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# Anti-Atopic Dermatitis Activity of *Cornus walteri* and Identification of the Bioactive Compounds

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**Abstract:** Atopic dermatitis (AD) is a chronic inflammatory disease characterized by hyperactivated immune reactions in the skin. AD is a prevalent health concern in developing countries, with a particularly high incidence among children. Despite ongoing research on AD, prevention and treatment strategies for patients remain inadequate. In this study, the inhibitory effects of *Cornus walteri* on AD were investigated. *C. walteri* (Cornaceae), commonly known as "Walter's dogwood," is a deciduous shrub used as a traditional medicine to treat dermatologic inflammation caused by lacquer poisoning. However, the role of *C. walteri* in AD remains largely unknown. To evaluate its anti-AD potential, we investigated the anti-inflammatory activity of the MeOH extract of *C. walteri* stems (CWS) using the HaCaT human keratinocyte cell line. CWS reduced the secretion of AD-related chemokines, eotaxin-3/CCL26 and thymus and activation-regulated chemokine (TARC/CCL17). In addition, CWS also inhibited the mRNA expression of macrophage-derived chemokine (MDC/CCL22) and upregulated filaggrin, which plays an essential role in skin barrier functions. To identify the bioactive constituents of CWS, phytochemical investigation of CWS led to the isolation of potential bioactive constituents (**1**–**6**), including four triterpenoids, one steroid and one diterpene analog, the structures of which were identified as lupeol (**1**), betulinic acid (**2**), 5$\alpha$-stigmast-3,6-dione (**3**), 3-*O*-acetylbetulin (**4**), betulinic acid methyl ester (**5**) and norphytan (**6**) through nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography (LC)–mass spectrometry (MS) analysis. The isolated compounds (**1**–**6**) were evaluated for their inhibitory activities against eotaxin-3 expression. Compounds **1**, **2** and **3** significantly reduced the levels of eotaxin-3. These findings provided experimental evidence that CWS, particularly active compounds **1**, **2** and **3**, could be further utilized as potential therapeutic agents to treat AD.

**Keywords:** Walter's dogwood; atopic dermatitis; keratinocytes; chemokine; filaggrin



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## 1. Introduction

Atopic dermatitis (AD) is a chronic skin disease caused by dysregulation of the immune system [1]. It is one of the most common inflammatory skin disorders in children, and its incidence is increasing in adults [2,3]. The skin of patients with AD is characterized by excessive immune responses and the overexpression of various cytokines/chemokines. Increased chemokines in lesional skin recruit eosinophils and activate T-helper cells [4,5].

In particular, eotaxin-3/CCL26, thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) are essential biomarkers for AD and are upregulated in patients with AD [6,7]. They trigger allergic symptoms, such as itchiness and weaken the epidermal barriers, causing dry skin. Additionally, these symptoms

lead to psychological stress, negatively affecting the quality of life [8,9]. Moreover, there are limited therapeutic options, and the current drugs are mainly immunosuppressive steroids that have various side effects [10]. Therefore, there is a great need to develop novel agents that can suppress the expression of AD mediators.

*Cornus walteri* Wanger (Cornaceae), known as "Walter's dogwood," is a deciduous shrub, which is distributed in Asian countries, including China and Korea, and it is utilized as raw material for agricultural tools and high-grade furniture. It has historically been used as a traditional medicine to treat dermatological inflammation caused by lacquer poison [11]. In addition, its leaves have been used as an antidiarrheal in traditional Korean medicine [12]. In previous pharmacological studies, the extract of *C. walteri* has been found to reduce nitric oxide production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages [13].

