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Anti-acne effects of *Castanea crenata* bur extract and identification of active compound

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Abstract

Acne vulgaris is a common disease of the pilosebaceous unit. Hyperseborrhea, a follicular colonization by *Cutibacterium acnes* and a complex inflammatory state are pathogenic factors of acne vulgaris. In the present study we investigated the anti-acne efficacy of *Castanea crenata* bur extract (CBE) in vitro and searched active compound for mitigating hyperseborrhea. In sebocytes, CBE inhibited the sebum synthesis through downregulation of sterol response element-binding protein-1 and peroxisome proliferator-activated receptor γ expression. CBE also inhibited the 5-alpha reductase activity which is associated with androgen-induced sebum production. Moreover, CBE showed anti-inflammatory effect in *C. acnes* and free fatty acid-induced inflammatory condition through suppressing Toll-like receptor 2 activity. Anti-inflammatory effect was also observed in keratinocytes via inhibition of NF-κB translocation into nuclei. Finally, we identified the ellagic acid as an active compound for inhibiting sebum production in CBE. These findings suggest that CBE have potential to be a multi-target agent for acne vulgaris and a good source of ellagic acid as an anti-sebum compound.

Keywords: *Castanea crenata*, Sebocyte, Sebum synthesis, Anti-inflammation, 5-alpha reductase

Introduction

Acne vulgaris is a chronic inflammatory skin disease related to the pilosebaceous unit. In acne lesions, hyperseborrhea, dysseborrhea, elevated dihydrotestosterone (DHT) levels, abnormal follicular keratinization responsible for the comedone formation, an imbalance in skin microflora, particularly an increase of *Cutibacterium acnes* (*C. acnes*) colonization, and an inflammation have been observed [1].

Sebum is produced by sebocytes which constitute a major part of the sebaceous glands (SGs). Sebocytes start proliferation at the junctional zone of SGs and hair follicles (HFs) and differentiation begin after migration to the maturation zone of the SGs. Fully differentiated sebocytes secrete sebum through a lysis process, which called a holocrine secretion. Sebum production is affected by steroid hormones, vitamin A derivatives, insulin-like growth factor 1 (IGF-1), and free fatty acids (FFAs). Sterol response element-binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs) are key transcriptional regulators of lipid synthesis in sebocytes [2].

Sebum is a mixture of lipids mainly composed of triglyceride (TG) and FFAs [3]. TG is hydrolyzed into FFAs by bacteria, especially *C. acnes*, as well as genuine FFAs secreted from sebocytes. Excessive sebum production is a major event in puberty acne, which contributes to comedone formation, sebogenesis, and inflammation [4]. Palmitic acid (PA), saturated fatty acid, is recently discovered to play an important role in upregulation of lipid contents and inflammatory response of the skin [5].

Higher androgen level is known to contribute to acne vulgaris through activation of sebum production. Testosterone, the major sex hormone in men, is converted to DHT by 5α-reductase. DHT is an agonist of

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the androgen receptor (AR) with about two-to four-fold higher affinity than testosterone. Binding with AR in the cytoplasm lead to translocate AR into the nucleus and then subsequently activates the lipid metabolism related gene expression. Thus, the downregulation of 5α-reductase has been considered as a target for acne vulgaris by inhibiting excessive sebum production [6].

During acne lesion development, inflammation is observed at most stages. *C. acnes* is one of the major bacteria responsible for acne and plays a critical role in induction and maintenances of inflammation. In keratinocytes, *C. acnes* is reported to activate inflammation through Toll-like receptor 2 (TLR2) and nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) pathways [7]. Along with the inhibition of sebum production in sebocytes, the downregulation of inflammation induced by *C. acnes* is an additional therapeutic alternative to remedy and prevents acne vulgaris.

Castanea crenata Siebold and Zucc (*C. crenata*) is classified in the *Fagaceae* family and inhabits northeast asian countries including Korea and Japan. The extracts from various parts of this species have been reported to exert antioxidant, antimicrobial, and anti-allergic activities [8]. *C. crenata* bark contains tannin used as preservatives, the outer and inner shells of *C. crenata* have anti-adipogenic activity, and other parts like leaves, flowers, and the inner skin abound with phenolic and flavonoid compounds with antioxidant properties [9]. *C. crenata* bur (chestnut bur), also known as involucre, is a waste product of chestnuts and has been used as traditional medicine for erysipelas, pertussis, and bronchitis. The anti-viral activity of the flavonoid glycoside derived from chestnut bur was reported by Kim et al. [10], but the effects on the skin have not been elucidated yet. In this study, we investigated the anti-acne properties of *C. crenata* bur extract on the three well described acne triggers: hyperseborrhea (sebum production), androgenic hormone level (5α-reductase activity), and inflammation induced by *C. acnes* and FFAs, and then elucidated the active compound for inhibiting sebum synthesis.

Materials and methods

Preparation of *C. crenata* bur extract

Dried *C. crenata* bur purchased from the Pungcheon Ginseng Medicinal Herbs Farming union (Korea, 33 g) was extracted with distilled water (320 mL) for 3 h at 100 ℃ and filtered. The extract was concentrated in a rotary evaporator at 55 ℃ and dried using a freeze dryer at 50 ℃ for 24 h. The yield of *C. crenata* bur extract is 2.8%. The extract dissolved in distilled water was named *C. crenata* bur extract (CBE) and used in all experiments.

