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Paedoksan ameliorates allergic disease through inhibition of the phosphorylation of STAT6 in DNCB-induced atopic dermatitis like mice

Sang Heon Lee[1,2†], Youngse Oh[3†], Sim-Kyu Bong[1], Jin Woo Lee[1], No-June Park[1,4], Young-Joo Kim[1], Hyun Bong Park[2], Yong Kee Kim[5], Seung Hyun Kim[3*] and Su-Nam Kim[1,4*]

Abstract

Various allergic diseases such as atopic dermatitis (AD), allergic rhinitis, and asthma are considered incurable conditions that have yet to be fully conquered. Paedoksan (PDS), an herbal preparation consisting of 14 medicines, displays effective anti-inflammatory and anti-allergic properties, yet its underlying molecular mechanism is unknown. This study aims to uncover PDS's mechanism for treating allergic diseases and suggest its therapeutic potential. Through a network pharmacological prediction, its impact on signal transducer and activator of transcription 6 (STAT6) regulation, a sub-mechanism of interleukin 4 (IL-4), a major inflammatory cytokine involved in degranulation and allergy, was investigated in RBL-2H3 cells and an atopic mouse model. PDS inhibits immunoglobulin E (IgE)-induced degranulation and STAT6 phosphorylation evoked by IL-4 in granulocytes. The downregulation of phospho-STAT6 and thymic stromal lymphopoietin (TSLP) by PDS was confirmed in 2,4-dinitrochlorobenzene (DNCB)-induced mouse skin. The results demonstrate that PDS exhibited remarkable effects on degranulation and STAT6 phosphorylation in RBL-2H3 cells, as well as in an atopic mouse model. Furthermore, the main active components from PDS based on chromatographic analysis showed good accordance with PDS's effects on RBL-2H3 cells. In summary, these findings collectively suggest that PDS holds the potential to effectively suppress inflammatory and allergic reactions by obstructing the target IL-4 protein and its downstream effects, as elucidated through a network pharmacological analysis.

Keywords Paedoksan, Network pharmacology, Atopic dermatitis, STAT6, IL-4

†Sang Heon Lee and Youngse Oh contributed equally to this work.

*Correspondence:
Seung Hyun Kim
kimsh11@yonsei.ac.kr
Su-Nam Kim
snkim@kist.re.kr
[1] Natural Products Research Institute, Korea Institute of Science and Technology, 679 Saimdang-Ro, Gangneung-Si, Gangwon-Do 25451, Republic of Korea
[2] Department of Biology, College of Natural Sciences, Gangneung-Wonju National University, Gangneung 25457, Republic of Korea
[3] College of Pharmacy, Yonsei Institute of Pharmaceutical Science, Yonsei University, Incheon 21983, Republic of Korea
[4] Division of Biomedical Science and Technology, KIST School, University of Science and Technology, Seoul 02792, Republic of Korea
[5] College of Pharmacy, Sookmyung Women's University, Seoul 04610, Republic of Korea

Introduction

Allergic inflammation occurs due to the progression of an immune response induced by an allergen and can cause various diseases containing atopic dermatitis (AD), asthma, allergic rhinitis, urticaria, and anaphylaxis [1]. Mast cells are major mediators in allergic inflammatory responses [2]. Cross-binding of FcεR1-particular immunoglobulin E (IgE) on the outer membrane layer of mast cells leads to the release of inflammatory factors, such as histamine, leukotrienes, and prostaglandins, causes immediate symptoms of type 1 hypersensitivity reactions that are characterized by an increase in mucus production due to vasodilation and mucosal irritation [3].

Steroids or anti-histamines used to treat allergic diseases cause serious side effects and cannot be used long-term. Therefore, a new type of natural therapeutic material needs to be developed to reduce side effects during long-term use [4]. Traditional herbal medicine (THM) has been used to treat skin diseases for hundreds of years before the introduction of Western medicine. According to herbal medicine research published over the past 20 years, many THM compounds have been found to inhibit IgE, mast cell activation, and inflammatory cytokine signaling [5].

Paedoksan (PDS, baidu-san in Chinese or haidoku-san in Japanese) has been traditionally used as a treatment of body aches, headaches, fever in Korean and Chinese traditional medicines. PDS is used as it is, but as several herbs are added or mixed, it is also used in various forms of addition and subtraction formulas. According to a recent study, a modification treatment of PDS known as Lizhongtang plus baidusan and Gamipaedok-san produce suppressive effects on allergic rhinitis and atopic dermatitis via modulation of the inflammatory response [6, 7]. In this study, we used a fourteen herbal formula PDS, which is composed of *Schizonepeta tenuifolia* Spike, *Saposhnikovia divaricata* Radix, *Angelica decursiva* Radix, *Aralia continentalis* Radix, *Citrus aurantium* Fructus, *Bupleurum falcatum* Radix, *Cnidium officinale* Rhizoma, *Ostericum koreanum* Radix and Rhizoma, *Platycodon grandiflorum* Radix, *Poria cocos* Sclerotium, *Atractylodes lancea* Rhizoma, *Perilla frutescens* Leaf, *Citrus unshiu* Peel, and *Pueraria lobate* Radix [8, 9]. Not only its formular overlaps with the aforementioned Gamipaedok-san in some of herbal ingredients, but there are also several reports describing the anti-allergy effects of some the individual herbs [10–12]. Although the effects of PDS on allergic diseases has been found, the molecular mechanism is not known.

Considering the herbal composition of PDS and the study of PDS modification, it can be expected that PDS might ameliorate allergic disease and affect the allergic inflammation-related targets mentioned above. However, the fact that one of herbal ingredients can ameliorate allergic reactions does not mean that it can play the same role in the PDS formula since PDS is prepared by extracting all ingredients together. Moreover, as seen in most of the THM formulations, the complexity of PDS's chemical composition, multi-targets, and their interactions makes it very difficult to predict allergic response-related molecular mechanisms. These difficulties often occur in the study of a single herb in addition to THM mixtures owing to its characteristics of multi-compound, multi-target, and multi-pathway actions. Recently, a network pharmacological analysis provided a new prospective strategy for elucidating unclear molecular mechanisms of THM [13]. In this respect, it is great significance to study whether PDS can ameliorates allergic disease through any molecular mechanisms. Here, we also applied this strategy to more efficiently predict the key components, target proteins, and their related signaling pathway against allergic disease. Furthermore, we employed an experimental verification involving high-performance liquid chromatography/quadrupole time-of-flight/mass spectrometry (HPLC/QTOF/MS) and gene expression analysis and in-vivo assays based on established analytical networking.

In this study, we concentrated on inflammatory T-helper (Th)2 cytokine, interleukin 4 (IL-4) among 11 targets based on analysis data of the network pharmacology, and we demonstrated that PDS inhibits IgE-mediated mast cell degranulation and the signal transducer and activator of transcription 6 (STAT6) phosphorylation in mast cells. In addition, the improved efficacy of PDS toward STAT6 phosphorylation and serum IL-4 and IgE was confirmed in the DNCB mouse AD-like mouse model. Therefore, our results suggest that PDS can be used as a new agent for natural therapeutic material for allergic diseases.