The leaves of this plant were reported to exhibit protective effects against photoaging induced by UVB in human reconstituted skin [11]. In our continued quest to discover bioactive phytochemicals from natural resources [14–19], our group investigated the potential bioactive constituents of *C. walteri* stems (CWS) [20–23], which have been underdeveloped and underutilized. In our previous studies, it was reported that the MeOH extract of CWS exhibited significant cytotoxic activity [20]; cytotoxic triterpenoids and δ-valerolactones were also reported [20–22]. Recently, we reported that benzyl salicylate isolated from the MeOH extract of CWS exhibited nephroprotective effects against nephrotoxicity induced by cisplatin in renal tubular epithelial (LLC-PK1) cells. However, the effects of *C. walteri* on AD remain largely unknown.

In this study, we investigated whether the MeOH extract of CWS can ameliorate AD in human keratinocytes. CWS reduces the secretion and gene expression of chemokines and enhances the mRNA expression of the skin structural protein filaggrin [24]. To identify potential bioactive phytochemicals based on the findings of CWS activity, phytochemical analysis of the MeOH extract was performed, which led to the isolation of six constituents (**1**–**6**), including four triterpenoids, one steroid and one diterpene analog.

The structures of the isolated compounds (**1**–**6**) were determined by the interpretation of nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography–mass spectrometry (LC/MS) analysis. Additionally, the isolated compounds (**1**–**6**) were evaluated for their cell viability and eotaxin-3 reducing effects on HaCaT cells. Herein, we describe our evaluation of the anti-AD activities of the MeOH extract of *C. walteri*, the isolation and structural elucidation of compounds **1**–**6** and the identification of bioactive compounds.

## 2. Results and Discussion

### *2.1. Effect of CWS against AD Chemokine Expression in Human Keratinocytes*

We first treated the HaCaT cells with 2.5–20 μg/mL MeOH extract of CWS and evaluated cell viability. No apparent cytotoxicity of CWS against HaCaT cells was observed (Figure 1A). The chemokine eotaxin-3/CCL26 is a critical factor in AD pathology and is significantly upregulated by interleukin-4 (IL-4) and IL-13 [4]. To examine the anti-AD potential of CWS, its effect on eotaxin-3 expression was examined after IL-4 and IL-13 treatment.

The production level of eotaxin-3 increased in the IL-4/IL-13-stimulated group, while CWS decreased IL-4/IL-13-induced eotaxin-3 in a dose-dependent manner (Figure 1B). Another pro-inflammatory chemokine, TARC/CCL17, is elevated in patients with AD, and IFN-γ is a major inducer of TARC [25]. IFN-γ-stimulated TARC levels decreased with CWS treatment in a dose-dependent manner (Figure 1C). These results suggested that CWS effectively reduced chemokines in AD-like HaCaT cells.