Sebocytes culture and Nile red staining for lipid synthesis

Human sebocytes purchased from Celprogen (Torrance, CA, USA) were cultured in Human Sebocyte Complete Growth Media (Celprogen) at 37 °C in a 5% CO_2 incubator. Human sebocytes were differentiated with 100 μM palmitic acid (Sigma-Aldrich, St. Louis, MO, USA) with or without CBE or ellagic acid for 48 h. The treated cells were washed using 1 × PBS and fixed with 4% formaldehyde for 10 min at room temperature (RT). After washing three times, the cells were stained with 10 μg/mL Nile red (Sigma-Aldrich) diluted in 1 × PBS at a 1:100 ratio for 15 min at RT. The nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) solution and fluorescence was detected at excitation and emission wavelengths of 550 nm and 630 nm, respectively.

Triglyceride (TG) ELISA assay

TG released from sebocytes were measured by using ELISA (Triglyceride Quantification Assay Kit, Abcam, Cambridge, UK) according to the manufacturer's instructions. The reaction mixture was added and shaken for 60 min at RT protected from light and the absorbance was measured at 570 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

Western blot analysis of SREBP-1 and PPARγ

For western blot analysis of SREBP-1 and PPARγ, the sebocytes were seeded into a 60 mm dish and incubated in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) containing 1% serum and treated with CBE at 1, 10 and 50 μg/mL at 37 °C for 3 days. Cells were collected from the dishes and lysed with lysis buffer (PRO-PREP, iNtRON Biotechnology, Seongnam, Korea). After sonication, the cell lysate was centrifuged at 16,200×*g* for 10 min and the pellet was removed. Proteins were quantified by the Bradford assay and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membranes using a transfer kit (iBlot2, iBlot gel transfer stacks, Thermo Fisher Scientific) and blocked with 5% skim milk solubilized in 1 × PBS containing 0.1% Tween 20 (PBST, Sigma-Aldrich) at RT for 1 h. Then, the membranes were incubated with PPARγ antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA), SREBP1 antibody (1:1000) (Novus Biologicals, Centennial, CO, USA), and GAPDH (1:1000) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4 ℃ overnight. After washing with PBST three times, the membrane was incubated with mouse or rabbit IgG secondary antibody (1:1000, Cell Signaling Technology) at RT for 1 h and the reaction was developed with

chemiluminescence substrate (GloBrite ECL Reagent Kit, R&D System, Detroit, MI, USA). The detected proteins were normalized to the individual non-phosphorylated form or GAPDH.

5α-reductase activity assay

Active solution (0.5 mL) was prepared with or without a CBE in 1 mmol/L dithiothreitol (Thermo Fisher Scientific), pH 6.5, 40 mmol/L potassium phosphate (Sigma-Aldrich), 100 μmol/L NADPH (Roche Holding AG, IN, USA), and 3.5 μmol/L testosterone (ChemFaces, Hubei, China) for 20 min at RT. Then, 20 μg of rat liver microsomes (Sigma-Aldrich) was added and incubated for 30 min at 37 ℃ to start the reaction. After lyophilizing the reaction solution, it was dissolved in methanol and filtered through a 0.2-micron filter (Pall, NY, USA). Finally, testosterone was measured by HPLC (Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, Waters, MA, USA) on a C18 column (4.6 × 150 mm, 2.6 μm, Phenomenex, Torrance, CA, USA) at RT, and a UV detection wavelength of 245 nm. The analysis was conducted with an isocratic mobile phase of 0.1% trifluoroacetic acid in water: acetonitrile (50:50, v/v) at a flow rate of 1.0 mL/min.

Preparation of the conditioned medium of *C. acnes*

The strain of *C. acnes* ATCC 6919 used in this study was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *C. acnes* was anaerobically cultured in Reinforced Clostridial Medium (RCM, Difco, Franklin Lakes, NJ, USA) at 37 ℃ for 48 h. *C. acnes* was cultured in a 96-well microplate with or without CBE, respectively. *C. acnes* was adjusted to 1×10^7 colony-forming units (CFU)/mL in RCM. CBE was dissolved in distilled water and prepared in media at concentrations of 10, 50, 100, 500, 1000, and 2000 μg/mL. Each well was inoculated with 1×10^5 CFU/mL of the bacterial suspension and the microplate was incubated for 18 h. After collecting the conditioned medium, the supernatant was filtrated through a 0.22-micron pore-sized filter (EMD Millipore, Temecula, CA, USA) and treated with 1/100 dilution in cells. The conditioned medium recovered by culturing *C. acnes* was named *C. acnes* CM, and the conditioned medium with CBE was named *C. acnes* CBE, respectively (Fig. 1A, B).

TLR2/NF-κB/SEAP activity assay

HEK293-hTLR2 cells (HEK293-Blue™ hTLR2, InvivoGen, San Diego, CA, USA) was used to determine the effect of CBE on TLR2. The TLR2 reporter cell line is a stably co-transfected cell line which expresses full-length human TLR2 and the secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of an NF-κB response element. HEK293-hTLR2 cells were cultured in DMEM with 4 mM L-glutamine, 4500 mg/L glucose, and sodium pyruvate supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a 5% CO_2 incubator. HEK293-hTLR2 cells were seeded in 48-well plates and cultured in serum-free DMEM with *C. acnes* CM or *C. acnes* CBE. After 6 h, cultured medium was collected and reacted with QUANTI-Blue™ solution (InvivoGen), then incubated at 37 ℃ in a 5% CO_2 incubator for 30 min. SEAP activity was measured by reading the optical density (OD) at 620 nm with a microplate reader.

