Inhibition of Proinflammatory Cytokines in *Cutibacterium acnes*-Induced Inflammation in HaCaT Cells by Using *Buddleja davidii* Aqueous Extract

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Acne is an inflammatory skin disorder; although some anti-inflammatory medicines for treating acne are available in a market, they have considerable side effects; therefore, new treatment options are needed. In the present study, among the 16 aqueous extracts of plants collected from Jeju Island in Korea which are used to test anti-inflammatory activity, *B. davidii* showed the strong decline of the proinflammatory cytokine expression against the inflammatory process caused by *C. acnes* in Human HaCaT keratinocyte cells. *B. davidii* downregulated the expression of 57% of COX-2, 41% of iNOS, and proinflammatory cytokines 29% of TNF-α, 32% of IL-1β, 21% of IL-6, and 35% of IL-8. Furthermore, *B. davidii* inhibited NF-κB and MAPK signaling cascades in keratinocytes that activated by toll-like receptor 2 (TLR-2) in response to *C. acnes*. Given those results, *B. davidii* is a potential agent to reduce the proinflammatory cytokine expression against *C. acnes*-induced inflammation and might provide an alternative to the current medications.

1. Introduction

Acne is a chronic inflammatory disorder of the sebaceous follicles on the chin, cheek, forehead, and back. Cysts and scarring can occur when the disease is severe. Although acne is a skin disease, it can lead to psychological issues that affect patient quality of life. In acne pathogenesis, *C. acnes* has been identified as one of the main causative agents. *C. acnes*, a commensal Gram-positive anaerobic, colonizes the duct of the sebaceous follicle, causing an innate immune response [1].

Inflammation is the body’s initial complex response to infection. The processes of inflammation caused by *C. acnes* begin when peptidoglycan (PGN) and lipoteichoic acid (LTA) recognized by binding to toll-like receptors (TLRs) activate the TLR signaling pathway and trigger important intracellular signaling pathways including the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) pathways. This causes nuclear translocation of transcription factors, such as activator protein-1 (AP-1) and NF-κB, ultimately leading to production of a variety of proinflammatory cytokines including tumor necrosis factor (TNF) α, interleukin (IL) 1β, IL-8, and IL-6 [2, 3].

Adverse effects associated with anti-inflammatory drugs have become increasingly common and include increased risk of gastrointestinal ulcers, bleeding, heart attack, and kidney disease associated with nonsteroidal anti-inflammatory drugs (NSAIDs) [4]. Some anti-inflammatory drugs interfere with deactivated and hyperactivated cellular signaling networks or transcription factors [4]. Therefore, to limit adverse effects, the next generation of anti-inflammatory drugs needs to target either enzymes or signaling pathways that are only operational in affected tissues or a small subset of inflammatory target genes.

Medicinal plants have long been used as sources of therapeutically active compounds, which is also a popular traditional medicine to treat inflammatory diseases. The use of traditional medicines for treatment of inflammation could
provide safer alternatives compared with existing conventional treatments. Evidence suggests that herbal medicines have a lower rate of side effects compared with conventional medicines [5]. Furthermore, medicinal plants used for dermatological purposes with low or no side effects are gaining popularity in both traditional medicine and the cosmetic industry. *Buddleja davidi* is a perennial herbaceous plant that belongs to the Scrophulariaceae family with a pan tropical distribution across South Asia, Africa, and America. Its roots, leaves, and flowers are used in folk medicine for antioxidant, anti-inflammatory, antiviral, antiplasmodium, and antifungal effects [6, 7].

The purpose of the present study was to determine the anti-inflammatory activity of *B. davidii* against *C. acnes* infection and to elucidate its mechanism of action in human keratinocyte cell line.

2. Materials and Methods

2.1. Preparation of Bacteria. *C. acnes* (CCARM9009) were purchased from the Culture Collection of Antimicrobial Resistant Microbes (CCARM) at Seoul Women's University in Korea. *C. acnes* were grown in Reinforced Clostridial Medium (RCM) agar (Becton, Dickinson and Company, USA) for 48–72 hours at 37°C under anaerobic conditions in a GasPak™ EZ anaerobic container system (Becton, Dickinson and Company, USA). *C. acnes* were cultured in RCM broth to 100 MOI and then heat-killed at 80°C for 30 minutes before use in further experiments [2].

