



Topical application of *Kochia scoparia* inhibits the development of contact dermatitis in mice

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ABSTRACT

Ethnopharmacological relevance: *Kochia scoparia* (Chenopodiaceae) has been reported to have anti-nociceptive, anti-inflammatory, anti-allergic, and anti-pruritic actions. This study investigated the anti-inflammatory effects of externally applied *Kochia scoparia* water extract (KSW) in 2,4-dinitrochlorobenzene (DNCB)-induced contact dermatitis mouse model.

Materials and methods: To develop atopic dermatitis-like skin lesions, 100 μ L of 1% DNCB in acetone/olive oil (4:1) had been applied for three days on shaved dorsal skin. 1% KSW was topically applied to DNCB-induced mice. After KSW treatment, histological analysis was measured by hematoxylin eosin staining. The cytokine and pro-inflammatory expressions were examined using reverse transcription polymerase chain reaction and western blotting analysis.

Results: Histological studies showed that hyperplasia of the epidermis and dermis in the KSW treated group was markedly decreased as compared with the DNCB group. The expression levels of pro-inflammatory cytokine such as IL-1 β , and TNF- α mRNA were significantly reduced by topical application of KSW, whereas these cytokines were increased in DNCB-induced dorsal skin. In addition, NF- κ B expression was inhibited by KSW treatment in DNCB-induced mice. Similarly, KSW treatment significantly suppressed the expression of several MAP kinases, including ERK1/2, p38, and JNK compared to their expression in DNCB-induced mice.

Conclusions: These findings indicated that KSW ameliorates contact dermatitis via inhibition of the production of several inflammatory mediators. Therefore, external application of KSW may be used for the treatment of contact dermatitis as an alternative therapy.

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1. Introduction

Contact dermatitis (CD) is one of the most common skin diseases and is characterized by a significant skin inflammatory reaction resulting from exposure to irritants or allergens from the outdoor (Kamsteeg et al., 2010). As the world becomes more industrialized and the population grows, the incidence of CD is continually increasing, with a prevalence rate of approximately 10–20% worldwide (Cahill et al., 2004). Many studies have shown that skin barrier disturbances are the initial step in the development of CD, as the skin is the body's largest and most exposed interface with the environment (Fluhr et al., 2008).

CD has been medically treated using topical corticosteroids (Guilpain and Le Jeune, 2012). However, side effects such as skin atrophy, striae distensae, and perioral dermatitis in sensitive areas have prevented long-term use of corticosteroids (Kawai et al., 2007). Moreover, treatments for these side effects remain a challenge. Recently, new plant-derived compounds have been reported as potential alternatives for CD treatment (Guo et al., 2008; M.H. Kim et al., 2013). Treatments using natural compounds are expected to prevent the onset of inflammatory diseases and ameliorate allergic symptoms (Norris, 2005; Y.Y. Choi et al., 2013). Accordingly, many studies have suggested that using natural medicines can decrease inflammation in skin disease (Yamaura et al., 2011; J.H. Kim et al., 2013).

The dried fruit of *Kochia scoparia* (Chenopodiaceae) is used medicinally in Korea as a main ingredient in traditional herbal formulas indicated for external and internal applications for skin diseases and rheumatoid arthritis (Lee et al., 2011; Choi et al.,

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2002). It has been reported that *Kochia scoparia* has a peripheral anti-nociceptive effect mediated by its anti-inflammatory actions and this effect can be partially attributed to momordin Ic, a principal saponin constituent of *Kochia scoparia* (Matsuda et al., 1997). In addition, momordin Ic showed the pro-apoptotic and gastroprotective effects (Matsuda et al., 1999; Wang et al., 2013). Also *Kochia scoparia* has been mentioned as a treatment for liver disorders and used in traditional medicine for the alleviation effect of jaundice and edema (Agrawal and Beohar, 2010). In addition, methanol extract of the *Kochia scoparia* fruit appeared to be a potential therapeutic agent for treating lipopolysaccharide (LPS)-induced sepsis syndrome (Shin et al., 2004). Although therapeutic potentials of *Kochia scoparia* for anti-inflammatory activity have been investigated, its effect in contact dermatitis is poorly understood.