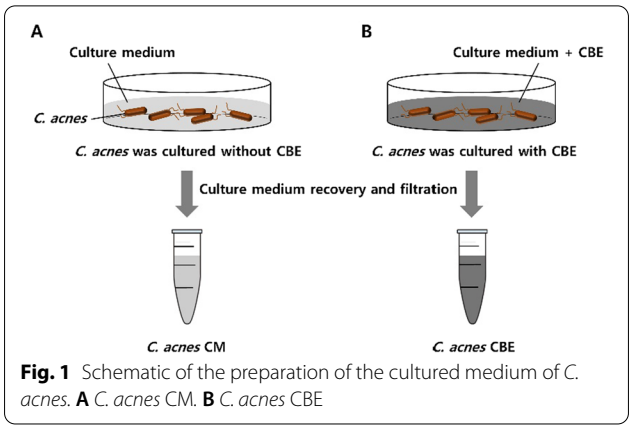

Fig. 1 Schematic of the preparation of the cultured medium of *C. acnes*. **A** *C. acnes* CM. **B** *C. acnes* CBE

Immunocytochemistry assay of NF-κB p65 nuclear translocation

NF-κB p65 nuclear translocation was detected to confirm inflammatory process activation in the human epidermal keratinocytes (HEKn). HEKn (ATCC, Manassas, VA, USA) were cultured in EpiLife™ Medium with 60 μM calcium (Gibco, Carlsbad, CA, USA) and Human Keratinocyte Growth Supplement (HKGS, Gibco) with CBE and *C. acnes* CM at 37 ℃ in a 5% CO_2 incubator for 1 h. After washing with PBS, the cells were fixed with 4% formaldehyde (Sigma-Aldrich), washed using PBS three times, and permeabilized with 0.2% Triton X-100 (BIOSESANG, Seongnam, Korea). Then cells were washed three times and blocked with 5% albumin (Albumin Fraction V, bioWORLD, Dublin, OH, USA) in PBS. The cells were treated with NF-κB p65 polyclonal antibody (1:200) (eBioscience™, Thermo Fisher Scientific) overnight at 4 ℃, then with rabbit IgG antibody tagged with Alexa Fluor 488 (1:200) (Merck, Darmstadt, Germany). The nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific) before detecting NF-κB p65 translocation using a fluorescence microscope (EVOS® FL, Thermo Fisher Scientific).

Western blot analysis of phospho-IκBα and IκB

For western blot analysis of phospho-IκBα and IκBα, the HEKn were seeded into a 60 mm dish and incubated in DMEM containing 1% FBS and treated with CBE at 1, 10 and 50 μg/mL and *C. acnes* CM at 37 °C for 3 days. Then, follow the same steps as above. The membranes were incubated with phospho-IκBα (Ser32) antibody (1:1000), IκBα antibody (1:1000) (Cell Signaling Technology), at 4 ℃ overnight.

Activity guided identification of active compound

C. crenata bur was extracted with boiling water in a reflux system. The aqueous extract (10.15%) was suspended in EtOH (70%, v/v), then the soluble part was evaporated and partitioned by chloroform, ethyl acetate, and *n*-butanol, depending upon the solvent polarity. Thus, the ethyl acetate fraction was further separated by methanol precipitation. After precipitation, the soluble part was separated using silica gel and Sephadex LH-20 column chromatography, and the precipitated fraction was dissolved in dimethyl sulfoxide (DMSO). Figure 6A shows the schematic of the fractionation of CBE.

The HPLC system used in this study was a Waters 2695 (Waters, Milford, MA, USA) system equipped with a Waters 996 Photodiode Array (PDA) Detector. Empower 2 software was used to control the analytical system and perform the data collection and processing. HPLC–PDA analysis was performed on a Phenomenex Luna C18 (2) (4.6 × 150 mm, 5 μm) reverse-phase column protected by a C18 guard column from Phenomenex, Inc. (Torrance, CA, USA). The sample injection volume was 10 μL. The signal was monitored at 240 nm. The elution system used for the HPLC–PDA assay was a binary high-pressure gradient elution system with mobile phase A (0.1% trifluoroacetic acid (TFA) in H_2O) and mobile phase B (acetonitrile). The elution gradient was 10% organic phase B, held for 7 min; then, 10–55% organic phase B in 21 min (linear gradient); 55–70% organic phase B in 4 min (linear gradient); then return to the starting condition in 3 min and re-equilibration for 5 min. The flow rate was 1.0 mL/min. Each analysis required 40 min, including the re-equilibration time.

Statistical analysis

All experiments were conducted at least three times, and the data are expressed as the mean ± standard deviation (SD). The data were analyzed by the student's t test. A *P* value less than 0.05 was considered statistically significant.

Results

Effect of CBE on sebum synthesis of sebocytes

Excessive sebum production known as hyperseborrhea is the major causative step of acne vulgaris. To confirm the inhibitory effect of CBE on sebum synthesis, we used a PA-induced differentiation model of sebocytes. PA has been reported to associated with sebum synthesis and inflammation in acne [3]. As shown in Fig. 2A, CBE significantly inhibited the PA-induced sebum production at a concentration of 10 and 50 μg/mL. Since TG is a major component of sebum, we also quantified the secreted TG levels by using ELISA. As expected, CBE decreased the level of TG in a dose dependent manner (Fig. 2B). Then, to explore the regulatory mechanism of CBE on lipid synthesis, we assessed the expression level of SREBP-1 and PPARγ, which are well-known transcriptional factors of lipid synthesis. As a result, we found that CBE decreased the expression levels of SREBP-1 and PPARγ induced by PA (Fig. 2C). These results suggest that CBE inhibited the PA-induced sebum production by downregulation of SREBP-1 and PPARγ expression in sebocytes.

