Anti-allergic effects of sesquiterpene lactones from the root of *Aucklandia lappa* Decne

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Abstract. *Aucklandia lappa* Decne, a well-known traditional herbal medicine, is used for the treatment of asthma, rheumatism, coughs, tuberculosis and numerous other diseases. The present study evaluated the inhibitory effects of the three sesquiterpene lactones costunolide, dehydrocostus lactone, and alantolactone, isolated from a 70% methanolic extract of *Aucklandia lappa*, on the expression of chemokine mRNA in HaCaT human keratinocyte cells. The cytotoxicities of the compounds on HaCaT cells were evaluated using a Cell Counting Kit8 assay. Furthermore, the inhibitory effects of the three compounds on chemokine expression in tumor necrosis factor (TNF)-α- and interferon (IFN)-γ-stimulated HaCaT cells were analyzed by reverse transcription-polymerase chain reaction analysis. Treatment with the compounds caused a significant reduction in the mRNA expression of a range of chemokines, including TARC/CCL17, MDC/CCL22, RANTES/CCL5 and interleukin-8 in TNF-α and IFN-γ-stimulated HaCaT cells. The present study indicated that costunolide, dehydrocostus lactone and alantolactone may have the potential to be used for treating inflammatory skin disorders by suppressing chemokine expression.

Introduction

Aucklandiae Radix, the root of *Aucklandia* (*A.*) *lappa* Decne, syn. *Saussurea lappa* C.B. Clarke, which belongs to the family of *Asteraceae*, has been officially documented as 'Mokhyang' in the Korean Herbal Pharmacopoeia (1-4). *A. lappa* has been traditionally used to treat anorexia, nausea and abdominal pain (5). Several biological properties of *A. lappa*, have been confirmed by scientific studies, including its anti-oxidative (6),

anti-ulcer (7), anti-cancer (8), anti-viral (9) and hepatoprotective effects (10). A recent study by our group reported that *A. lappa* alleviates inflammatory chemokine production in HaCaT cells and house dust mite-induced atopic-like dermatitis in Nc/Nga mice (11). In addition, it has been reported that sesquiterpene lactones from *A. lappa* have anti-cancer (12,13), anti-ulcer (14,15), and anti-inflammatory (16) effects. However, to the best of our knowledge, the effects of active components of *A. lappa* against atopic dermatitis have not yet been investigated. The present study investigated the effects of the three sesquiterpene lactones costunolide, dehydrocostus lactone and alantolactone on the expression of Thelper type 2 chemokines, which are essential factors in the development of atopic dermatitis, in HaCaT human keratinocytes.

Materials and methods

Preparation of 70% methanolic extract. The roots of *A. lappa* used in the present study were purchased from HMAX (Jecheon, Korea) in July 2009. The botanical identity of this sample was confirmed taxonomically by Professor Je-Hyun Lee (Department of Herbology, College of Korean Medicine, Dongguk University, Gyeongju, Republic of Korea). A voucher specimen (no. 2009-KIOM62) has been deposited at the Herbal Medicine Formulation Research Group (K-Herb Research Center, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea). Dried roots of *A. lappa* (100 g) were extracted three times with 70% (v/v) methanol (1 l) (JT Baker, Phillipsburg, NJ, USA) for 90 min with heating under reflux. The extracted solution was filtered through filter paper and the solvent was evaporated at 40˚C using a Büchi R-210 rotary evaporator (Büchi, Flawil, Switzerland) under vacuum to dryness, followed by freeze-drying (PVTFD10R; IlShinBioBase Co., Ltd., Dongducheon, Korea). The yield of the freeze-dried 70% methanolic extract obtained was 28.57% (28.57 g).

