved in the pathogenesis of human AD; therefore, an animal model with HDM-induced AD is more effective in mimicking human AD. Der p1, which is a major allergen of HDM, induces TSLP expression *in vitro* in human bronchial epithelial cells [13,14]. Epicutaneous application of HDM induced the expression of TSLP in patients with AD [15]. Thus, an animal model with HDM-induced AD is suitable for investigating the TSLP-mediated symptoms of AD.

A recent study showed that an extract of *P. ussuriensis* Maxim. leaves (PUL) ameliorates DNCB-induced dermatitis in NC/Nga mice [16]. However, the changes in the levels of various cytokines such as IL-1 β , IL-4, IL-6, and IL-13, but not of TSLP, were examined in the study. Moreover, an animal model of hapten-induced AD may not be enough to estimate the overall effect of the PUL extract on AD. Therefore, we determined whether PUL extract and its fractions alleviate the itch sensation in two distinct mouse models of AD induced by DNFB or

HDM. Furthermore, we investigated the specific extracts and/or fractions of PUL that are involved in the TSLP-mediated itch signaling pathway.

2. Materials and methods

2.1. Reagents

Mouse TSLP was purchased Sino Biological Inc. (Beijing, China). DNFB was purchased from Sigma Aldrich (St. Louis, MO, USA). For treatment with HDM, we purchased Biostir® AD (Biostir, Osaka, Japan) an ointment derived from *Dermatophagoides farinae* (HDM allergens).

2.2. Plant collection, extraction, and sample preparation

PULs were collected and extracts were prepared according to the method described by Cho et al. [16]. Briefly, the PULs were collected from Hwacheon-gun and Gangwon-do regions of Korea. The sample was identified and authenticated by Dr. Kim Seung Chul, Department of Biological Sciences, Sungkyunwan University (Suwon, Korea) and submitted to the herbarium (voucher number SU-160706001). PULs (5 kg) were dried at room temperature and were extracted in 70% ethanol at room temperature for 1 day. Then, the material was filtered and the solvent was evaporated under reduced pressure thus yielding the “total extract” of PUL (525 g). We identified fourteen chemical constituents of PUL by HPLC-Q-TOF, and the major compounds were further quantified by HPLC. HPLC analysis has indicated that the main constituents of the leaf extract of PUL are arbutin, chlorogenic acid, and rutin (Supplementary data in Cho et al., 2018). The total ethanol extract was then subjected to liquid–liquid partition successively with hexane, chloroform, ethyl acetate, butanol, and water, which yielded soluble fractions derived from hexane (55 g), chloroform (23 g), ethyl acetate (41 g), butanol (5 g), and water (287 g). Further, open column chromatography was performed by applying the ethyl acetate fraction on a sephadex LH-20 open column chromatography and eluting with a stepped gradient consisting of methanol and water. The samples obtained were then analyzed using thin-layer chromatography (TLC), and subfractions that showed similar patterns on the TLC (ethyl acetate: methanol: water = 100: 13.5: 10) were combined. We classified 14 distinct subfractions from ethyl acetate fraction and named them

sequentially as Pf1 to Pf14. The overall process for the preparation of PUL extract is illustrated in Fig. 1.

2.3. Animals

Adult male ICR mice were purchased from Koatech (Pyeongtaek gyeonggi-do, Korea). Female NC/Nga mice were purchased from Charles River (Yokohama, Japan). All mice were 8–12 weeks of age. The animals were housed with food and water available *ad libitum* and kept under controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity (60–80%) conditions in a 12-h light/12-h dark cycle. All experiments were performed in accordance with the guidelines for animals used in biological studies of Gachon University, Incheon, Korea.

2.4. Gene transfection

HEK293T cells in a lysine-coated 8-well chamber (Lab-Tek; Nunc, Rochester, NY, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . The cells were subcultured every 3 to 4 days with fresh DMEM for maintenance. Then, the cells were transfected with appropriate genes using Viafect transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Mouse IL-7R α , TSLPR, and TRPA1 were cloned from the mouse dorsal root ganglia (DRG) cDNA and subcloned into pcDNA3.1 plasmid. Each gene was transfected at a final concentration of 2 mg, and all experiments were performed 24 h after transfection. All genes showed 100% sequence identity to those in the NCBI GenBank database.