Materials and methods

Chemicals and cells

2,4-Dinitrochlorobenzene (DNCB) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). IL-4 and IgE enzyme-linked immunosorbent assay (ELISA) kits were obtained from Invitrogen (Frederick, MD, USA). Power SYBR® Green Master Mix was purchased from Applied Biosystems (Foster, CA, USA). Anti-STAT6 and anti-phosphorylated STAT6 (p-STAT6) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rat basophilic leukemia (RBL-2H3) cells were obtained from the Korean Cell Line Bank (KCLB No. 22256, Seoul, Korea), EL4 (a murine T lymphoblast cell line) from the American Type Culture Collection (ATCC, TIB-39) and HaCaT immortalized human keratinocyte

cell line (catalog no. T0020001; AddexBio, San Diego, CA, USA) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco Lab., Grand Island, NY, USA) with 10% (v/v) fetal bovine serum (FBS) and 100 U of penicillin and 100 μg/mL of streptomycin at 37 °C in 5% CO_2 incubator. Human multiple myeloma cells (U266B1: ATCC, TIB196TM) were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco Lab.) medium supplemented with 15% FBS, 1 mM of sodium pyruvate, 2 mM of L-glutamine, 100 U of penicillin, and 50 μg/mL of streptomycin at 37 °C in a 5% CO_2 incubator.

Paedoksan preparation

PDS extract was prepared by Hanpoong Pharmaceutical Co., Ltd. (Jeonju, Korea) according to Good Manufacturing Practices. The herbal formula of PDS is a complex containing 14 herbal medicines as listed in Additional file 1: Table S1 (Additional file 1: Table S1). Atractylodes Rhizome (20 g), Cnidium Rhizome (20 g), Platycodon Root (20 g), Bupleurum Root (20 g), Peucedani Radix (20 g), Ostericum Root (20 g), Aralia Continentalis Root (20 g), Aurantii Fructus Immaturus (20 g), Perilla Leaf (20 g), Pueraria Root (10 g), Saposhnikovia Root (10 g), Poria (10 g), Schizonepeta Spike (10 g) and Citrus Unshiu Peel (10 g) were mixed, added to a tenfold volume of distilled water, and decocted from 90 to 100 °C for 3 h. The solution was filtered through a filter paper with 5 μm pore size, evaporated to dryness, and vacuum-dried to yield a powder (25 g). The yield of PDS extract was 21.69%. PDS was dissolved in distilled water and filtered through a 0.45 μm sterile filter for in vivo and in vitro studies. Puerarin was used as an analytical marker compound of PDS and was quantified at 1.90 mg/g. Standard components (purity ≥ 98.0%) were purchased from Avention (Incheon, Korea), Sigma-Aldrich Co. and ChemFaces (Wuhan, Hubei, China).

Network pharmacological analysis

The chemical ingredients of PDS were manually curated by databases, including PubMed (https://pubmed.ncbi.nlm.nih.gov/) and a database of medicinal materials and chemical compounds in Northeast Asian traditional medicine (TM-MC, https://informatics.kiom.re.kr/) as described by Kim et al. [14]. All collected chemical components were evaluated for their drug-likeness (DL) and oral bioavailability (OB) using the quantitative estimate of drug-likeness (QED) method [15]. Physicochemical properties were obtained from SwissADME (https://www.swissadme.ch/) database to calculate QED value of each compound [16]. The chemical compounds satisfying the cut-off values (QED ≥ 0.4 and OB = TRUE) were selected as the expected active compounds. To obtain the predicted targets, the SMILES strings of selected compounds were inputted into the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) [17]. The allergic disease-related targets were collected in GeneCards database (https://www.genecards.org; [18]). Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/) was used to draw a Venn diagram to detect common targets. The common targets were uploaded into a STRING database (https://string-db.org; [19]) to obtain a protein–protein interaction (PPI) relationship. Protein interactions with a combined score ≥ 0.7 were copied into Cytoscape (3. 9. 0, Seattle, WA, USA) to construct a PPI network. With this PPI network, the three parameters (degree, betweenness centrality, and closeness centrality) were used to assess topological features of key targets [20]. Gene ontology and Kyoto Encyclopedia of Genes and Genomes (GO and KEGG, respectively) pathway enrichment analysis of the key targets was performed using the DAVID Bioinformatics Resources 6.8 database (https://david.ncifcrf.gov/home.jsp; [21]. The analysis parameter (p-value < 0.05) was set as a threshold value to identify distinct signaling pathways. The pathway enrichment analysis results were visualized using the ImageGP (http://www.ehbio.com/ImageGP). An integrated network of compounds, key targets, and pathway was established and analyzed using the Cytoscape software.

HPLC-QTOF-MS analysis

To determine PDS's key components using HPLC/QTOF/MS, the concentrated samples were dissolved in an ethanol–water mixture (50%, v/v) to a concentration of 10 mg/mL for PDS and 1 mg/mL for reference standard compounds. HPLC was performed on an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Mass spectrometry was carried out an Agilent 6530 QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface and analyzed in the positive mode. The ESI parameter were set to specific conditions: (1) mass range, 100–3200 m/z; (2) drying gas temperature, 300 °C; (3) pressure of nebulizer, 35 psi; (4) drying gas flow, 8.0 L/min; (5) capillary voltage, 3500 V; (6) sheath gas temperature, 350 °C; and (7) collision energy 20, 40, and 60 eV. To obtain the exact mass, calibration was performed with an Agilent tune mix (Agilent technologies, Palo Alto, CA, USA) from 100 to 1600 Da. Separation was performed using a Waters® ACQUITY UPLC BEH C18 (2.1 mm i.d. × 100 mm, 1.7 μm). The mobile phase consisted of 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B with a set gradient elution profile: 5–60% B (0–35 min), 60–100% B (35–50 min) at a flow rate of 0.3 mL/min. Each sample was injected using a volume of

5.0 μL, and the column temperature was maintained at 25 °C. All MS data were acquired using the MassHunter Qualitative analysis software (version B.06.00, Agilent Technologies, Santa Clara, CA, USA) to confirm mass accuracy throughout the chromatographic run. The "Find by Auto MS/MS" function of the analysis software was used to extract all the MS/MS spectrum and identify compounds based on accurate m/z value, isotope distribution, fragment ions, and ultraviolet (UV) spectrum compared to reference standard compounds.

Animals

Six-week-old female SKH-1 hairless mice were procured from Orient Bio (Seongnam, Korea). All animals were accommodated in wire cages at 20 to 22 °C at 50% ± 10% relative moisture and 12 h illumination (06:00–18:00) and allowed to consume standard laboratory feed and water freely. All experiments were performed according to procedures approved by the Korea Institute of Science and Technology (KIST) Institutional Animal Care and Use Committee (IRB Code No. 2020-001; KIST, Seoul, Korea).