High-performance liquid chromatography (HPLC) analysis. Reference compounds costunolide, dehydrocostus lactone and alantolactone were purchased from ChemFaces (Wuhan, China). The purities of the three sesquiterpene lactones were ≥98.0% according to HPLC analysis. HPLC-grade solvents methanol, acetonitrile and water were obtained from JT

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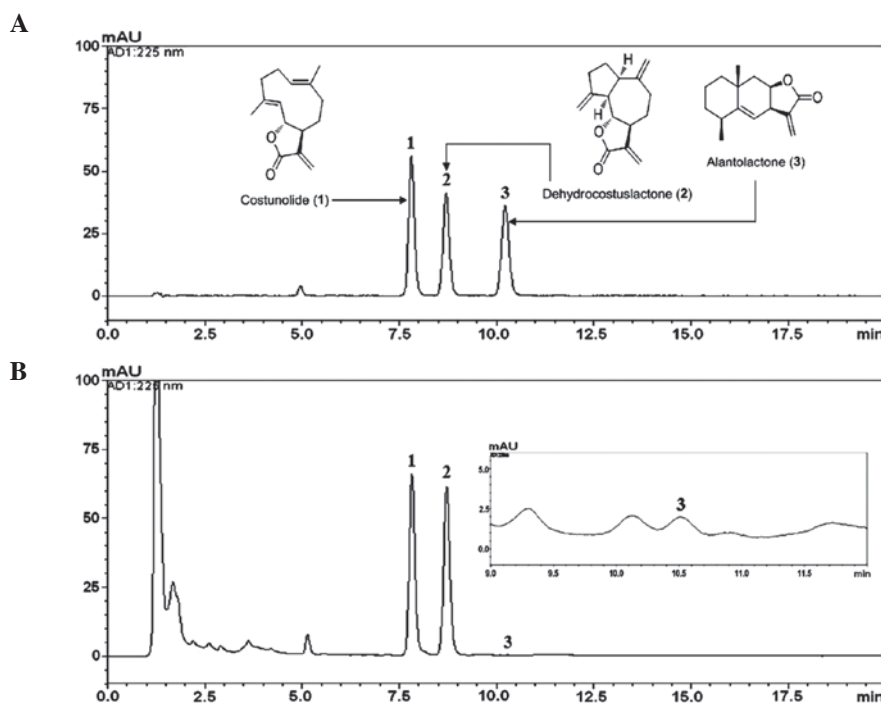E-mail: hkshin@kiom.re.kr

Figure 1. High performance liquid chromatograms of (A) standard solution and (B) *Aucklandia lappa* extract.

Baker. Glacial acetic acid (analytical grade) was purchased from Merck KGaA (Darmstadt, Germany). The sample was analyzed using a Shimadzu Prominence LC-20A series HPLC apparatus (Shimadzu Co., Kyoto, Japan) consisting of a solvent delivery unit (no. LC-20AT), on-line degasser (no. DGU-20A3), column oven (no. CTO-20A), auto sample injector (no. SIL-20AC) and PDA detector (no. SPD-M20A). Data were collected and processed using LCsolution software (version 1.24; Shimadzu Co.). The stationary phase used for the separation of the compounds was a reverse-phase SunFire™ C_{18} analytical column (Waters, Milford, MA, USA; 150x4.6 mm and 5 μm particle size). The mobile phase was composed of water (A) and acetonitrile (B) with isocratic elution (i.e., 40% A and 60% B). The flow rate was 1.0 ml/min, the column temperature was maintained at 35°C, and the injection volume was 10 μl. The detection wavelength for quantification covered the range of 190-400 nm and recorded at 225 nm. To prepare the stock solutions, reference compounds were accurately weighed and dissolved in methanol to a concentration of 1.0 mg/ml; the samples were stored below 4°C. The concentration ranges of test samples for the generation of calibration curves were 1.56-200.00, 2.34-300.00 and 0.23-30.00 μg/ml for costunolide, dehydrocostus lactone and alantolactone, respectively. For HPLC analysis of the 70% methanolic extract, 20 mg extracted solid was dissolved in 10 ml 70% methanol and dissolved by sonication for 30 min. The solution was filtered through a 0.2-μm membrane filter (Woongki Science, Seoul, Korea) prior to injection into the HPLC instrument.