Effect of CBE on 5α-reductase activity

Androgen-induced sebum production is considerably related to 5α-reductase enzyme activity, which converts testosterone to DHT. DHT, the active androgenic form of testosterone, has a higher affinity for the AR than testosterone. Therefore, the inhibition of 5α-reductase can play a critical role in improving DHT-mediated acne through decreasing sebum synthesis. To explore the inhibitory effect of CBE on 5α-reductase activity, we measured the remaining testosterone in an enzymatic reaction using testosterone and rat liver microsomes containing 5α-reductase. The higher amount of testosterone in reaction mixture means that the activity of 5α-reductase is inhibited. As shown in Fig. 3, CBE inhibited the conversion of testosterone to DHT in a dose dependent manner. Finasteride, used as a positive control, also showed inhibitory effect on 5α-reductase. The result suggests that CBE can be effective to mitigate acne vulgaris by inhibition of 5α-reductase activity.

Effect of CBE on *C. acnes* and FFA-induced TLR2 activation in HEK293-hTLR2 cells

TLR2 ligands present on *C. acnes* have been known to initiate inflammatory gene expression and contribute to the vicious inflammatory circle of acne. To explore the anti-inflammatory effect of CBE on TRL2 activity, we used the HEK293-hTLR2 cells in *C. acnes* and FFAs induced inflammation model. We first check whether CBE can inhibit the level of virulence factors secreted from *C. acnes*, we used the *C. acnes* CM and *C. acnes* CBE. We applied the collected *C. acnes* CM and *C. acnes*

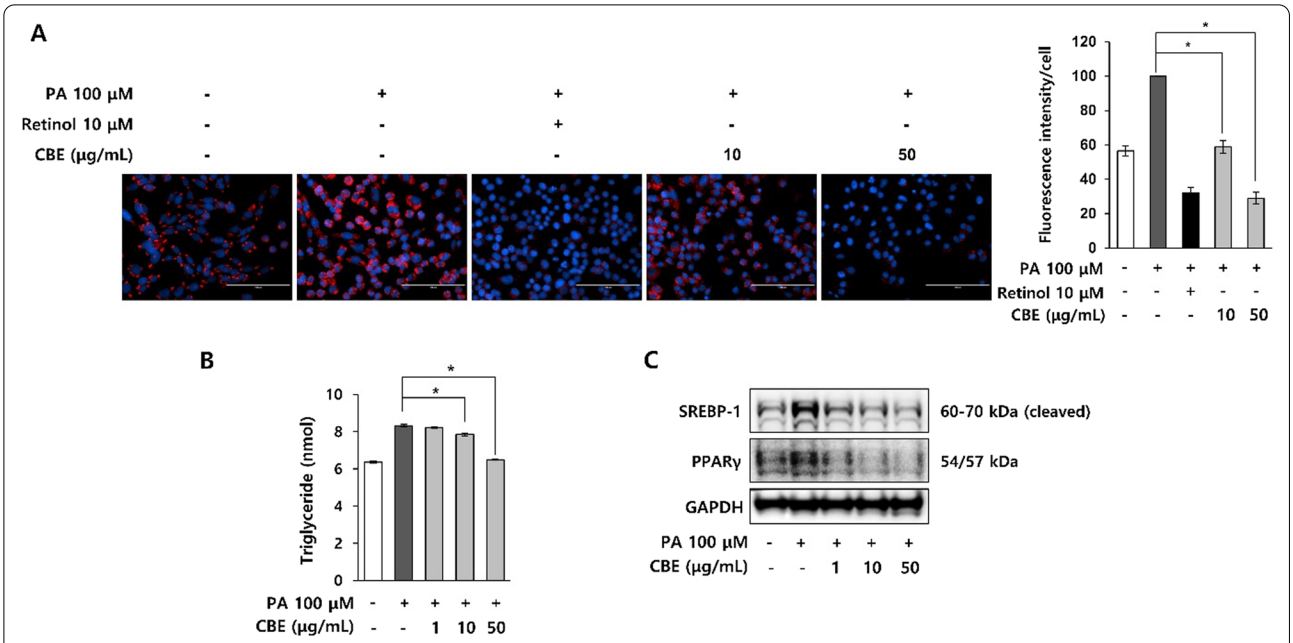

Fig. 2 CBE suppresses sebum production, and TG, SREBP-1 and PPARγ expression induced by PA in sebocytes. **A** Sebocytes were treated PA with or without retinol or CBE and incubated for 48 h. To detect sebum synthesized in the sebocytes, Nile red staining was performed, and sebum droplets were observed by fluorescence microscopy. Scale bar is 100 μm. The fluorescence intensity of the droplets was calculated using ImageJ. **B** Sebocytes were cultured and incubated for 72 h with or without CBE. TG levels were measured using the TG ELISA Kit. **C** Prepared sebocytes lysates were treated. Protein levels of SREBP-1, PPARγ and GAPDH were detected. The levels of SREBP-1 and PPARγ were normalized to that of GAPDH (n = 3). These data are presented as the mean ± SD, **P* < 0.05 compared to PA-treated control

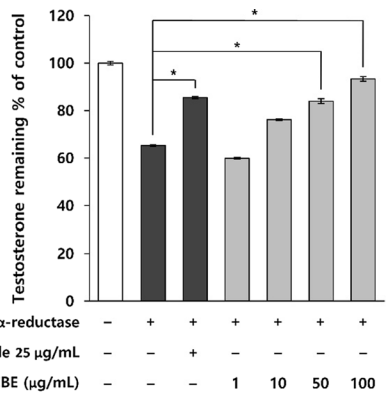

Fig. 3 CBE inhibits 5α-reductase activity. The liver microsomal fraction containing 5α-reductase activity was put in contact to finasteride or CBE. After lyophilizing the reaction solution, the remaining testosterone was analyzed by HPLC (n = 3). The data are presented as the mean ± SD, **P* < 0.05 compared to 5α-reductase-treated control