Based on the combined findings of previous studies, we investigated the anti-inflammatory activity of the water extract of *Kochia scoparia* (KSW). Furthermore, our findings contribute to the understanding of cutaneous inflammation. We also discuss the external application of KSW that have been considered as a means of manipulating pro-inflammatory mediator production with a focus on improving skin health.

2. Materials and methods

2.1. Preparation of KSW

Kochia scoparia was purchased from Omni Herb, Inc. as a dried herb (Andong-si, Gyeongbuk, south) and authenticated by Dr. Woong Mo Yang, who is a national-licensed doctor of Korean medicine and a professor of College of Korean Medicine at Kyung Hee University. The dried fruit of *Kochia scoparia* (250 g) was extracted with 250 mL boiling distilled water for 1 h, and the extract was then concentrated under low pressure. After filtration, an aqueous solution of the extract was concentrated in a rotary evaporator, freeze-dried for three days, and stored in aliquots at 4 °C. The yield of dried extract from starting crude materials was about 17.9% (w/w) for 44.8 g dry weight. A voucher specimen (#*Kochia scoparia* water extract: KSW001) was deposited in our laboratory, and 1% KSW extract was dissolved in phosphate buffered saline (PBS) for animal treatment.

2.2. Standardization of KSW

KSW was characterized based on the content of momordin Ic and determined using HPLC. Briefly, the extracts were dissolved in ethanol (1 g/mL) and injected into HPLC (Agilent 1100 Series) by auto-sampler. A ZORABAX Eclipse (150 × 4.6 mm², 5 μm,) column was used for the chromatographic separation analysis. The mobile phase consisted of water and acetonitrile. Separation was achieved by a linear gradient elution from 5% in 0 min, 5% in 8 min, 50% in 15 min, 100% in 28 min and 100% in 35 min at a flow rate of 0.5 mL/min. The separation temperature was set at 25 °C and the detection wavelength was 210 nm.

2.3. Animal treatment

Female BALB/c mice, 7 weeks of age, were obtained from SLC, Inc. (Hamamatsu, Japan). Animals were housed in an animal room at 23 °C with a relative humidity of 55% and a 12-h light/dark cycle. The mice were given an unlimited amount of water and food throughout the duration of the experiment. Animal care and experimental procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education and Welfare, NIH publication # 78-23, 1996). Animals were randomized into three groups ($n=5$ per group): Nor (mice treated with vehicle alone), DNCB (mice sensitized with DNCB) and DNCB+KSW (mice sensitized with DNCB and treated with KSW). After a 7-day adaptation period, the hair on the upper back was shaved and the experiment was immediately started. The barrier function of the shaved dorsal skin was disrupted by applying 100 μL of 4% sodium dodecyl sulfate (SDS). Then, 100 μL of 1% DNCB in acetone/olive oil (4:1, v/v) was applied to the dorsal skin for three days to induce dermatitis, followed by no treatment for five days. After the first challenge, the treatment was repeated with 4% SDS (in distilled water) for removing the skin barrier and 0.5% DNCB. Mice in the KSW group were then topical treated with 1% KSW for 14 days. Mice were sacrificed on day 17 of the experiment. Skin tissues from the backs of the mice were excised and subjected to histological analysis, reverse transcription polymerase chain reaction (RT-PCR) and western blot examination.

2.4. Histological analysis

Dorsal skins were fixed in 4% formaldehyde for 24 h. Formalin-fixed, paraffin-embedded skin tissues were sectioned at a thickness of 4 μm, and the sections were stained with hematoxylin and eosin (H&E). Images were taken at a magnification of 100 ×, and the thicknesses were measured using Leica Application Suite (LAS; Leica Microsystems, Buffalo Grove, IL, USA).

