

# Anti-inflammatory activity of *Kochia scoparia* fruit on contact dermatitis in mice

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**Abstract.** The mature fruit of *Kochia scoparia* (L.) Schrad. is widely administered in China and Korea as a medicinal herb for treatment of skin diseases, diabetes mellitus and rheumatoid arthritis. The present study investigated the effects of methanol extracts of *K. scoparia* dried fruit (MEKS) on ear swelling, histopathological changes (such as epidermal acanthosis, spongiosis and immune cell infiltration) and cytokine production in 1-fluoro-2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis mice. Topical application of MEKS inhibited DNFB-induced ear thickness and weight increases, as well as DNFB-induced epidermal acanthosis, spongiosis and immune cell infiltration. In addition, treatment with MEKS significantly decreased the levels of tumor necrosis factor- $\alpha$ , interferon- $\gamma$  and monocyte chemoattractant protein-1 in inflamed tissues. These data indicate that the mature fruit of *K. scoparia* has the potential to be administered for the treatment of inflammatory skin diseases and that the anti-inflammatory action of *K. scoparia* is involved in the inhibition of type 1 T helper cell skewing reactions.

## Introduction

Contact dermatitis (CD), the predominant inflammatory skin disease worldwide, is a type of eczematous eruption that occurs following contact with foreign substances (1,2). The histopathological features of CD include epithelial hyperkeratosis, acanthosis and spongiosis that present with perivascular inflammatory cell infiltration, primarily consisting of T cells in the epidermis and upper dermis (3).

Following exposure to a skin irritant, various cells, including activated keratinocytes, dendritic cells, dermal fibroblasts, and endothelial cells, release pro-inflammatory and inflammatory cytokines/chemokines (4). The cytokines/chemokines induce the dilatation of blood vessels and inflammatory cell infiltration, resulting in epidermal acanthosis and spongiosis, however, the precise mechanism remains to be elucidated (4). Tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$  and monocyte chemoattractant protein (MCP)-1 are key primary cytokines in the activation cascade and pathogenesis of CD (4,5).

The fruit of *Kochia scoparia* (L.) Schrad., which is also designated *Bassia scoparia*, *Bassia sieversiana* or *Kochia alata*, is administered to treat skin diseases, diabetes mellitus and rheumatoid arthritis in Chinese and Korean traditional medicine (6,7). Furthermore, it is frequently administered to treat urticaria in Taiwan (8). Previous studies have indicated that *K. scoparia* and its components exert anti-inflammatory (9) and anti-allergic (10,11) activities. Choi *et al* (12) recently reported that the water extract of *K. scoparia* inhibits the development of CD in mice.

The aim of the current study was to evaluate the anti-inflammatory effects of methanol extracts of *K. scoparia* dried fruit (MEKS) on 1-fluoro-2,4-dinitrobenzene (DNFB)-induced CD. For this reason, the effects of MEKS on ear thickness and weight, the histopathological changes in ear tissue samples, and the cytokine and chemokine levels of inflamed tissues were assessed *in vivo*.

## Materials and methods

**Preparation of MEKS.** The mature fruit of *K. scoparia* was purchased from Hwalim Medicinal Herbs (Pusan, Korea). A total of 100 g *K. scoparia* dried fruit was immersed in 1,000 ml methanol and sonicated for 30 min, following which the samples were extracted for 48 h. The extract was subsequently filtered through Whatman filter paper No. 20 (Advantech, Milpitas, CA, USA) and evaporated under reduced pressure using a vacuum evaporator (N-1000V-W; Eyela Co., Ltd., Tokyo, Japan), following which the condensed extract was lyophilized using a freeze dryer (Labconco Corporation, Kansas City, MO, USA) and 4.46 g lyophilized powder was obtained (yield, 4.46%). An aliquot of the extract (MEKS)

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was deposited at the Department of Pharmacology, School of Korean Medicine, Pusan National University (Yongsan, South Korea; voucher no. MH2013-006).

