

Ashwagandha root extract exerts anti-inflammatory effects in HaCaT cells by inhibiting the MAPK/NF- κ B pathways and by regulating cytokines

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Abstract. A paste composed of the boiled leaves and roots of the Ashwagandha plant is used to cure ulcer and swelling in Ayurvedic medicine. However, the effects of the hot water extract of Ashwagandha roots (ASH-WEX), which is also used in Ayurveda, on skin have not been fully elucidated. Therefore, the present study investigated the anti-inflammatory activity of ASH-WEX on skin, by using the human keratinocyte cell line HaCaT. The results indicated that ASH-WEX significantly inhibited mRNA expression of inflammatory cytokines, including interleukin (IL)-8, IL-6, tumor necrosis factor (TNF- α), IL-1 β and IL-12, and promoted the mRNA expression of the anti-inflammatory cytokine transforming growth factor (TGF)- β 1 in HaCaT cells. In addition, ASH-WEX inhibited the lipopolysaccharide-induced phosphorylation of p38 and c-Jun N-terminal kinase, as well as the nuclear translocation of nuclear factor (NF)- κ B p65. Downregulation of TNF- α mRNA and upregulation of TGF- β 1 mRNA were also observed *in vivo* following ASH-WEX treatment of mouse skin. In conclusion, the present study demonstrated that the anti-inflammatory effect of ASH-WEX may be due to its ability to suppress the NF- κ B and mitogen-activated protein kinase pathways, and to modulate cytokine expression. These results suggest that ASH-WEX can potentially protect against skin inflammation.

Introduction

Human skin is divided into epidermal, dermal and fatty subcutaneous tissues. The epidermis, which is comprised mainly of keratinocytes, is classified into multiple layers, the stratum corneum, granular layer, spinous layer and basal layer, based on the differentiation stage of the keratinocytes (1). Keratinocytes are known to have an important role in inflammation (2). Indeed, many inflammation-related skin diseases, such as allergic contact dermatitis, psoriasis and atopic dermatitis, are highly associated with the function of keratinocytes and cytokines (3). Keratinocytes are also a major source of inflammatory mediators, including members of the tumor necrosis factor (TNF)- α and interleukin (IL) families (2). Overproduction of pro-inflammatory mediators may lead to an abnormal inflammatory response. For this reason, potential anti-inflammatory agents can be used to suppress pro-inflammatory mediators (4).

Multiple studies have discovered anti-inflammatory and wound healing activities in substances extracted from plants (5,6). The effects of Ashwagandha (*Withania Somnifera*) leaves and roots, one of the most popular herbal treatments in the traditional Ayurvedic medicine in India, are considered to include enhancements in physical strength, energy, resistance to various stresses, and immunity. Since the roots of the Ashwagandha plant are also known to be beneficial in treating ulcers, leucoderma and scabies, they have also been applied topically to heal skin sores and reduce swelling (7). These findings suggest that one or more components of Ashwagandha have active physiological effects on the skin. Studies on topical treatment with Ashwagandha organic and aqueous extracts have confirmed their chemopreventive effects on skin cancer (8) and their melanin regulatory effects (9). In addition, Ashwagandha, in the form of a paste of boiled leaves and roots, is considered to have wound-healing abilities in Ayurveda (10).

It has been determined that the main organic solvent extract of Ashwagandha is the water-insoluble Withaferin-A, and that Withaferin-A exhibits anti-inflammatory, antiangiogenesis, antimetastasis and anticancer activities (11). In Ayurveda, however, most herbal remedies are applied as a hot-water extract. Whether the healing effects of

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Abbreviations: TNF, tumor necrosis factor; IL, interleukin; ASH-WEX, Ashwagandha root water extract; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NF- κ B, nuclear factor- κ B; TGF, transforming growth factor; PCR, polymerase chain reaction; MAPK, mitogen-activated protein kinase

Key words: Ashwagandha, anti-inflammation, mitogen-activated protein kinase, nuclear factor- κ B, cytokine modulation

Withaferin-A would be observed by applying a hot-water extract of Ashwagandha (ASH-WEX) to skin remains largely unexplored.

Recently, a report determined that ASH-WEX had physiological activities and effects that were different than those of Withaferin-A, and were unrelated to Withaferin-A (12). Bhat *et al.* (13) also reported that Ashwagandha tea increased natural killer-cell activity, possibly due to various effects of the components of the tea in cytokine secretion. The present study hypothesized that ASH-WEX may modulate the expression of cytokines when used as a topical treatment for skin. Thus, the anti-inflammatory effects and the intracellular mechanisms of ASH-WEX were investigated *in vitro* and *in vivo*.

Materials and methods

Materials. Ashwagandha roots were a kind gift from Dr Reiji Nishio (Toray Industries, Inc., Tokyo, Japan). Lipopolysaccharide (LPS) and MTT were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Primary antibodies for p38 (cat. no. 9212S), phosphorylated (P)-p38 (cat. no. 9211S), c-Jun N-terminal kinase (JNK) (cat. no. 9252S), P-JNK (cat. no. 9251S), extracellular signal-regulated kinase (ERK) (cat. no. 9102S), P-ERK (cat. no. 9101S), nuclear factor (NF)- κ B, p65 (cat. no. 4764S), histone H3 (cat. no. 9715) and β -actin (cat. no. 4967S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). TNF- α polyclonal antibody (bs-2081R) was purchased from Bioss Antibodies, Inc. (Woburn, MA, USA). TGF- β 1 polyclonal antibody (cat. no. 5559-100) was purchased from BioVision, Inc. (Milpitas, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. 7074) (Cell Signaling Technology, Inc.) was used as a secondary antibody. Medetomidine hydrochloride and atipamezole hydrochloride were purchased from Wako Pure Chemical Industries, Ltd. Vetorphale was purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan) and midazolam was purchased from Sandoz (Tokyo, Japan).