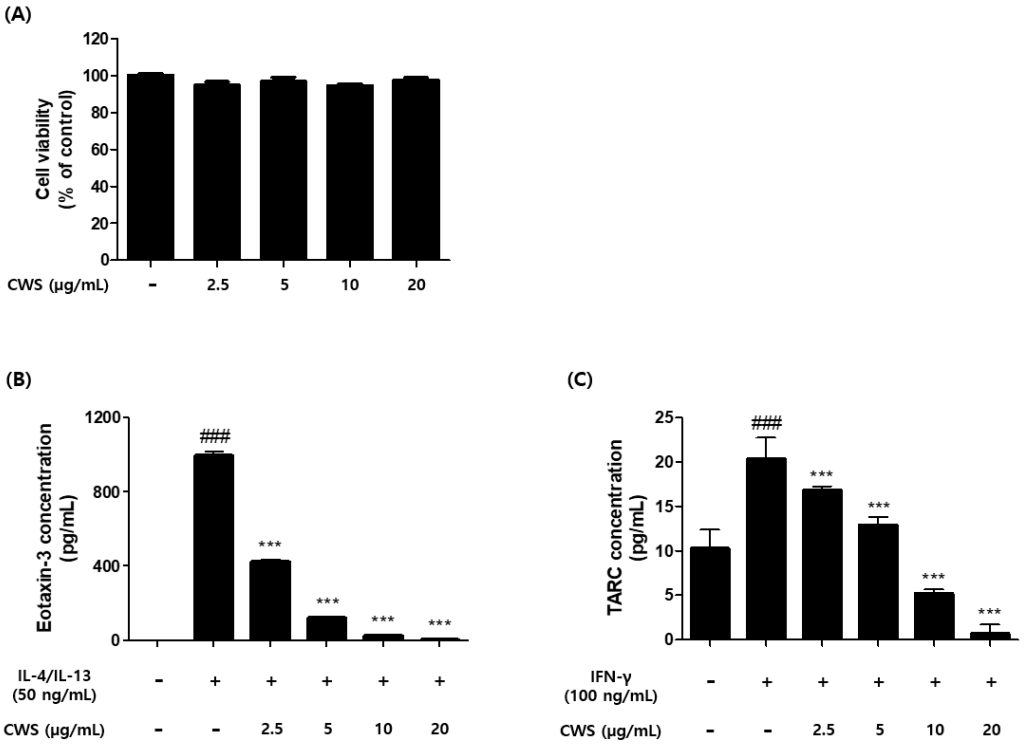


**Figure 1. Effect of CWS treatment on AD chemokines in human keratinocytes (HaCaT cells).** (**A**) Cell viability was measured after treatment of CWS (0, 2.5, 5, 10 and 20 μg/mL) for 48 h. (**B**) HaCaT cells were treated with varying concentrations of CWS and induced with IL-4/IL-13 (50 ng/mL each). The production of eotaxin-3 in culture supernatants was measured by ELISA ($n$ = 3). (**C**) HaCaT cells were treated with varying concentrations of CWS and induced with IFN-γ (100 ng/mL). The production of TARC in culture supernatants was measured by ELISA ($n$ = 3). The data are expressed as the mean ± standard deviation ### $p < 0.001$ indicate a significant difference between the induced group and untreated control; *** $p < 0.001$ indicate a significant difference between the sample treatment group and induced group.

### *2.2. Effects of CWS on AD-Related Gene Expression in HaCaT Cells*

Filaggrin (FLG) is a structural skin component that participates in epidermal differentiation and skin barrier maintenance [26]. As FLG is known to be downregulated in the skin of patients with AD [27], we examined the effect of CWS on FLG expression. Treatment with the pro-inflammatory cytokines TNF-α and IFN-γ led to a decrease in FLG mRNA expression (Figure 2A). However, co-treatment with CWS rescued TNF-α/IFN-γ-mediated suppression of FLG levels (Figure 2A). Additionally, the mRNA expression of TNF-α-induced MDC/CCL22 was decreased by CWS treatment (Figure 2B). These results suggested that CWS can efficiently regulate the gene expression of AD-related proteins in HaCaT cells.

### *2.3. Isolation and Identification of the Compounds*

The bioactive MeOH extract of CWS was sequentially employed for preparative HPLC to obtain four fractions (F1–F4) according to the separation of the peaks. Chemical analysis of the F4 fraction was performed by repeated process of column chromatography and semi-preparative HPLC purification, which yielded six compounds (Figure 3). The structures of the isolated compounds (Figure 4) were determined to be lupeol (**1**) [28], betulinic acid (**2**) [29], 5α-stigmast-3,6-dione (**3**) [30], 3-*O*-acetylbetulin (**4**) [31], betulinic acid methyl ester (**5**) [32] and norphytan (**6**) [33] by the interpretation of NMR spectroscopic data and comparing their NMR data (Figures S1–S8) with previously reported data and MS data obtained by LC/MS analysis.