**Animals.** A total of 44 male 6-week-old BALB/c mice were purchased from Samtaco (Incheon, Korea). The mice were housed under specific pathogen-free conditions with a 12-h light/dark cycle and free access to standard rodent food and water. All animal experiments were approved by the Animal Care and Use Committee of Pusan National University and performed according to institutional guidelines (PNU-2011-000406).

**Induction of CD and experimental design.** Mice were sensitized by applying 50  $\mu$ l DNFB (0.1%, v/v; Sigma-Aldrich, St. Louis, MO, USA), in a vehicle of acetone:olive oil (AOO; 4:1), onto the shaved back of each mouse for three consecutive days. Four days following sensitization, each mouse was challenged by applying 30  $\mu$ l DNFB (0.2%, v/v) in AOO onto the dorsal surface of each ear every two days (four applications in total). MEKS solution (30, 100 or 300  $\mu$ g/ear) was applied onto the dorsal surface of each ear for seven consecutive days. Healthy mice (n=6) were treated with the vehicle, AOO and only AOO was topically applied (non-treated normal mice; NOR). Control mice (n=8) were sensitized and challenged with DNFB, following which AOO was topically applied (non-treated CD mice; CTL). MEKS-treated mice (n=8) were sensitized and challenged with DNFB, and 30, 100 or 300  $\mu$ g/ear (1%, w/v) of MEKS was topically applied. Dexamethasone (DEX; Sigma-Aldrich)-treated mice (n=6) were sensitized and challenged with DNFB, exposed to 75  $\mu$ g/ear DEX and served as a positive control. The experimental design is summarized in Fig. 1.

**Measurement of ear thicknesses and weights.** Mice were anesthetized with 30 mg/kg Zoletil<sup>®</sup> (Virbac, Carros, France) and sacrificed by cervical dislocation, following which the thickness of each ear was measured using vernier calipers (Mitutoyo, Kanagawa, Japan). The pieces of ear (5 mm in diameter) obtained via dermal punch were weighed using a microbalance (US/EL-2000S; Sartorius AG, Göttingen, Germany).

**Histopathological examination.** Following assessment of ear thicknesses and weights, ear tissues (4  $\mu$ m) were resected, formalin-fixed (Sigma-Aldrich) and embedded in paraffin (Leica Microsystems GmbH, Wetzlar, Germany). Sections were cut and stained with hematoxylin and eosin (Sigma-Aldrich) to observe histopathological changes, such as epidermal acanthosis, spongiosis and immune cell infiltration. Stained tissue sections were observed using a light microscope (magnification, x200; DE/Axio Imager A1; Carl Zeiss AG, Oberkochen, Germany).

**Evaluation of epidermal acanthosis and immune cell infiltration.** To evaluate the epidermal acanthosis and immune cell infiltration, five non-overlapping fields per slide were randomly selected and images were captured with the light microscope. To measure the thickness of the epithelium, the vertical length between the basal lamina and top of

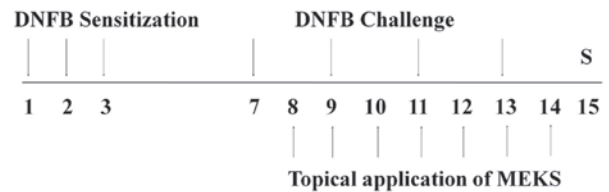


Figure 1. Experimental design. On days 1, 2 and 3 DNFB was applied to the shaved backs of mice in the treatment groups for sensitization. DNFB was then applied to the dorsal surface of each ear on days 7, 9, 11 and 13. MEKS and dexamethasone were topically applied for 7 consecutive days. All animals were sacrificed on day 15. DNFB, 1-fluoro-2,4-dinitrofluorobenzene; MEKS, methanol extracts of *Kochia scoparia* dried fruit; S, sacrifice.

the outermost stratum granulosum was quantified. For each slide, five lengths were measured at random using Motic Images Plus 2.0 (Motic Instruments, Richmond, BC, Canada), following which the mean epithelial thicknesses of all experimental groups were used for analysis. To evaluate immune cell infiltration, the immune cells were counted using a cell counting grid.

