

Original Article

Pyrus ussuriensis Maxim. leaves extract ameliorates DNCB-induced atopic dermatitis-like symptoms in NC/Nga mice

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ABSTRACT

Purpose: *Pyrus ussuriensis* Maxim. has been reported to treat the fever, cough, asthma, and chronic skin disease in Korean Medicine. However, there is no scientific evidence for the use of *Pyrus ussuriensis* Maxim. Leaves (PUL) extract or its mechanism of action in atopic dermatitis (AD). This study was performed to find the potential therapeutic effects of PUL on the progression of AD using *in vitro* and *in vivo* experimental models.

Methods: We examined the effects of PUL on the production of nitric oxide (NO) in RAW 264.7, Interleukin 6 (IL-6) and Interleukin 1 β (IL-1 β) in tumor necrosis factor α (TNF- α) -induced HaCaT cells, respectively. The PUL extract was topically administered to the 2,4-Dinitrochlorobenzene (DNCB) -treated NC/Nga mice. The potential effects of PUL extract were evaluated by measuring the dermatitis score, scratching behavior and serum levels of immunoglobulin E (IgE). The Interleukin 4 (IL-4) and Interleukin 13 (IL-13) cytokines levels were also measured in the splenocytes. In addition, the major components from PUL were analyzed using high performance liquid chromatography (HPLC).

Results: PUL extract significantly reduced the level of NO in RAW 264.7 cells, as well as IL-6 and IL-1 β in TNF- α -induced HaCaT cells. It also reduced IL-4 and IL-13 levels in splenocytes. In DNCB-treated NC/Nga mice, PUL extract significantly ameliorated the dermatitis severity, scratching tendency and transepidermal water loss (TEWL) compared to the negative control. Also, it normalized skin barriers with decreased production of IgE in mice serum. The arbutin, chlorogenic acid, and rutin were identified as major constituents of the extract by HPLC analysis. These constituents may be involved either alone or together in the regulation of atopic dermatitis.

Conclusion: Our studies indicate that PUL ameliorates atopic dermatitis-like symptoms by suppressing the proinflammatory cytokines and immune stimuli in both *in vitro* and *in vivo* animal models. Therefore, these data suggest that PUL might be an effective natural resource for the treatment of AD.

Introduction

Atopic dermatitis (AD), a chronic allergic inflammatory skin condition, occurs because of a complex interaction among immunological, environmental, and genetic mechanisms (Bin and Leung, 2016). Importantly, it is one of the most common health problems worldwide and

affects 15–30% children and 2–10% adults. In particular, AD has a high prevalence in East Asian regions (Nuttan, 2015). In the pathogenesis of AD, a specific immune response is initiated in response to environmental pathogens and the infected body shows symptoms of disease. It is well known that macrophages are important in immune responses. Macrophage activation initiates various inflammatory responses

Abbreviations: PUL, *Pyrus ussuriensis* Maxim. Leaves; AD, Atopic dermatitis; NO, Nitric oxide; IL-6, Interleukin 6; IL-1 β , Interleukin 1 β ; TNF- α , tumor necrosis factor; DNCB, 2, 4-dinitrochlorobenzene; IgE, Immunoglobulin E; IL-4, Interleukin 4; IL-13, Interleukin 13; HPLC, High performance liquid chromatography; TEWL, Transepidermal water loss; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; LPS, Lipopolysaccharide; DMSO, Dimethyl sulfoxide; PBS, phosphate-buffered saline; H&E, Hematoxylin and eosin; SEM, Standard error of the mean; ANOVA, Analysis of variance

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including the production of proinflammatory cytokines such as Interleukin 1 β (IL-1 β), Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interleukin 13 (IL-13), and tumor necrosis factor α (TNF- α) (Lyons et al., 2015). These proinflammatory cytokines trigger inflammatory and immune responses in AD skin lesions. Clinical features of AD include pruritus, erythema, dryness, relapsing eczematous skin scratches, and abnormality in skin barriers with increased production of immunoglobulin E (IgE) (Gandhi et al., 2017). Therefore, these multiple factors are considered central pathological manifestations of the pathogenesis of AD. Owing to the involvement of multiple factors in the pathogenesis of AD, there is a need to develop a multitargeted therapy for treating AD. The current and emerging treatments for AD include the use of local or systematic corticosteroids, emollients, immunosuppressants, and antihistamines with novel mechanisms (Udkoff et al., 2017). Although these drugs are used widely for AD treatment, new therapeutics are needed that target AD through multiple mechanisms.

Skin changes that occur in human AD can be induced in an *in vivo* model in NC/Nga mice by exposing them to several environmental aeroallergens (Jung et al., 2010). For this purpose, a cytotoxic and electrophilic benzene derivative named 2, 4-dinitrochlorobenzene (DNCB) is used to induce stable AD-like skin diseases in NC/Nga mice (Fujii et al., 2009). Skin changes that occur in NC/Nga mice after exposure to aeroallergens are characterized by excessive itching and rubbing behavior, followed by the fast development of erythema, leathery patches of skin, and hemorrhages (Inagaki and Nagai, 2009). Histological investigation reveals the presence of hyperplasia in skin lesions and increased serum levels of IgE (Inagaki and Nagai, 2009).

There are various traditional and complementary medicines affecting multiple targets that are currently in use for the prevention of AD. These medicines have several advantages, including fewer side effects, lower cost, and better patient compliance (Dawid-Pač, 2013). However, until now, there were few candidates for clinical use.