Extraction of ASH-WEX. Several root pieces from different Ashwagandha roots were cut and powdered with ceramic mortar, and then added to Milli Q water (5 g powder in 50 ml water). After 2 h of heating with an electric heater (Tokyo Technological Laboratories, Tokyo, Japan) at 100°C, the extract was passed through a paper filter (GE Healthcare Life Sciences, Little Chalfont, UK). Following centrifugation at 180 x g for 5 min, the supernatants were filtered with a 0.2 μ m filter (Sartorius AG, Goettingen, Germany) and stored at -80°C until use. The final concentration of ASH-WEX was 100 mg/ml. ASH-WEX was diluted with Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or Milli Q prior to its use in the treatment of HaCaT cells or mouse skin, respectively.

Cell culture. The human keratinocyte cell line HaCaT was provided by Professor P. Boukamp and Dr N. Fusenig (German Cancer Research Center, Heidelberg, Germany) (14) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Biofill, Victoria, Australia), 100 U/ml penicillin

and 100 μ g/ml streptomycin at 37°C in a humidified chamber under an atmosphere of 95% air and 5% CO₂.

Animals. A total of 18 six-week-old male C57BL/6J mice (weight, ~20 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The mice were given ad libitum access to standard chow and water, and housed in our departmental animal room under specific pathogen-free conditions. All animals were kept in a controlled environment for 1 week prior to experiments and all experimental procedures were approved by the Tokyo Denki University Animal Care and Use Committee in accordance with their Ethical and Animal Experiment Regulations.

Cell viability assay. Cell viability was evaluated by MTT assay. HaCaT cells (1x10⁴ cells/well) were cultured in 96-well plates for 24 h (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then treated with different concentrations of ASH-WEX for 24 h. Cells were then incubated with 10 μ l of 5 mg/ml MTT for 1 h at 37°C. Finally, the supernatants were discarded and DMSO (100 μ l/well) was added to dissolve MTT formation. The absorbance was measured with a microplate reader (Awareness Technology, Palm City, FL, USA) at 570 nm.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) *in vitro*. HaCaT cells (1x10⁶ cells/dish) were incubated in a dish (diameter, 100 mm) for 24 h and then treated with ASH-WEX (0.25 or 1.5 mg/ml) for 24 h, and total RNA was isolated by TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PrimeScript Reverse Transcriptase (Takara Bio, Inc., Kusatsu, Japan) was used for the reverse transcription reaction. In brief, 2.5 μ l of Milli Q, 2 μ l of 5X PrimeScript buffer, 2 μ l of 2.5 mM dNTP, 2 μ l of 2.5 μ M Oligo dT (Sigma-Aldrich; Merck KGaA), 0.25 μ l of PrimeScript, 0.25 μ l of recombinant RNase inhibitor and 1 μ l of isolated RNA (1 μ g/ μ l) were combined as a single sample, incubated at 42°C for 30 min and 70°C for 15 min, and placed in short-term storage at 4°C in a thermal cycler (ASTEC, Shime, Japan). Reverse transcriptase-generated complementary DNA encoding IL-1 β , IL-6, IL-8, IL-12, TNF- α , and transforming growth factor (TGF)- β 1 genes were amplified by PCR using the following specific primers: IL-1 β forward, GCTGAGGAAGATGCTGGTTC and reverse, TCCATATCCTGTCCCTGGAG; IL-6 forward, CCTGAACCTTCCAAAGATGGC and reverse, TTCACCAGGCAAGTCTCCTCA; IL-8 forward, GTCCTTGTTCCACTGTGCCT and reverse, GCTTCCACATGTCCCTCACAA; IL-12 forward, GATGGCCCTGTGCCTTAGTA and reverse, TCCATATCCTGTCCCTGGAG; TNF- α forward, TCCTTCAGACACCCTCAACC and reverse, TCCTTCAGACACCCTCAACC; TGF- β forward, CCCTGGACACCAACTATTGC and reverse, GTCCAGGCTCCAAATGTAGG; and GAPDH forward, ATCATCAGCAATGCCTCCTG and reverse, GTGCTTACCACCTTCTTGA. In brief, a reaction solution [12.5 μ l of 2X GeneAce SYBR qPCR Mix α (Wako Pure Chemical Industries, Ltd.), 0.5 μ l of each primer, Milli Q, and 2.5 μ l of cDNA sample] was dispensed into optical 96-well reaction plates (Applied Biosystems; Thermo Fisher Scientific, Inc.), and set in an ABI PRISM 7000 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Then, amplification consisted

of 1 cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 30 sec and 60°C for 1 min, with a final dissociation stage at 95°C for 15 sec, 60°C for 20 min, and 95°C for 15 sec. Relative fold changes in mRNA expression were calculated using the $2^{-\Delta\Delta C_q}$ method, according to Livak and Schmittgen (15).

Western blot analysis. HaCaT cells (1×10^6 cells/dish) were incubated in a dish (diameter, 100 mm) for 24 h and then pretreated with ASH-WEX (5 mg/ml) for 20 h, followed by 4 h incubation with LPS (1 μ g/ml). To examine the mitogen-activated protein kinase (MAPK) pathway, total proteins were extracted with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X 100, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium orthovanadate, 1% protease inhibitor cocktail; pH 7.5) and western blot was performed as previously described (16). To investigate the NF- κ B translocation to the nucleus, the nuclear fractions were prepared as previously described (17) with slight modifications. HaCaT cells (1×10^6 cells/dish) were incubated a dish (diameter, 100 mm) for 24 h and then pretreated with ASH-WEX for 20 h, followed by 4 h incubation with LPS (1 μ g/ml). After the cells were collected and washed with PBS, the pellets were resuspended in 100 μ l of buffer A [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.4% (v/v) NP-40, 1% protease inhibitor; pH 7.9] and incubated on ice for 10 min. Following centrifugation at 15,000 \times g for 3 min, the supernatant was collected as a cytosolic fraction. The remaining pellets were washed with buffer A, resuspended in 60 μ l of buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1% protease inhibitor; pH 7.9) and stirred at 4°C for 2 h. The supernatants were collected as a nucleic fraction following centrifugation at 15,000 \times g at 4°C for 3 min. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). For each sample, 20 μ g of protein was subjected to 12% polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated in blocking solution (3% non-fat skimmed milk) for 1 h, and then probed with primary antibodies (1:1,000) at 4°C overnight. After washing with 0.1% Tween 20-PBS, the membrane was incubated with secondary antibody (1:2,000) for 1 h at room temperature. Finally, the membranes were visualized with enhanced chemiluminescence reagents (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.) following exposure for 1 min, and observed with an Image Quant LAS-4000 system (GE Healthcare Life Sciences). The relative density of bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA isolation, RT-qPCR and wound-healing effect of ASH-WEX in vivo. The anti-inflammatory effect of ASH-WEX *in vivo* was investigated using 7-week-old male C57BL/6J mice. The back of each mouse was shaved under anesthesia (mixed anesthesia of 0.75-mg/kg bw medetomidine hydrochloride, 4-mg/kg bw midazolam, and 5-mg/kg bw vetorphale in physiological saline and antagonist of 0.75-mg/kg bw atipamezole hydrochloride in physiological saline) (18), and a 0.5-cm diameter wound area was excised on both sides of the back using a dermal punch (Nipro, Osaka, Japan), for initiation of an inflammatory response. Each mouse was topically treated with Milli Q on one side (20 μ l; control side) and