β-Hexosaminidase release assay

The RBL-2H3 cells were seeded in a 24-well plate (5×10^5 cells/well) overnight and sensitized with anti-DNP-IgE (100 ng/mL) for 4 h. The 24-well plates were then washed with Siraganian buffer (119 mM of NaCl, 5 mM of KCl, 1 mM of $CaCl_2$, 0.4 mM of $MgCl_2$, 25 mM of piperazine-N,N′-bis(2-ethanesulfonic acid)[PIPES], 5.6 mM of glucose, pH 7.2). The cells were incubated with Siraganian buffer for 10 min and treated with PDS (10, 30, or 100 μg/mL). After treatment with PDS for 20 min and treated with DNP-BSA (1 μg/mL) for 30 min. To measure the amount of released β-hexosaminidase, the supernatants were transferred to 96-well plates and incubated with 1 mM substrate (1 mM 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M of citrate buffer) at 37 °C for 1 h. The reaction sample was measured using an M1000 (TECAN, Salzburg, Austria) plate reader at 405 nm after adding the stopping solution (0.1 M of $Na_2CO_3/NaHCO_3$, pH 10.0).

Quantitative real-time PCR (qPCR)

Total RNA was extracted using an easy-BLUE™ total RNA extraction Kit (iNTRON Biotechnology, Seoul, Korea). The extracted RNA was reverse-transcribed to cDNA using the RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Bremen, Germany). Several primers were selected: (1) IL-4, forward 5′-CAG GTC AAC ACC ACG GAG AA-3′ and reverse 5′-ACA TCT CGG TGC ATG GAG TC-3′; (2) thymic stromal lymphopoietin (TSLP), forward (5′-CGA CAG CAT GGT TCT TCT CA-3′) and reverse (5′-CGA TTT GCT CGA ACT TAG CC-3′); (3) IL-33, forward 5′-AGC CTT GTG TTT CAA GCT GG -3′ and reverse 5′-ATG GAG CTC CAC AGA GTG TTC-3′; and (4) glyceraldehyde 3 phosphate dehydrogenase (GAPDH), forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-CCA CCA CCC TGT TGC TGT A-3′. Specific PCR settings were used: (1) the initial denaturation for 15 s at 95 °C, (2) amplification for 40 cycles at 95 °C for 30 s and (3) at 60 °C for 30 s. A subsequent melting curve analysis was performed by increasing the temperature from 60 to 95 °C.

Western blot analysis

Total protein in RBL-2H3 cell lysates and mice dorsal tissues was extracted using lysis buffer (1× RIPA, phosphatase inhibitor and protease inhibitor). Protein quantification was measured by Bradford assay. Western blotting was conducted. Proteins were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinyldene fluoride (PVDF) membrane. The membrane was blocked by 5% skim milk for 1 h, and then incubated with primary antibodies overnight in 4 °C cold room. After incubation with primary antibodies (against STAT6 and p-STAT6, 1:1,000 in Tris-buffed saline [TBST]), membranes were washed four times with TBST and incubated with the secondary antibody (1:4000 in TBST) for 1 h at room temperature. Protein expression was analyzed using iBright CL1000 analysis program (Invitrogen) with ECL kits.

Induction of AD by DNCB in hairless mice

DNCB was dissolved in 99% acetone and olive oil in a volume ratio of 3:1 was applied on the dorsal skin of SKH-1 hairless mice. The first week, 1% DNCB was administered once a day. After 1 week, mice were challenged with 0.1% DNCB every 3 days for an additional 2 weeks. During the challenge with 0.1% DNCB, mice were given oral PDS (50 or 100 mg/kg, dissolved in 0.5% carboxymethyl cellulose) once a day. On the last day of an experiment, the SKH-1 hairless mice were sacrificed, and their blood samples and dorsal skin tissue were collected for further study.

Evaluation of the skin lesion (dermatitis score)

The asperity of dermatitis induced by DNCB treatment was evaluated before sacrifice. The development of excoriation/erosion, erythema/hemorrhage, edema, and/or scarring/dryness was scored on a scale of 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe). The sum of the scores of each item was used as the dermatitis score.

Histological examinations

For the histological evaluation, the dorsal skins of SKH-1 hairless mice were fixed with 10% formalin and processed for paraffin wax. Paraffin block sections of 10 μm were prepared using a microtome. Hematoxylin and eosin (H&E) and toluidine blue staining were performed using an optical microscope (Olympus CX31/BX51; Olympus Optical Co., Tokyo, Japan), and photographs were obtained using a TE-2000U (Nikon Instruments Inc., Melville, NY, USA) camera. The thicknesses of the epidermal layers were measured using a microscope equipped with a ruler and the LAS v4.8 (Leica Microsystem, Herbrugg, Switzerland) program.

Measurement of total serum IL-4 and IgE levels

Blood samples were collected from the abdominal aorta of the SKH-1 hairless mice and then centrifuged at 8000 rpm for 20 min at 4 °C to isolate the serum. Serum IL-4 and IgE concentrations were measured using ELISA kits (Invitrogen, CA, USA).

Statistical analysis

All results were obtained from at least two independent experiments and are expressed as mean ± standard deviation. Statistical analyses were carried out by a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post hoc analysis.

Results

Network pharmacology analysis predicts the active compounds, key targets, and pathways of Paedoksan regulating allergic disease

The advances in network pharmacology analysis has introduced a new approach for predicting unclear molecular mechanisms of traditional medicine, including complex components and their diverse targets with multiple-level actions [22]. To deduce the key compounds and molecular mechanisms for regulating allergic disease, 2710 chemical compounds found in 14 herbs in PDS were collected from two herbal medicine databases, PubMed and TM-MC. The candidate active compounds were estimated in terms of their oral bioavailability (OB) using the QED method. Among them, 1758 chemicals have an OB value of TRUE and QED value of 0.400 or higher. Excluding the duplicates of compounds, 157 chemical compounds were selected as the expected active compounds found in PDS (Additional file 1: Table S2).

To acquire associated target genes of PDS, the 1376 targets of expected active compounds and 37 allergic disease-related targets were collected from Target prediction databases, SwissTargetPrediction, Pubchem, and GeneCards. The 11 common target genes were selected as key targets (Fig. 1A). To analyze the topological score (degree, betweenness centrality, and closeness centrality) of key targets, a PPI network was constructed and analyzed (Fig. 1B). Based on the results of the PPI network analysis, IL-4 showed a high degree value in addition to a high relevance score (Table 1). In addition, a pathway enrichment analysis based on KEGG and GO gene sets of the 11 target genes showed that pathways associated with the allergic responses, such as immune and inflammatory responses (GO:0006955 and GO:0006954, respectively), were enriched (Fig. 1C). The KEGG pathway enrichment analysis also suggested the Toll-like receptor signaling pathway in which the targets were also enriched (Fig. 1D).

A compound-target-pathway (C-T-P) network was established to provide a comprehensive understanding of network pharmacology result (Fig. 1E). The C-T-P network consist of 178 nodes (157 expected active compounds, 11 key targets, and 10 pathways) and 252 edges. The seven red diamond nodes (quercetin, caffeic acid, apigenin, kaempferol, daidzein, genistein, and luteolin) represent the highest degree value of other expected active compounds, and the red edge represents their interactions with key target genes, thereby indicating that they are important nodes and edges in the network. Taken together, it can be deduced that PDS regulates allergic disease by the actions of its key compounds on IL-4-associated biological pathways. Based on these results, we employed an experimental verification involving HPLC/QTOF/MS and gene expression analyses and in vivo assay based on established analytical networking.