CBE to HEK293-hTLR2 cells for 6 h and then measured the TLR2 activity. As a result, we observed that *C. acnes* CBE significantly decreased the TLR2 activity compared to *C. acnes* CM (Fig. 4A). Then we found that CBE can attenuate the *C. acnes* CM induced inflammation in HEK293-hTLR2 cells. As shown in Fig. 4B, CBE inhibited the *C. acnes* CM-induced TLR2 activation in a dose dependent manner. These results suggest that CBE can exert anti-inflammatory effect by inhibiting the secretion of virulence factors from *C. acnes* as well as direct action on *C. acnes*-induced inflammatory pathway.

PA, a FFA well-known to trigger inflammation in acne vulgaris, induces activation of TLR2 pathway and secreting IL-1β related to inflammasome [5, 11]. We found that PA stimulates the TLR2 signaling pathway and this activation was effectively reduced by CBE (Fig. 4C). The result indicates that CBE can be also effective in inflammatory acne vulgaris lesions by preventing the inflammatory responses induced by PA. Therefore, it is expected that CBE can be effectively applied for relieving inflammation occurring in inflammatory acne vulgaris lesions.

Effect of CBE on *C. acnes*-induced NF-κB activation in epidermal keratinocytes

Keratinocytes are important in the innate immune activation of inflammatory acne lesions on the skin. To investigate whether CBE exert anti-inflammatory activity against *C. acnes* on HEKn, we performed an immunocytochemistry for NF-κB p65 translocation and western blot analysis for IκBα expression. As shown in Fig. 5A, upon treatment with *C. acnes* CM, the nuclear

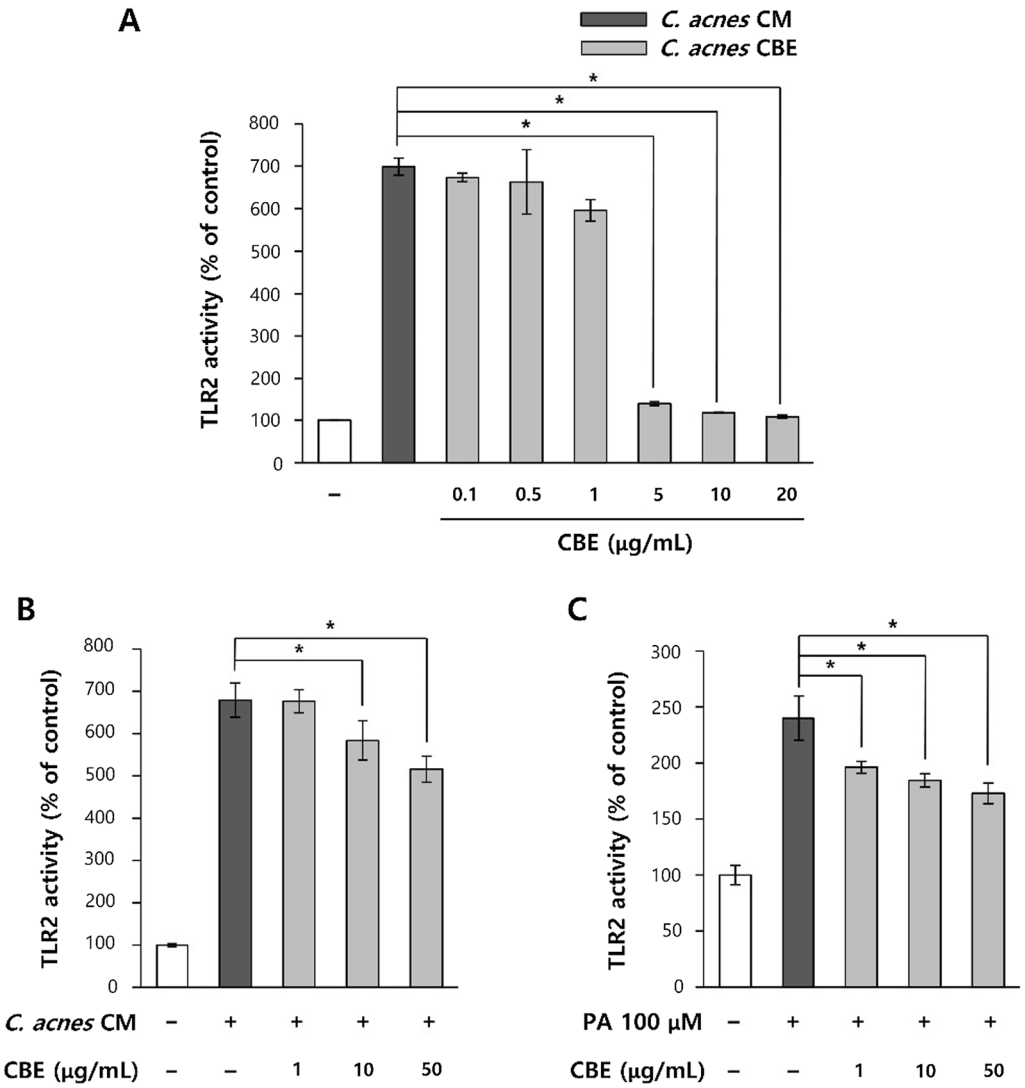

Fig. 4 CBE inhibits TLR2-mediated inflammatory pathway induced by *C. acnes* and PA in HEK293-TLR2 cells. **A** The HEK293-TLR2 cells were cultured in serum-free conditions and treated with *C. acnes* CM or *C. acnes* CBE. **B** The HEK293-TLR2 cells were cultured in serum-free conditions, treated with *C. acnes* CM, and CBE were added for 6 h. **C** The HEK293-TLR2 cells were cultured in serum-free conditions and PA was treated with or without CBE for 18 h. TLR2 activity was measured through the secreted alkaline phosphatase. The values are expressed compared to the control, which was considered to be 100% (n = 3). The data are presented as the mean ± SD, **P* < 0.05 compared to the control group