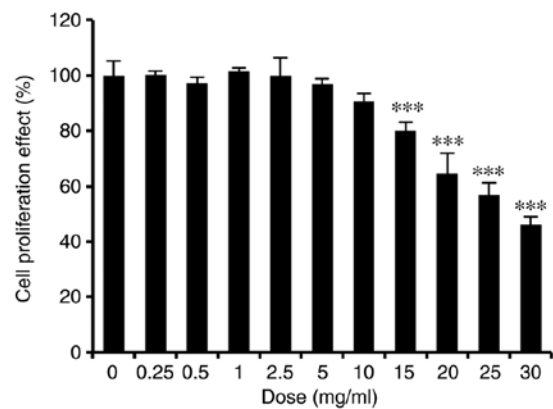


Figure 1. Effect of ASH-WEX on cell viability. HaCaT cells were treated with the indicated concentration of ASH-WEX for 24 h. Cell viability was assessed by MTT assay. The quantified data are shown as the mean \pm standard deviation of three independent experiments. ***P<0.001 vs. untreated cells. ASH-WEX, Ashwagandha root water extract.

with ASH-WEX on the other side (20 μ l; 10 mg/ml; treatment side). Following treatment, two types of tape (Tegaderm Roll 16006S, 3M Health Care, Tokyo, Japan; Dressing tape, Kyowa, Osaka, Japan) were placed at the sites, in order to avoid direct contact with the wound and to prevent evaporation. The tapes were peeled off gently and changed following topical treatment. Each mouse was housed separately, and treated every 24 h. The day when the wounds were made and treated with ASH-WEX or Milli Q was designated as day 0. The wounds were scanned and photographed prior to treatment and every day, until day 11. Wound closure was calculated as follows: wound closure of Day n = Day n wound area / Day 0 wound area. The weight of each mouse was measured every day following treatment, with a Table Top High Precision Balance (A&D Company, San Jose, CA, USA). Relative wound closure, expressed as the wound size ratio of day n/day 0, was quantified using ImageJ software.

To investigate cytokine expression, mice were sacrificed on day 5, and a 1x1 cm area of injured skin was removed and used for RNA extraction. Total RNA was isolated by TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Following reverse transcription, RT-qPCR was performed, as aforementioned. The following primers were used: TNF- α forward, CCACCACGCTCTTCTGTCTA and reverse, CAC TTGGTGGTTTGCTACGA; TGF- β forward, TTGCTTCAG CTCCACAGAGA and reverse, TGGTTGTAGAGGGCAAGG AC; and GAPDH forward, AACCTTGGCATTGTGGAAGG and reverse, ACACATTGGGGGTAGGAACA.

Hematoxylin and eosin (H&E) staining. Seven-week-old male C57BL/6J mice were treated with ASH-WEX or Milli Q as described above. On day 5, six mice were sacrificed and a 1x1 cm area including the entire wound and surrounding unwounded skin were collected for H&E staining. Mouse skin samples were fixed in 4% paraformaldehyde at 4°C for 72 h. The samples were dehydrated and embedded in paraffin and skin sections were cut at a thickness of 5 μ m. Following deparaffinization and rehydration, the samples were stained with H&E and the epidermal and dermal thicknesses were measured by ImageJ. Digital images were captured with an