Cell culture. The HaCaT human keratinocyte cell line was obtained from CLS Cell Lines Service GmbH (Eppelheim, Baden-Württemberg, Germany). The HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM,

Gibco-BRL, Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), penicillin (100 μg/ml; Gibco-BRL) and streptomycin (100 μg/ml; Gibco-BRL) in an incubator containing 5% CO_2 at 37°C.

Cytotoxicity assay. Cell viability was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) following the manufacturer's instructions. HaCaT cells ($1x10^3$ cells/well) were incubated in 96-well plates with costunolide or dehydrocostus lactone at 0, 1.25, 2.5, 5 or 10 μM or with alantolactone at 0, 0.625, 1.25, 2.5 or 5 μM for 24 h. CCK-8 reagent was added to each well and cells were incubated for an additional 4 h. The absorbance was measured at 450 nm using a Benchmark plus microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viable cells was calculated as follows: Cell viability (%) = (mean absorbance in test wells/mean absorbance in untreated control well) x100.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Inc.). HaCaT cells ($1x10^6$ cells/well) were cultured to 80-90% confluency in 6-well plates. When the cells reached confluence, the cells were washed and treated with costunolide, dehydrocostus lactone or alantolactone in 1 ml serum-free medium (Gibco-BRL) supplemented with tumor necrosis factor (TNF)-α and interferon (IFN)-γ (each 10 ng/ml; R&D Systems Inc., Minneapolis, MN, USA) for 24 h. Silymarin (Sigma-Aldrich Inc., St. Louis, MO) was used as a positive control drug. Total RNA (1 μg) was then converted into cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc.) containing oligo-dT primers.
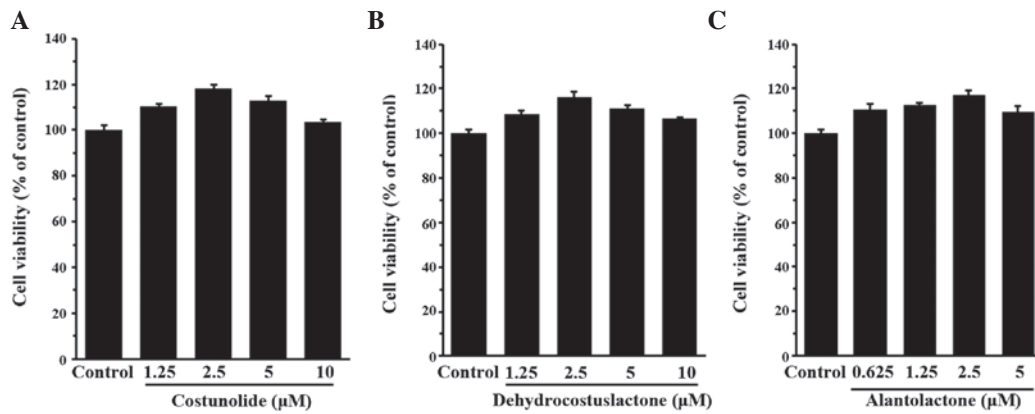
Figure 2. Cytotoxicity of the components of *Aucklandia lappa* extract on HaCaT cells. Cells were seeded onto 96-well plates and treated with various concentrations of the components of *Aucklandia lappa* extract for 24 h. (A) Costunolide, (B) dehydrocostus lactone and (C) alantolactone. Cell viability was assessed using a Cell Counting kit-8 assay. Values are expressed as the mean ± standard error of the mean of three independent experiments.
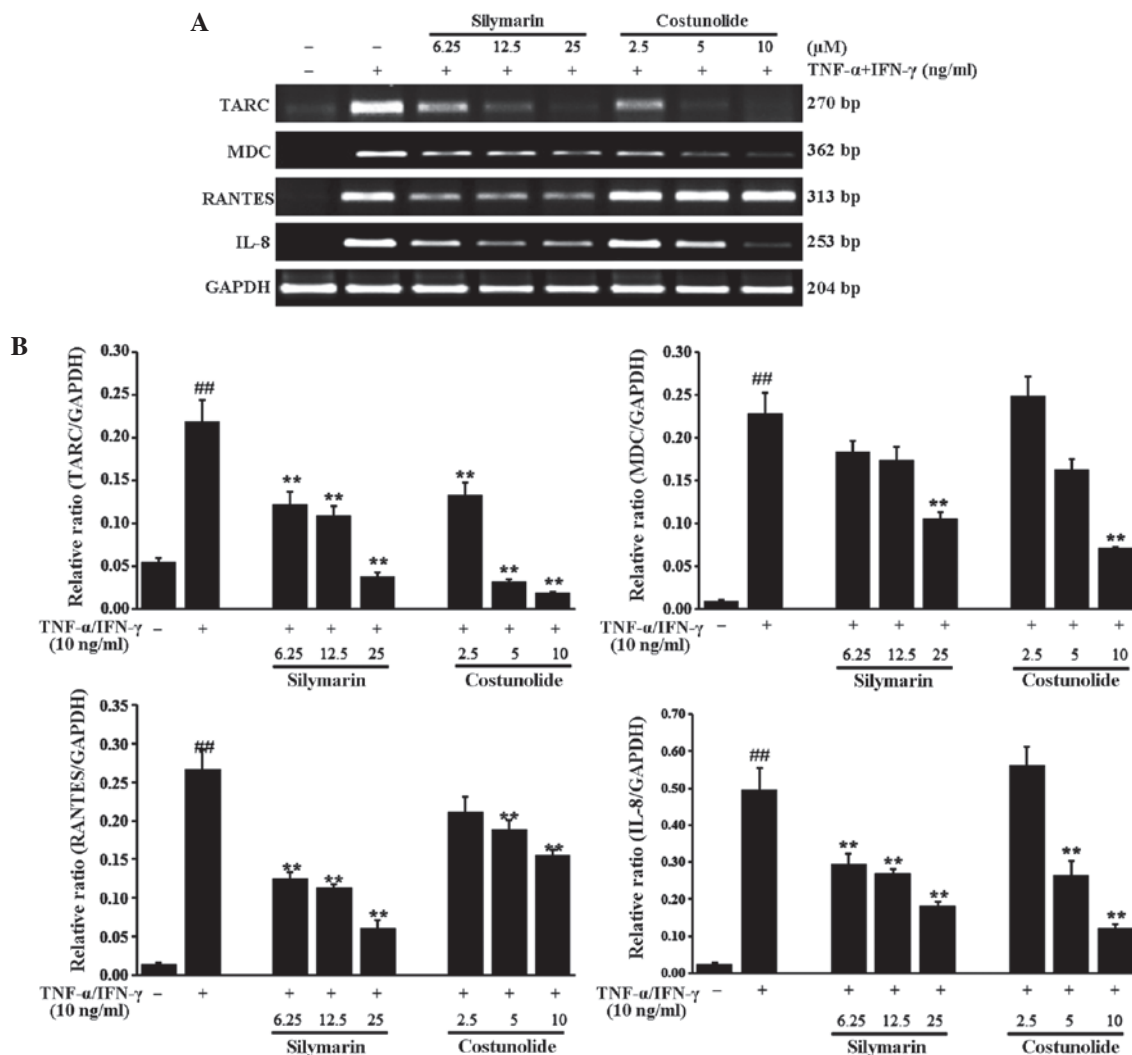