2.5. Primary culture of mouse DRG neurons

The DRG neurons were primarily cultured as described previously [7]. Briefly, the isolated mouse DRG were incubated for 30 min at 37°C with 1 mg/mL collagenase (Worthington Biochemical, Lakehold, NJ, USA), followed by an additional 30 min incubation at 37°C with 2.5 mg/mL trypsin (Gibco, Gangnam, Korea). Subsequently, the DRG neurons were cultured in neurobasal medium containing 10% fetal bovine serum, 50–100 ng/mL of nerve growth factor (Invitrogen, Gaithersburg, MD), and 100 U/mL of Zell Shield (Minerva Biolabs, Berlin, Germany) by plating on poly-L-lysine-treated 8-well glass slides, and they DRG neurons were incubated for 4 days at 37°C under 95% humidity and 5% CO_2 .

2.6. Measurement of intracellular calcium level changes

The level changes of intracellular calcium in either DRG primary cultures or HEK293T cells were measured using an inverted microscope (ECLIPSE Ti-U; Nikon, Tokyo, Japan). Right before the measurement, the culture media were replaced with normal bath solution (140 mM NaCl, 5.0 mM KCl, 2 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glucose, and 5.5 mM HEPES [pH 7.4]). Then, the cells were incubated with a calcium-specific fluorescent dye Fluo-3 AM (5 mM, Invitrogen) for 1 h in the presence of 0.1% Pluronic F-127 (Invitrogen). Subsequently, the medium was washed out, and the cells were treated with specific compounds to evoke changes of intracellular calcium levels. The calcium-specific fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 515 nm. Microscopic images were created using the Nikon NIS Elements software at 1.5-s intervals. Changes in intracellular calcium levels were expressed as the F/F_0 ratio, where F_0 is the initial fluorescence intensity. Calculation of the ratio was performed using the ImageJ program (National Institutes of Health (NIH), Bethesda, MD, USA) using custom-made scripts.

2.7. Scratching behavior tests in mice

The animal model of DNFB-induced AD was established by painting the skin of male ICR mice repetitively with 150 μL of 0.15% DNFB dissolved in acetone on the shaved nape four times (day 1, 4, 11, and 18). The control mice were painted with acetone in the same manner. Immediately after painting with DNFB, we counted bouts of scratching by hind limbs 1 h. The antipruritic effect was examined by daily intraperitoneal administration of the extracts (2 g/kg) 30 min before painting with DNFB. For establishing the animal model of HDM-induced AD, female NC/Nga mice were shaved on the back, and the hair were completely removed using a depilatory. After wiping off the depilatory, 100 mg of Biostir® AD was evenly applied on the shaved nape and both ears. Before application of Biostir® AD for the second time, the regrown hairs were shaved, and 150 μL of 4% sodium dodecyl sulfate solution was applied on the shaved nape and both ears. The mice were dried with a hair drier in cooling mode, and the solution was air-dried for 2–3 h. Then, 100 mg of Biostir® AD was evenly applied on the nape of mice on days 4, 9, 11, 16, and 18, which constitutes a total of six repetitive treatments at a two-week interval for three weeks. Immediately after treatment with Biostir® AD, we counted scratching by hind limbs for 1 h. The antipruritic effect was examined after daily oral administration of the extracts (2 g/kg). All scratching behaviors were recorded using a video camera (HDR-CX560; Sony, Tokyo, Japan). The counting and analysis were performed by an experimenter blinded to the treatment condition.

2.8. Evaluation of the skin lesion

The severity of dermatitis induced by treatment with HDM was evaluated once a week immediately before each treatment. The development of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion was scored on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe). The sum of the individual scores was taken as the dermatitis score. The dermatitis score was analyzed on day 0, 3, 5, 10, 12, 17, 19, and 21 after the start of each treatment.

2.9. Measurement of TSLP levels

TSLP levels from HDM-treated female NC/Nga mice at day 21 were measured by mouse TSLP enzyme-linked immunosorbent assay (ELISA) kit (Catalog# MTLPO0, R&D Systems, Minneapolis, MN, USA). After sera from 5 to 6 HDM-treated mice at day 21 were prepared, and the mouse TSLP levels were measured by exactly following manufacturer's protocol.

2.10. Statistical analysis

All the values are expressed as mean $\pm$ standard error mean. One-way analysis of variance (ANOVA) with Dunnett's post-test or unpaired Student's *t*-test were used for statistical analysis. Statistical significance was determined when *p* values were < 0.05 . GraphPad Prism 6.0 was used for statistical analysis.