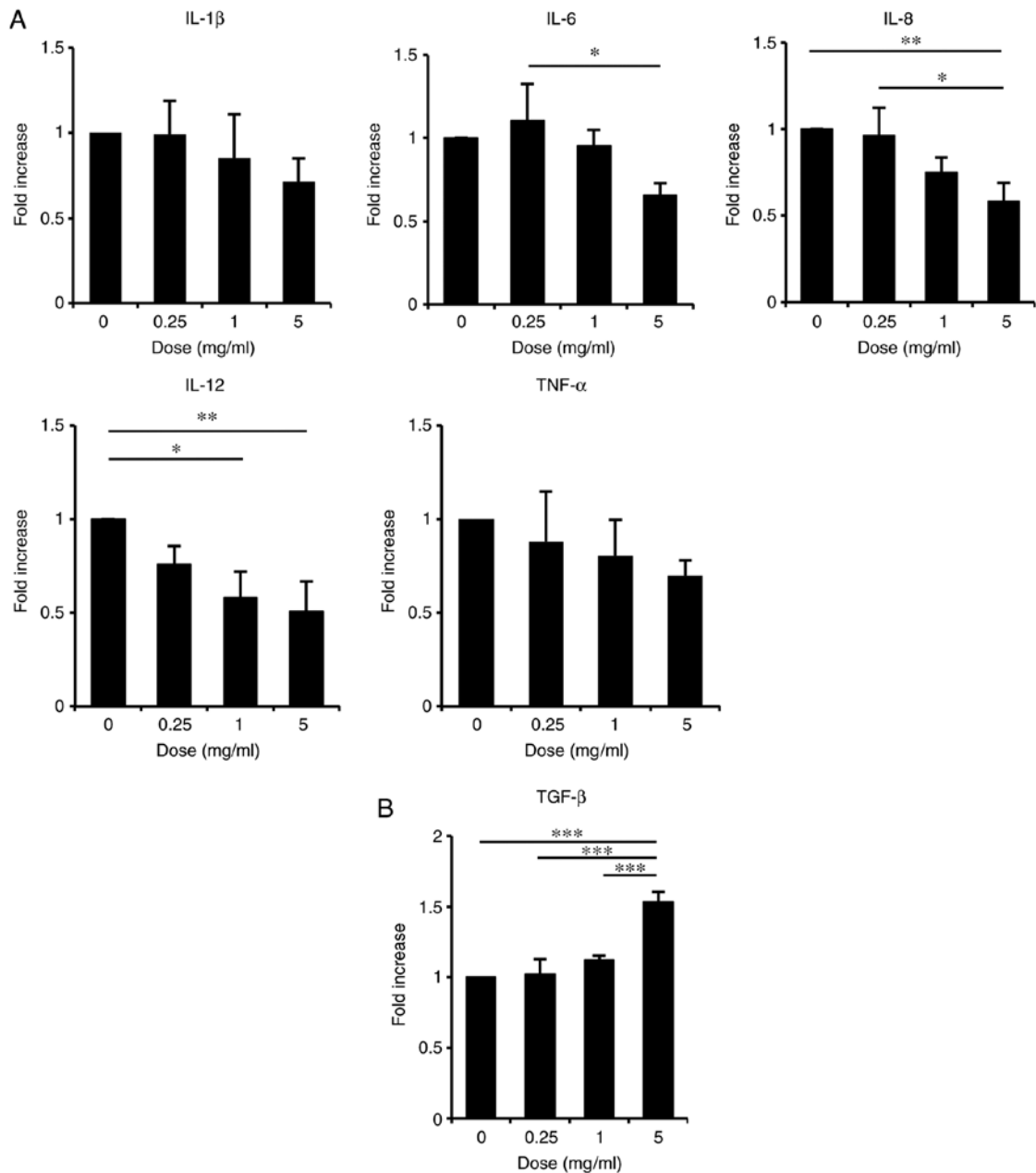


Figure 2. Differential expression of cytokine mRNA in ASH-WEX-treated HaCaT cells. HaCaT cells were treated with the indicated concentration of ASH-WEX for 24 h, then the mRNA expression levels of (A) pro-inflammatory and (B) anti-inflammatory cytokines were analyzed. The quantified data are shown as the mean \pm standard deviation of three independent experiments. GAPDH was used as a control. *P<0.05, **P<0.01 and ***P<0.001 with comparisons indicated by lines. ASH-WEX, Ashwagandha root water extract; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) and DP714 digital camera (Olympus Corporation).

Statistical analysis. The Student's t-test was used to compare paired groups. One-way analysis of variance was used for multi-group analysis, followed by Bonferroni test as a post hoc test (Prism 7, ver. 7.0d, GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Low toxicity effect of ASH-WEX on HaCaT cells. Cells of the human keratinocyte line HaCaT have features similar to

normal human cells, and thus are widely applied in inflammatory skin-related research. In the present study, HaCaT cells were used to investigate the anti-inflammatory effects of ASH-WEX. To determine whether ASH-WEX may have a toxic effect on HaCaT cells, an MTT assay was performed. The results demonstrated that ASH-WEX was not toxic to HaCaT cells up to a dose of 10 mg/ml (Fig. 1).

ASH-WEX treatment downregulates inflammatory cytokines, while it upregulates anti-inflammatory cytokines. Cytokines released from keratinocytes have vital roles in many inflammation-related skin diseases (19). In order to examine whether ASH-WEX might regulate the cytokine expression in keratinocytes, the expression levels of several cytokines

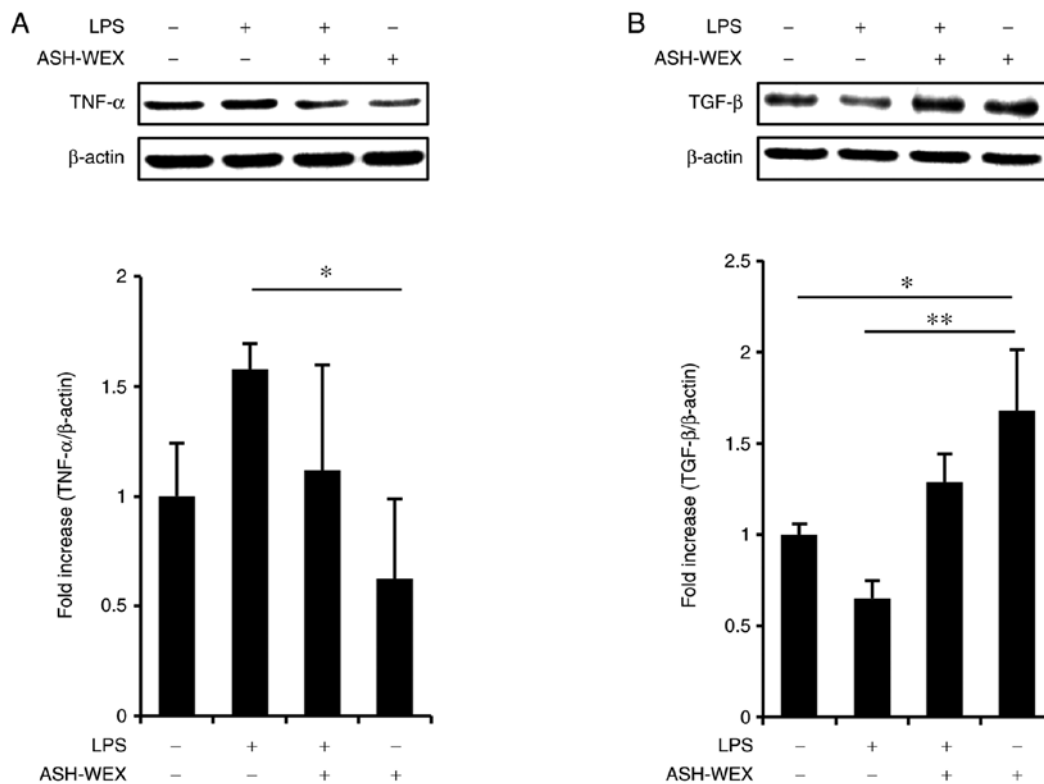


Figure 3. Differential expression of cytokine protein in ASH-WEX-treated HaCaT cells. HaCaT cells were treated with 5 mg/ml of ASH-WEX for 12 h and then with LPS (1 μ g/ml) for 12 h. (A) TNF- α and (B) TGF- β protein expression levels were determined by western blot analysis. The relative density of the TNF- α , TGF- β and β -actin bands were estimated and the mean \pm standard deviation of three independent experiments are presented as quantified data. * P <0.05 and ** P <0.01 with comparisons indicated by lines. ASH-WEX, Ashwagandha root water extract; LPS, lipopolysaccharide; TNF, tumor necrosis factor; TGF, transforming growth factor.