**Measurement of cytokine production.** Cytokine levels in the ear tissue samples were measured according to the cytometric bead array (CBA) method with the Mouse Inflammation CBA kit (BD Biosciences, San Jose, CA, USA). Resected ear tissue samples were lysed and homogenized with protein extraction solution (Pro-Prep; Intron Biotechnology, Seoul, Korea) using a bullet blender (BB2516; Next Advance, Averill Park, NY, USA) to obtain tissue lysates. The levels of TNF- $\alpha$ , IFN- $\gamma$ , interleukin (IL)-10 and MCP-1 were measured in 50  $\mu$ g each lysate using the CBA kit. All experimental procedures were conducted according to the manufacturer's protocols.

**Measurement of body and spleen weights.** Body and spleen weights were measured on day 15 using the microbalance. The effects of MEKS on changes in spleen weights were analyzed as the spleen/body weight ratio.

**Statistical analysis.** A Mann-Whitney U test was used for all statistical comparisons, and Prism 5 for Windows version 5.01 (GraphPad Software Inc., La Jolla, CA, USA) was used for all analyses. All data are presented as the mean  $\pm$  standard deviation and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**MEKS decreased the DNFB-induced changes in ear thickness and weight.** Topical application of DNFB induced ear swelling, which is a major feature of CD. These increases in the thickness and weight of ear tissues were inhibited in a dose-dependent manner by topical application of MEKS (ear thickness:  $P < 0.001$ , MEKS treatment on left ear;  $P < 0.01$ , MEKS treatment on right ear;  $P < 0.001$ , DEX treatment on both ears. Ear weight:  $P < 0.05$ , 100 or 300  $\mu$ g/ear MEKS treatment;  $P < 0.001$ , 75  $\mu$ g/ear DEX treatment) as presented in Fig. 2.

**MEKS inhibited epidermal spongiosis in the inflamed tissue.** Histological examination in the tissue sections of

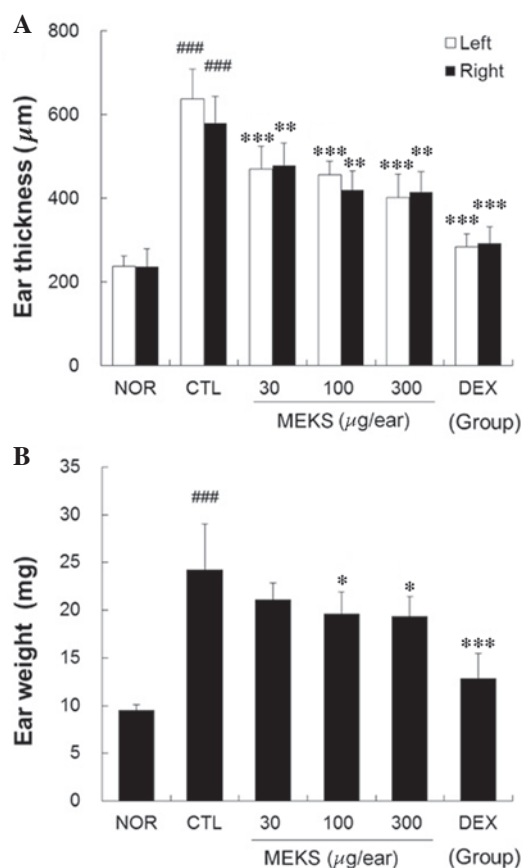


Figure 2. Effect of MEKS on ear thickness and weight in CD mice. Reduction of ear (A) thickness and (B) weight, in response to topical application of MEKS, was analyzed using vernier calipers and a microbalance on day 15. Values are presented as means  $\pm$  standard deviation. ###P<0.001 vs. NOR; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. CTL. CD, contact dermatitis; NOR, non-treated normal mice; CTL, non-treated CD mice; DEX, dexamethasone; MEKS, methanol extracts of *Kochia scoparia* dried fruit.

the NOR group demonstrated normal epidermal thickness and a smaller degree of immune cell infiltration into the dermis (Fig. 3A). Repeated application of DNFB induced small and large subcorneal and intraepidermal vesicles, diffused spongiotic changes and intercellular edema, which are characteristics of CD (Fig. 3B). Topical application of MEKS inhibited epidermal spongiosis in the inflamed tissue (Fig. 3C-E).