Pyrus ussuriensis Maxim., a member of the Rosaceae family, is one of the three major cultivated species of the genus pear, which also includes *Pyrus calleryana* and *Pyrus pyrifolia*. These species are primarily distributed in China, Japan, and Korea (Li et al., 2014). The pear is considered a fruit and an herbal medicine with anti-inflammatory, antioxidant, antitussive, and diuretic properties (Lee et al., 2010). Traditionally, it is used to treat various diseases, including fever, cough, asthma, lung disease, gastric ulcer, and constipation. It is also effective for whooping cough in children. In addition, *Pyrus ussuriensis Maxim.* has been reported to have strong antioxidant activity (Qu et al., 2016) and anti-inflammatory effects (Li et al., 2014). Therefore, it may be useful as an alternative treatment for AD. Nevertheless, there is no scientific evidence for its potential effect in AD. Therefore, the current study aimed to evaluate the therapeutic effects of PUL for AD via a multitargeted approach using various macrophages and keratinocyte cell lines as *in vitro* and *ex vivo* experimental models. Furthermore, an *in vivo* study was performed to determine the potential effects of PUL in the treatment of DNCB-induced AD-like skin lesions in NC/Nga mice.

Materials and methods

Chemical and reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), antimycotics/antibiotics (Lonza, Walkersville, MD, USA). Lipopolysaccharide (LPS), TNF- α , gries reagent, 5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), dexamethasone, arbutin, chlorogenic acid, and rutin (purity $\geq$ 95%) were used by Sigma-Aldrich chemicals (St. Louis, MO, USA). RBC lysis buffer (Intron Biotechnology, Sungnam, Korea). IL-6, IL-1 β , IL-4, IL-13, and IgE secretions using an enzyme-linked immunosorbent assay by ELISA development kit (R&D System, Minneapolis, MN, USA and eBioscience, San Diego, CA, USA).

Plant collection, extraction, and sample preparation

PUL was collected from Hwacheon-gun in the Gangwon-do Province of South Korea in July 2016. The sample was identified and authenticated by Dr. Kim Seung Chul, Department of Biological Sciences, Sungkyunwan University, Suwon, South Korea and submitted to the herbarium with Voucher Number SU-160706001. The sample was dried at room temperature. Thereafter, 5 kg of plant material was powdered, soaked in 70% ethanol for three days, extracted, filtered, and concentrated using a rotary evaporator. The freeze-dried extract was kept in the refrigerator at -4°C until use. The yield was 10.5%.

Cell culture

For this study, two cell lines, RAW 264.7 and human HaCaT cells, were purchased from the Korean Cell Line Bank, Seoul, Korea. RAW 264.7 cells were used for nitric oxide (NO) evaluation and human HaCaT cells were used to evaluate IL-6 and IL-1 β expression. Cells were cultured and maintained in DMEM containing 1% penicillin (1×10^5 U/L), streptomycin (100 mg/ml), and 10% FBS at a temperature of 37°C in a humidified incubator with 5% CO_2 , according to the method described by Robbins et al. (Hwang et al., 2016).

Cell viability assay

An MTT assay was used to measure cell viability according to the method described by Hwang et al. (2016). Cells were cultured in a 96 well plate and treated with different concentrations of PUL with or without LPS. After 24 h, media were removed and MTT solution was added. The cells were incubated again for an additional 1 h. After incubation, the media were removed and 200 μl of DMSO was added to each well. The optical density was recorded at 570 nm.

Measurement of NO production induced by LPS in RAW 264.7 cells

The inhibitory effect of PUL on LPS-induced NO was evaluated in RAW 264.7 cells according to the method described by Han et al. with some modification (Han et al., 2016). Cells were seeded in a 96 well plate at a density of 4×10^4 cells/well and pretreated of PUL extract, followed by LPS activation for 24 h. The NO level was measured in culture media using Gries reagent. Fifty microliters of conditioned medium was mixed with 50 μl of Gries reagent and absorbance was recorded at 570 nm.

Measurement of IL-6 and IL-1 β in TNF- α -induced HaCaT cells

To measure the secretion of TNF- α -induced IL-6 and IL-1 β , HaCaT cells were seeded in a 24 well plate at a density of 2×10^5 cells/well in DMEM according to the method described by Yang et al. with some modification (Yang et al., 2016b). The cells were incubated for 24 h and then stimulated with TNF- α in the presence of PUL extract and its major constituent such as arbutin, chlorogenic acid, and rutin. After 24 h of incubation, the supernatant was collected and harvested to further evaluate IL-6 and IL-1 β secretions.

Isolation of splenocytes

Splenocytes were isolated according to the method described by Falcone et al. with some modification (Falcone et al., 1998). Spleen from C57BL/6 mice was removed aseptically and homogenized in RPMI 1640 medium supplemented with 10% FBS and 1% antimycotics/antibiotics. The homogenate was centrifuged at 1000 rpm and erythrocytes were lysed by adding RBC lysis buffer. And then, the cells were washed twice with PBS.

Measurement of IL-4 and IL-13 cytokines

For the measurement of IL-4 and IL-13 (Yang et al., 2016a), splenocytes (3×10^6 cells/ml) treated with PUL were incubated in a plate bound with anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml) antibodies for 24 h. The levels of IL-4 and IL-13 in the culture supernatant were determined using a commercially available ELISA kit according to the manufacturer's protocol.