Figure 3. Effect of costunolide on TNF-α- and IFN-γ-stimulated expression of chemokine mRNA in HaCaT cells. (A) Reverse transcription PCR was performed to determine the mRNA expression levels of TARC, MDC, RANTES and IL-8. (B) Bar graphs representing the intensities of the PCR bands. Values are expressed as the mean ± standard error of the mean of three independent experiments. ##P<0.01 vs. vehicle control cells; **P<0.01 vs. TNF-α- and IFN-γ-stimulated cells. PCR, polymerase chain reaction. IFN, interferon; TNF, tumor necrosis factor.

Diethylpyrocarbonate-treated water was added to a final volume of 20 µl followed by incubation at 42°C for 30 min using a Bio-Rad iCycler apparatus (Bio-Rad Laboratories,

Inc.). For PCR amplification, the following gene-specific primers were used: TARC forward, 5'-ACTGCTCCAGGG ATGCCATCGTTTTT-3' and reverse, 5'-ACAAGGGGA
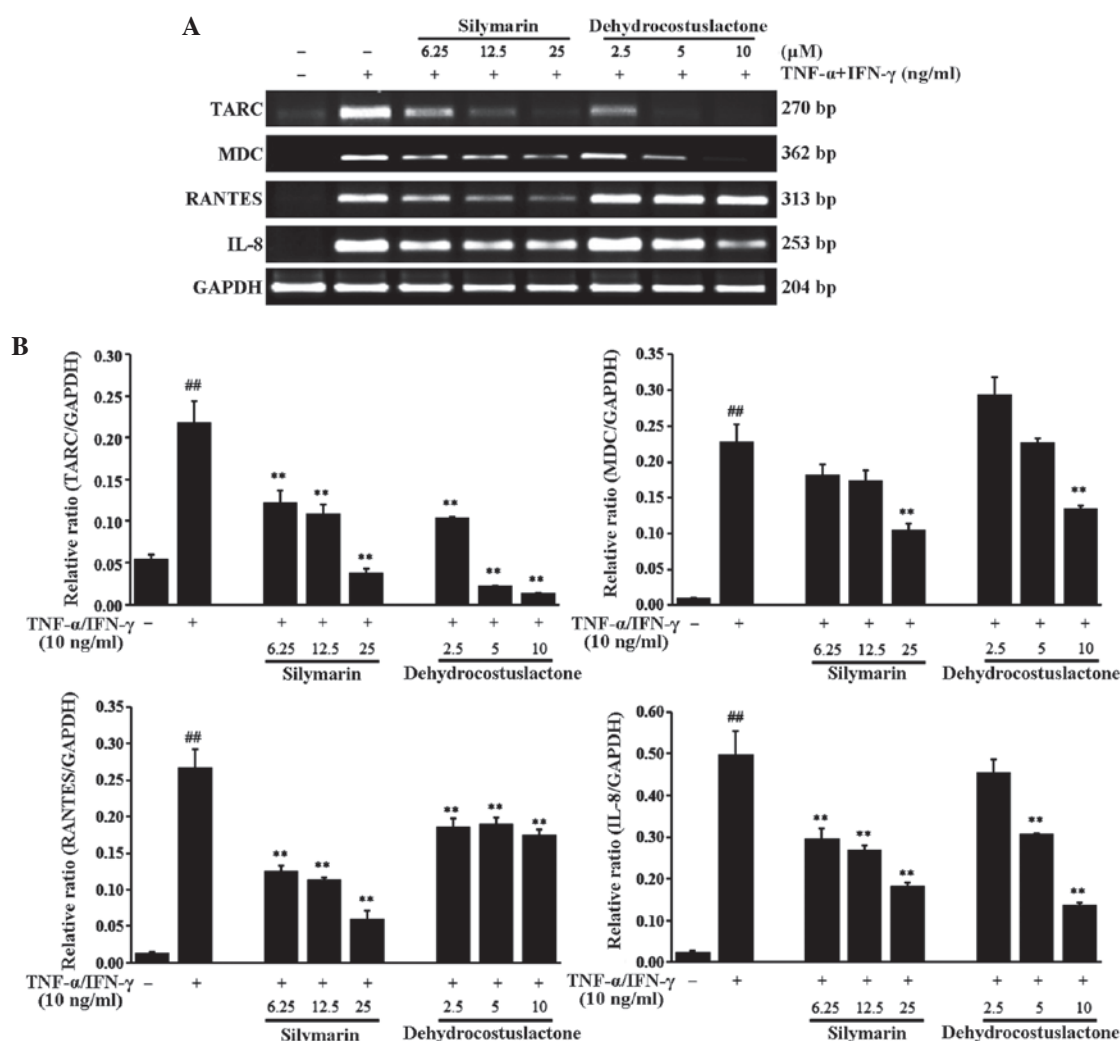
A

B

Figure 4. Effect of dehydrocostus lactone on TNF-α- and IFN-γ-stimulated expression of chemokine mRNA in HaCaT cells. (A) Reverse transcription PCR was performed to determine the mRNA levels of TARC, MDC, RANTES and IL-8. (B) Bar graphs representing the intensities of the PCR bands. Values are expressed as the mean ± standard error of the mean of three independent experiments. ##P<0.01 vs. vehicle control cells; **P<0.01 vs. TNF-α- and IFN-γ-stimulated cells. PCR, polymerase chain reaction. IFN, interferon; TNF, tumor necrosis factor.