2.5. Total RNA isolation and quantitative reverse transcription-PCR

Total RNA was extracted from dorsal skins using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instruction and quantified by determining the OD at 260 nm. Isolated total RNA (1 μg) was then reverse-transcribed into cDNA using commercially available cDNA synthesis kits (Invitrogen Corp., Carlsbad, Buffalo Grove, IL, USA). In all assays, cDNA of each group was amplified using a program listed as follows: stage 1: 45 °C for 60 min; stage 2: 95 °C for 5 min. Amplification of cDNA was conducted with Taq polymerase (Promega) and primers specific for IL-1β, TNF-α mRNA and GAPDH. The primers used for amplifying were designed using Primer Express Software (Applied Biosystems) and listed in Table 1. Optimum conditions for RT-PCR were established using routine methods. The conditions used for IL-1β and TNF-α were 35 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 2 min, and final extension at 72 °C for 10 min. The intensity of gene expression was calculated and normalized to the levels of housekeeping genes (GAPDH) mRNA. The relative changes in target gene expression were analyzed by Quantity One software.

Table 1

RT-PCR sequence of target designed by the software of applied Bioscience Prism 7000 system.

Target gene	Forward primer	Reverse primer	Reverse primer
GAPDH	5'-GGC ATG GAC TGT GGT CAT GA -3'	5'-TTC ACC ACC ATG GAG AAG GC-3	376
IL-1β	5'-CTC TAG ACC ATG CTA CAG AC-3'	5'-TGG AAT CCA GGG GAA ACA CTG-3'	291
TNF-α	5'-GGT GCA ATG CAG AGC CTT CC-3'	5'-CAG TGA TGT AGC GAC AGC CTG G-3'	173

2.6. Western blot analysis

To examine the effects of KSW on the production of nuclear factor κ B (NF- κ B) and mitogen-activated protein (ERK1/2, JNK and p38), western blot analysis was performed. The frozen tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 1% nonidet P-40, 0.5% sodium deoxycholate, and 150 mM NaCl) containing protease inhibitors (Hoffmann-La Roche Inc., Nutley, NJ, USA). After vortexing for 15 s, the tissue homogenate was incubated on ice for 15 min. This process was repeated four times. Protein fractions were obtained as pellets after centrifugation at 10,000g for 30 min at 4 °C. The protein concentration was measured using a protein assay reagent (Bio-Rad, Hercules, CA, USA), and 30 μ g protein was denatured with SDS buffer. Each sample was separated on a 10% SDS-polyacrylamide gel, and the proteins were then electro transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked by incubation with Tris-Buffered Saline and Tween 20 (TBS-T) buffer containing 5% skim milk for 1 h at room temperature (RT). The membrane was then lightly washed with TBS-T and incubated overnight at 4 °C with primary antibody (NF- κ B, Anti-ERK1/2, phospho-ERK1/2, anti-p38, phospho-p38 MAPK, anti-SAPK/JNK and phospho-SAPK/JNK; diluted 1:1000 in TBS-T). Anti-rabbit alkaline phosphatase-conjugated secondary antibody (diluted 1:2000 in TBS-T) was then added, followed by incubation for 1 h at RT. The proteins were then visualized using an enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia, Piscataway, NJ, USA). The relative band density was determined using a computerized densitometry system and normalized to the β -actin and signal from a blot developed under similar conditions.

2.7. Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). Differences between treatment groups were examined using a one-way analysis of variance (ANOVA) and Student's *t*-tests. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Phytochemical analysis of KSW by HPLC

Examination of the HPLC chromatograms of momordin Ic (Fig. 1A) and KSW (Fig. 1B). Comparison of the peak was made on the basis of their ultraviolet absorption spectra and retention times, which showed that the peak for KSW (B) corresponded to momordin Ic (A), which is a component of the dried fruit of *Kochia scoparia*. The retention time of momordin Ic was 19.2 min. The content of momordin Ic in of KSW water extract was 1.45% (Fig. 1). This result was consistent with previous report (Balsevich et al., 2009).