**MEKS inhibited epidermal acanthosis and immune cell infiltration.** Repeated application of DNFB induced acanthosis, hyperkeratosis and focal crust formation in the epidermis. In addition, DNFB induced diffuse acute and chronic immune cell infiltration, blood vessel dilation and perivascular eosinophil infiltration into the dermis. Topical application of MEKS effectively relieved epidermal acanthosis and immune cell infiltration in a dose-dependent manner (epithelial thickness: P<0.05, 100  $\mu$ g/ear MEKS treatment; P<0.01, 300  $\mu$ g/ear MEKS treatment; P<0.001, 75  $\mu$ g/ear DEX treatment. Immune cell infiltration: P<0.001, 100 or 300  $\mu$ g/ear MEKS treatment or 75  $\mu$ g/ear DEX treatment) as presented in Fig. 4.

**MEKS reduced expression levels of TNF- $\alpha$ , IFN- $\gamma$  and MCP-1 in ear tissue samples of CD mice.** Marked increases

in TNF- $\alpha$ , IFN- $\gamma$  and MCP-1 production were observed in the CTL group. These increases were effectively reduced in a dose-dependent manner by topical application of MEKS (TNF- $\alpha$  and IFN- $\gamma$ : P<0.05, 100  $\mu$ g/ear MEKS treatment; P<0.01, 300  $\mu$ g/ear MEKS treatment; P<0.001, 75  $\mu$ g/ear DEX treatment. MCP-1: P<0.01, 300  $\mu$ g/ear MEKS treatment or 75  $\mu$ g/ear DEX treatment). Treatment with MEKS did not affect the production of IL-10, although DEX treatment reduced the IL-10 level significantly when compared with that of the NOR and CTL groups (P<0.05, 75  $\mu$ g/ear DEX treatment) as presented in Fig. 5.

**MEKS did not affect spleen/body weight ratio in CD mice.** The effects of MEKS on enlargement of the spleen were estimated in terms of the spleen/body weight ratio. The spleen/body weight ratio in the CTL group was significantly elevated compared with the NOR group (P<0.001). Treatment with MEKS did not affect the spleen/body weight ratio in CD mice. However, this ratio was significantly reduced in the DEX group when compared with that of the CTL and NOR groups (Fig. 6; P<0.001).

## Discussion

The present study demonstrated the anti-inflammatory effects of MEKS on CD. MEKS effectively prevented ear thickness and weight increases, as well as epidermal acanthosis, spongiosis and immune cell infiltration in inflamed tissues. In addition, the expression levels of TNF- $\alpha$ , IFN- $\gamma$  and MCP-1 were dose-dependently reduced in response to MEKS. These findings indicate that MEKS treatment prevents the inflammatory reactions, which leads to the inhibition of ear thickness and weight increases in inflamed tissues.