2.2. Plant Material and Extraction. Candidate plants, the herb medicine with anti-inflammation properties, were obtained from Jeju Island in South Korea. The plants were cleaned with distilled water and then dried using a freeze dryer (iLShin, Korea). After drying, 300 g of the dried herb was extracted three times in 3 liter of distilled water with refluxing at 80°C for 8 hours. 500 ml extract was concentrated under reduced pressure at 50°C using a rotary evaporator (EYELA, Japan) connected to a refrigerated bath circulator (Jeio Tech, Korea) [8]. The decoction was filtered using muslin cloth followed by Whatman (GE Healthcare, USA) grade 1 filter paper, lyophilized using a freeze dryer (iLShin, Korea), and stored at 4°C before further use [9]. To prepare the sample for experiments, the stock of extract powder (10 mg) was dissolved in 1 ml of dimethyl sulfoxide (DMSO).

2.3. Cell Culture. Human HaCaT keratinocyte cells [10] were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin at 37°C in a humidified incubator under 5% CO₂.

2.4. Quantitative Real-Time PCR. HaCaT cells (seeded at a density of 5 × 10⁵ per well in a six-well plate) were incubated for 24 hours, pretreated with various concentrations (12.5, 25, 50 and 100 μg/ml) of aqueous extract of plants for 1 hour, and subsequently treated with heat-killed *C. acnes* (100 MOI, 5 × 10⁷ CFU per well) for 24 hours. Total RNA was isolated from the HaCaT cells using TRIzol reagent (Life Technology, Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. A reverse transcriptase (NanoHelix, Korea) reaction was prepared using 1 μg of RNA to obtain cDNA. The cDNA was used as the template for real-time PCR (qRT-PCR), which was carried out with *QGreen* 2X SybrGreen qPCR Master Mix (CellSafe, Korea). The primer sequences used to detect TLR-2, iNOS, COX-2, TNF-α, IL-8, IL-6, IL-1β, and GAPDH are listed in Table 1. GAPDH was used as an endogenous control. The delta-delta Cq formula is used to calculate the gene expression.

2.5. Western Blot Analysis. HaCaT cells were treated with *B. davidii* aqueous extract and heat-killed *C. acnes* as described above. The cells were then lysed using radioimmunoprecipitation assay buffer (RIPA) lysis buffer containing 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), and complete protease inhibitor cocktail (BIOMAX, Korea). Protein concentration was determined with the Bradford (Sigma-Aldrich, USA) assay using bovine serum albumin as a standard and detected using a UVITEC imaging system (Uvitec Ltd, UK). Protein samples (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) for 1 hour at room temperature and then subsequently incubated with primary antibodies including p-NF-κB/NF-κB (sc-101752/8242S), p-p38/p38 (sc-17852/sc-535), p-JNK/JNK (sc-6254/sc-7345), and GAPDH (sc-25778) (Santa Cruz Biotechnology, Inc, USA) at 4°C overnight. Membranes were then shaken with secondary antibodies for 1 hour at room temperature. Signals were obtained using ECL reagent and detected with UVITEC imaging system equipment. The relative protein expression of p-NF-κB and NF-κB, p-p38 and p-38, and p-JNK and JNK (relative to GAPDH) from western blot data was determined using ImageJ.

2.6. Cell Viability Assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed to determine cell viability [3]. HaCaT cells were seeded at a density of 10⁴ per 100 μl culture media in 96-well plates. *B. davidii* aqueous extract was added at various concentrations from a dilution series from 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, and 1.5625 μg/ml. After incubation for 24 hours, the cells were incubated with a 0.1% MTT solution in the cell culture medium for 4 hours at 37°C in a humidified incubator under 5% CO₂. The MTT solution was discarded, and DMSO was added to solubilize the MTT-formazan crystals produced in the live cells. After incubating in the dark for 2 hours, absorbance was measured at 540 nm [16].
2.7. Statistical Analysis. All data are presented as mean ± standard deviation (SD) of at least three independent experiments and were analyzed using one-way ANOVA. Student’s t test was used when only two groups were compared. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$.