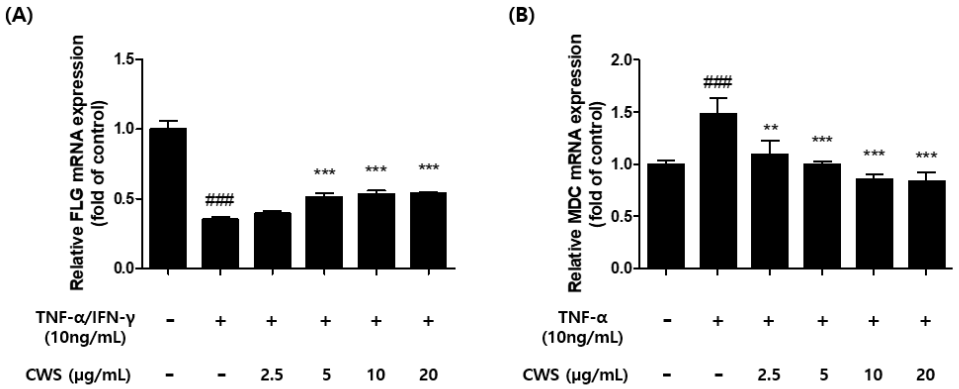


**Figure 2. Effects of CWS on mRNA expression of AD-related skin structural protein and chemokine in HaCaT cells.** Cells were treated with varying concentrations of CWS and induced with TNF-α and IFN-γ (10 ng/mL each) or TNF-α only (10 ng/mL). The mRNA expression levels of (**A**) FLG and (**B**) MDC were examined by RT-PCR. The data are expressed as the mean ± standard deviation ### $p < 0.001$ indicates a significant difference between the induced group and untreated control; ** $p < 0.01$, *** $p < 0.001$ indicates a significant difference between the sample treatment group and induced group.