3. Results

3.1. PUL extract decreased the scratching behavior in DNFB-treated mice

To confirm the antipruritic effects of the PUL extract and its fractions, we first used a mouse model of DNFB-induced AD. For this end, the total ethanol extract and various fractions from PUL were intraperitoneally administered 30 min before application of DNFB on the back of the mice, and the scratching behaviors of the animals were counted after 10 min. In the initial stages, such as on days 1 and 4, the total ethanol extract and fractions showed no decrease in the scratching behaviors of the DNFB models (Fig. 2A). Ironically, the aqueous

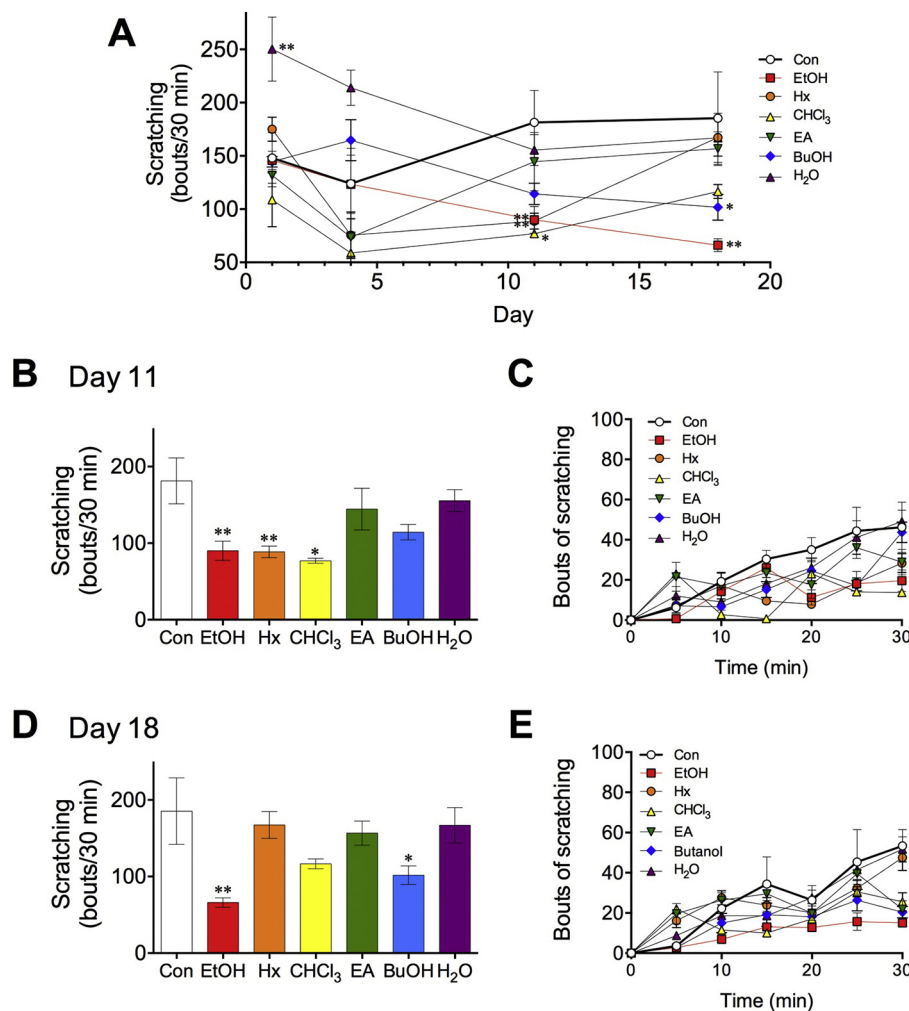


Fig. 2. Antipruritic effects of *Pyrus ussuriensis* Maxim. leaves (PUL) extract/fractions in dinitrofluorobenzene (DNFB)-treated mice. Various extract/fractions of *Pyrus ussuriensis* Maxim. leaves (PUL) were intraperitoneally administered before treatment with dinitrofluorobenzene (DNFB), and bouts of scratching were counted for 30 min and both total and time-course bouts of scratching were assessed. (A) Summary for total bouts of scratching in DNFB-treated mice on respective days. (B) Comparison of scratching bouts on day 11. (C) Time-dependent changes in scratching bouts on day 11. (D) Comparison of scratching bouts on day 18. (E) Time-dependent changes in scratching bouts on day 18. * $p < .05$, ** $p < .01$.