3. Results

3.1. Screening for Potential Anti-Inflammatory Activity of Aqueous Extract of Plants. The effect of plant aqueous extract on the expression of genes is given in Table 2.

The inflammatory process increases the expression of COX-2 and NO synthase, which produce inflammatory mediators [2, 17]. In addition, proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 trigger and rapidly amplify the inflammatory response to limit the spreading of the infection [2, 18]. In this study, 16 plants were extracted from Jeju Island (Korea) that were tested for their anti-inflammatory activity against C. acnes-induced inflammation. The percentage of gene expression described in Table 2 was calculated based on the ratio of gene expression of C. acnes-infected HaCaT cells treated with aqueous plant extract over one without the plant aqueous extract as an increasing 100% compared with C. acnes-free HaCaT cells. Reduced values represent the inhibition of the expression of C. acnes-infected HaCaT cells treated with aqueous plant extract. The results show that plant extracts reduced the expression of inflammatory mediators such as COX-2, NO, and proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8.

3.2. Suppression of C. acnes-Induced TLR-2 Expression in HaCaT Cells by B. davidii Aqueous Extract. C. acnes contribute to the inflammation in acne through activation of toll-like receptors (TLRs), in particular, TLR-2 [2, 19]. In the initial screening, B. davidii aqueous extracts displayed the strong decline of the proinflammatory cytokine expressions in C. acnes-treated HaCaT cells (Table 2). The effects of B. davidii aqueous extract on expression of the pattern recognition receptor TLR-2 showed C. acnes induced the expression of TLR-2 while treated with 50 μg/ml of B. davidii aqueous extracts and reduced the TLR-2 mRNA expression by 30% compared with C. acnes-treated HaCaT cells without B. davidii aqueous extract (Figure 1).

3.3. B. davidii Aqueous Extract Reduced NF-κB Phosphorylation in C. acnes-Treated HaCaT Cells. TLR-2 signaling leads to the activation of the NF-κB pathway, and then NF-κB is released and translocates to the nucleus where it regulates expression of an array of genes, which in turn stimulates the release of proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α [2–4]. The NF-κB pathway was activated by C. acnes, indicated by increased expression of toll-like receptor 2 (TLR-2) (Figure 1) compared with C. acnes-free. B. davidii aqueous extract dose-dependently reduced the amount of p-NF-κB and NF-κB protein which could be due to the decrease of the gene expression, as determined using western blotting (Figure 2). These results suggest that the anti-inflammatory activity of B. davidii aqueous extract in HaCaT cells with C. acnes-induced inflammation might be due to suppression of the NF-κB signaling pathway.

3.4. Inhibition of C. acnes-Induced p38 and JNK Expression and Phosphorylation by B. davidii Aqueous Extract. The mitogen-activated protein kinase (MAPK) signaling pathways play an essential role in regulating the production of multiple inflammatory mediators [2, 4, 20]. The effects of B. davidii aqueous extract on C. acnes-induced MAPK pathway activity were examined using western blotting for p-JNK and p-p38 (Figure 3). C. acnes-induced MAPK activity in HaCaT cells and treatment with B. davidii aqueous extract extracts dose-dependently suppressed this MAPK activity. These results indicate that B. davidii aqueous extract exerts its anti-inflammatory actions via inhibition of the MAPK signaling pathway.

3.5. B. davidii Aqueous Extract Inhibited the C. acnes-Induced Proinflammatory Cytokine Expression-Induced HaCaT Cells. The primary proinflammatory cytokines are IL-1β, IL-6, IL-8, and TNF-α that play important roles in inflammation [4, 18]. To assess the effects of B. davidii aqueous extract on C. acnes-induced proinflammatory cytokine expression, HaCaT cells were pretreated with indicated concentrations (12.5, 25, 50, and 100 μg/ml) of B. davidii aqueous extract for 1 hour prior to addition of heat-killed C. acnes (100 MOI) for 24 hours. C. acnes-treated HaCaT increased the expression...
However, the expression of IL-1β, IL-6, IL-8, and TNF-α was dose-dependently declined in C. acnes-treated cells after treatment with B. davidii aqueous extract (Figure 4). IL-1β, IL-6, IL-8, and TNF-α expression was decreased by approximately 32%, 21%, 35%, and 29% at 50 μg/ml, respectively, compared with untreated cells. Hence, 50 μg/ml of B. davidii aqueous extract is the highest concentration of the extract shows the sharpest effect. These results indicate that B. davidii aqueous extract could have anti-inflammatory effects through inhibiting proinflammatory cytokine expression.