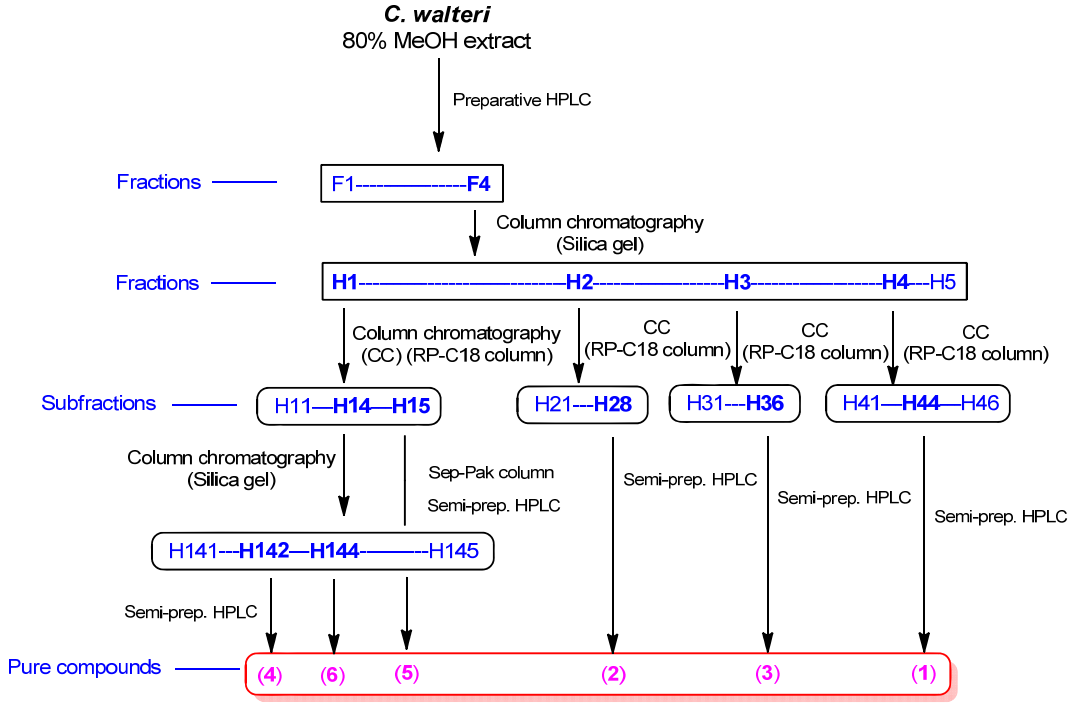


**Figure 3.** Separation scheme of compounds **1**–**6**.

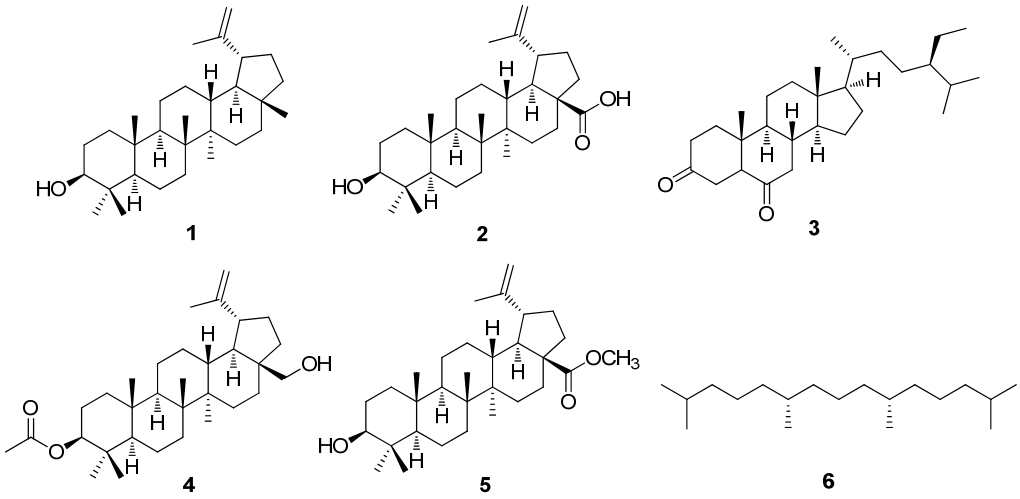


**Figure 4.** Chemical structure of compounds **1**–**6**.

*2.4. Effects of the Isolated Compounds **1**–**6** on IL-4/IL-13-Induced Eotaxin-3 Expression*

The effects of the isolated compounds **1**–**6** on cell viability were examined. While some compounds did not display any noticeable cytotoxicity at the tested concentrations, compounds **1**, **2** and **3** reduced cell viability at 20 μM to 84.5, 84.8 and 81.9%, respectively (Figure 5A). Therefore, further experiments were performed at concentrations up to 10 μM. The levels of eotaxin-3 were measured to compare the anti-AD potential of compounds **1**–**6**. At 5 μM, compound **2** exhibited a superior inhibitory effect against IL-4/IL-13-induced eotaxin-3 levels compared with the other compounds (Figure 5B). In addition, 10 μM of compounds **1** and **3** showed the strongest suppressive effect on eotaxin-3 expression (Figure 5B).

Compounds **5** and **6** displayed relatively weaker suppression compared with compounds **1**–**3** (Figure 5B). These results indicate that the isolated compounds **1**–**6** have the potential to inhibit AD, and compounds **1**–**3** may be the major active compounds in CWS responsible for the anti-AD effect. Interestingly, to the best of our knowledge, lupeol (**1**), betulinic acid (**2**), 5α-stigmast-3,6-dione (**3**), 3-*O*-acetylbetulin (**4**), betulinic acid methyl ester (**5**) and norphytan (**6**) have not been previously reported to be involved in AD, suggesting that they are novel molecules that could suppress AD. Therefore, further studies are needed to investigate the anti-AD effects of these compounds and their underlying molecular mechanisms.