TGGGATCTCCCTCACTG-3'; MDC forward, 5'-AGGACA GAGCATGGCTCGCCTACAGA' and reverse, 5'-TAATGG CAGGGAGCTAGGGCTCCTGA-3'; RANTES forward, 5'-CCCCGTGCCGAGATCAAGGAGTATTT-3' and reverse, 5'-CGTCCAGCCTGGGGAAGGTTTTTGTA-3'; IL-8 forward, 5'-GTGGCTCTCTTGGCAGCCTTCCTGAT-3' and reverse, 5'-TCTCCACAACCCTCTGCACCCAGTTT-3'; and GAPDH forward, 5'-GTGATGGCATGGACTGTGGT-3' and reverse, 5'-AAGGGTCATCATCTCTGCCC-3'. The PCR reaction mixture contained 1 µl cDNA and 1.56 µl γTaq PCR master mix (cat. no. EBT-1014; Elpis Biotech, Inc., Daejeon, Korea), which contained 1.5 mM MgCl$_2$, 0.1 µM of each forward and reverse primer and 7.44 µl water in a final volume of 10 µl. The thermocycling program comprised initial denaturation at 94˚C for 5 min, followed by 25 cycles of denaturation at 94˚C for 30 sec, annealing at 64˚C for 1 min, extension at 72˚C for 1 min 30 sec for all of the chemokines, and 25 cycles of denaturation at 94˚C for 30 sec, annealing at 52˚C for 1 min, and extension at 72˚C for 1 min 30 sec for GAPDH. A final extension step was conducted at 72˚C for 7 min. The amplified products were separated by 1.5% agarose gel and visualized using Loading STAR staining (A750; DYNE Bio, Seongnam, Korea). The relative expression levels of TARC, MDC, RANTES and IL-8 mRNA were normalized to those of GAPDH mRNA using a Chemi-Doc Band Analysis system (Bio-Rad Laboratories, Inc.).

Statistical analysis. Values are expressed as the mean ± standard error of the mean. All of the experiments were performed at least three times. One-way analysis of variance was used to detect significant differences between the control and treatment groups. Dunnett's test was used for multiple comparisons using GraphPad InStat version 3.10 (GraphPad Software Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a significant difference between values.