fraction even increased the scratching behavior on day 1 after treatment with DNFB (aqueous fraction: 250.3 ± 30.13 bouts, $n = 6$ vs. control: 148.0 ± 23.69 bouts, $n = 6$) and the reasons for this increase in scratching behavior were unknown (Fig. 2A). However, the total ethanol extract of PUL started to show a marked decrease in bouts of scratching from day 11 (total ethanol extract: 90.00 ± 12.62 bouts, $n = 6$ vs. control: 181.4 ± 30.00 bouts, $n = 5$), which implied the presence of antipruritic constituents in the total ethanol extract of PUL (Fig. 2B). In addition, the antipruritic effects of the extract/fractions were continuously observed throughout the 30-min measurement period (Fig. 2C). As shown in Fig. 2D, the total ethanol extract of PUL showed a significant decrease in the bouts of scratching on day 18 as well (total ethanol extract: 66.17 ± 5.997 bouts, $n = 6$ vs. control: 185.4 ± 43.49 bouts, $n = 5$). Although fractions from hexane, chloroform, and butanol showed some inhibitory effects on either day 11 or 18 (Fig. 2B and D), it is the total ethanol extract that consistently showed significant suppressive effects on DNFB-induced scratching behaviors in mice on both days 11 and 18. Thus, our results showed that the total ethanol extract of PUL have antipruritic effect in the mouse model of DNFB-induced AD, which was consistent with the results reported previously [16].

3.2. The total ethanol extract and the ethyl acetate fraction of PUL suppressed the scratching behavior and improved dermatitis in the HDM-induced AD mice

We then investigated whether the PUL extract can inhibit itch sensation in a different mouse AD model. To this end, HDM was

regularly applied on the back of the mice to induce AD-like symptoms, and changes in the bouts of scratching and skin lesions were recorded throughout the experimental period. For comparison, the ethyl acetate fraction of PUL was also used to compare the effect of the total ethanol extract of PUL.

Unlike the control group (369.0 ± 81.62 bouts, $n = 5$), mice administered intraperitoneally with either the total ethanol extract (114.2 ± 56.29 bouts, $n = 5$) or the ethyl acetate fraction (106.8 ± 46.87 bouts, $n = 6$) showed a significant decrease in the bouts of scratching on day 18 (Figs. 3A and B). However, while the total ethanol extract showed no improvement in dermatitis of the HDM-treated mice (6.600 ± 1.435 , $n = 5$) compared to that by the control (8.000 ± 1.225 , $n = 5$), the ethyl acetate fraction unexpectedly showed a significant improvement in dermatitis on day 21 (3.400 ± 0.8718 , $n = 5$) (Figs. 3C and D). More importantly, serum levels of TSLP were significantly decreased compared to control (26.73 ± 5.876 pg/mL, $n = 5$) when the total ethanol extract (8.228 ± 1.271 pg/mL, $n = 5$) or the ethyl acetate fraction (5.437 ± 0.8988 pg/mL, $n = 6$) were administered. Taken together, our results showed that the ethyl acetate fraction of PUL not only has antipruritic effects, but also has beneficial effects on dermatitis in HDM-treated mice.

3.3. Ethyl acetate subfractions of PUL exhibited inhibitory effect on TSLP-induced calcium increase

Since the ethyl acetate fraction of PUL showed promising effects on dermatitis in HDM-treated mice, we further examined whether the