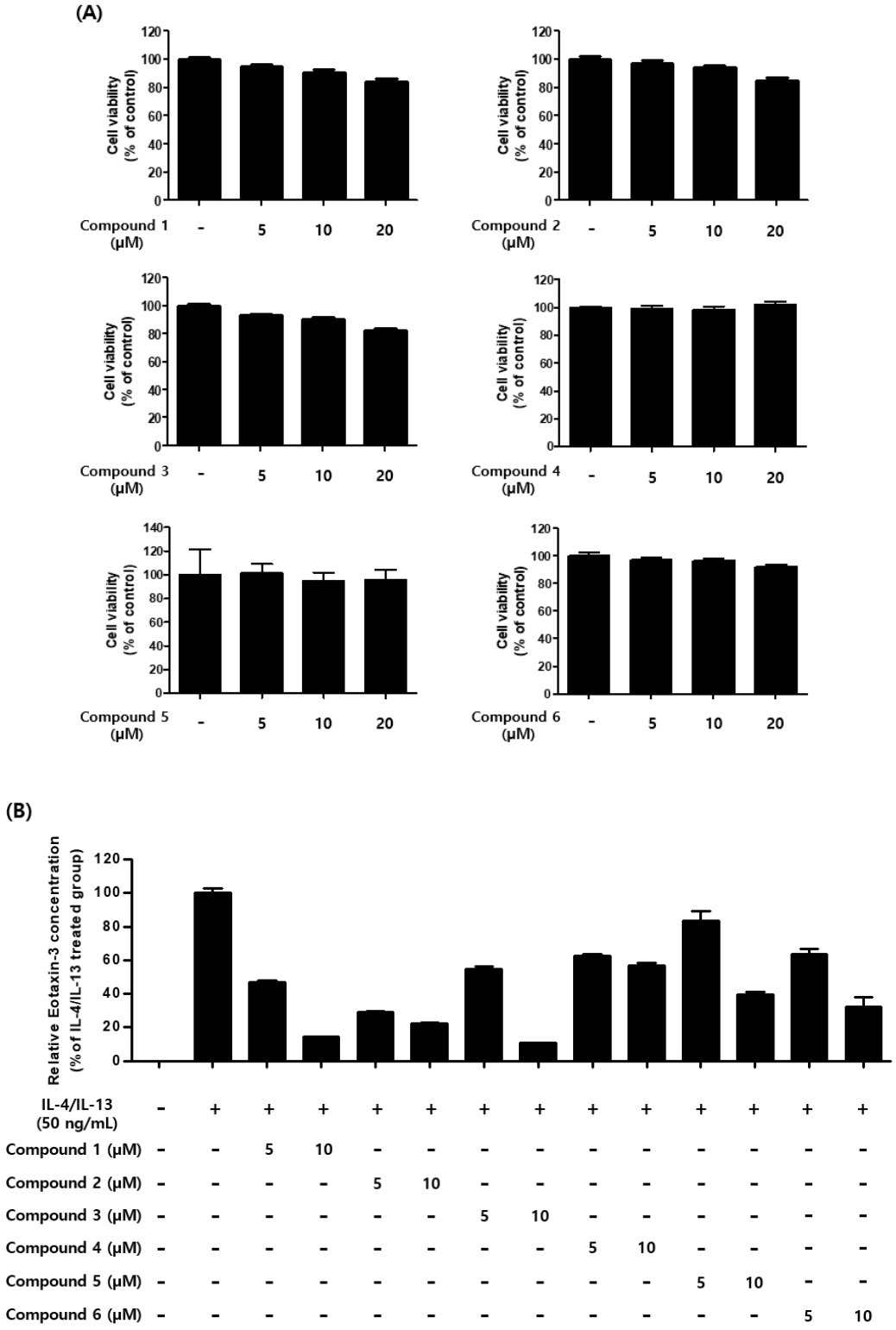


**Figure 5. Effect of the isolated compounds 1–6 on IL-4/IL-13-induced AD-like human keratinocytes (HaCaT cells).** (**A**) Cell viability was measured after treatment of compounds **1–6** (0, 5, 10 and 20 μM) for 48 h. (**B**) Cells were treated with compounds **1–6** and induced with IL-4/IL-13 (50 ng/mL each). The production of eotaxin-3 in culture supernatants was measured by ELISA ($n = 3$). The data are expressed as the mean ± standard deviation.