3.2. Effects of KSW on skin thickening and hyperplasia

We examined whether application of KSW affected the interrupted skin barrier in CD using H&E staining. No inflammation was detected in the normal control group. In contrast, the DNCB group showed hyperplasia and hyperkeratosis of the epidermis and dermis (69.4% and 60.9%, respectively), which was worse than that measured in the normal control group ($P < 0.001$; Fig. 2). The KSW-treated group showed markedly diminished hyperplasia and thickening of the epidermis and dermis (39.2% and 35.6%, respectively) as compared with the DNCB group ($P < 0.001$; Fig. 2).

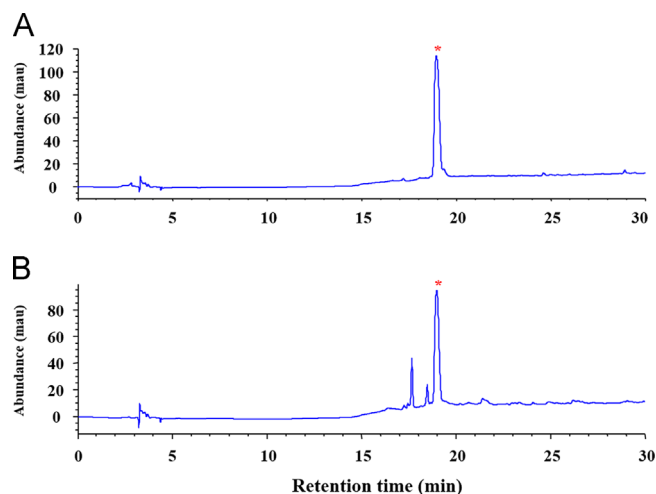


Fig. 1. High-performance liquid chromatography (HPLC) profile of standard materials on KSW. HPLC chromatograms of (A) authentic standard (Momordin Ic) and (B) KSW. The peaks for KSW corresponded to standard compound, which are components of *Kochia scoparia*.

3.3. Inhibitory effects of KSW on pro-inflammatory cytokines

After DNCB application for indicated time periods, the total RNA was extracted from dorsal skin and the expressions of the IL-1 β and TNF- α mRNA were determined using RT-PCR. Expressions of IL-1 β and TNF- α mRNA were increased in DNCB group compared to normal group ($P < 0.001$). KSW treatment significantly reduced the expression of IL-1 β (60.9%, $P < 0.001$) and TNF- α (54.2%, $P < 0.01$) compared to DNCB group (Fig. 3).

3.4. Inhibitory effects of KSW on NF- κ B expression

To determine the mechanisms underlying the effects of KSW, we quantified the protein expression levels of NF- κ B by western blot analysis. DNCB mice had 73.8% higher expression of NF- κ B than the normal control group. On the other hand, we observed that NF- κ B expression was markedly suppressed by 33.6% in KSW treatment mice, as compared to DNCB group ($P < 0.01$; Fig. 4).

3.5. Inhibitory effects of KSW on MAP kinases expressions

DNCB stimulated MAP kinase activity, as demonstrated by the increased phosphorylation of several MAP kinases. Similar to the NF- κ B expression results, KSW treatment significantly suppressed the expression of ERK1/2, p38 and JNK, by 69.4%, 35.9%, 68.9% respectively, as compared with DNCB-induced mice ($P < 0.001$; Fig. 5).

4. Discussion

Inflammation can be a normal physiological self-regulated response or underpin the pathology of cutaneous diseases (Homey et al., 2006). Therefore, a detailed understanding of the mediators involved in inflammation can be valuable for directing efforts to identify new therapeutics (J.H. Choi et al., 2013; Im et al., 2011). The present data revealed the anti-inflammatory effects of KSW via its external application with regard to several inflammatory symptoms and the expression of several pro-inflammatory mediators.