Inflammatory reactions, such as immune cell infiltration and pro-inflammatory cytokine production, are important in the pathophysiology of CD and may serve as therapeutic targets. During progression of CD, IL-1 and TNF- $\alpha$  markedly upregulate various chemokines, resulting in the recruitment of leukocytes (5). In addition, IFN- $\gamma$  is indicative of type 1 T helper skewing reaction of T cells and rapidly promotes the secretion of mediators, including chemokine (C-X-C motif) receptor 3 agonist and MCP-1, when it is present alone or together with TNF- $\alpha$  (13,14). The results of the present study are consistent with those of a previous study by Choi *et al* (12), who demonstrated that topical application of *K. scoparia* extract inhibited the expression of IL-1 $\beta$  and TNF- $\alpha$  messenger RNA. In the present study, the production of TNF- $\alpha$ , IFN- $\gamma$ , MCP-1 and IL-10 was evaluated using proteins obtained from samples of lysed tissues. Treatment with MEKS effectively decreased expression levels of TNF- $\alpha$  and IFN- $\gamma$  in inflamed tissues. MCP-1, also termed chemokine (C-C motif) ligand 2, is a small chemokine that belongs to the CC chemokine family, which recruits monocytes, T cells and dendritic cells to inflammatory sites (15,16). In the present study, treatment with 300  $\mu$ g/ear of MEKS significantly inhibited MCP-1 production in samples of ear tissue. These results indicate that the underlying mechanism of MEKS inhibition of immune cell infiltration involves decreasing MCP-1 production.

The effects of MEKS on CD were further demonstrated by histopathological analysis, which indicated that epidermal