were examined in HaCaT cells treated with ASH-WEX by RT-qPCR. The results demonstrated that, when cells were treated with ASH-WEX at concentrations <5 mg/ml which were non-toxic in HaCaT cells (Fig. 1), the mRNA expression levels of the inflammatory cytokines IL-8, IL-6 and IL-12 were significantly decreased in a dose-dependent manner (Fig. 2A). The mRNA levels of TNF- α and IL-1 β also appeared decreased, although this effect was less significant (Fig. 2A). By contrast, treatment with ASH-WEX at concentrations <5 mg/ml significantly increased the mRNA expression levels of the anti-inflammatory cytokine TGF- β 1 in a dose-dependent manner (Fig. 2B). These results indicated that ASH-WEX might have an anti-inflammatory effect on keratinocytes.

HaCaT cells possess Toll-like receptor (TLR) 2 and TLR4 (20), and the expression of pro-inflammatory cytokines, including IL-8, IL-6, TNF- α , IL-1 β and IL-12, are modulated by TLR signaling (21). Therefore the TLR4 agonist, LPS, was used as a model of inflammation and the effects of ASH-WEX cytokine production were investigated at the protein level by western blotting. The results demonstrated that treatment with 5 mg/ml ASH-WEX inhibited the LPS-induced TNF- α upregulation (Fig. 3A), but significantly increased TGF- β 1 levels (Fig. 3B). These results were in accordance with the mRNA results (Fig. 2).

ASH-WEX treatment inhibits the MAPK/NF- κ B pathways. The present data suggested that ASH-WEX might have

anti-inflammatory properties by suppressing the expression of inflammatory cytokines. It is well established that the NF- κ B and MAPK signaling pathways are strongly associated with inflammatory cytokine expression in HaCaT cells (22,23). LPS stimulation activates NF- κ B by modulating TLR4 (24). LPS can also activate the MAPK pathway in HaCaT cells (22). To determine whether ASH-WEX suppressed the LPS-induced MAPK and NF- κ B pathways, a western blot analysis was performed. The results revealed that treatment with 5 mg/ml ASH-WEX inhibited the LPS-induced phosphorylation of p38 (Fig. 4A) and JNK (Fig. 4B), but upregulated the phosphorylation of ERK (Fig. 4C). Next, it was investigated whether ASH-WEX inhibited LPS-induced nuclear transduction of NF- κ B, and the results demonstrated that treatment with 5 mg/ml ASH-WEX inhibited the LPS-induced nuclear translocation of p65 (Fig. 4D), which is a significant mediator of the NF- κ B signaling pathway.

Effect of ASH-WEX on cytokine expression and wound-closure effect in vivo. Finally, the anti-inflammatory effect of ASH-WEX was investigated *in vivo*. It is well known that external stimuli, such as wounds, initiate inflammation in the skin (25). In the present study, wounded mouse skin was employed as an *in vivo* inflammation model in order to investigate the anti-inflammatory effects of ASH-WEX. On each mouse, ASH-WEX was topically applied to a wound on the left side of the back skin and solvent Milli Q was topically applied to a wound at the right side of the back skin every 24 h until