Ethical declaration

All animal procedures were performed according to the guidelines for the Care and Use of Laboratory Animals (LCDI-2017-0038) and were authenticated by the Institutional Animal Care and Use Committee of Gachon University, Incheon, Korea.

Animal study

Four-week-old NC/Nga female mice were purchased from Orient Bio (Gyeonggi-do, Korea). Mice were kept in individual properly ventilated cages under a specific pathogen-free environment at a temperature of $22 \pm 2^\circ\text{C}$. The mice were exposed to a 12 h light/dark cycle. mice were randomly divided into five groups of nine animals as follows: a, normal control (vehicle); b, DNCB + vehicle; c, DNCB + positive control (dexamethasone 0.1%); d, DNCB + PUL 1%; e, DNCB + PUL 2.5%. For induction of AD-like symptoms and skin lesions, DNCB was applied to the dorsal skin and the back of both ears of the NC/Nga mice. At day 6, animals were shaved. At day 7, 200 µl of 1% DNCB dissolved in an acetone + olive oil mixture (3:1, v/v) was applied to the dorsal skin. Each 200 µl was applied to the back of both ears two times in a week and this was continued for five weeks. After five weeks, all five groups were treated according to their above-assigned group. The following changes were measured on the last day of the experiment: (Fig. 3A).

TEWL, hydration, and erythema of the epidermal layer of skin

Transepidermal water loss (TEWL) is a term used to measure the amount of water that passes from inside an animal's body through the epidermal layer of skin to the surrounding environment via processes of diffusion and evaporation (Noguchi et al., 2017). A Dermalab Combo system (C40000.03-189, Cortex Technology, Hadsund, Denmark) was used to measure TEWL from mouse dorsal skin under specific conditions at a temperature of approximately $21\text{--}22^\circ\text{C}$ and humidity of 50–55%. The probe was placed on the skin and stabilized for approximately 30 s, then the measurement was recorded. The microprocessor was used to analyze the data and the data are presented in $\text{g}/\text{m}^2/\text{h}$. The hydration and erythema of the epidermal layer of skin were measured using the Dermalab Combo system (C40000.03-189, Cortex Technology, Hadsund, Denmark). The measurement was performed from the seven weeks of the experiment.

Skin lesions and dermatitis score

Mice were anesthetized using 2% isoflurane and images of the skin lesions were captured on the last day of the experiment using a digital camera (Coolpix A300, Nikon imaging Korea, Seoul, Korea). The level of dermatitis was evaluated once a week according to a previously described method with a slight modification (Noguchi et al., 2017). A score was calculated for each of the four symptoms, including A, edema; B, erythema/hemorrhage; C, erosion/excoriation; and D, dryness. The score was recorded as follows: 0 for none, 1 for mild, 2 for moderate, and 3 for severe conditions. The overall sum indicated the clinical severity.

Scratching behavior

Scratching behavior was determined according to a previously described method (Noguchi et al., 2017) by calculating the time spent on rubbing by each infected animal with the help of hind paws over 20 min. Scratching behavior was recorded on the last day of the experiment using a digital camera (Coolpix A300, Nikon imaging Korea, Seoul, Korea).

Measurement of IgE chemokine

IgE chemokine was measured according to a previously described method with some modification (Yang et al., 2016a). Blood and dorsal skin were collected on the last day of the experiment and kept at -80°C until use. An ELISA kit was used for the measurement of serum IgE levels.

HPLC analysis of PUL

High performance liquid chromatography (HPLC) analysis was performed using a Waters system (Waters Corp., Milford, MA, USA) connected to separation modules (e2695) with a photodiode array detector. One gram of dried powdered PUL was extracted using 10 ml of methanol. The sample was sonicated at a temperature of 40°C for 60 min. After centrifugation, the sample was filtered and methanol was added to a final volume of 10 ml. The samples were kept at -4°C until use. For HPLC analysis, 1 ml of sample was used and content analysis was performed at 280 nm. Samples were separated using a C18 column (250, 4.6 mm $\times$ 5 µm). The mobile phase consisted of A) methanol and B) 0.1% acetic acid with the gradient flow as follows: 0–20 min, 5–15% A; 20–40 min, 15–25% A; 40–60 min, 25–35% A; 60–80 min, 35–45% A; 80–100 min, 45–55% A; 100–110 min, 55–95% A; 110–115 min, 95–5% A with a flow rate of 1 ml/min. The column temperature was set at 30°C . The injected volume was 10 µl. Arbutin, chlorogenic acid, and rutin were used as standard compounds at a concentration of 1 mg/ml of each standard to identify the peaks.

Statistical analysis

SPSS statistics software was used for statistical analysis and the data was expressed as a mean $\pm$ standard error of the mean (SEM). For comparison of differences among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's honest significance test was used. The differences among the multiple groups were considered significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results

PUL reduced the NO production induced by LPS in RAW 264.7 cells

An inhibitory effect of PUL was noted in LPS-treated RAW 264.7 cells. The PUL extract showed promising effects by lowering NO production to 69.9% compared to negative control at a dose of 100 µg/ml. The inhibitory effect on NO production was dose-dependent and there was no cellular toxicity (Fig. 1A and B).

PUL reduced IL-6 and IL-1β in TNF-α-treated HaCaT cells

An inhibitory effect of PUL on IL-6 and IL-1β production was observed in TNF-α-treated human HaCaT cells. The PUL extract showed promising effects by lowering IL-6 levels to 48.4% and IL-1β levels to 89.7% compared to negative control at a dose of 25 µg/ml. The inhibitory effect of PUL on inflammatory cytokines was dose-dependent (Fig. 1C and D).