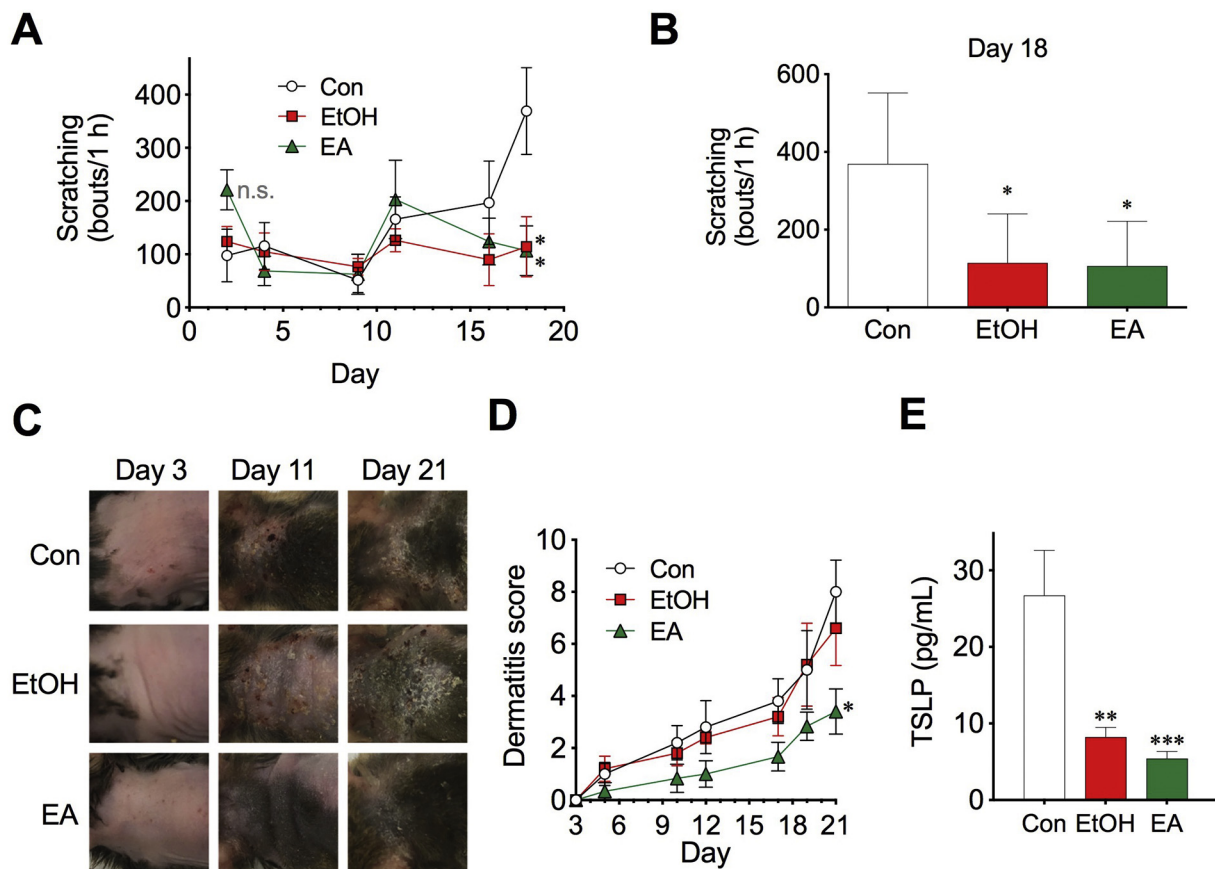


Fig. 3. The total ethanol extract and ethyl acetate fraction of *Pyrus ussuriensis* Maxim. leaves (PUL) are effective in house dust mite (HDM)-treated mice. (A) Summary for total bouts of scratching in house dust mite (HDM)-treated mice on respective days. (B) Significant decrease in bouts of scratching after oral administration of the total ethanol extract or ethyl acetate fraction on day 21. (C) Representative images of the changes in the skin on the afflicted regions of the back. The group receiving the ethyl acetate fraction group showed a marked improvement in the skin lesions. (D) Changes in the dermatitis score among separate groups of HDM-treated mice. On day 21, only the group receiving the ethyl acetate fraction showed a decrease in the score of dermatitis. (E) TSLP levels in the serum of HDM-treated mice at day 21. * $p < .05$, ** $p < .01$, *** $p < .001$, n.s. not significant.

ethyl acetate fraction of PUL may exert inhibitory effects on the TSLP-mediated itch pathway. To this end, subfractions from the ethyl acetate fraction (Pf1 to Pf14, Fig. 1) were obtained and examined if there are any inhibitory effects over TSLP-induced calcium influx. The TSLP-induced calcium signaling pathway involves three key proteins, namely, TSLPR, IL-7R α , and TRPA1 [9,17]. Thus, the HEK293T cells were transiently transfected with three cDNAs simultaneously. The TSLP-induced increase in calcium levels was the highest in the cells simultaneously transfected with three cDNAs (hereafter known as “TSLPR/IL-7R α /TRPA1”) (Fig. 4A). Thus, we used the TSLPR/IL-7R α /TRPA1 cell line throughout the experiment. We pretreated the cells with 10 μ g/mL of each subfraction for 5 min, and TSLP (5 ng/mL) was applied onto TSLPR/IL-7R α /TRPA1 cells. Compared to the control ($100.0 \pm 5.645\%$, $n = 721$), it was found that the Pf6 subfraction almost completely abolished the TSLP-induced calcium increase (peak response of Pf6: $2.513 \pm 0.6510\%$, $n = 1224$) (Figs. 4B and C). In addition, although other subfractions such as Pf9 and Pf13 showed marginal inhibitory effects on the TSLP-induced responses, the effects of these subfractions were not as significant as that of Pf6.