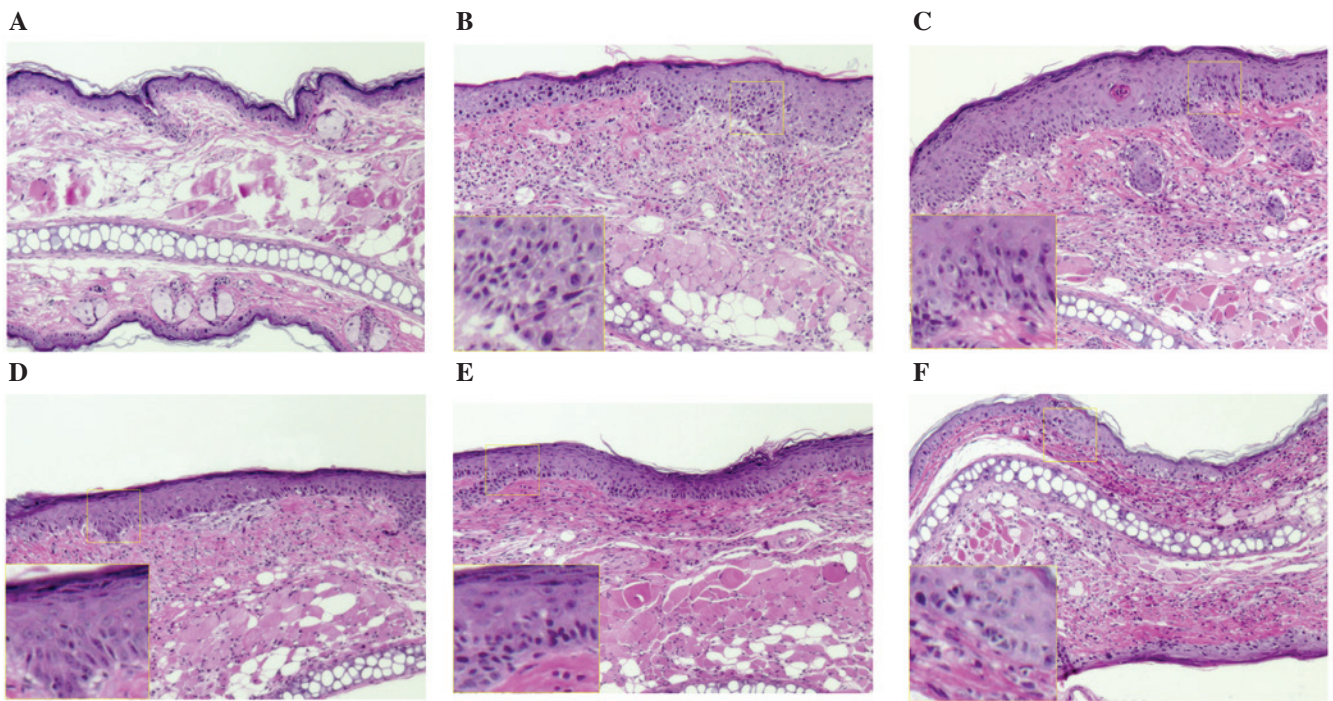


Figure 3. Effect of MEKS on histopathological changes in CD mice. Ear tissues were stained with hematoxylin and eosin and observed using a light microscope. (A) NOR group, (B) CTL group, (C) 30  $\mu\text{g}/\text{ear}$  MEKS-treated group, (D) 100  $\mu\text{g}/\text{ear}$  MEKS-treated group, (E) 300  $\mu\text{g}/\text{ear}$  MEKS-treated group and (F) 75  $\mu\text{g}/\text{ear}$  dexamethasone-treated group. The insets represent spongiotic areas. Magnification,  $\times 200$ . MEKS, methanol extracts of *Kochia scoparia* dried fruit; CD, contact dermatitis; NOR, non-treated normal mice; CTL, non-treated CD mice.

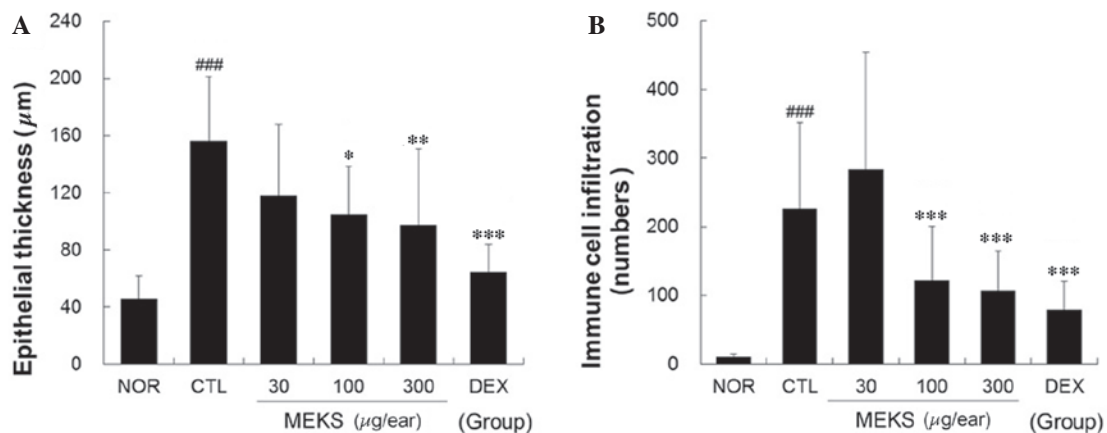


Figure 4. Effects of MEKS on epidermal acanthosis and immune cell infiltration in inflamed tissue. (A) The epithelial thickness and (B) number of infiltrated immune cells were observed under a light microscope. All values are presented as means  $\pm$  standard deviation.  $###P < 0.001$  vs. NOR;  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$  vs. CTL. MEKS, methanol extracts of *Kochia scoparia* dried fruit; DEX, dexamethasone; NOR, non-treated normal mice; CTL, non-treated contact dermatitis mice.

hyperplasia, spongiosis and immune cell infiltration were decreased by topical application of MEKS. Keratinocytes at the site of CD overexpress numerous cytokines and chemokines, such as  $\text{TNF-}\alpha$ ,  $\text{IFN-}\gamma$ , MCP-1 and IL-10 (17-19). It has been previously reported that  $\text{IFN-}\gamma$  is important in the development of skin hypertrophy, and  $\text{TNF-}\alpha$  and MCP-1 function as stimulants of immune cell recruitment around blood vessels (17-19). In the present study, the topical application of MEKS inhibited the histopathological features of CD via suppression of  $\text{TNF-}\alpha$ ,  $\text{IFN-}\gamma$  and MCP-1 expression. These observations and the changes in cytokine production in the MEKS-treated tissue samples, suggest that MEKS is an

anti-inflammatory agent against the Th1 skewing reaction, thus, reducing inflammatory reactions, such as epidermal acanthosis, spongiosis and immune cell infiltration.