Paedoksan inhibits allergic disease related mediators

Based on the network pharmacology analysis, we identified IL-4 associated with the expected active compound targets of PDS and allergic disease-related genes. Furthermore, previous studies have demonstrated that some THM constituents significantly reduce the levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-4 and -6 [23]. In this study, we concentrated on elucidating molecular mechanism of PDS as an anti-AD agent on inhibition of IL-4.

Meanwhile, the IgE class switching of B-cell plays a critical role in degranulation [24]. IgE production was measured by induction of U266B1 cells using IL-4 and lipopolysaccaride (LPS). However, PDS did not affect IgE production (Additional file 1: Fig. S1A). Expression of IL-4 and -33 was confirmed in HaCaT keratinocytes and RBL-2H3 cells, but no effect due to PDS was found. Considering that PDS does not lead to a decrease in IL-4 and -33 in HaCaT keratinocytes, EL-4 cells, and RBL-2H3 cells, we investigated the STAT6 pathway, a downstream effector of IL-4 (Additional file 1: Fig. S1B–D).

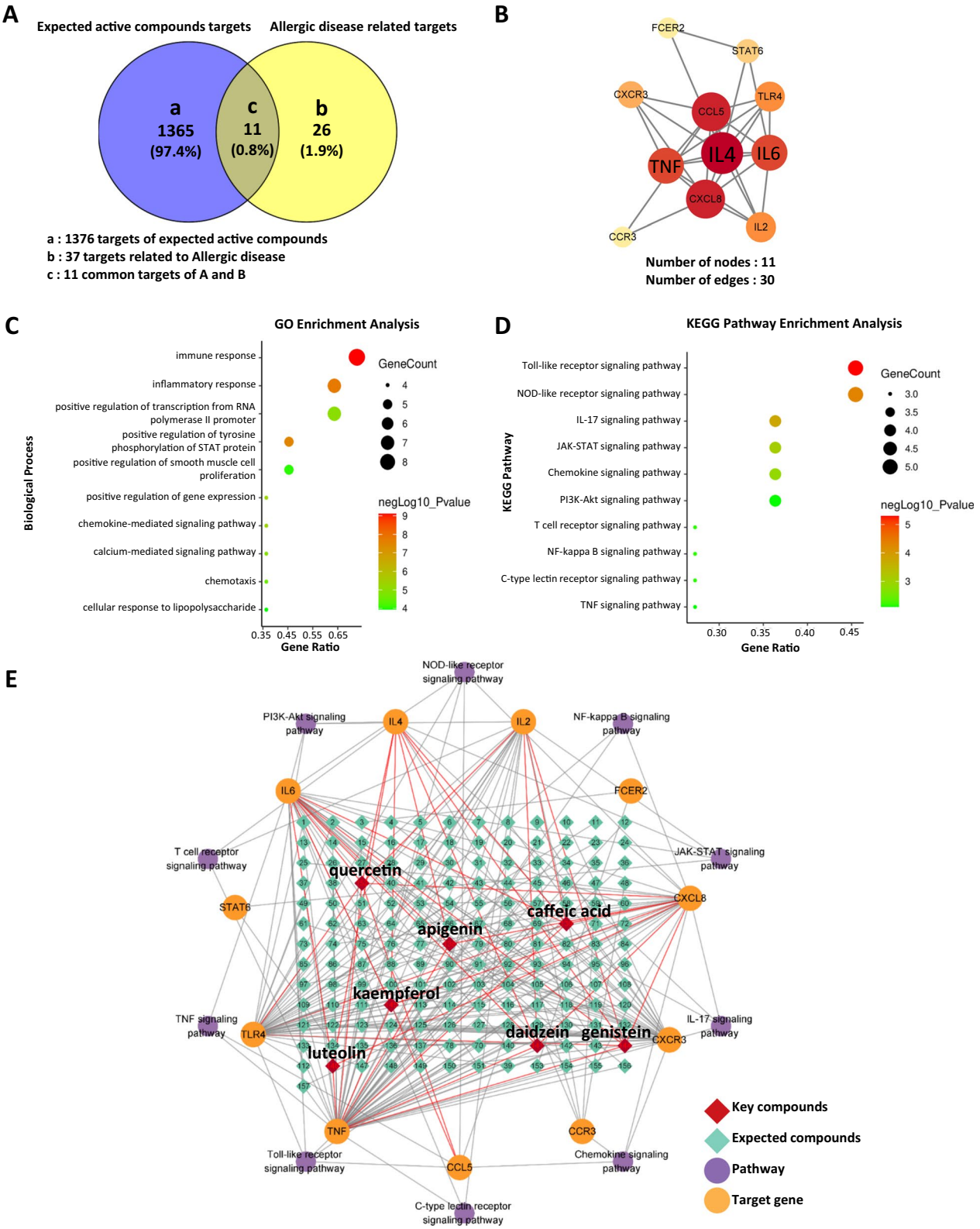

Fig. 1 Network pharmacological analysis of Paedoksan (PDS). **A** Venn diagram for 1376 target genes of PDS and 37 allergic disease related target genes. The 11 common target genes were selected key targets. **B** Protein–protein interaction (PPI) networks (**C** and **D**) Bubble chart of gene ontology (GO) (**C**) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (**D**) enrichment analysis for the 11 key targets. **E** Expected active compounds-key targets-pathways (C–T–P) network

Table 1 Key targets of PDS based on PPI network topological analysis

No.	Uniprot ID	Gene	Degree	Relevance score
1	P05112	IL-4	9	25.38
2	P13501	CCL5	8	15.59
3	P10145	CXCL8	8	17.62
4	P05231	IL-6	7	15.86
5	P01375	TNF	7	24.28
6	P60568	IL-2	5	15.54
7	O00206	TLR4	5	12.89
8	P49682	CXCR3	4	12.54
9	P42226	STAT6	3	11.81
10	P51677	CCR3	2	11.18
11	P06734	FCER2	2	12.32

The anti-allergic inflammatory potential of PDS was then investigated using a degranulation analysis using RBL-2H3 cells. In RBL-2H3 cells, IgE/DNP BSA treatment produced an increase in β-hexosaminidase release, and PDS treatment yielded a concentration-dependent inhibition (Fig. 2A). These data show that PDS could lead to an improvement in degranulation-mediated allergic inflammation.

STAT6 phosphorylation is important for IgE/DNP-BSA-mediated cytokine release in mast cells. RBL-2H3 cells were sensitized with IgE (100 ng/mL), treated DNP-BSA (10 ng/mL) and after 10 min, measured protein expression. STAT6 phosphorylation was increased in IgE/DNP-BSA treat groups, whereas PDS treated group inhibit STAT6 phosphorylation (Fig. 2B). Similarly, quercetin, caffeic acid, apigenin, kaempferol, daidzein, genistein, and luteolin selected as targets in network pharmacology showed degranulation and STAT6 phosphorylation inhibition such as positive control in most of the compounds (Additional file 1: Fig. S2A, B).