Thus, our results found that the Pf6 subfraction from the ethyl acetate PUL fraction has strong inhibitory effect on TSLP-induced calcium increase in the cells expressing their own specific receptors, which implies the antipruritic effects of this subfraction in the TSLP-mediated itch pathway.

3.4. An ethyl acetate subfraction of PUL inhibited the histamine-independent TSLP-induced calcium increase in the DRG

We finally examined the putative antipruritic effect of Pf6 subfraction using primary cultures of mouse DRG cells. Our results showed that compared to the control ($100.0 \pm 8.587\%$, $n = 209$), the Pf6 subfraction had no inhibitory effect on the increase in calcium levels induced by histamine ($92.47 \pm 7.794\%$, $n = 115$), which is in line with previous findings that TSLP-induced itch is not histamine-dependent (Fig. 5A). However, the Pf6 subfraction suppressed the TSLP-mediated increases of calcium levels in the DRG ($74.14 \pm 9.590\%$, $n = 62$) when compared to the control ($100.0 \pm 3.341\%$, $n = 281$). This is consistent with the calcium imaging data obtained previously in an *in vitro* study performed using TSLPR/IL-7R α /TRPA1 cells (Fig. 4). Thus, our results indicate that Pf6 subfraction derived from PUL extract may inhibit the TSLP-induced itch responses in a histamine-independent manner on a sensory neuronal level.

4. Discussion

AD is a skin disease associated with a severe vicious itch-scratch cycle. Patients often rely on the repetitive use of topical corticosteroids, which cause various side effects such as skin thinning, enlarged blood vessels, and mild addiction. Other alternative agents for the treatment of AD are immunosuppressive agents such as cyclosporin, tacrolimus, and pimecrolimus. However, the use of these agents is associated with severe side effects such as increased burning sensation and increased

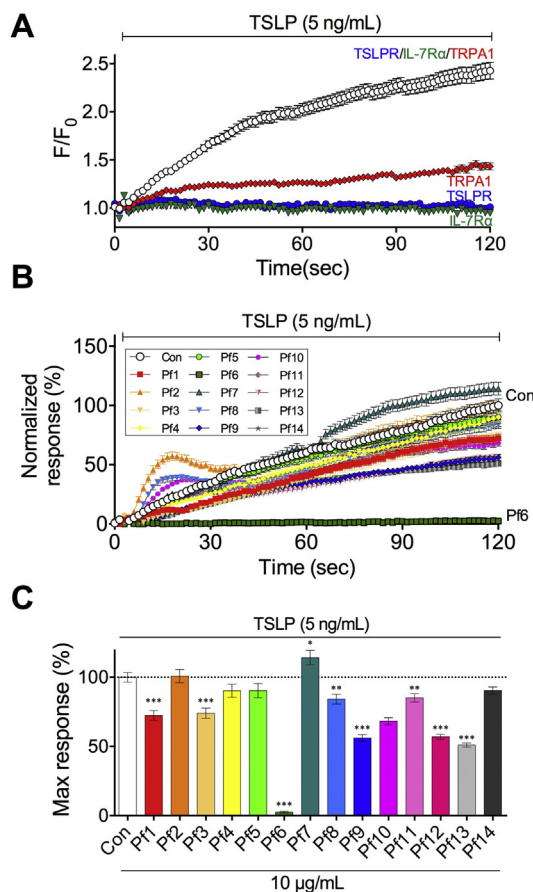


Fig. 4. Subfractions of the *Pyrus ussuriensis* Maxim. leaves (PUL) extract have inhibitory effects on thymic stromal lymphopoietin (TSLP)-induced increase in calcium levels. (A) Treatment with mouse TSLP (5 ng/mL) induced a strong increase in calcium levels particularly in the HEK293T cells expressing all three cDNAs (TSLPR/IL-7Rα/TRPA1). (B) Time-dependent changes in TSLP-induced calcium levels after pretreatment of TSLPR/IL-7Rα/TRPA1 cells with specific subfractions of the *Pyrus ussuriensis* Maxim. leaves (PUL) extract (Pf1–14, 10 µg/mL). (C) Summary of peak responses of the subfractions. * $p < .05$, ** $p < .01$, *** $p < .001$.

risks of melanoma. In addition, antihistamine agents often fail to suppress the severe itch sensation in AD patients. To date, an optimal therapeutic agent that can alleviate the symptoms of AD without severe side effects has not been discovered, and thus, further studies are required to identify novel and alternative agents for alleviating the symptoms of AD. Recent studies have shown that TSLP plays an important role in the pathogenesis of AD [9], and thus, it can be used as a novel therapeutic target for alleviating the symptoms of AD.