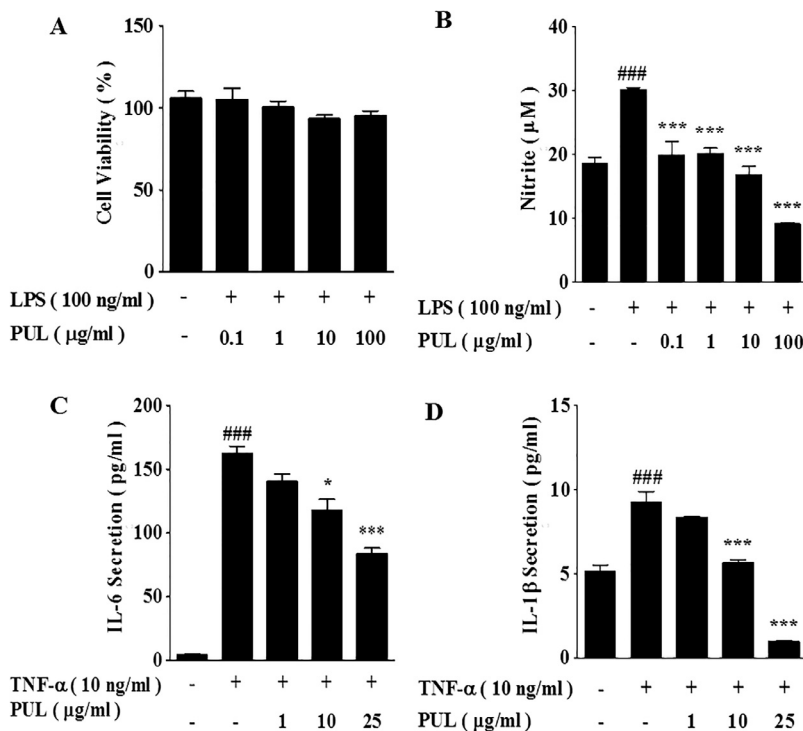


Fig. 1. Effect of PUL extract on cell viability, NO production in RAW 264.7 macrophages and IL-6, IL-1β secretion in HaCaT cell were treated with various doses of extract as indicated. (A) Cell viability. (B) Effect on LPS-induced NO production. (C) Effect on TNF-α-induced IL-6 production. (D) Effect on TNF-α-induced IL-1β production. (A,B) Results are expressed as mean ± SEM, ****p* < 0.001 vs. LPS group, ###*p* < 0.001 vs. untreated group. (C,D) **p* < 0.05 and ****p* < 0.001 vs. TNF-α-treated group. N = 3.

PUL reduced IL-4 and IL-13 in splenocytes

To test the effects of PUL on Th2 cytokine production, including IL-4 and IL-13, splenocytes were treated with PUL at various concentrations. There was no cytotoxicity (data not shown) and the splenocytes were stimulated with anti-CD3 and anti-CD28 for 24 h. The levels of IL-4 and IL-13 significantly increased up to approximately 95.10 and 479.43 pg/ml, respectively, whereas naïve cells showed minimum levels. In the presence of 8 μg/ml PUL, productions of IL-4 and IL-13 were suppressed by 23.68% and 68.95%, respectively, compared with that in anti-CD3 and anti-CD28 stimulated cells. (Fig. 2A and B).

PUL improved skin TEWL in DNCB-induced NC/Nga mice

To determine the effects of PUL on DNCB-induced skin TEWL in the epidermal skin layer of NC/Nga mice, skin hydration was evaluated between weeks 5 and 11. On the last day of the experiment, a decrease in skin hydration was observed in the DNCB-induced NC/Nga mice compared with that in the normal control (6.72 ± 1.00 g/m²h). In contrast, a marked decrease in skin TEWL was recorded after treatment with dexamethasone 0.1% (18.06 ± 1.04 g/m²h), PUL 1% (18.5 ± 0.74 g/m²h), and PUL 2.5% (17.25 ± 0.39 g/m²h) in DNCB-treated mice (30.98 ± 5.23 g/m²h) (Fig. 3B).

PUL improved skin hydration in DNCB-induced NC/Nga mice

To determine the effects of PUL on DNCB-induced skin hydration in the epidermal skin layer of NC/Nga mice, skin hydration was evaluated between weeks 5 and 11. On the last day of the experiment, a decrease in skin hydration was observed in the DNCB-induced NC/Nga mice compared with that in the normal control (90.57 ± 8.59 μs). In contrast, a marked improvement in skin hydration was recorded after treatment with dexamethasone 0.1% (121.25 ± 14.40 μs), PUL 1% (72.83 ± 2.67 μs), and PUL 2.5% (81 ± 8.91 μs) in DNCB-treated mice (43.28 ± 3.40 μs) (Fig. 3B).

PUL decreased erythema in DNCB-induced NC/Nga mice

To determine the effects of PUL on DNCB-induced erythema through the epidermal skin layer in NC/Nga mice, erythema was measured between weeks 5 and 11. On the last day of experiment, an increased erythema level was observed in the DNCB-induced NC/Nga mice compared with normal control (6.61 ± 0.58 RI), whereas a markedly decreased erythema level was recorded after treatment with dexamethasone 0.1% (11.25 ± 0.76 RI), PUL 1% (9.21 ± 0.51 RI), and PUL 2.5% (8.58 ± 0.34 RI) in DNCB-treated mice (9.83 ± 0.77 RI) (Fig. 3B).