Results

Quantitative determination of three components of A. lappa extract. Each compound in the HPLC chromatogram was identified by comparing the retention times and ultraviolet
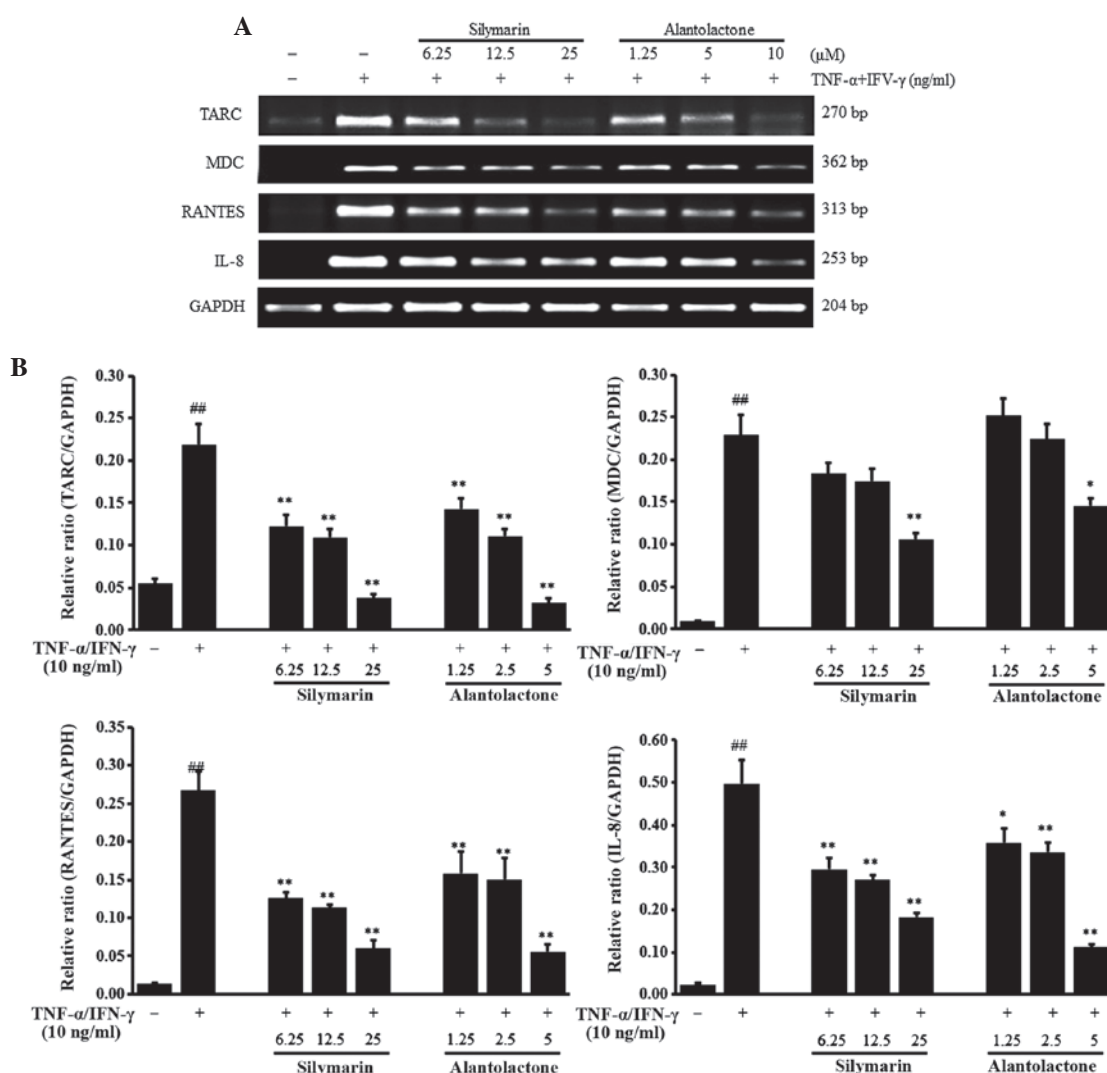
Figure 5. Effect of alantolactone on TNF-α- and IFN-γ-stimulated expression of chemokine mRNA in HaCaT cells. (A) Reversetranscription PCR was performed to determine the mRNA levels of TARC, MDC, RANTES and IL-8. (B) Bar graphs representing the intensities of the PCR bands. Values are expressed as the mean ± standard error of the mean of three independent experiments. ##P<0.01 vs. vehicle control cells; *P<0.05 and **P<0.01 vs. TNF-α- and IFN-γ-stimulated cells. PCR, polymerase chain reaction. IFN, interferon; TNF, tumor necrosis factor.

absorption spectra with those of the reference standards. The retention times of costunolide, dehydrocostus lactone and alantolactone under the optimized conditions were 7.81, 8.70, and 10.22 min, respectively (Fig. 1). Each regression equation ($y=ax+b$) was calculated based on the ratio of peak area (y) and concentration (x, μg/ml) of costunolide, dehydrocostus lactone and alantolactone. Standard curves plotted for the three compounds showed high linearity, with $r^2 \geq 0.9999$ in eight different concentration ranges tested. The established analytical HPLC method was applied for the simultaneous quantification of the three compounds in the methanolic extract of *A. lappa*. The amounts of costunolide, dehydrocostus lactone and alantolactone in the extract were 17.32, 28.26, and 0.01 mg/g, respectively.