The present study is essentially an extension to a previous study, which showed the antipruritic effect of the PUL extract for the first time [16]. Our study differs from the previous study in that the previous study used only DNCB-treated NC/Nga mice as an animal model of AD. Typically, haptens such as DNCB and DNFB are small molecules that penetrate the epidermis and induce immune responses, which will lead to contact dermatitis. Acute topical application of these haptens on mice evokes typical Th1 responses. Repeated application of the haptens results in switching the immune fate toward Th2 responses, which are characteristic of AD-like symptoms [10]. Animal models of DNFB-induced AD present crusting and desquamation that are not typically observed in humans with AD [10]. Therefore, although an animal model of hapten-induced AD has advantages such as low cost and ease of reproducibility, this model does not entirely represent the complex aspects of AD. Thus, we used an additional animal model of AD, particularly a mouse model of HDM-induced AD. Ewald et al. compared the

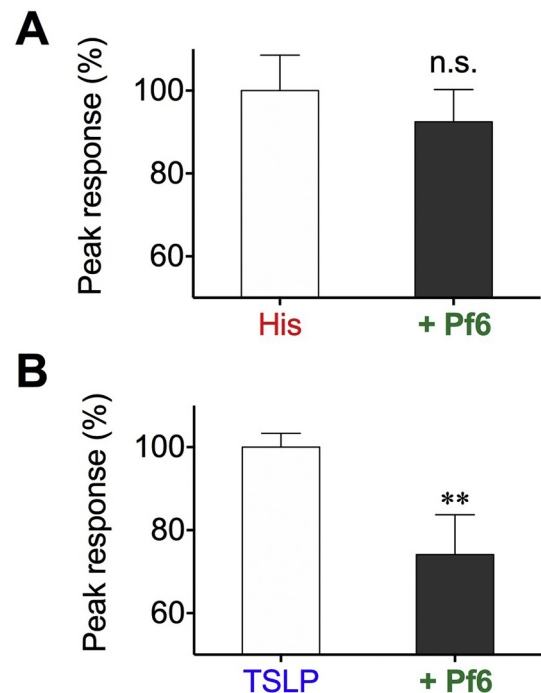


Fig. 5. Pf6 inhibited the thymic stromal lymphopoietin (TSLP)-induced increase in calcium levels in a culture of mouse dorsal root ganglia (DRG) neurons. (A) Pf6 (10 µg/mL) pretreatment did not inhibit the increase in calcium levels induced by histamine (100 µM). (B) Pf6 significantly inhibited the increase in calcium levels induced by thymic stromal lymphopoietin (TSLP, 5 ng/mL). * $p < .01$, n.s. not significant.

transcriptomic profiles of various animal models of AD to those of human AD, and their results showed that the animal model with IL-23-induced AD followed by the one with HDM-induced AD had the highest similarity with human AD [18]. Further, the IL-23-treated model is similar to the model used for studying human psoriasis; therefore, the HDM-treated model is an ideal model that mimics the features of human AD [10]. Under this view, the strength of the present study would be that it examined the antipruritic effects of the PUL extract/fractions not only in the DNFB model but also in the HDM model, which is believed to better reflect human AD.

The present study clearly found that the total ethanol extract of PUL showed consistent inhibition of scratching behaviors in the DNFB model in both day 11 and day 18 (Fig. 2). Similarly, an inhibitory effect of the total ethanol extract on the scratching behavior was found in the HDM model as well (Fig. 3A and B). In fact, it was only the total ethanol extract of PUL that consistently decreased scratching behaviors in the two distinct animal AD models, corroborating a hypothesis that the PUL extract indeed has a putative antipruritic effect.