### 3.6. Cytotoxicity of B. davidii Aqueous Extract in HaCaT C.

To determine the cytotoxic effects of B. davidii aqueous extracts on HaCaT cells, the MTT assay was used. The cells were treated with B. davidii aqueous extracts for 24 hours at a concentration ranging from 0.39 μg/ml to 100 μg/ml, and the B. davidii aqueous extracts had no significant cytotoxic effects on HaCaT cells (Figure 5).

### 4. Discussion

Inflammation has been suggested as a key factor involved in the development and aggravation of acne vulgaris, although the exact pathogenesis of acne vulgaris has not been elucidated. It has been verified that C. acnes play a major role in the development of inflammatory acne lesions [1, 4]. C. acnes promote the activation of TLR-2 in keratinocytes, aggravating the inflammation reactions [2, 4]. In fact, the C. acnes infection begins to activate monocyte TLR-2, a major signaling pathway induced by activation of NF-κB or MAPKs signaling pathway, which in turn gives rise to expression of cytokines, chemokines, adhesion molecules, and

### Table 2: Effect of plant aqueous extract on the expression of genes related to inflammatory mediators and proinflammatory cytokines in C. acnes-treated HaCaT cells (unit: %).

| Herb                        | iNOS | COX-2 | TNF-α | IL-1β | IL-6 | IL-8 |
|-----------------------------|------|-------|-------|-------|------|------|
| Akebia quinata              | 160  | 218   | 81    | 123   | 131  | 87   |
| Angelica japonica A. Gray   | 67   | 26    | 58    | 65    | 38   | 87   |
| Artemisia campestris        | 38   | 53    | 44    | 73    | 126  | 105  |
| Buddleja davidii            | 41   | 57    | 29    | 32    | 21   | 35   |
| Ceramium kondoi Yendo       | 173  | 148   | 115   | 151   | 117  | 95   |
| Chelidonium majus L. asiaticum Ohwi | 61   | 54    | 90    | 173   | 112  | 121  |
| Elaeagnus glabra            | 50   | 48    | 114   | 211   | 180  | 183  |
| Fallopia japonica (Houtt.) Ronse Decr. | 167  | 238   | 72    | 87    | 109  | 114  |
| Hydrangea macrophylla       | 120  | 106   | 18    | 22    | 97   | 25   |
| Hydrangea serrate           | 178  | 116   | 86    | 111   | 132  | 161  |
| Hydrangea serrata Seringe   | 110  | 90    | 75    | 66    | 83   | 66   |
| Illicium verum              | 16   | 73    | 31    | 46    | 61   | 44   |
| Platycladus orientalis      | 156  | 114   | 31    | 122   | 15   | 182  |
| Pyllacantha fibrosa         | 25   | 77    | 69    | 62    | 36   | 69   |
| Rubus coreanus              | 60   | 58    | 95    | 113   | 145  | 179  |
| Sapindus mukorossi          | 102  | 99    | 60    | 169   | 127  | 107  |

Control 1: HaCaT with C. acnes-free and without plants aqueous extract. Control 2: HaCaT with C. acnes and without plant aqueous extract (increasing 100% compared with control 1).
granulopoiesis factors \([4, 18, 20]\). On the basis of this information, \(B.\) \textit{davidii} aqueous extract effectively prevents the inflammation caused by \(C.\) \textit{acnes} through inhibition of the TLR-2 to NF-\(\kappa\)B signaling cascade in keratinocytes (Figures 1 and 2).