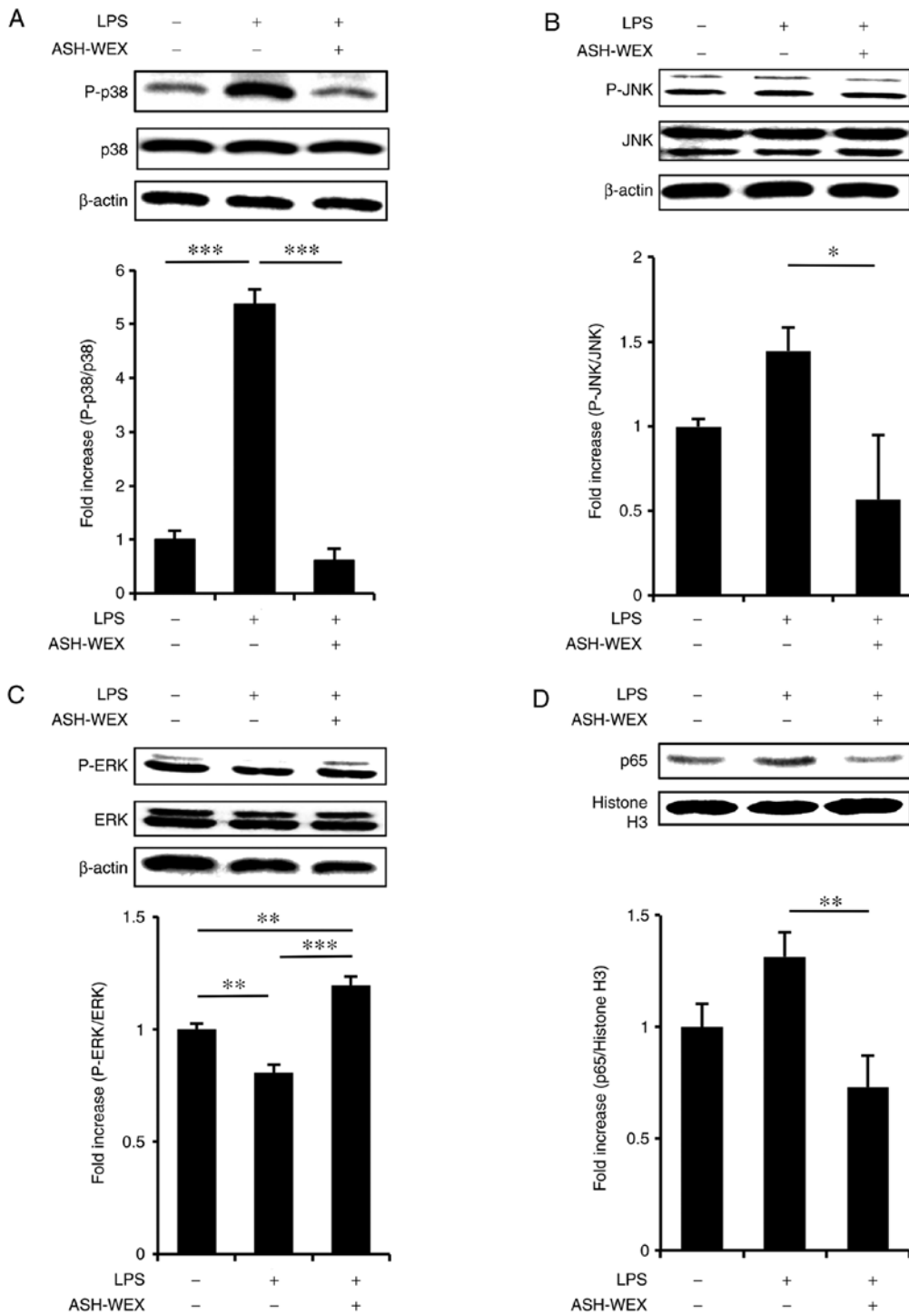


Figure 4. ASH-WEX treatment attenuates the LPS-induced inflammatory response signaling. HaCaT cells (1×10^5 cells) were treated with 5 mg/ml of ASH-WEX for 20 h and then with LPS (1 μ g/ml) for 4 h. (A) The levels of p38 MAPK phosphorylation, (B) JNK phosphorylation, and (C) ERK phosphorylation were measured by western blotting. β -actin was used as internal control. (D) The protein levels of p65 NF- κ B in the nuclear fraction of the treated cells were determined by western blotting. Histone H3 was used as internal control. The relative density of the bands was estimated and the mean \pm standard deviation of three independent experiments is presented as quantified data. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with comparisons indicated by lines. ASH-WEX, Ashwagandha root water extract; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NF- κ B, nuclear factor- κ B; P-, phosphorylated.

day 5. Thereafter, the skin was removed and used to assay the mRNA expression levels of cytokines by RT-qPCR. The results demonstrated that ASH-WEX treatment inhibited the mRNA expression of the pro-inflammatory cytokine TNF- α , while it increased the mRNA expression of the anti-inflammatory cytokine TGF- β 1 (Fig. 5). These results further demonstrated that

ASH-WEX may regulate the inflammatory response associated with wound closure (26).

Finally, a wound-healing assay was performed *in vivo* to examine the properties of ASH-WEX. The results demonstrated that, on days 1-5, the ASH-WEX treatment side exhibited a significantly decreased wound area compared

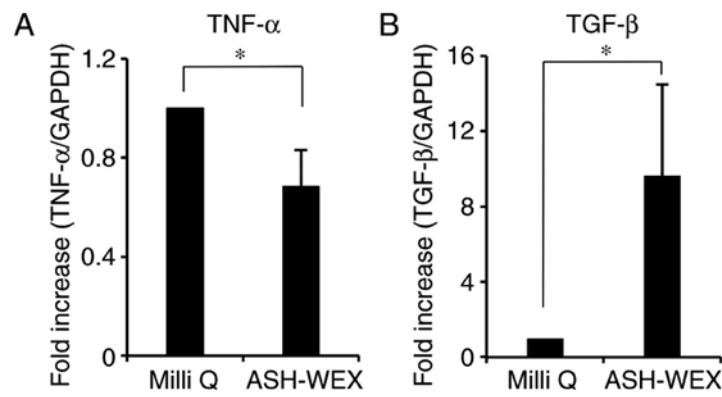


Figure 5. Differential expression of cytokine mRNAs in ASH-WEX-treated mouse skin. The back of each mouse was shaved and a 0.5 cm diameter wound area was excised on both sides of the back. Mice were treated with Milli Q on one side and ASH-WEX on the other, once every 24 h. Mice were sacrificed, and the RNA was isolated on day 5. (A) TNF- α and (B) TGF- β mRNA expressions were analyzed by reverse transcription-quantitative polymerase chain reaction. The quantified data are shown as the mean \pm standard deviation of three independent experiments. GAPDH was used as a control. * $P < 0.05$. ASH-WEX, Ashwagandha root water extract; TNF, tumor necrosis factor; TGF, transforming growth factor.