Several studies have demonstrated that the DNCB application model could be adopted to induce a CD-like phenomenon in mice, which has been reported as a reliable model demonstrating

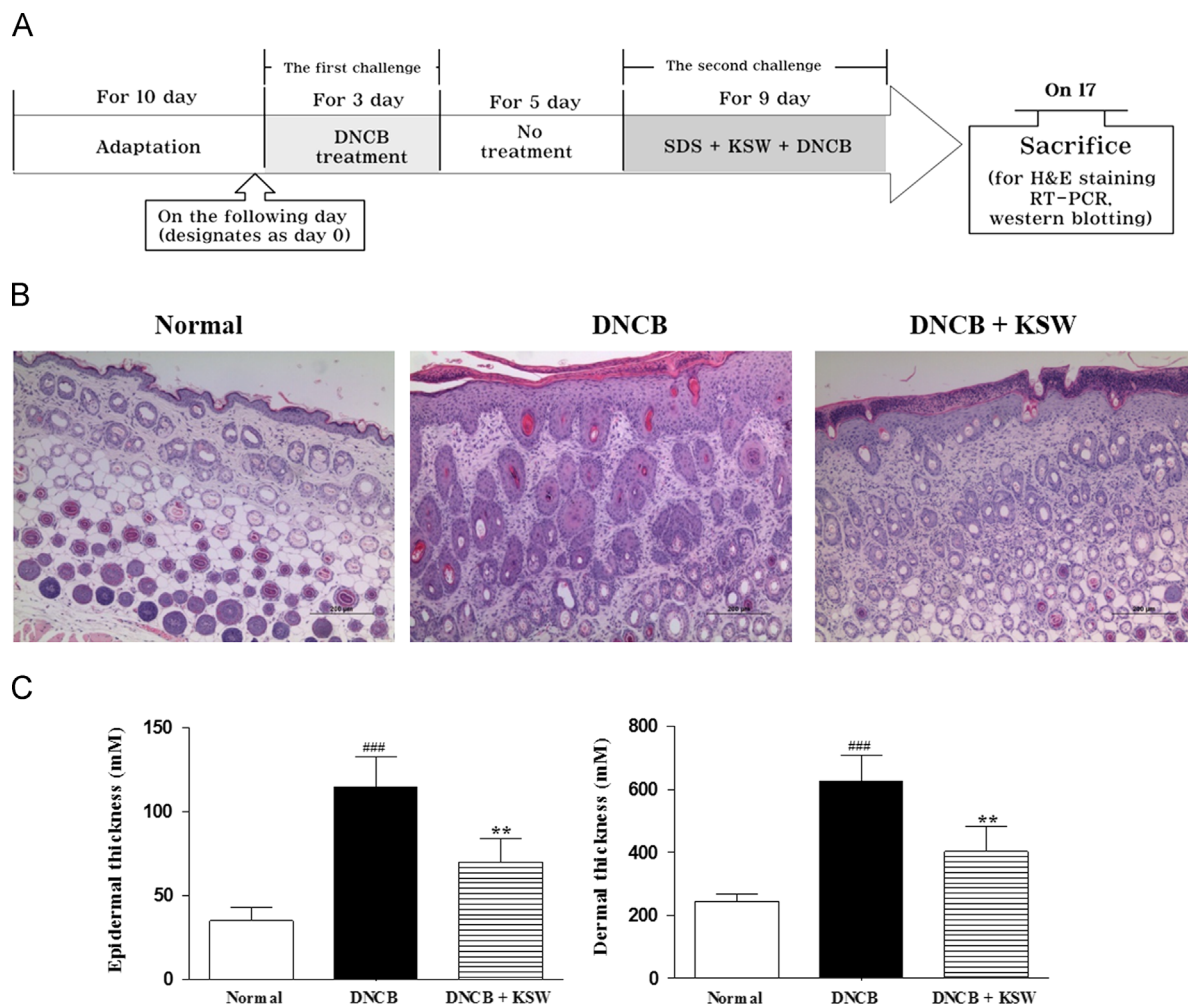


Fig. 2. Experiment design and effects of KSW extract on skin lesions and histological changes in DNCB-induced AD-like symptoms mice. (A) Experiment design and (B) histopathological findings by H&E staining of skin section ($n=5$, magnification: $100\times$). Each data represent the mean $\pm$ SEM. [#] Indicates significance for the difference between normal control group and LPS group ($^{###}P < 0.001$). ^{*} Indicates significant difference from LPS group ($^{**}P < 0.01$).