translocation of NF-κB p65 was increased in keratinocytes, while treatment with CBE mitigated the *C. acnes* CM-induced NF-κB p65 translocation. NF-κB p65 translocation is related to IκBα, which binds to NF-κB and prohibits the translocation of NF-κB to the nucleus. Once IκBα is phosphorylated by IKK, IκBα is degraded and the translocation of NF-κB p65 occurs. Therefore, we analyzed the expression of phosphorylated IκBα in HEKn. In Fig. 5B, *C. acnes* CM increased the phosphorylation of IκBα, which was inhibited by CBE in a dose dependent manner. These data suggest that CBE suppress *C. acnes*-induced inflammation by inhibiting NF-κB pathway in HEKn.

Identification of ellagic acid as an active compound in CBE

To elucidate the active compound of CBE responsible for sebum production, we performed a bioassay-guided fractionation (Fig. 6A). Among the fraction obtained, we found that the ethyl acetate fraction most effectively inhibits sebum production (Additional file 1: Figure S1). The inhibitory activity on sebum production was found in the precipitate (Additional file 1: Figure S2), and the

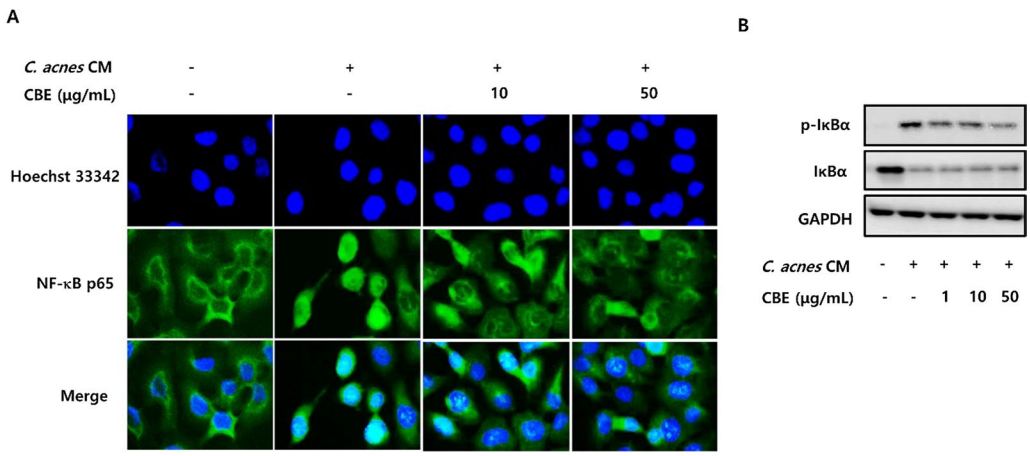

Fig. 5 CBE decreased *C. acnes* CM-induced NF-κB p65 translocation and IκBα phosphorylation in HEKn. **A** Immunofluorescence of NF-κB p65 subunit translocation in HEKn cultured in *C. acnes* CM or *C. acnes* CBE. The blue and green fluorescence indicates the nucleus and NF-κB p65 subunit, respectively. **B** After cell lysis, phosphorylated IκBα, IκBα, and GAPDH protein expression were detected by western blots. The protein levels were normalized to that of GAPDH, n = 3

main active compound was determined to be ellagic acid using HPLC analysis (Fig. 6B). The content of ellagic acid was 1.1% in the aqueous extract, but it reached 64% in the fraction R10. To examine the effect of ellagic acid on sebocytes differentiation, sebocytes were treated with various concentrations (1, 10, and 50 μg/mL) of ellagic acid, and the level of lipid synthesis was analyzed by Nile red staining. Figure 7 shows that ellagic acid decreases the fluorescence intensity by 76%, 52%, and 36% at 1, 10, and 50 μg/mL, respectively, compared to the PA-treated control. These results demonstrate that ellagic acid is an active compound of CBE that inhibits the sebum accumulation induced by PA in sebocytes.

Discussion

Various pharmacological treatments have been considered as anti-acne therapies. Among them, retinoids have been widely used for a long time. Retinoids exert anti-acne efficacy through inhibition of *C. acnes*-induced cytokine secretion by reducing TLR2 pathway and downregulation of sebum production [12, 13]. Despite significant efficacy of retinoids on acne therapies, several adverse effects such as pruritus, erythema, peeling, burning sensation, cheilitis, xerosis, and local irritation has been consistently reported [14]. For this reason, it is very meaningful to develop safe anti-acne agents derived from natural materials that can replace retinoids. In this study, we demonstrated that CBE reduces lipid accumulation by decreasing the expression of SREBP-1 and PPARγ, a regulator of fatty acid synthesis in sebocytes. And it was confirmed that EA from CBE was an active ingredient with an inhibitory effect on sebum synthesis. CBE also inhibited 5α-reductase activity and suppressed the TLR-mediated inflammatory response leading to the activation of NF-κB induced by *C. acnes* and FFA. In addition, CBE downregulates the NF-κB translocation and IκBα phosphorylation induced by *C. acnes*. Therefore, it is expected that CBE will have the ability to reduce comedolytic activity by reducing the NF-κB pathway in keratinocytes.