Compared to the previous study that only used the total ethanol extract of PUL [16], the present study also used various fractions of PUL for further examination. Although administration of certain fractions of PUL (e.g. hexane, chloroform, and butanol fractions) marginally reduced bouts of scratching in DNFB-treated mice, it is intriguing that the ethyl acetate fraction of PUL showed beneficial effect only in the HDM but not in the DNFB model. To be honest, the finding that the ethyl acetate fraction had an antipruritic effect in the HDM model was beyond our expectation, since it had no effect in the DNFB model (Fig. 2). Moreover, as shown in Figs. 3C and D, the ethyl acetate fraction also significantly ameliorated the skin condition of the HDM model. Because of this unexpected strong antipruritic and beneficial effect of the ethyl acetate fraction, further separation of the ethyl acetate fraction into subfractions (Pf1–Pf14) was performed to investigate which subfraction holds the key component critical for the effect. As a result, it was

found that a specific subfraction Pf6 had the strongest inhibitory effect via TSLP-mediated manner (Figs. 4 and 5). Further studies are mandatory to identify molecular entities that are responsible for the effect found in Pf6.

Although it is challenging to identify why the ethyl acetate fraction had no effect on the DNFB model, the discrepancy may be attributed to the difference in the strains and/or sex of mice used for treatment with DNFB (ICR, male) or HDM (NC/Nga, female). In fact, it has been reported that the difference in the strains and sex in mice can markedly alter the itch sensitivity [19,20]. It is noteworthy that we used *female NC/Nga* mice to ensure the proper development of AD-like symptoms using the Biostir® AD ointment. In fact, we first attempted to apply the Biostir® AD ointment on *male ICR* mice to match the strain and sex of the DNFB model. However, induction of AD-like model with male ICR mice by Biostir® AD ointment was not successful (data not shown). These results were consistent with those reported previously that epicutaneous application of HDM on intact skin does not sensitize and initiate development of lesions in BALB/c or C57BL/6 mice [21,22]. Therefore, since it was mandatory to use *female NC/Nga* mice for proper development of HDM-induced AD, the difference in the strain as well as sex in mice between DNFB and HDM models could be a possible reason for the lack of effect when the ethyl acetate fraction of PUL was treated on DNFB model. Still, it mandates further investigation to clarify the discrepancy of the ethyl acetate extract of PUL.

TSLP binds to its specific membrane receptor complex, which is a heterodimer of two different subunits, namely, TSLPR and IL-7R α [17]. Moreover, the heterodimeric TSLP receptor in the peripheral sensory neurons is functionally linked to the TRPA1 ion channel, which plays a critical role in the itch sensation [9]. To examine the inhibitory effects of various subfractions (Pf1-Pf14) on the TSLP-mediated responses, we transiently transfected HEK293T cells with three cDNAs (TSLPR, IL-7R α , and TRPA1) (Fig. 4A). The cell line that expressed all the three cDNAs (TSLPR/IL-7R α /TRPA1) was remarkably sensitive to TSLP treatment whereas the one expressing TRPA1 alone showed marginal responses to TSLP. Further studies are required to determine whether the responses observed in cells expressing TRPA1 alone were specifically evoked by TSLP by pretreatment with a TRPA1-specific antagonist. We observed no increase in calcium levels after treatment with TSLP in HEK293T cells expressing either TSLPR or IL-7R α (Fig. 4A). Further, our results showed that treatment with TSLP induced a gradual increase in calcium levels over a 2-min period, but pretreatment with certain subfractions (e.g., Pf2, Pf8, and Pf10) induced an instant increase in calcium levels in less than 30 s after treatment with TSLP (Fig. 4B). The immediate increase in calcium levels could be attributed to direct activation of TRPA1. Moreover, a possibility cannot be ruled out that each subfraction contains varying amounts of constituents that may either decrease or increase the calcium levels. Our results showed that pretreatment with Pf6 showed a marked inhibition of the TSLP-induced increase in calcium levels (Fig. 4C). Moreover, treatment with Pf6 showed a marked inhibition of the TSLP-induced increase in calcium levels in primary cultures of DRG neurons (Fig. 5), which suggests that Pf6 may possess the core constituents responsible for the inhibitory effects of the ethyl acetate fraction of PUL. We aim to identify the specific constituents present in the Pf6 subfraction in our future studies. In addition, further studies should be performed to determine whether the putative core compound has antipruritic and anti-inflammatory effects in various models of AD.

In summary, our results showed that PUL extract/fractions have beneficial effects in animal models of DNFB- and HDM-induced AD. The ethyl acetate fraction of PUL, in particular, the Pf6 subfraction exhibited potent antipruritic and anti-inflammatory effects probably via

the TSLP-mediated pathway. Our results may provide insights for the development of novel agents derived from PULs for the treatment of AD.

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