STAT6 phosphorylation leads to up-regulation of inflammatory cytokines, such as CCL26 and TSLP, chemokines, and down-regulation of skin barrier factors. To prove the effect of the PDS on this finding, we measured STAT6 phosphorylation protein expression in keratinocytes. HaCaT keratinocytes treated with IL-4 (20 ng/mL) were measured by western blot. Treatment of HaCaT keratinocytes with IL-4 led to an increase in the expression of STAT6 phosphorylation compared to that in untreated cells. However, the PDS treatment group did not inhibit STAT6 phosphorylation by IL-4 (Additional file 1: Fig. S2C). It was confirmed that it was not possible to suppress IL-4-induced STAT6 phosphorylation in HaCaT keratinocytes, and the effect of PDS was confirmed for CCL26, which causes inflammatory reactions by recruiting eosinophils among its downstream mechanism. IL-4 induction led to an increase in CCL26 expression, but the effect of PDS on CCL26 was insignificant (Additional file 1: Fig. S2D). Synthetically our data

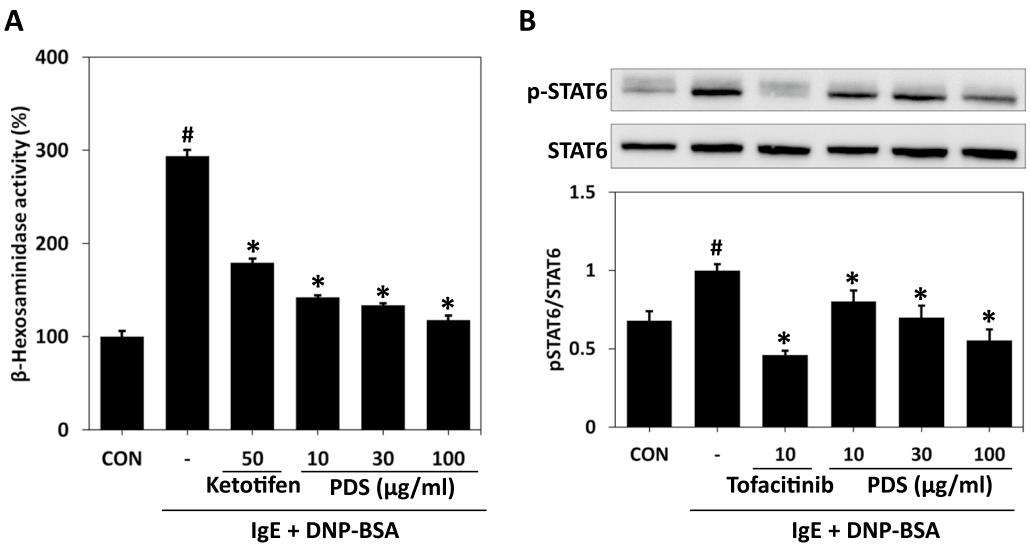

Fig. 2 Effects of PDS on allergic disease-related mediators. **A** RBL-2H3 cells were sensitized with anti-DNP IgE (100 ng/mL), treated with indicated doses of PDS, and challenged with DNP-BSA (1 μg/mL). β-Hexosaminidase release was calculated relative to the control (CON) group. Data are presented as the mean ± S.D. of the duplicates. $^{\#}p < 0.05$ significantly greater than CON. $^{*}p < 0.05$ significantly lower than the anti-DNP IgE + DNP-BSA-treated group. **B** Phosphorylation levels of signal transducer and activator of transcription (STAT6) in RBL-2H3 cells in response to IgE (100 ng/mL) and DNP-BSA (10 ng/mL) in the presence of indicated concentration of PDS. Results were normalized to signal transducer and activator of transcription (STAT6) expression in RBL-2H3 cells. Each bar represents the mean ± standard deviation (S.D.) of the duplicates. $^{\#}p < 0.05$ significantly greater than CON. $^{*}p < 0.05$ significantly lower than the anti-DNP IgE + DNP-BSA-treated group

indicate that PDS can lead to improvements in allergic reactions by targeting STAT6 phosphorylation regulation in IgE-induced mast cells.

Paedoksan improves allergic symptoms in DNCB induced hairless mice

Oral medication was given at 50 or 100 mg/kg to confirm the effect of PDS on the DNCB-induced AD-like mouse model. As a result of applying DNCB to SKH-1 hairless mouse for 1 week, major symptoms such as excitation/emotions, scaring/dryness, edema, and erythema/hemorrhage appeared. Subsequently, repeated administration of PDS to mice for 2 weeks showed improvement in AD-like lesions (Fig. 3A). Dermatitis scores were evaluated for PDS-induced improvements on AD-like symptoms, such as eythema/hemorrhage, scaring/dryness, edema, and excitation/emotions. As a result, the dermatitis score was improved depending on the concentration of the PDS (Fig. 3D). In the case of transepidermal water loss (TEWL), which is an indicator of skin moisture loss, the score slightly declined in the PDS-treated group. On the final day of the experiment, TEWL levels decreased in the PDS-treated group (Fig. 3E).

SKH-1 hairless mouse dorsal skin was stained with H&E to assess the effects of PDS on epidermal thickness. The thickness of the epidermis increased in the DNCB treatment group compared to the control group. Oral administration of PDS led to an improvement in epidermal hyperplasia (Fig. 3B, F). To examine skin infiltration of mast cells, lesion skin was stained with toluidine blue. In the DNCB-treated group, mast cells showed more infiltration, but the results showed a significant improvement when treated with PDS (Fig. 3C, G). These results verify that oral administration of PDS led to effective improvement in allergic inflammatory skin diseases in the DNCB-induced AD-like mouse model.

Paedoksan leads to recovery in allergic disease-related serum and tissue factors

An increase in serum IgE and IL-4 is a feature of the DNCB-induced AD-like mouse model. IgE and IL-4 increased in the DNCB treatment group compared to the control group. In the PDS treatment group, both IgE and IL-4 in the serum showed a decrease (Fig. 4A, B). The STAT6 phosphorylation in PDS treatment, which exhibited an anti-allergic effect in mast cells and keratinocytes, was analyzed using a WB analysis in AD lesion skin tissue. STAT6 phosphorylation had significantly increased in the DNCB-treated group compared to the untreated control group. Contrary to the results, STAT6 phosphorylation was the same as the control group in the PDS-treated dorsal tissue (Fig. 4C) after which mRNA levels of the inflammatory cytokine TSLP, which is activated by the STAT6 pathway, were measured. The mRNA levels of TSLP had significantly increased compared to the control group, but the PDS-treated group showed the same level of expression as the control group (Fig. 4D). Therefore, PDS appears to lead to an improvement in allergic inflammation by causing a reduction in STAT6 phosphorylation and expression of its sub-transcription factors in DNCB-induced AD-like models.

HPLC/QTOF/MS analysis of Paedoksan

The optimal HPLC/QTOF/MS conditions were applied to determine the seven key compounds of PDS based on a network pharmacology analysis (Fig. 5, Additional file 1: Fig. S3). After comparing by the m/z, retention time, and UV spectrum of standard reference compounds, five key compounds (daidzein, luteolin, quercetin, apigenin, and kaempferol) were identified. However, apigenin and genistein had the same retention times and m/z values. So we compared UV spectrum of peak four with the standard reference UV spectrum of apigenin or genistein, thereby we confirmed that the peak 4 was apigenin (Additional file 1: Fig. S4). Besides, caffeic acid was not detected by LC/MS. The chemical information and content, such as compound name, molecular formula, retention time, adduct, calculated molecular ions, measured molecular ions (m/z), error (ppm), and origin quantified by LC/MS analysis are listed in Table 2.