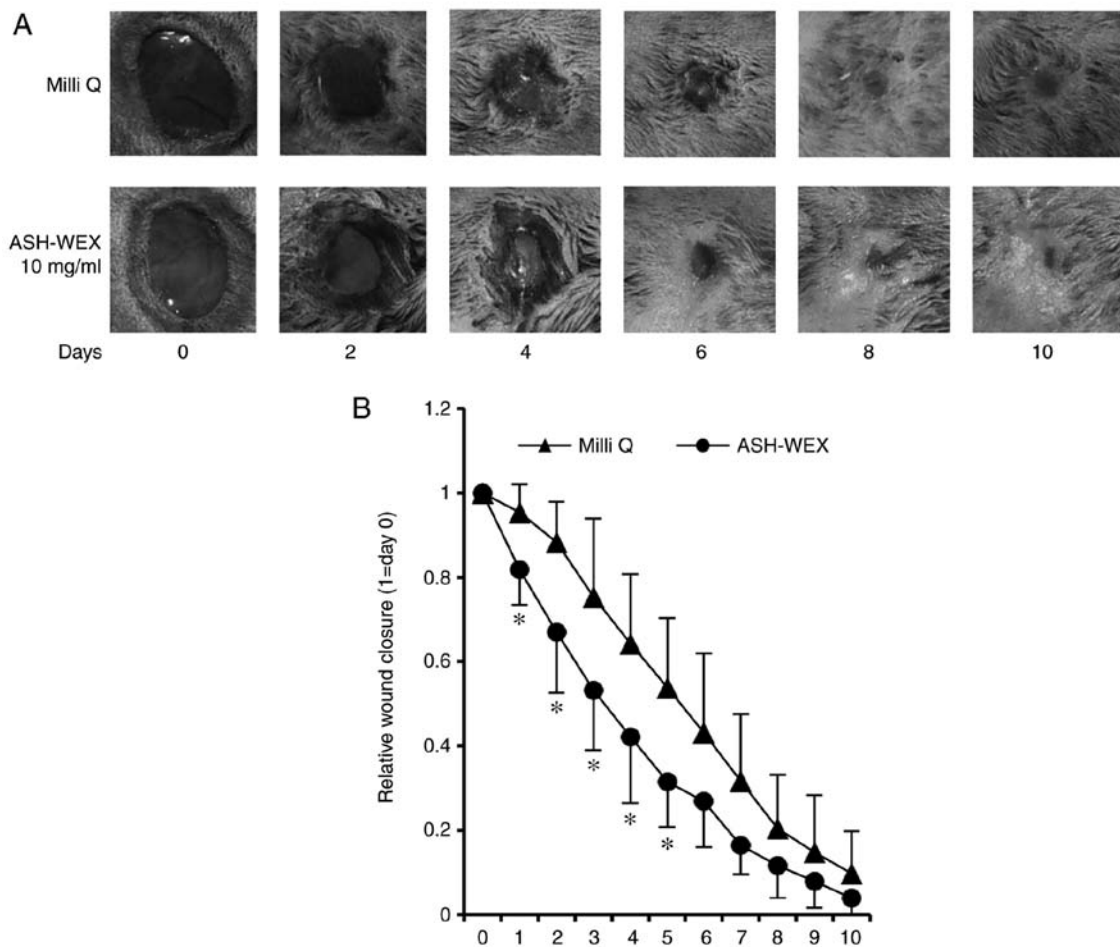


Figure 6. Effect of ASH-WEX treatment on wound closure in mice. The back of each mouse was shaved and a wound area 0.5 cm in diameter was excised on both sides of the back. The wounds were treated with Milli Q on one side and ASH-WEX on the other, once every 24 h. (A) Representative photographs of wound sites from six independent experiments. (B) Quantification of wound area per treatment group is shown as the mean \pm standard deviation of six independent experiments. * $P < 0.05$ vs. the control-treated side. ASH-WEX, Ashwagandha root water extract.

with the control Milli Q- treated side (Fig. 6). These results suggest that ASH-WEX treatment accelerated the speed of wound-closure in the early stage, which is the inflammation phase of wound healing. In addition, the ASH-WEX-treated skin displayed a thinner epidermis and thinner dermis

compared with the control skin (Fig. 7). Furthermore, less aggregation of immune cells was observed in the ASH-WEX-treated skin compared with the control-treated skin (Fig. 7). Of note, ASH-WEX treatment did not affect the weight of the mice (data not shown).

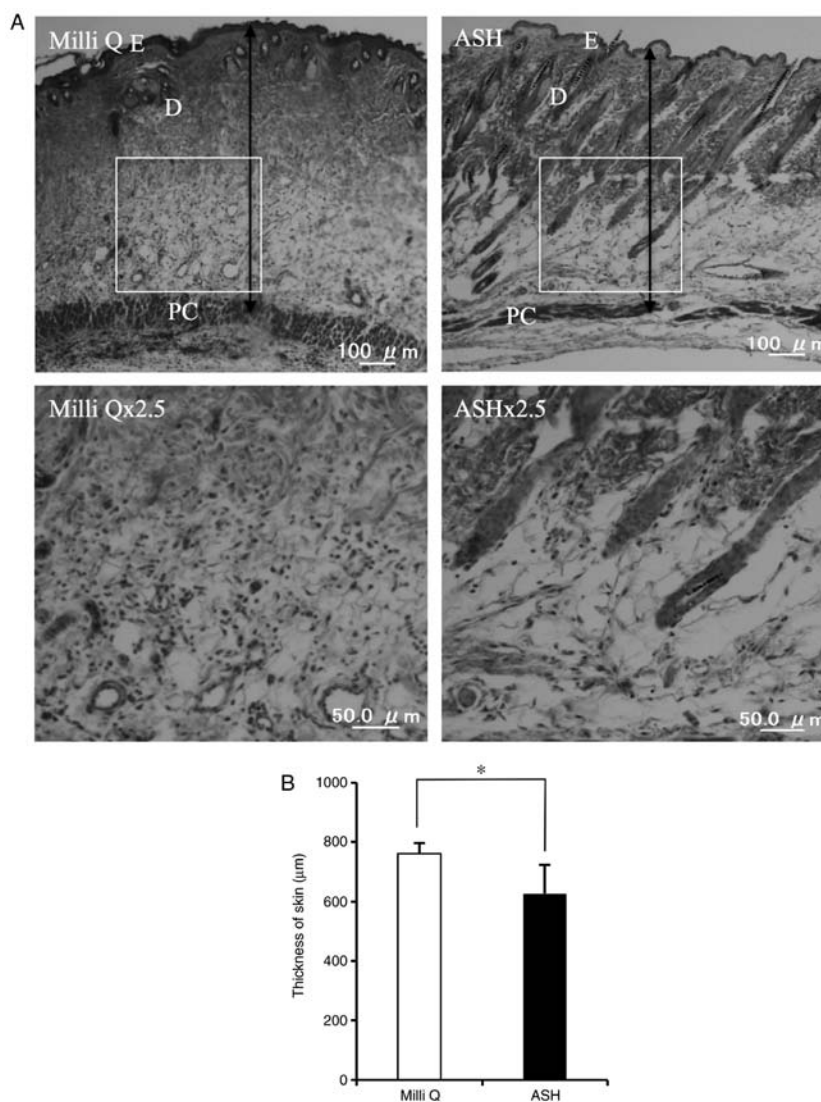


Figure 7. Histological changes in the wounded mouse skin following ASH-WEX treatment. The back of each mouse was shaved and a 0.5 cm diameter wound area was excised on both sides of the back. Mice were treated with Milli Q on one side and ASH-WEX on the other, once every 24 h. The skin wound samples were collected and stained with H&E and the epidermal and dermal thickness were measured by ImageJ software. (A) Representative images of H&E-stained skin sections from the Milli Q-treated (Milli Q) or ASH-WEX-treated (ASH) wound area. The lower images represent a 2.5x enlargement of the boxed areas of the corresponding upper images. Black-arrowed lines indicate the thickness of skin. (B) Quantified data of the thickness of skins are shown as the mean \pm standard deviation of four independent experiments. * $P < 0.05$ vs. the control-treated side. ASH-WEX, Ashwagandha root water extract D, dermis; E, epidermis; PC, panniculus carnosus muscle.