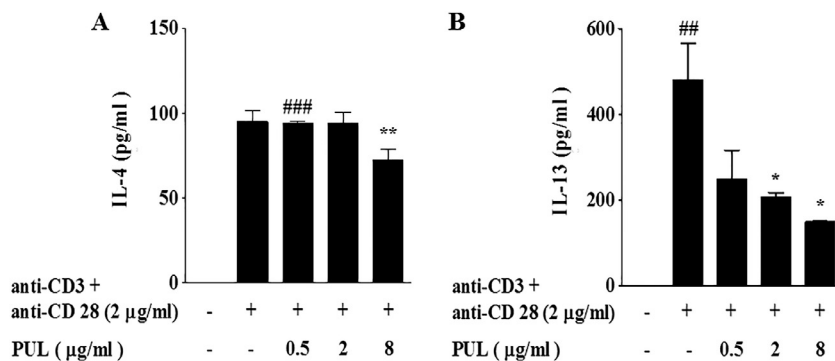


Fig. 2. Effects of PUL extract on cytokine production in splenocytes. Splenocytes were treated with various doses of extract as indicated for 1 h, then stimulated with plate-coated anti-CD3 and anti-CD28 for 24 h. The levels of (A) IL-4 and (B) IL-13 in splenocytes were determined using ELISA. The data were determined in triplicate and represent the mean ± SD. ###*p* < 0.001 vs. untreated group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. anti-CD3 and anti-CD28 group using one-way ANOVA.

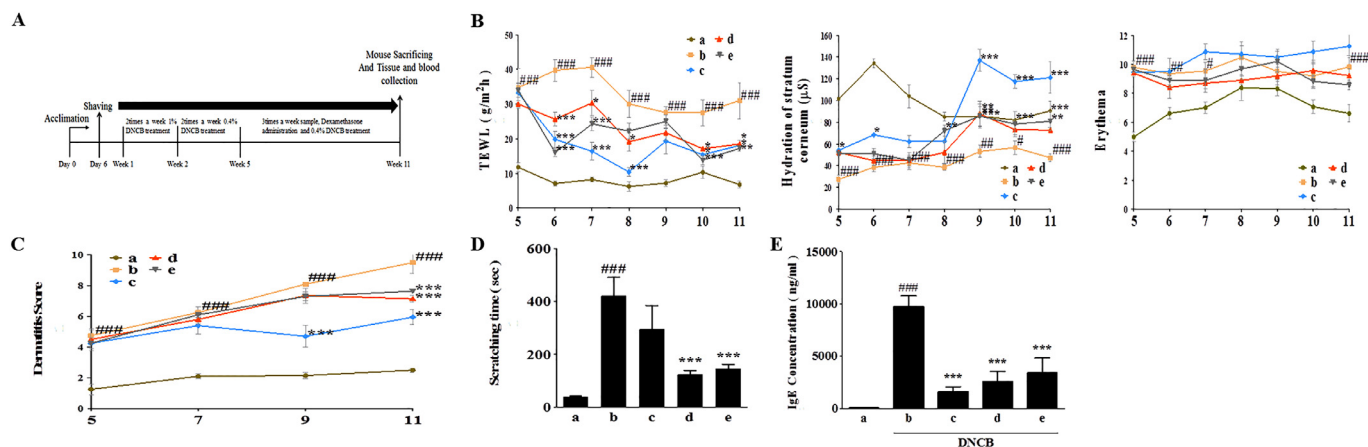


Fig. 3. Experimental schedule and effects of PUL extracts on the pathological signs, TEWL, stratum corneum hydration and erythema, and IgE kit assay of atopic dermatitis in DNCB-induced NC/Nga mice. NC/Nga mice were treated dorsally with PUL. (A) Experimental schedule for the induction of AD lesions. (B) TEWL, Stratum corneum hydration, and Erythema. (C) Weekly clinical skin severity scores. (D) Scratching time on the last day of the experiment. (E) IgE levels were evaluated on the last day of the experiment using ELISA kits. Data represent the mean $\pm$ SEM. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. DNCB-only treated group; ### p < 0.001 vs. untreated group. N = 7. (a) untreated group; (b, c, d, e) DNCB-treated groups; (c) positive control (Dexamethasone 0.1%) treated group; (d) PUL 1% treated group; (e) PUL 2.5% treated group. AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; TEWL, transepidermal water loss.

PUL ameliorated DNCB-induced AD-like symptoms in NC/Nga mice

To determine the effects of PUL on DNCB-induced AD-like symptoms in NC/Nga mice, skin lesion images were captured, and the dermatitis level was evaluated. On the last day of the experiment, the dorsal skins of DNCB-treated NC/Nga mice exhibited a significantly severe level of erythema, erosions, and dryness. Reduced AD-like symptom severity was found in the DNCB-treated NC/Nga mice treated with dexamethasone 0.1% and PUL (Fig. 4A). A higher dermatitis score was observed in DNCB-treated NC/Nga mice (9.5 ± 0.5) compared with the normal controls (2.5 ± 0.1) (Fig. 3C). In contrast, a significantly reduced dermatitis level was observed in those treated with dexamethasone 0.1% (5.95 ± 0.35), PUL 1% (7.15 ± 0.15), and PUL 2.5% (7.65 ± 0.05) (Fig. 3C). Treatment with PUL at a dose of 2.5% resulted in a 19.5% decrease compared to negative control.