Discussion

PDS has been used as a traditional medicine for colds, headaches, and body aches in Korea and China. In previous study, *Perilla frutescens* leaf, which is a component of PDS, alleviates atopic dermatitis symptoms in DNFB-induced animal model [25]. In addition, several studies in which it was reported that some THMs were examined in terms of its effect on allergic disease [23]. However, a few studies elucidating molecular mechanisms against atopic dermatitis of herbal medicines have been published [26]. On the other hand, it is difficult to interpret the molecular mechanisms owing to the chemical and pharmacological complexity of herbal medicines. In this respect, the application of network pharmacology analysis made it practical to predict molecular mechanisms, such as expected active compounds, key target genes, and their related pathways. In present study, network interactions were constructed and integrated with the QED, OB evaluation, PPI network analysis, and GO and KEGG enrichment analysis. In network pharmacology analysis, "degree" refers to the number of interactions in the network. Nodes with a high degree are considered more influential within the network, as they have a greater number of interactions with other nodes. In a compounds-target-pathway network, a

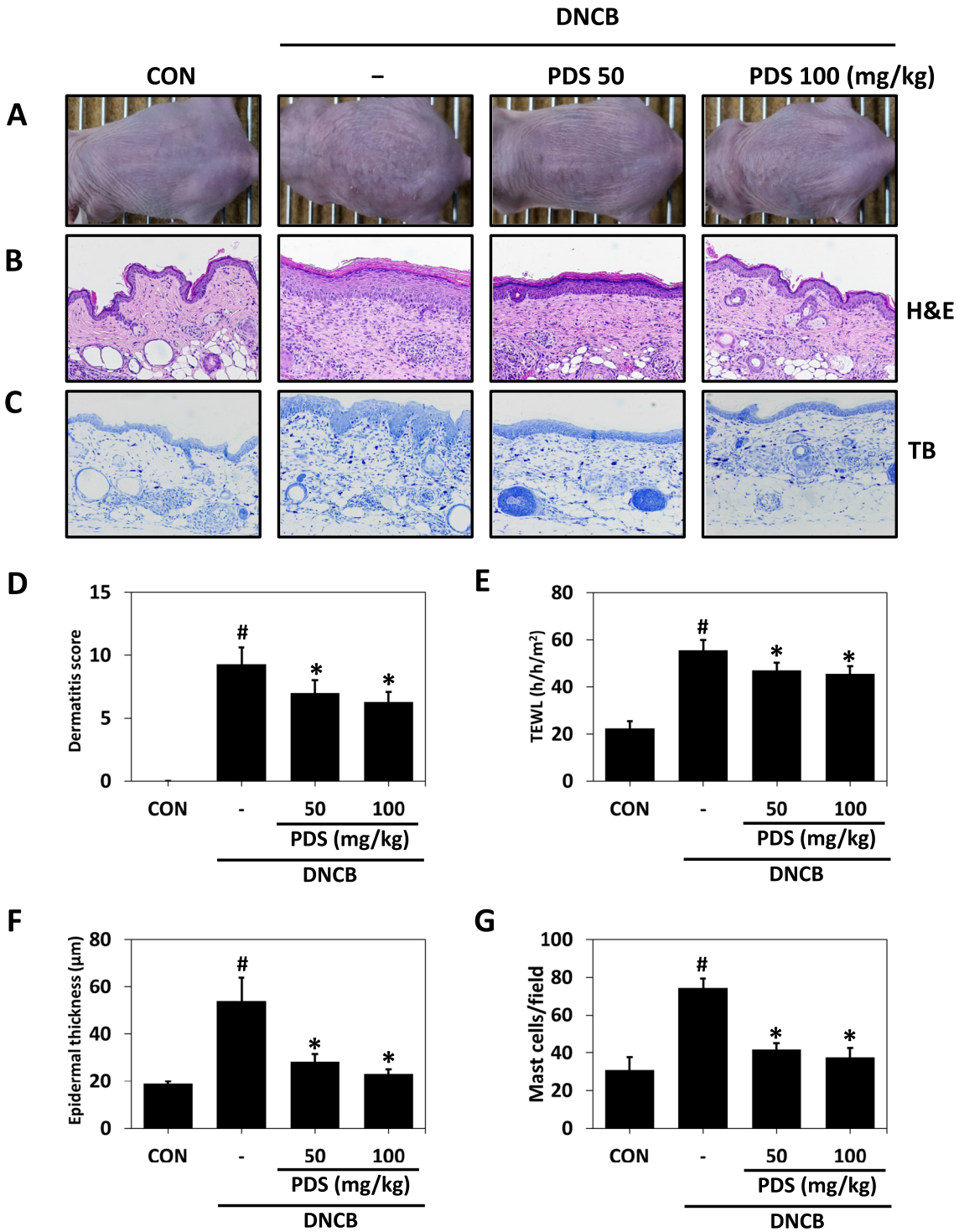

Fig. 3 Effects of PDS on 2,4-dinitorchlorobenzene (DNCB)-induced AD-like lesions in the dorsal skin of hairless mice. **A** Clinical features of PDS treatment (n = 5 in each group). Histopathological features stained by hematoxylin and eosin (H&E) (**B**) and toluidine blue (**C**) of dorsal lesions. **D** Dermatitis score was evaluated by symptoms of lesion such as excoriation/erosion, scarring/dryness, edema, and erythema/hemorrhage. **E** Level of trans epidermal water loss (TEWL). Measurement of epidermal thickness (**F**) and infiltration of mast cells (**G**) in dorsal lesions. The number of infiltrated mast cells in field were counted. Each bar represents the mean ± S.D. (n = 5 in each group). $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ versus the DNCB group