DEX and MEKS prevented ear swelling, diminished epidermal acanthosis, spongiosis and immune cell infiltration and decreased the levels of  $\text{TNF-}\alpha$ ,  $\text{IFN-}\gamma$  and MCP-1 in ear tissue samples. MEKS did not affect the expression of IL-10, however, DEX significantly reduced the expression levels of all cytokines examined in the present study, including IL-10, which has a suppressive role in CD and atopic dermatitis (20). Treatment with DEX also led to a significant reduction in the spleen/body weight ratio when compared with the CTL

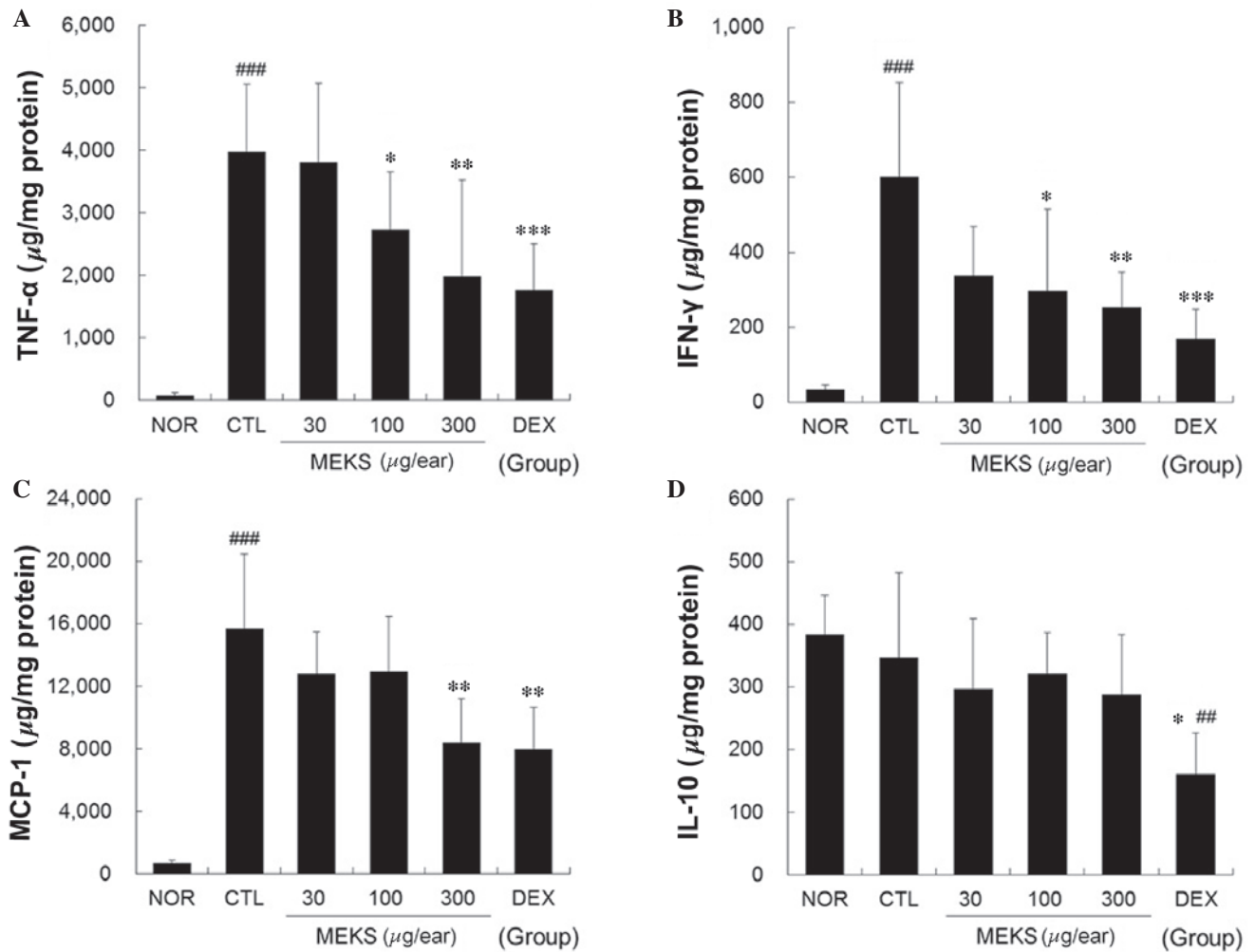


Figure 5. Effect of MEKS on expression levels of TNF- $\alpha$ , IFN- $\gamma$ , MCP-1 and IL-10 in CD mice. The expression levels of (A) TNF- $\alpha$ , (B) IFN- $\gamma$ , (C) MCP-1 and (D) IL-10 in the ear tissues were analyzed using the cytometric bead array method. A total of 50  $\mu$ g tissue lysates was used to measure the cytokine levels. All values are presented as means  $\pm$  standard deviation. ##P<0.01, ###P<0.001 vs. NOR; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. CTL. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ ; interferon- $\gamma$ ; MCP-1, monocyte chemoattractant protein-1; IL-10, interleukin-10; NOR, non-treated normal mice; CD, contact dermatitis; CTL, non-treated CD mice; MEKS, methanol extracts from *Kochia scoparia* dried fruit; DEX, dexamethasone.

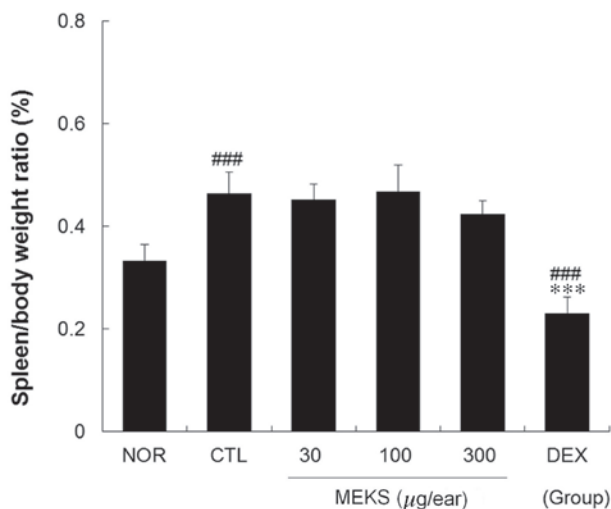