PUL reduced scratching tendency in DNCB-induced NC/Nga mice

To determine the antipruritic effects of PUL on DNCB-induced scratching behavior in NC/Nga mice, animals were monitored and the time spent by each mouse on rubbing their dorsal skin and ears with

their hind paws was calculated as scratching time. On the last day of the experiment, a markedly increased scratching time was observed in DNCB-treated NC/Nga mice (419.77 ± 71.56 s) compared with that of the normal controls (36.55 ± 6.23 s), whereas a significantly reduced scratching time was observed after dexamethasone 0.1% (293.30 ± 90.90 s), PUL 1% (122.58 ± 16.32 s), and PUL 2.5% (144.21 ± 17.06 s) treatment in DNCB-treated mice (419.77 ± 71.56 s) (Fig. 3D). Treatment with PUL at a dose of 1% resulted in a 70.8% decrease in scratching time compared to negative control.

PUL reduced serum IgE levels in DNCB-induced NC/Nga mice

To determine the effects of PUL on DNCB-induced serum IgE levels in NC/Nga mice, a blood sample was collected on the last day of the experiment and IgE levels were evaluated. A markedly increased level of serum IgE was observed in DNCB-treated NC/Nga mice (9750.86 ± 516.38 ng/ml) compared with the normal control value (43.47 ± 14.76 ng/ml), whereas a significantly reduced serum IgE level was recorded after dexamethasone 0.1% (1590.27 ± 191.87 ng/ml), PUL 1% (2559.44 ± 341.45 ng/ml), and PUL 2.5%

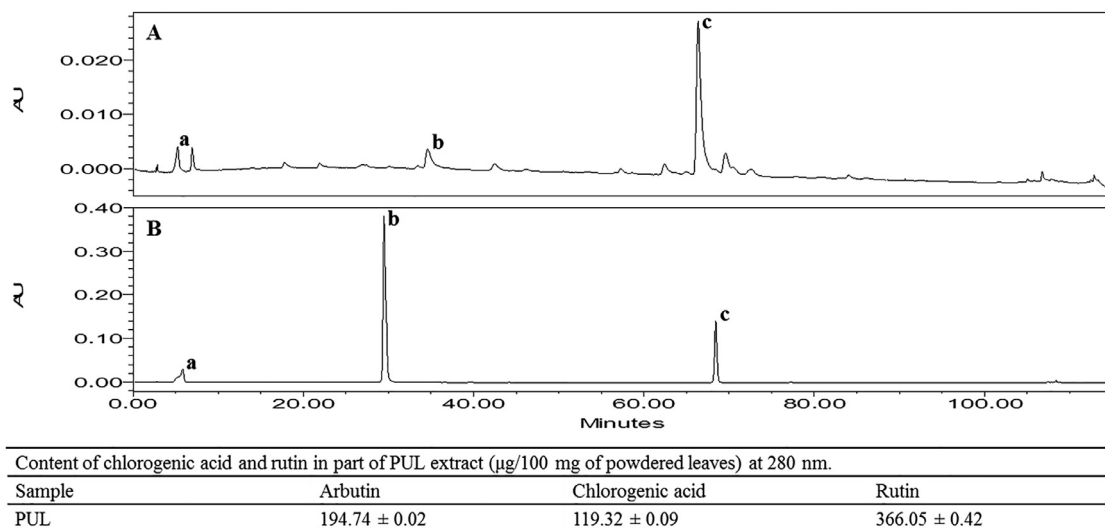


Fig. 4. (A) Chromatogram of PUL extract; (B) chromatogram of standard mixture; a) Arbutin; b) Chlorogenic acid; c) Rutin.