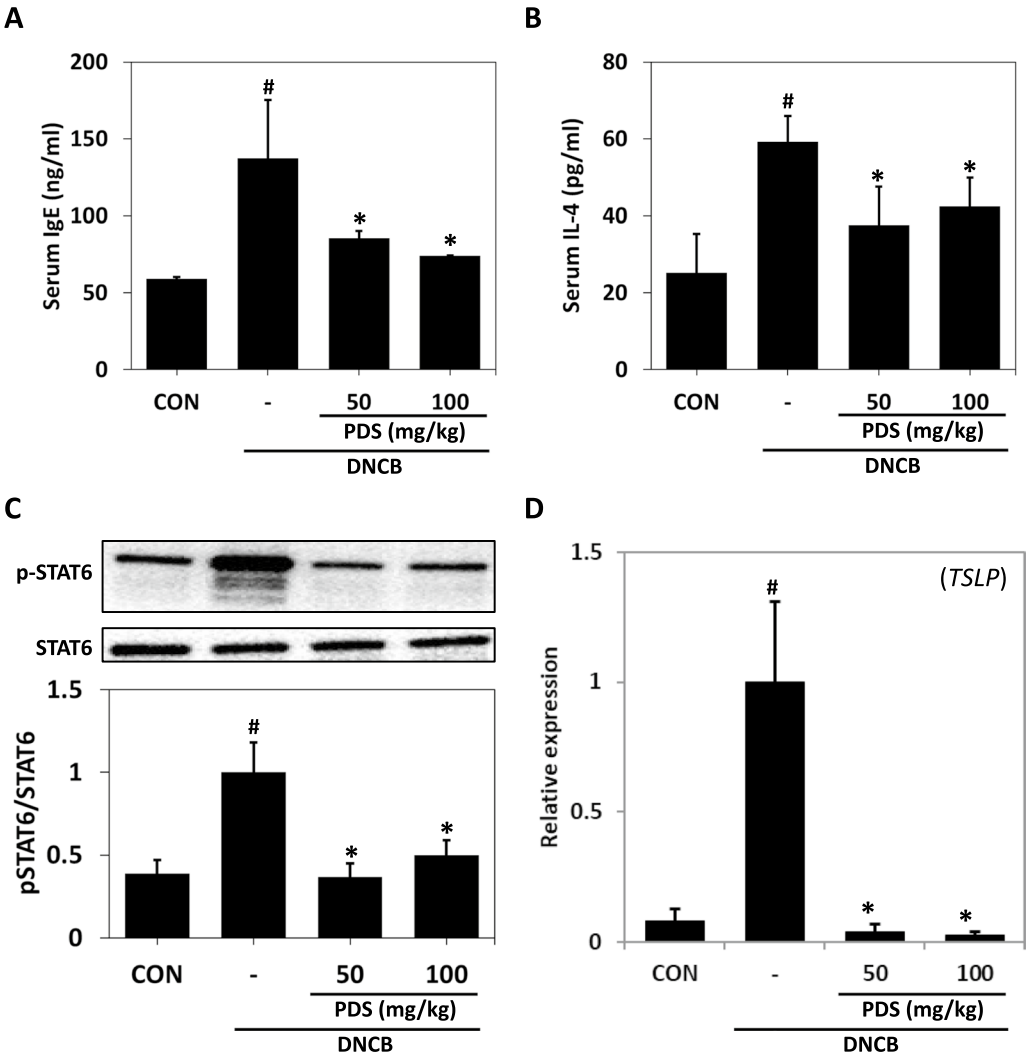

Fig. 4 Effects of PDS on the serum and tissue factors related to allergic disease in DNCB-induced AD-like mice. Concentrations of serum IgE (**A**) and IL-4 (**B**) were examined with enzyme-linked immunosorbent assay (ELISA). Phosphorylation of STAT6 in lesion tissues was analyzed by western blot (**C**). The mRNA level of TSLP in the tissue was progressed using qPCR (**D**). Data are presented as the mean ± S.D. (n = 5 in each group). $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. DNCB group

compound with a high degree would be one that interacts with many target proteins, indicating that it can represent critical points in the network that are responsible for mediating disease pathways [13]. When we constructed 157 expected compounds-11 target gene network, the 7 active compounds showed high degree value. The ideal approach would be to experiment with all expected active compounds in the PDS. However, considering the complex chemical composition of the PDS, it is necessary to select the most possibly and practically feasible compounds for experimentation. Although this approach may have limitations, it is suggested as an efficient strategy in multi-herb extracts such as TCM of complex constituents [13, 22]. Notably, in validation process, the compounds identified as promising through network pharmacology analysis have been experimentally validated for their effects on allergic disease-related mediators in RBL-2H3 and HaCaT cells. As a result, the anti-allergic disease effects of PDS were inferred to possibly correlate with 7 key compounds and 11 key targets, which included IL-4 as the highest degree value. According to these deduction, we performed the experimental validation (Additional file 1: Fig. S5).

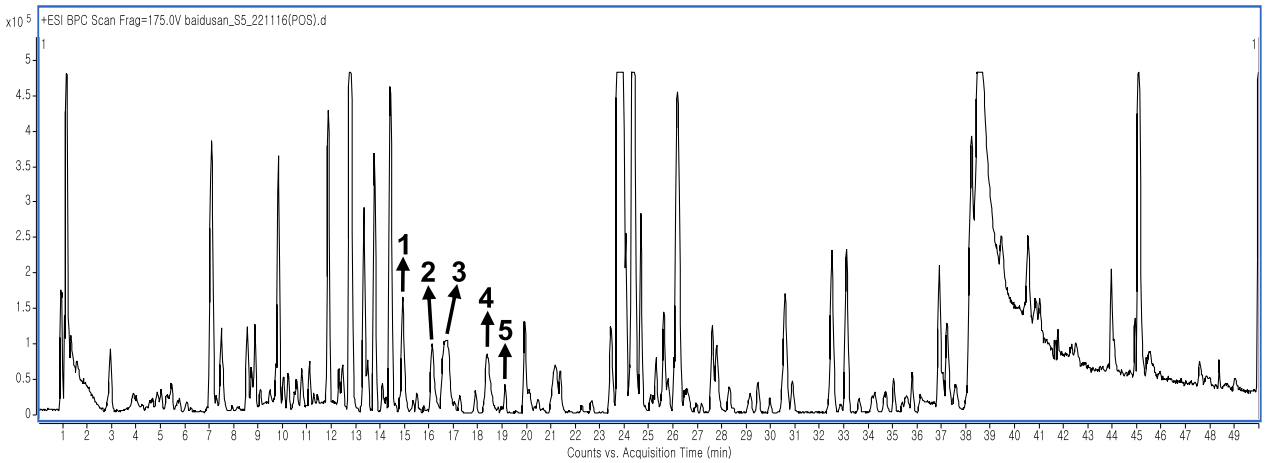

Fig. 5 Liquid chromatography/mass spectrometry (LC/MS) chromatogram of PDS. The representative base peak chromatogram of the PDS in positive electrospray ionization (ESI) mode. 1: daidzein, 2: luteolin, 3: quercetin, 4: apigenin, 5: kaempferol

This study focuses on investigating the effects of PDS on anti-allergic inflammation. Allergic diseases have the same symptoms, involving elevated Th2-type cytokines and IgE. Recent studies indicate that T-cells differentiated with Th2 play a facilitating role in activation of B cells, mast cells, and eosinophils that produce IgE antibodies. IgE is a major agent that causes inflammatory mediators, including prostaglandins, leukotrienes, and histamine in allergic inflammation.

To investigate the inhibitory effect of PDS on IgE production, U266B1 B-cells were stimulated with IL-4 and LPS. However, PDS did not inhibit IgE production in U266B1 cells. Next, the effects of PDS on degranulation caused by IgE production was investigated. Degranulation was induced by IgE and DNP-BSA in RBL-2H3 cells, and as a result, degranulation effectively improved.

It is known that STAT6 plays a major role in IgE/FcεR1-mediated cytokine release in allergic inflammatory responses [27]. STAT6 phosphorylation was induced by stimulating IgE/DNP-BSA in RBL2H3 cells, and it was confirmed that the PDS caused a significant lowering of STAT6 phosphorylation. Furthermore, STAT6 is known as a regulator of the IL-4-dependent immune response [28]. IL-4 is an inflammatory cytokine, which is important in the progression of allergic reactions. IL-4 is related to the generation of an ε isotype switch and the secretion of IgE by B lymphocytes in the progress of allergic inflammation. The IgE-mediated allergic inflammatory response is enhanced by the up-regulation of IgE receptors on the outer membrane layer by IL-4. In addition, IgE up-regulation by IL-4 leads to activation of STAT6 phosphorylation in mast cells. STAT pathways form a major mechanism in allergic disease progression by mediating critical responses in mast cells and playing a role in supporting mast cell progress and survival [29]. Among the STAT protein family, STAT6 is important for Th cell differentiation and subsequent responses [30]. STAT6 is activated by the combining of inflammatory cytokines IL-4 and its receptor. STAT6 phosphorylation induces dimerization and nuclear translocation to activate gene transcription of inflammatory cytokines, such as TSLP, IL-33, and chemokines, such as C-C motif ligand 26 (CCL26) [31–34].