## 3. Materials and Methods

### 3.1. Plant Extraction and Isolation of Compounds

CWS (2.5 kg), collected from Jeju Island, Korea, were dried and chopped. Crude extracts were prepared using 80% aqueous MeOH (3 × 5 h) under reflux. The filtrate was concentrated under a vacuum to obtain the MeOH extract (210 g). The MeOH extract (150 g) was fractionated by preparative HPLC (MeOH/$H_2O$, from 10% MeOH to 100% MeOH for 120 min) using an Agilent Eclipse XDB-C18 column (250 × 21.2 mm, 7 μm) to obtain four fractions (F1–F4) according to the separation of peaks. The F4 fraction (9.3 g) was chromatographed on a silica gel (300 g) column eluting with hexane-EtOAc (3:1 to 1:1, gradient system) to yield fractions F41–F45 according to TLC analysis.

Fraction F41 (4.1 g) was separated using an RP-C18 silica gel open column chromatography using 100% MeOH to obtain five subfractions (F411–F415). An additional five subfractions (F4141–F4145) were acquired from subfraction F414 (290 mg) by silica gel open column chromatography using a gradient solvent system of hexane-EtOAc (16:1 to 1:1, $v/v$). Subfraction F4142 (48 mg) was purified using a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) using an isocratic solvent system of hexane-EtOAc (28:1, $v/v$) to afford compound **4** (39 mg).

Compound **6** (29 mg) was isolated from subfraction F4144 (50 mg) using a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) eluting with an isocratic solvent system of hexane-EtOAc (12:1, $v/v$). Subfraction F415 (90 mg) was separated using a silica Sep-Pak column eluting with an isocratic solvent system of hexane-EtOAc (10:1, $v/v$) and further isolated using a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) with an isocratic solvent system of hexane-EtOAc (20:1, $v/v$) to obtain compound **5** (7 mg).

Fraction F42 (2.5 g) was loaded onto an RP-C18 silica gel open column and separated by elution with 100% MeOH to obtain eight subfractions (F421–F428). Subfraction F428 (98 mg) was isolated using a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) with an isocratic solvent system of hexane-EtOAc (8:1, $v/v$) to afford compound **2** (23 mg). Fraction F43 (1.7 g) was separated by an RP-C18 silica gel open column and fractionated by elution with 100% MeOH to obtain six subfractions (F431–F436).

Compound **3** (8 mg) was purified from subfraction F436 (120 mg) by a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) with an isocratic solvent system of hexane-EtOAc (4:1, $v/v$). Fraction F44 (1.1 g) was applied to an RP-C18 silica gel open column and fractionated by elution with 100% MeOH to give six subfractions (F441–F446). Subfraction F444 (115 mg) was isolated using a semi-preparative HPLC (Apollo Silica column, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min) with an isocratic solvent system of hexane-EtOAc (2:1, $v/v$) to yield compound **1** (21 mg).

### 3.2. Cell Culture

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Gibco BRL, New York, NY, USA) and 1% penicillin/streptomycin (Corning Inc., New York, NY, USA) at 37 °C in a 5% $CO_2$-humidified incubator.

### 3.3. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed as previously reported [34]. HaCaT cells were seeded in 12-well culture plates and incubated in a $CO_2$ incubator. The culture medium was changed to serum-free DMEM, and the cells were incubated for an additional 24 h. Cells were treated with CWS (2.5, 5, 10 and 20 μg/mL) or compounds **1**–**6** (5, 10 and 20 μM) and then incubated for 1 h. Next, the cells were treated with cytokines. The culture supernatants were collected and stored in a deep freezer.