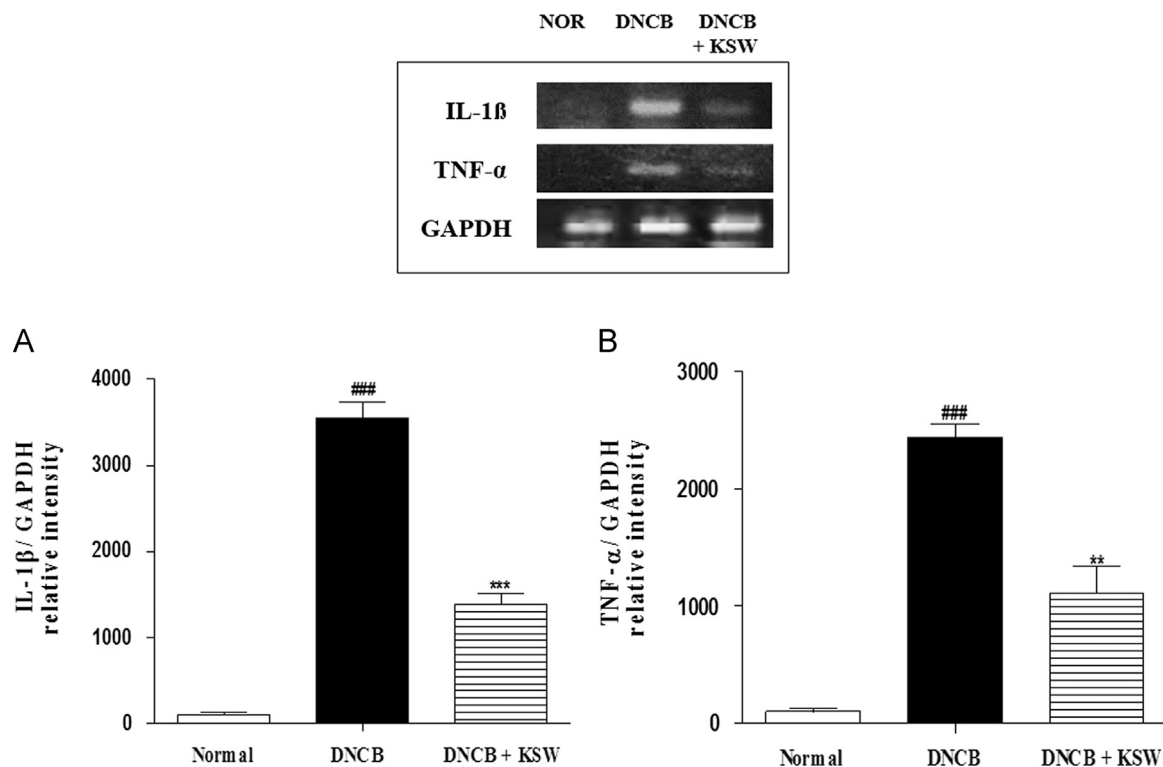


Fig. 3. Topical applications of KSW suppress the gene expressions of IL-1 β and TNF- α in DNCB-induced AD-like symptoms. Each data represent the mean $\pm$ SEM. [#] Indicates significance for the difference between normal control group and LPS group ($^{###}P < 0.001$). ^{*} Indicates significant difference from LPS group ($^{***}P < 0.001$, $^{**}P < 0.01$).

similar skin alterations as those seen in human CD (Chiang et al., 2012). Hyperplasia, skin erosion, epidermal necrosis, and hyperproliferation are hallmarks of skin inflammatory diseases, such as CD (de Jongh et al., 2008). Based on these previous reports, we confirmed the development of CD-like skin lesions on mice through repeated topical application of DNCB. Our histological analysis showed that the dermis and epidermis were thickened in the DNCB mice compared to normal mouse skin, but hyperplasia and thickening of the epidermis and dermis were significantly reduced in the DNCB+KSW group (39.2% and 35.6%, respectively). Namely, topical application of KSW significantly improved the severity of CD-like skin lesions by suppressing skin thickening and hyperkeratosis. These improvements could be partially attributed to the anti-inflammatory properties of KSW.