Discussion

The present study investigated the anti-inflammatory and cytokine modulatory effect of ASH-WEX *in vitro*. HaCaT cells were used to examine the cytokine modulatory effect of ASH-WEX, and the results revealed that ASH-WEX treatment inhibited the MAPK and NF- κ B pathways to decrease the expression of pro-inflammatory cytokines and increase the expression of anti-inflammatory cytokines. Finally, ASH-WEX treatment was demonstrated to also exert an anti-inflammatory effect *in vivo*.

The present results indicated that ASH-WEX treatment resulted in a decrease of pro-inflammatory cytokine expression in HaCaT cells at both the mRNA and protein level. To investigate the mechanism underlying these effects, the MAPK pathway and its downstream pathway NF- κ B were investigated, which are strongly related to pro-inflammatory

cytokine release in monocytes (27). Ultraviolet electromagnetic radiation (UV) and bacteria can activate the MAPK and NF- κ B pathways (4,28). Tang *et al* (4) confirmed that the anti-inflammatory effect of glycolic acid is due to its ability to inhibit UVB-induced mRNA expression of inflammatory cytokines and NF- κ B nuclear translocation in HaCaT cells. In addition, LPS can activate NF- κ B and MAPK signaling in HaCaT cells (20,21). In the present study, LPS-treated HaCaT cells were used to determine whether ASH-WEX suppressed the LPS-induced MAPK and NF- κ B pathways. The results demonstrated that ASH-WEX indeed inhibited the LPS-induced phosphorylation of the MAPKs p38 and JNK, as well as the nuclear translocation of p65 NF- κ B. These results are in agreement with the study of Wang *et al* (29), in which the anti-inflammatory agent Baicalein inhibited LPS-induced p38 MAPK and NF- κ B activation. There has been at least one report that Ashwagandha roots exert an antibacterial

effect (30). Based on the present results, ASH-WEX may also have antibacterial activity.

Multiple plants possess wound-healing activity due to the anti-inflammatory effects of active components in their extracts (31). It is well known that external stimuli, such as wounds, initiate inflammation in the skin. Indeed, one of the main and early phases of wound healing is the inflammatory phase, in which many pro-inflammatory mediators, such as TNF- α , are released in the wound area to initiate inflammation (32). This inflammatory response is indispensable for wound repair. However, an excessive or prolonged inflammation phase will delay wound healing (33). TGF- β 1 serves a vital role in wound healing, and increased levels of TGF- β 1 signal the transition from inflammatory phase to proliferation phase, which is another essential stage of wound healing (34). In the present study, at days 1-5 following ASH-WEX treatment, the size of the wound decreased significantly compared with the control-treated wound. Furthermore, histological analysis revealed that the immune cells were less aggregated in the ASH-WEX-treated skin compared with the control skin. These data suggested that the control side remained in the inflammatory phase, while the ASH-WEX-treated side rapidly progressed to the proliferation phase. Ghilissi *et al* (35) suggested that the wound-healing activity of *Artemisia campestris* aqueous extract *in vivo* may be due to the reduction of the inflammatory phase, and the rapid transition to the proliferation phase by its anti-inflammatory effect. Collectively, these findings suggest that ASH-WEX may be a promising wound-healing agent, and thus the wound-healing activity of ASH-WEX warrants further investigation.

Both the organic and the aqueous extract of Ashwagandha have anti-inflammatory activity (36,37). Previous phytochemical studies have confirmed that Ashwagandha contains alkaloids, flavonols, withanolides, glycowithanolides, steroidal lactones, sterols and phenolic acids (7,38-40). The most bioactive ingredients from the root include sitoindosides (VII, VIII, IX, X) withanine, withananine, and ashwagandhanolide (41). However, most of the bioactive components of Ashwagandha have been isolated from its organic layer extracts. There are few studies about the bioactive ingredients of ASH-WEX. One of the existing reports on this subject indicates that the main component of ASH-WEX is Withaferin-A (42), but, when the solubility of purified Withaferin-A in water was tested by heating for 2 h, the same condition used to extract ASH-WEX, the Withaferin-A powder remained in the solution. Another study reported that 5 withanolides and one glyco-withanolide were the active constituents of ASH-WEX (43). Withanolides have anti-inflammatory activities (44). To the best of our knowledge, the anti-inflammatory effects of withanolides on HaCaT cells remain unclear. Recently, a study reported that triethylene glycol (TEG) was present in the aqueous extract of Ashwagandha, and particularly concentrated in the leaves (12). In the present study, therefore, we tested whether TEG modulates the levels of cytokine expression in HaCaT cells and revealed that TEG suppresses the LPS-induced expression of the inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-8 (data not shown). TEG also inhibited LPS-induced nuclear transduction of NF- κ B (data not shown). From this result, TEG might be one of the bioactive components of ASH-WEX that induce anti-inflammation. However, the anti-inflammatory

effects of TEG and its wound-healing activities require further investigation.

In conclusion, the present study demonstrated that ASH-WEX had an anti-inflammatory effect, which it exerted by suppressing the NF- κ B and MAPK pathways, and by modulating the cytokine expression in HaCaT cells. ASH-WEX treatment increased the mRNA expression of the anti-inflammatory cytokine TGF- β 1 and decreased the mRNA expression of the pro-inflammatory cytokine TNF- α *in vivo*. Thus, ASH-WEX could be a promising anti-inflammatory agent for skin, as well as a potential wound-healing therapeutic agent.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Author's contributions

AS, TS and YN designed the experiments; AS performed the experiments. AS and YN collected and analysed data. TS critically revised the manuscript for intellectual content. AS and YN wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Tokyo Denki University Animal Care and Use Committee in accordance with their Ethical and Animal Experiment Regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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