C. acnes is found in sebaceous glands producing sebum, which is used as the primary energy source of *C. acnes*. *C. acnes*-induced inflammation is an important step in various types of acne lesions by activating TLRs on keratinocytes and macrophages [15]. *C. acnes* secretes inflammatory factors called virulent molecules or factors like co-hemolytic Christie-Atkins-Munch-Peterson (CAMP) factors, lipase, sialidases, hyaluronate lyase (HYL), and porphyrins, which are recognized by pattern recognition receptors. TLR2 recognizes pathogen-associated molecular patterns (PAMPs) secreted from *C. acnes* and activates NF-κB signalling, stimulating the transcription of pro-inflammatory cytokines in the nucleus [16]. Therefore, virulence factors are considered as major triggers of inflammation in acne. In this study, we indirectly observed the effect of CBE on the secretion of virulence factors from *C. acnes* by measuring TLR2 activity in *C. acnes* induced inflammation model. CBE inhibited TLR2 activity by inhibiting the secretion of virulence factors from *C. acnes* (Fig. 4A). Since CBE did not show any antimicrobial activity against *C. acnes* (Data not shown), the effect of CBE on TLR2 activation may derived from the

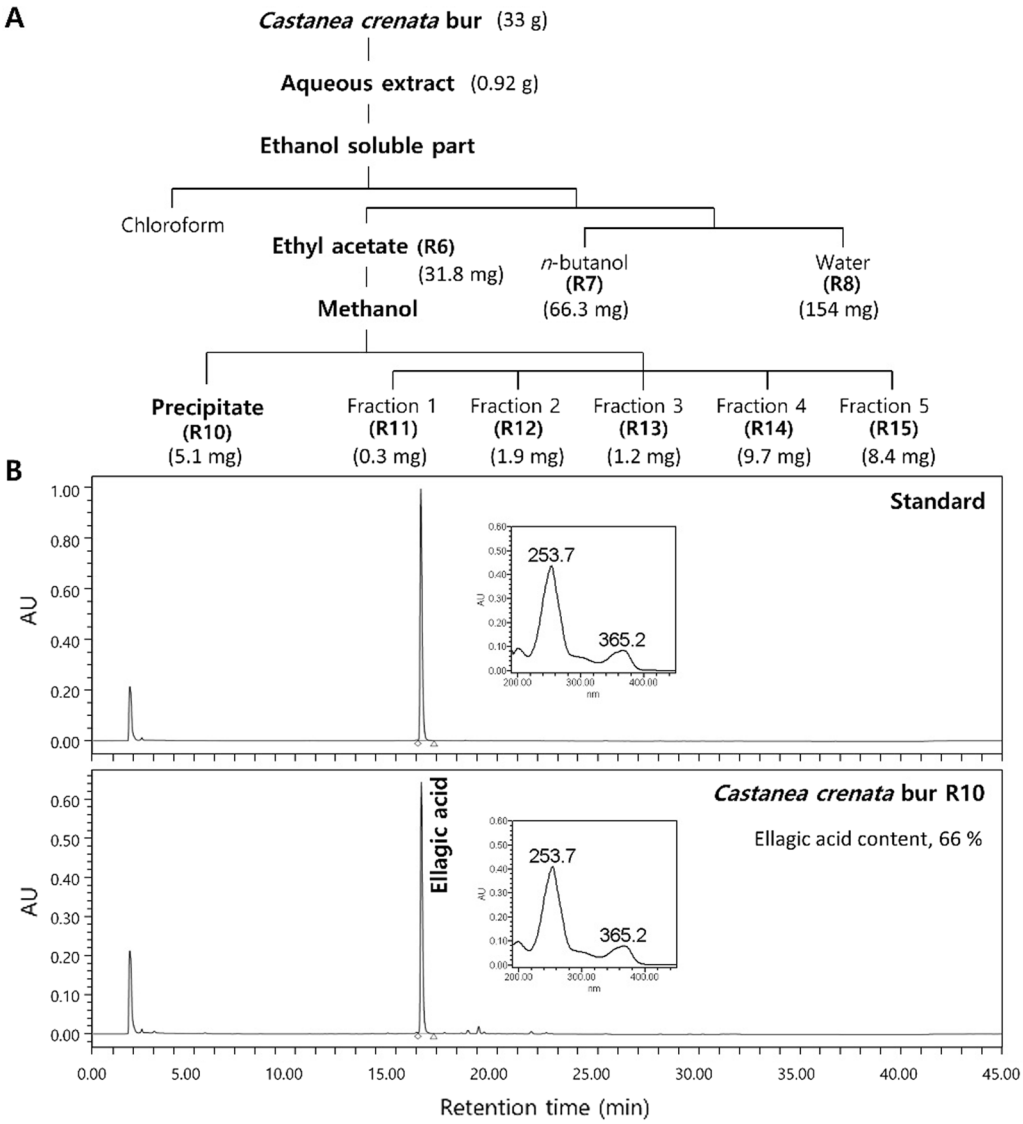

Fig. 6 CBE was fractionated and analyzed by HPLC. **A** Schematic of the fractionation of CBE. **B** Ellagic acid peak in the fraction R10 (lower) and in the ellagic acid standard (upper)

reduction of virulence factors secretion from *C. acnes*. Moreover, CBE directly inhibited the TLR2 activity induced by *C. acnes* CM (Fig. 4B).