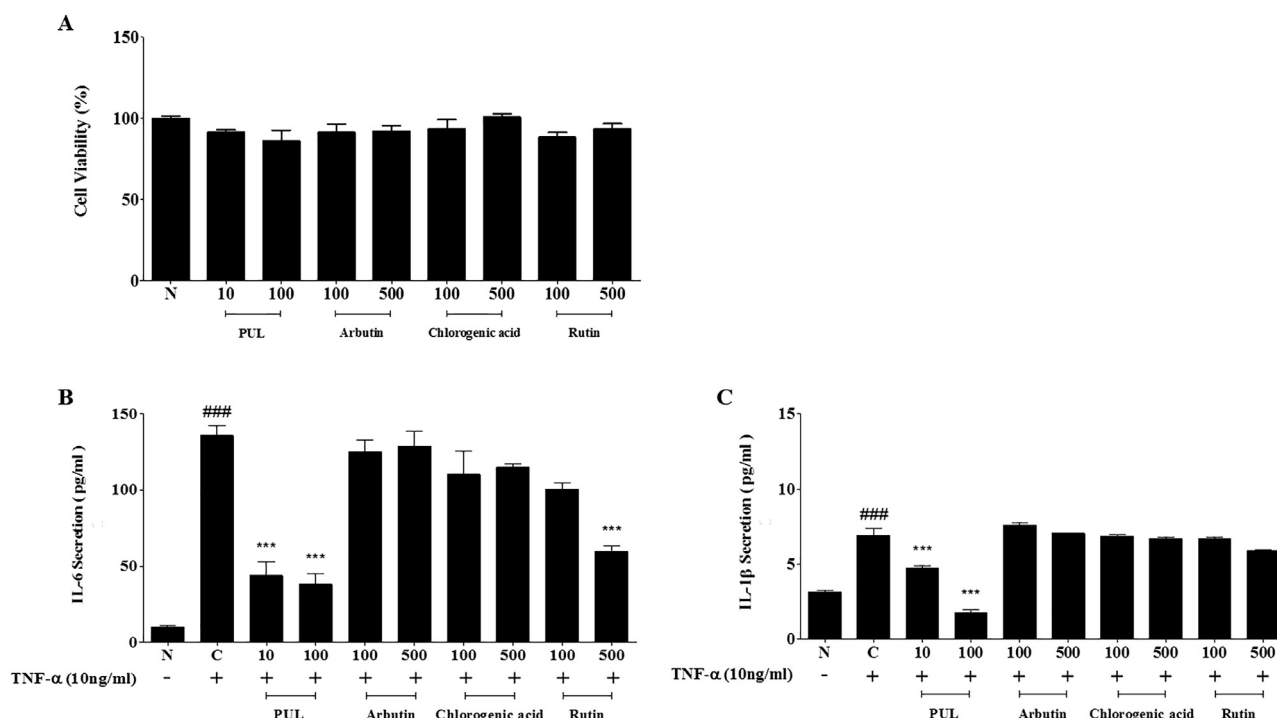


Fig. 5. Effect of PUL, arbutin, chlorogenic acid, and rutin on Cell viability (A), IL-6 (B), and IL-1 β (C) induced by TNF- α in HaCaT cells. Results were expressed as mean $\pm$ SEM, *** p < 0.001 vs. LPS group, ### p < 0.001 vs. untreated group. (B,C) * p < 0.05 and *** p < 0.001 vs. TNF- α -treated group. n = 3.

(3404 $\pm$ 580.51 ng/ml) treatment in DNCB-treated mice (Fig. 3E). Treatment with PUL at a dose of 1% resulted in a 73.8% decrease in IgE level compared to negative control.

HPLC analysis of PUL extract

HPLC-UV analysis indicated the major constituents at 280 nm and the observed peaks were assigned as arbutin (1), chlorogenic acid (2), and rutin (3) by comparing their retention time with standard compounds in the chromatogram (Fig. 4). The content analysis indicated that PUL contained 194.74 $\pm$ 0.02 μ g/100 mg arbutin, 119.32 $\pm$ 0.09 μ g/100 mg chlorogenic acid, and 366.05 $\pm$ 0.42 rutin μ g/100 mg of powdered PUL (Fig. 4).

PUL reduced IL-6 and IL-1 β in TNF- α -treated HaCaT cells

An inhibitory effect of PUL extract and its major constituents on IL-6 and IL-1 β production was observed in TNF- α -treated human HaCaT cells and no significant activity were found in comparison with the PUL extract (Fig. 5).

Discussion

AD, an inflammatory skin disorder, is increasingly common among children worldwide (Lyons et al., 2015). Owing to multiple causative factors associated with the complex pathogenesis of AD, the “one target, one molecule, one gene” concept common in current drug development could not be followed and novel drugs with multitargeted mechanisms are required for the treatment of AD. In addition, currently used anti-atopic agents have severe side effects that limit their use (Kang et al., 2016). Recently, it has been reported from various experiments and observations that natural immunomodulators derived from traditional herbal medicine contain multiple active compounds that can significantly affect multiple targets involved in the pathogenesis of AD (Tabassum and Hamdani, 2014; Tan et al., 2013). Previous studies with *Pyrus ussuriensis Maxim.* indicated that leaf extracts possessed

antioxidant and anti-inflammatory properties (Zhao et al., 2016).

Overexpression of IL-1 β , IL-4, IL-6, and IL-13 are linked to AD and are involved in the induction of pathological responses (Nedoszytko et al., 2014). LPS and TNF- α stimulate the pro-inflammatory cytokines, including NO, IL-6, and IL-1 β (Hay et al., 2012), that initiate inflammation. IL-4 and IL-13 are linked and considered crucial players in the pathogenesis of AD. Our results indicated that *Pyrus ussuriensis Maxim.*, one of the species of the genus *Pyrus*, suppressed AD in *in vitro* and *ex vivo* experimental models, possibly owing to the down-regulations of NO, IL-1 β and IL-6 in immune cells. These pro-inflammatory cytokines exert inflammatory effects, which are further involved in the regulation of various inflammatory and immune responses in AD skin lesions. The IL-4 and IL-13, cytokines with the closely related biological function, are secreted by Th2 cells, continually activate mast cells to produce more IgE. Therefore down-regulation of IL-4 or IL-13 is an important target which is associated with AD. In our study, the PUL significantly decreased the productions of IL-4 and IL-13 in splenocytes. Interestingly, the suppressive effect of PUL on IL-13 production was more prominent than those on IL-4 production at the low concentration. Also, a markedly decreased level of IgE by PUL was observed in DNCB-treated NC/Nga mice serum compared with the control group. Based on our data, we can hypothesize that the suppression of IL-4 and IL-13 in splenocytes by PUL may improve Th1/Th2 immune balance to downregulate IgE production. As a result, PUL may alleviate IgE- and mast cell-related allergy reaction in AD skin lesions. Similarly, PUL also may regulate the inflammation presented in the lungs with allergy asthma.