Skin inflammation is induced by the contact allergens involving the production of pro-inflammatory cytokines such as IL-1 β and TNF- α (Hoefakker et al., 1995; Dinarello, 2011). IL-1 β is known to be important in a number of severe inflammatory diseases and

most of these diseases can be completely controlled by anti-IL-1 β treatment (Goldbach-Mansky et al., 2006). TNF- α also is an important cytokine involved in the maintenance of inflammatory processes in the skin, and TNF- α is stressed by its capacity to induce IL-1 β (Groves et al., 1995). Consistent with these studies, we found that IL-1 β mRNA expression level was significantly increased by repeated DNCB application in mice, as well as TNF- α mRNA expression. KSW treatment significantly down-regulated IL-1 β and TNF- α mRNA expression by 60.9% ($P < 0.001$) and 54.2% ($P < 0.01$), respectively, compared with control mice. Thus, our results suggested that the anti-inflammatory activity of KSW was a function of the inhibition of IL-1 β mRNA expression with down-regulation of TNF- α mRNA.

NF- κ B is a central component in the cellular response to damage, stress, and inflammation, which has been implicated in the pathogenesis of many diseases as the result of pro-inflammatory cytokines (Kim and Shin, 2009; Smahi et al., 2002). In our experiment, KSW could remarkably down-regulate NF- κ B levels (33.6%, $P < 0.01$) in CD-like skin lesion, which likely affected anti-inflammatory processes at the affected site. Furthermore, we demonstrated that KSW had a potential role in the suppression of the NF- κ B signaling pathway which is mediated by TNF- α and IL-1 β production.

Another important signaling related to inflammation is the MAP kinase (MAPKs) pathway. MAPKs are a family of highly conserved signal transduction mediators that allow cells to respond to multiple extracellular inputs, such as hormones and growth factors acting through inflammatory cytokines (Imajo et al., 2006). These pathways are closely involved in diseases such as psoriasis and inflammation (Huang et al., 2010). Hence, MAPKs represent important targets for therapeutic intervention. Our results showed that the DNCB-induced phosphorylation of ERK1/2, JNK and p38 was significantly suppressed by KSW treatment. Especially, the observed reduction of ERK1/2, p38 (69.4%, 68.9%) expression by KSW treatment was particularly striking. ERK1/2 and p38 are activated in immune cells by inflammatory cytokines and play an important role in immune response activation (Chen et al., 2013; Iwasa et al., 2003). It is expected that the significant inhibition of p38 activity by KSW will affect immune-related disease. In addition, momordin Ic, a major component of *Kochia scoparia*, has been reported to have a pro-apoptotic effect through the MAPK mediated iNOS pathways (Wang et al., 2013). NO participation related with MAPK pathways may contact, interplay, and influence each other to regulate an inflammation. Thus, the

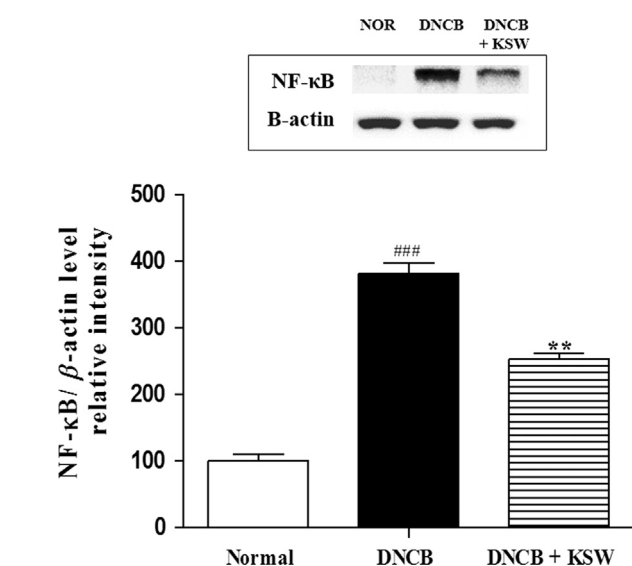


Fig. 4. Topical applications of KSW decrease the NF- κ B protein expressions in DNCB-induced AD-like symptoms in mice. Each data represent the mean $\pm$ SEM. # Indicates significance for the difference between normal control group and LPS group (### $P < 0.001$). * Indicates significant difference from LPS group (** $P < 0.01$).