Figure 6. Effect of MEKS on spleen/body weight ratio in CD mice. Body and spleen weights were measured on day 15 and the spleen/body weight ratio was calculated. All values are presented as means  $\pm$  standard deviation. ###P<0.001 vs. NOR; \*\*\*P<0.001 vs. CTL. MEKS, methanol extracts of *Kochia scoparia* dried fruit; CD, contact dermatitis; NOR, non-treated normal mice; CTL, non-treated CD mice; DEX, dexamethasone.

and the NOR groups, which is indicative of an immune reaction. These findings indicate that the underlying therapeutic mechanism of MEKS differs to that of DEX, particularly with regards to general immune suppression, which is one of the major side effects of corticosteroids, such as DEX.

The findings of the present study indicate that MEKS may be administered for the treatment of inflammatory skin diseases. In addition, the current study suggests that an anti-inflammatory mechanism of MEKS is involved in the inhibition of Th1 skewing reactions.

In conclusion, the present study demonstrated that MEKS reduces Th1 skewing reactions, such as production of TNF- $\alpha$ , IFN- $\gamma$  and MCP-1, resulting in decreased epidermal acanthosis, spongiosis and immune cell infiltration. Repeated administration of MEKS resulted in anti-inflammatory reactions leading to the inhibition of ear swelling. The effects of MEKS were similar to those of DEX, however, no general immune suppression was observed in response to MEKS treatment. The findings of the present study indicate that MEKS may be used to reduce or replace corticosteroid use with relative safety.

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