Furthermore, activation of IL-6, IL-1 β , IL-4, and IL-13 cytokines leads to increased IgE production along with the accumulation of eosinophils and increased epidermal hypertrophy (Matsunaga and Yamauchi, 2016). Our Histological results indicated that dexamethasone 0.1% and PUL treatment reduced the infiltration of inflammatory cells, decreased hypertrophy, and decreased serum IgE production. This suggested that PUL was shown to have significant suppressive activity on all the AD skin lesions involved in the reaction of allergy.

The represent clinical features of AD include pruritus, erythema, dryness, relapsing eczematous skin scratches, and abnormality in skin barriers. (Lyons et al., 2015). Our results indicated that the topical application of PUL reduced DNCB-induced AD-like complex features in NC/Nga mice. Treatment with PUL markedly reduced the increased severity of skin lesions, the level of dermatitis, and scratching time. Using a digital camera, we counted the scratching behaviors of the animals. Digital imaging provided a more objective and accurate evaluation of scratching behaviors as compared with visual counting. Treatment with PUL at a dose of 1% resulted in a 70.8% decrease in scratching time compared to negative control. Interestingly, this scratching tendency by treatment of PUL was more decreased than that of dexamethasone 0.1% treatment. Itching is one of the most persistent symptoms of atopy. But, this study did not clarify the mechanism of action to ameliorate the itching of the sample. Nevertheless, we suggest that the PUL may control the skin lesions and improve the quality of life via inhibition of scratching behaviors.

For protection from environmental pathogenesis, the epidermis provides a skin barrier. A defective physical barrier makes the skin more permeable to pathogens and allergens, which causes increased TEWL, decreased skin hydration, and ultimately increases itching intensity or erythema in AD. Recent studies have reported that TEWL is linked to AD disease activity (Marsella et al., 2011). In our experiments, an increase in TEWL was found in DNCB-treated mice compared with that in the negative control mice. However, significantly reduced TEWL, increased skin hydration, and decreased erythema were found after dexamethasone 0.1% and PUL treatment in DNCB-treated mice, indicating that PUL may be beneficial for maintaining the skin barrier function and controlling AD severity. Our results clearly suggest that PUL ameliorates the development of AD-like skin lesions and scratching behaviors in NC/Nga mice due to complex mechanisms.

Recently, herbal medicine has gained attention owing to the presence of various therapeutically active constituents, including phenols and flavonoids, in skin disorders and AD (Działo et al., 2016; Parveen et al., 2014). These ingredients are often present in food and herbal products and exhibit multiple biological activities. We identified fourteen chemical constituents of PUL by HPLC-Q-TOF (Supplementary data) 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. Previous studies revealed that rutin suppressed AD through inhibition of IL-4 and IL-13 (Ganeshpurkar and Saluja, 2017). Chlorogenic acid was shown to possess antibacterial, antioxidant, and antiallergic activities. Furthermore, chlorogenic acid was found to inhibit the production of inflammatory cytokines and chemokines (Ha et al., 2014; Tsang et al., 2016). Interestingly, arbutin exhibits antioxidant, anti-tyrosinase, and anti-melanin activity in the skin (Tada et al., 2014) and its anti-AD effect has not yet been explained. The findings indicate that these constituents may affect multiple factors linked to the pathogenesis of AD and help to ameliorate AD.

Major compounds such as arbutin, chlorogenic acid, and rutin were analyzed against IL-6 and IL-1 β . However, no significant activity was found. All these highlight the potential activity of PUL may be due to the some other compounds. Therefore, it is recommended that further studies should be performed bioactivity guided isolation.

In conclusion, Our outcomes from *in vitro* and *in vivo* models of AD suggested that PUL may be a good therapeutic source providing a multi-targeted approach for reducing AD development. The further studies should be conducted to explore the specific bioactive constituents of PUL that contribute to the anti-AD activity. And then we should determine their therapeutic potential, either alone or in combination, as anti-AD agents, along with their possible signaling pathway.

Conflict of interest

None to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.05.006.

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No.	Tentative identification	RT	Molecular formula	Exact mass	Molecular mass (m/z)	Reference
Positive mode						
1	Arbutin	4.4	C12H16O7	272.25	273.1	(Qiu et al., 2018)
2	5,7,3',5'-tetrahydroxyflavobe	52.07	C15H12O6	288.252	289.07	
3	Apigenin	52.18	C15H24O5	270.24	271.06	
4	3-(2,4,6-trihydroxyphenyl)-1-(4-hydroxyphenyl)-propan-1-one	60.54	C15H14O5	274.09	275.09	
5	Trilobatin	60.60	C21H24O10	436.41	437.14	
6	Rutin	64.59	C27H30O16	610.53	309.04	
7	Quercetin	74.16	C15H10O7	302.23	303.05	
8	Astragalin	74.27	C21H20O11	448.38	449.11	
9	Orobol	83.57	C15H10O6	286.24	287.07	
Negative mode						
10	Chlrorogenic acid	29.818	C16H18O9	354.31	309.04	(Qiu et al., 2018)
11	Catechin	41.683	C15H14O6	290.27	289.07	
12	Artselaeroside A	72.76	C19H28O10	416.41	415.16	
13	Daidzein	78.96	C15H10O4	254.2	253.05	
14	Tricin 4'-O-[threo-β-guaiacyl-(7''-O-methyl)-glyceryl] ether	114.21	C28H28O11	540.17	539.16	