Cytotoxicity of A. lappa extract components. The present study assessed the cytotoxic effects of the three components of the *A. lappa* extract on HaCaT cells (Fig. 2). The non-toxic concentrations of the three components were determined to be 10, 10, and 5 μM for costunolide, dehydrocostus lactone and

alantolactone, respectively. Non-toxic concentrations (>90% compared with the control) of the three components were applied in the biological assays.

Inhibitory effects of costunolide, dehydrocostus lactone and alantolactone on the chemokine mRNA levels in TNF-α- and IFN-γ-stimulated HaCaT cells. The effects of the three components of *A. lappa* extract on mRNA levels of the Thelper cell type 2 cytokines TARC, MDC, RANTES and IL-8 were assessed in TNF-α and IFN-γ-stimulated HaCaT cells. Stimulation with TNF-α and IFN-γ significantly increased the expression of TARC, MDC, RANTES and IL-8 mRNA in HaCaT cells (Figs. 3-5). Treatment of the cells with compound costunolide (Fig. 3), dehydrocostus lactone (Fig. 4) and alantolactone (Fig. 5) significantly reduced the expression of TARC, MDC, RANTES and IL-8 mRNA in a dose-dependent manner. Silymarin, a positive control, decreased the expression of TARC, MDC, RANTES and IL-8 mRNA compared with that following stimulation with TNF-α and IFN-γ in a dose-dependent manner.

Discussion

Chemokines have a pivotal role in immune responses and inflammatory reactions. Various inflammatory cytokines stimulate the production of chemokines and specific inflammatory chemokines are found in the serum of patients with atopic dermatitis (17). Among them, TARC/CCL17, MDC/CCL22 and RANTES/CCL5 are typical inflammatory chemokines that are predominantly expressed in various immune cells, including lymphocytes, dendritic cells, keratinocytes and eosinophils (18). Previous studies showed that levels of these chemokines in serum and skin lesions of patients with atopic dermatitis are elevated, suggesting that chemokines produced by keratinocytes may be key molecules that attract inflammatory lymphocytes to the skin (19). IL-8 is another important mediator in atopic dermatitis and previous studies have demonstrated that the amount of IL-8 is closely associated with the severity of atopic skin lesions (20).

The present study investigated the inhibitory effects of three components of *A. lappa*, including costunolide, dehydrocostus lactone and alantolactone on chemokine expression at the mRNA level in TNF-α and IFN-γ-stimulated HaCaT cells. All three sesquiterpene lactones markedly reduced TNF-α and IFN-γ-induced expression of TARC, MDC and IL-8 in a dose-dependent manner. While costunolide and dehydrocostus lactone had weak inhibitory effects on RANTES expression, alantolactone markedly suppressed the mRNA expression of RANTES mRNA in TNF-α and IFN-γ-stimulated HaCaT cells. These results indicated that these three components of *A. lappa* may have activity against skin inflammation through regulation of chemokine expression in keratinocytes.

Several studies have demonstrated the anti-inflammatory actions of sesquiterpene lactones from medicinal herbs, including *Milleria quinqueflora* (21), *Tithonia diversifolia* (22), *Ixeris dentate* (23) and *Eupatorium perfoliatum* (24), suggesting potential use of these herbs as anti-inflammatory agents. Similarly, the present study reported the anti-inflammatory effects of sesquiterpene lactones from *A. lappa*. Further investigation is necessary to verify the results of the present study and to evaluate the suitability of sesquiterpene lactones from *A. lappa* for the treatment of skin inflammation, including atopic dermatitis.

In conclusion, the present study demonstrated that the costunolide, dehydrocostus lactone and alantolactone, components of *A. lappa*, reduced the mRNA expression levels of chemokines TARC, MDC, RANTES and IL-8 in TNF-α and IFN-γ-stimulated HaCaT cells. These results indicated that the three compounds may be the active components of *A. lappa* that inhibit the production of chemokines. Further investigation is required to elucidate the detailed mechanisms of action of the sesquiterpene lactones, and the compounds should be subjected to toxicological tests using an *in vivo* experimental model.

Acknowledgements

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