The concentrations of eotaxin-3 and TARC in the supernatant were determined using human CCL26/Eotaxin-3 DuoSet ELISA kits or human CCL17/TARC DuoSet ELISA

kits (R&D Systems Inc., Minneapolis, MN, USA). The absorbance was evaluated using a Varioskan multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

### 3.4. Real-Time Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). cDNA was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) in a CFX Connect Optics Module (Bio-Rad Laboratories, Inc., Hercules, CA, USA). mRNA expression levels were monitored using FLG primers (forward: 5′-TGAAG CTATGACACCACTGA-3′, reverse: 5′-TCC CCT ACG CTT TCT TGT CCT-3′), MDC/CCL22 primers (forward: 5′-GAA GCC TGT GCC AAC TCT CT-3′, reverse: 5′-GGG AAT CGC TGA TGG GAA CA-3′) and Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) (forward: 5′-CCA TCA CCA TCT TCC AGG AG-3′, reverse: 5′-ACA GTC TTC TGG GTG GCA GT-3′).

### 3.5. Cell Viability Assay

HaCaT cells were seeded in six-well plates. The culture medium was replaced with serum-free DMEM the following day. After 24 h, cells were treated with varying concentrations of CWS or compounds **1**–**6** (5, 10 and 20 μM) for 48 h. Cells were fixed and stained with sulforhodamine B (Sigma-Aldrich, St. Louis, MO, USA) and then dissolved in 10 mM Tris. The absorbance was evaluated at 554 nm using a Varioskan multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

### 3.6. Statistical Analysis

The data are presented as the mean ± standard deviation. Differences in data among groups were analyzed using a one-way analysis of variance using GraphPad Prism 5 software (San Diego, CA, USA). Statistical significance was defined as $p < 0.05$.

## 4. Conclusions

In this study, the MeOH extract of CWS exerted anti-AD potential in an AD-like HaCaT model. Further phytochemical examination of the MeOH extract resulted in the isolation of six compounds, lupeol (**1**), betulinic acid (**2**), 5*α*-stigmast-3,6-dione (**3**), 3-*O*-acetylbetulin (**4**), betulinic acid methyl ester (**5**) and norphytan (**6**). The structures of the isolates were elucidated through NMR and LC/MS analyses. These isolated compounds also showed anti-AD effects by reducing chemokines in HaCaT cells. In particular, lupeol (**1**), betulinic acid (**2**) and 5*α*-stigmast-3,6-dione (**3**) exhibited superior inhibitory effects against AD. Further studies aimed at identifying the molecular mechanisms of these active compounds will contribute to the management of patients with AD.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12178857/s1, General experimental procedure; Plant material; NMR and physical data for compounds **1**–**6**. Figure S1: $^{1}$H NMR spectrum of lupeol (**1**) ($CDCl_3$, 700 MHz), Figure S2: $^{13}$C NMR spectrum of lupeol (**1**) ($CDCl_3$, 175 MHz), Figure S3: $^{1}$H NMR spectrum of betulinic acid (**2**) ($CDCl_3$, 700 MHz), Figure S4: $^{1}$H NMR spectrum of 5*α*-stigmast-3,6-dione (**3**) ($CDCl_3$, 700 MHz), Figure S5: $^{13}$C NMR spectrum of 5*α*-stigmast-3,6-dione (**3**) ($CDCl_3$, 175 MHz), Figure S6: $^{1}$H NMR spectrum of 3-*O*-acetylbetulin (**4**) ($CDCl_3$, 700 MHz), Figure S7: $^{1}$H NMR spectrum of betulinic acid methyl ester (**5**) ($CDCl_3$, 700 MHz), Figure S8: $^{1}$H NMR spectrum of norphytan (**6**) ($CDCl_3$, 700 MHz).

**Author Contributions:** Conceptualization, Y.-R.C., S.B. and K.H.K.; formal analysis, B.S.L., Y.-R.C., M.J., Y.S.J. and J.-C.K.; investigation, B.S.L., Y.-R.C., M.J. and Y.S.J.; writing—original draft preparation, B.S.L., Y.-R.C. and K.H.K.; writing—review and editing, S.B. and K.H.K.; visualization, B.S.L., Y.-R.C. and K.H.K.; supervision, S.B. and K.H.K.; project administration, S.B. and K.H.K.; funding acquisition, S.B. and K.H.K. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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