Recently, it has been studied that the MAPK signaling pathway plays a critical role in regulation of the inflammatory response and coordination of the induction of many genes encoding inflammatory mediators \([13, 19, 20]\). \(B.\) \textit{davidii} aqueous extract suppressed mitogen-activated protein kinase (MAPK) by inhibiting p-p38 and p-JNK in HaCaT cells treated with \(C.\) \textit{acnes} (Figure 3). Activation of the TLR-2-mediated downstream MAPK signaling pathway is responsible for production of inflammatory cytokines. MAPK activation has been found in clinical acne lesions, indicating that inhibition of MAPK signaling is important for the pathogenesis of acne vulgaris in general \([4, 20]\). Use of MAPK inhibitors is

\begin{figure}[h]
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\caption{Reduction of the protein ratio of NF-\(\kappa\)B in \(C.\) \textit{acnes}-treated HaCaT cells by \(B.\) \textit{davidii} aqueous extracts. HaCaT cells were pretreated with the indicated concentration of \(B.\) \textit{davidii} aqueous extracts for 1 hour before treatment with heat-killed \(C.\) \textit{acnes} (100 MOI) for 24 hours. Western blot analysis was carried out to evaluate the level of phosphorylated NF-\(\kappa\)B or NF-\(\kappa\)B. (a) \(B.\) \textit{davidii} aqueous extracts reduced the phosphorylation of NF-\(\kappa\)B and \(C.\) \textit{acnes}-induced inflammation. (b) Relative protein expression of p-NF-\(\kappa\)B and NF-\(\kappa\)B (relative to GAPDH) was determined using densitometry. Control was \(C.\) \textit{acnes}-free without \(B.\) \textit{davidii} aqueous extract. All data were expressed as mean \(\pm\) SD. * \(p < 0.05\) compared with \(C.\) \textit{acnes} treated cells only.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Inhibition of \(B.\) \textit{davidii} aqueous extract on MAPKs expression in \(C.\) \textit{acnes}-treated HaCaT cells. Western blot analysis was used to check the amount of protein of p-p38, p38, p-JNK, and JNK. HaCaT cells were pretreated with the indicated concentration of \(B.\) \textit{davidii} aqueous extracts for 1 hour before treatment with heat-killed \(C.\) \textit{acnes} (100 MOI) for 24 hours. (a) Total protein expression and phosphorylation of p38 and JNK were reduced by treatment with \(B.\) \textit{davidii} aqueous extracts. (b) Relative protein expression of p-p38, p38, p-JNK, and JNK (relative to GAPDH) was determined using ImageJ. All data were expressed as mean \(\pm\) SD. * \(p < 0.05\), ** \(p < 0.01\) compared with \(C.\) \textit{acnes}-treated cells only.}
\end{figure}
emerging as an attractive alternative to anti-inflammatory drugs because they decrease synthesis of proinflammatory cytokines [20]. The results of this study suggest that B. davidii aqueous extract modulates the expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) in the inflammation process through the inhibition of the NF-κB and MAPK kinase pathways (Figures 4 and 6). Hence, B. davidii aqueous extract might be a potential MAPK inhibitor to treat the inflammation caused by bacterial infections.

Previously, R. coreanus, S. mukorossi, and P. orientalis aqueous extract had been reported to be anti-inflammatory [21–23]; however, in this study, R. coreanus, S. mukorossi, and P. orientalis aqueous extract did not exhibit their anti-inflammatory activity (Table 2). In addition, A. japonica A. Gray, B. davidii, I. verum, and P. fibrosa aqueous extract...
showed an inhibition potential to the inflammation caused by C. acnes (Table 2). Here, B. davidii aqueous extract showed a stronger inhibition toward the inflammatory process caused by C. acnes than I. verum which was reported in the previous studies [15], A. japonica A. Gray, and P. fibrosa (Table 2).

B. davidii is a well-known, traditional, herbal medicine and a promising candidate for the treatment of acne and other inflammatory diseases [6, 7]. A better understanding of the mechanism of B. davidii’s anti-inflammatory activity would not only offer insight into inflammation homeostasis but also provide opportunities for the discovery of new anti-inflammatory compounds with fewer side effects in the future.

In conclusion, this study provides the first evidence that B. davidii has the anti-inflammation property through inhibiting two pathways that are NF-κB and MAPK signaling pathways, which are activated by TLR-2, leading to the reduced expression of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8.

**Data Availability**

No data were used to support this study.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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