In the epidermis, the keratinocytes inflammation through the activation of TLR2 is considered to have an important role in comedogenesis. PAMPs-induced TLR2 activation through NF-κB pathway stimulates normal human epidermal keratinocytes to release IL-1α, which promotes hypercornification like the TLR agonist-treated effect in isolated sebaceous glands [7]. Therefore, down-regulating NF-κB activation in keratinocytes may contribute to reducing comedogenesis. In this study, we confirmed that CBE decreased *C. acnes* CM-induced inflammation by inhibiting IκBα phosphorylation, which subsequently inhibits NF-κB p65 translocation to nuclei in HEKn (Fig. 5). Therefore, CBE could reduce comedogenesis related to inflammation in acne vulgaris lesions by reducing TLR2 signaling through the NF-κB pathway.

Ellagic acid is a heterocyclic compound usually found in fruits and nuts of plant and has been reported to show anti-inflammatory, antioxidant, antiviral, anticancer and anti-adipogenic activity [17, 18]. For example, ellagic acid reduces pro-inflammatory factors like TNF-α, IL-6, and IL-1β in the sera of rats and recovers D-galactose-reduced antioxidant activity by upregulating superoxide dismutase, catalase, and glutathione

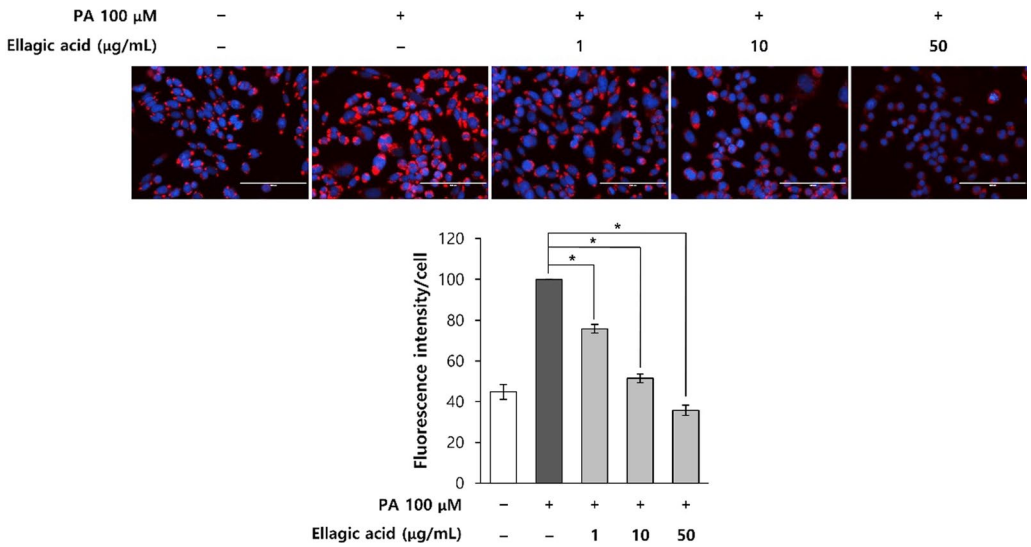

Fig. 7 Ellagic acid suppressed sebum production induced by PA in sebocytes. Sebocytes treated PA with or without ellagic acid and incubated for 48 h. Sebum accumulation was detected by Nile red staining and sebum droplets were observed by fluorescence microscopy. Scale bar is 100 μm. The fluorescence intensity of the droplets was measured using ImageJ (n = 3). The data are presented as the mean ± SD, *P < 0.05 compared to the control group

peroxidase. It can also regulate cholesterol metabolism by suppressing SREBP-2 in rat livers as a bioactive compound of unripe Rubuscoreanus [19]. Recently, ellagic acid was shown to alleviate UVB-induced skin wrinkles by reducing phototoxicity and preventing collagen degradation [20]. However, its effects on acne have not yet been elucidated. In this study, we found that ellagic acid inhibits sebum accumulation in sebocytes. Further study is needed to confirm the efficacy of ellagic acid on the other target of acne and regulatory mechanism of sebum synthesis.

Supplementary Information

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Additional file 1: Figure S1. Ethyl acetate fraction suppressed sebum production induced by PA in sebocytes. Sebocytes treated PA with or without fractions, ethyl acetate (R6), n-Butanol (R7) and water (R8) respectively, and incubated for 48 h. Sebum accumulation was detected by Nile red staining and sebum droplets were observed by fluorescence microscopy. Scale bar is 100 μm. The fluorescence intensity of the droplets was measured using ImageJ (n = 3). The data are presented as the mean ± SD, *P < 0.05 compared to PA-untreated control; **P < 0.05 compared to PA-treated control. **Figure S2.** Precipitate of methanol extraction suppressed sebum production induced by PA in sebocytes. Sebocytes were treated with PA with or without several fractions, precipitate (R10), fraction 1 (R11), fraction 2 (R12), fraction 3 (R13), fraction 4 (R14) and fraction 5 (R15) for 48 h. Sebum accumulation was detected by Nile red staining and sebum droplets were observed by fluorescence microscopy. Scale bar is 100 μm. The fluorescence intensity of the droplets was measured using ImageJ (n = 3). The data are presented as the mean ± SD, *P < 0.05 compared to PA-untreated control; **P < 0.05 compared to PA-treated control. **Figure S3.** LC-MS/MS spectrum of the fraction R10.

Authors' contributions

JY and EJ: designed the experiments; JY: conducted the experiments; KBR, HJ and EC: assisted in experiments; JY and KR: analysis of data; JY, KR and EJ: wrote the manuscript; HC and MF: critical revision of manuscript. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

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