Features of the DNCB AD-like mouse model include hypercornification, penetration of immune cells, and cytokine and IgE production in serum [35]. Treatment of PDS improved the atopic appearances, which were thickened by continuous inflammation and allergic reactions. Likewise, infiltration of immune cells, such as IgE- and inflammatory cytokine-activated mast cells was effectively suppressed.

The skin is a primary immune defense organ jointly composed of immune cells, such as keratinocytes and mast cells [36]. In the skin, STAT6 phosphorylation inhibits natural moisturizing factors and activates inflammatory signal factors, such as CCL26 and TSLP [37]. The main sources of TSLP are keratinocytes, epithelial cells, and fibroblasts [38]. TSLP stimulates dendritic cells to differentiate naive T cells into Th2 cells, known as AD developing cytokines, a late allergic inflammatory response [39, 40]. As a known study result, STAT6 phosphorylation and TSLP were induced in the dorsal lesional tissue of AD-like mice, and PDS very effectively improved STAT6 phosphorylation and TSLP mRNA levels.

Table 2 The chemical information and quantitative analysis of key compounds authentically in PDS

Peak no.	Compound name	Molecular formula	Degree in network	Correlating targets	Rt (min)	Adduct	Calculated molecular ions	Measured molecular ions (m/z)	Error (ppm)	Origin
1	Daidzein	$C_{15}H_{10}O_4$	5	TNF IL6 IL4 IL2 CXCL8	14.930	$(M+H)^+$	255.0651	255.0635	−1.9	*Pueraria* Root
2	Luteolin	$C_{15}H_{10}O_6$	4	TNF IL6 IL4 CXCL8	16.037	$(M+H)^+$	287.0550	287.0532	−6.2	*Perilla* Leaf *Schizonepeta* Spike
3	Quercetin	$C_{15}H_{10}O_7$	7	TNF TLR4 IL6 IL4 IL2 CXCL8 CCL5	16.8	$(M+H)^+$	303.0499	303.0494	−1.6	*Perilla* Leaf *Atractylodes* Rhizome *Cnidium* Rhizome *Aurantii* Fructus
4	Apigenin	$C_{15}H_{10}O_5$	4	TNF IL6 IL4 CXCL8	18.376	$(M+H)^+$	271.0601	271.0588	−4.7	*Perilla* Leaf *Pueraria* Root *Citrus Unshiu* Peel *Cnidium* Rhizome *Aurantii* Fructus
5	Kaempferol	$C_{15}H_{10}O_6$	5	TNF IL6 IL4 CXCL8 CCL5	19.09	$(M+H)^+$	287.0550	287.0535	5.2	*Aurantii* Fructus
6	Caffeic acid	$C_9H_8O_4$	6	TNF TLR4 IL6 IL4 IL2 CXCL8	n.d	$(M+H)^+$	181.0495	–	–	*Perilla* Leaf *Citrus Unshiu* Peel *Cnidium* Rhizome *Platycodon* Root
7	Genistein	$C_{15}H_{10}O_5$	6	TNF TLR4 IL6 IL4 IL2 CXCL8	n.d	$(M+H)^+$	271.0601	–	–	*Pueraria* Root

n.d: not detected

Although a compound can be thoroughly extracted when it is prepared from only one herb, sometimes its extraction efficiency can be drastically reduced due to various chemical and physical factors when it is extracted with other herbal materials. Thus, the presence of seven compounds that were suggested as expected active compounds based on network pharmacological analysis was validated using HPLC/QTOF/MS. Among the seven expected active compounds, caffeic acid and genistein were not detected under this experimental condition, while the remaining five expected anti-allergic active compounds, daidzein, luteolin, quercetin, apigenin, kaempferol, could be confirmed as component of in PDS based on HPLC/QTOF/MS. Furthermore, to ascertain the role of them in the anti-allergic activity of PDS, their effects were experimentally validated in vitro. As a result, luteolin, quercetin, and kaempferol from *Citrus aurantium*, *Stephanomeria tenuifolia*, *Atroctylodes lancea*, *P. frutescens*, and *Cynoglossum officinale* led to a significant improvement in STAT6 phosphorylation, implying their contribution to the anti-allergic activity of PDS. Recently, Xuanfei Baidu Decoction, a type of Baidu formula, recommended as a prescription for clinically treatment of coronavirus 2019 (COVID-19) in China, was reported to inhibit the IL-6/STAT3 signaling pathway. Although similar targets and biological pathways were suggested in the aforementioned study, both of the suggested active compounds, glycyrrhizic acid from *Glycyrrhiza uralensis* and polydatin from *Polygonum cuspidatum*, are components of the PDS formulation used here [41]. It suggests that the addition or subtraction of herbs from prescriptions of similar compositions can be a viable approach for targeting specific diseases and organs in treatment. It also demonstrates that compounds with similar activities may vary depending on the prescription, particularly when the target disease and organs are different.

In summary, we confirmed that PDS produced an inhibition of IgE-induced degranulation and STAT6 phosphorylation evoked by the inflammatory cytokine IL-4 in granulocytes. Similarly, inhibition of degranulation and STAT6 phosphorylation was demonstrated in the major five active components of PDS. In addition, inhibition of STAT6 and its downstream, thymic stromal lymphopoietin (TSLP), was confirmed in DNCB-induced mouse skin tissue, and inhibition of IL-4 and IgE was confirmed in blood. Taken together, these findings provide evidence indicating that PDS and compounds of PDS may have potential utility as novel sources of herbal medicine for the treatment of atopic dermatitis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00815-0.

Additional file 1. Additional methods; Detection of IL-33 in UVB-irradiated and LL-37-treated HaCaT keratinocytes; Assay of IgE from U266B1 cells, **Table S1:** Composition of PDS, **Table S2:** List of physicochemical properties, quantitative estimate of drug-likeness (QED), and oral bioavailability (OB) of expected active compounds from PDS, **Fig. S1:** Effects of PDS on allergic disease-related mediators, **Fig. S2:** Effects of compounds derived from PDS onallergic disease-related mediators in RBL-2H3 and HaCaT cells, **Fig. S3:** Comparison of standard reference compoundswith PDS through QTOF/MS, **Fig. S4**: HPLC chromatogram and UV spectrum of standard apigenin (A) and PDS (B), **Fig. S5:** Workflow of network pharmacology analysis and experimental validation.

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Author contributions
SNK, SHK, YJK, HBP and YKK conceived and designed the experiment. SHL, YO, SKB, JWL and NJP performed the experiment. SHL, YO, SHK and SNK analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript. To the best of our knowledge and belief, neither the entire paper nor any part of its content has been published or accepted elsewhere. It is not under consideration for publication in another journal at the time of submission.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
All authors declare no competing financial interests.

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