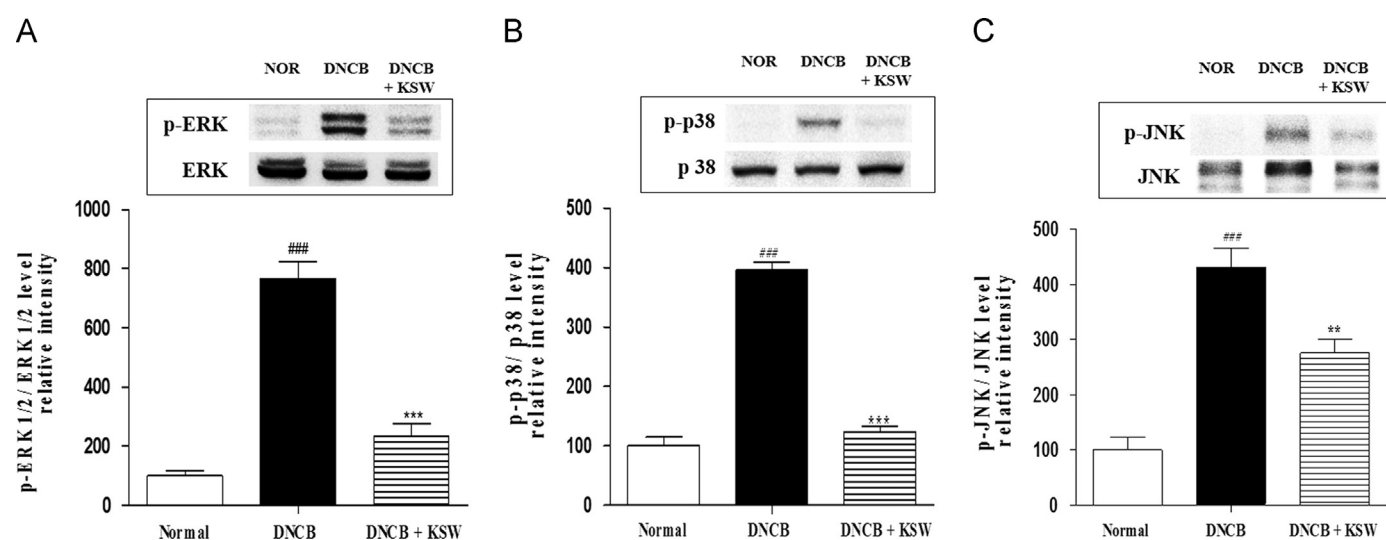


Fig. 5. Topical applications of KSW suppress the MAP kinase protein expressions in DNCB-induced AD-like symptoms. (A) phosphor-ERK1/2, (B) phosphor-p38 and (C) phosphor-JNK. Each data represent the mean $\pm$ SEM. # Indicates significance for the difference between normal control group and LPS group (### $P < 0.001$). * Indicates significant difference from LPS group (*** $P < 0.001$, ** $P < 0.01$).

effects of KSW on NO participation, cytokine production during immune responses as well as on inflammatory genes in the contact dermatitis are important questions for further studies.

5. Conclusion

In conclusion, our data demonstrated that topical application of KSW extract on skin tissues exerted anti-inflammatory effects against DNCB-induced CD. In histopathological analyses of the epidermis and dermis, the topical application of KSW inhibited skin thickening and hyperkeratosis, which was significantly decreased in DNCB-induced mice, and suppressed IL-1 β and TNF- α mRNA expression. Furthermore, we found that KSW inhibits DNCB-induced inflammation via regulation of NF- κ B and MAP kinases. It appears that the inhibition of MAPKs by external application of KSW may synergize with the inhibition of NF- κ B activation. Our data provide mechanistic evidence for the anti-inflammatory actions of KSW observed clinically upon its external application.

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