Spilanthol inhibits TNF-α-induced ICAM-1 expression and pro-inflammatory responses by inducing heme oxygenase-1 expression and suppressing pJNK in HaCaT keratinocytes

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Received January 20, 2018; Accepted May 31, 2018

DOI: 10.3892/mmr.2018.9245

Abstract. Spilanthol has been reported to possess antioxidant, anti-inflammatory, antimicrobial and antinociceptive properties. At present, the literature has reported the beneficial role of spilanthol on tumor necrosis factor-α (TNF-α)-stimulated HaCaT cells. The present study investigated the effects of spilanthol on the expression of TNF-α-induced intercellular adhesion molecule 1 (ICAM-1) and cyclooxygenase (COX)-2 in the human keratinocyte cell line HaCaT. Cells were pretreated with various concentrations of spilanthol (10-150 μM) followed by TNF-α to induce inflammation. Pretreatment with spilanthol decreased TNF-α-induced COX-2 expression by western blotting and suppressed the expression of pro-inflammatory mediators, including interleukin (IL)-6, IL-8 and monocyte chemotactic protein 1 using ELISA. Spilanthol also decreased the expression of TNF-α-induced ICAM-1 protein and mRNA assay by western blotting and RT-qPCR, respectively, in addition to the monocyte adhesiveness of HaCaT cells. Furthermore, spilanthol significantly suppressed the phosphorylation of c-Jun N-terminal kinase (JNK), while pretreatment with spilanthol enhanced heme oxygenase (HO)-1 protein expression by western blotting. These results demonstrated that spilanthol may exert its anti-inflammatory activity by suppressing the TNF-α-induced expression of ICAM-1, COX-2 and pro-inflammatory mediators by enhancing that of HO-1, and inhibiting the activation of the phosphorylated JNK signaling pathway. It is hypothesized that spilanthol may be a natural anti-inflammatory drug to attenuate skin inflammatory disease.

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Key words: spilanthol, intercellular adhesion molecule 1, heme oxygenase-1, keratinocyte, phosphorylated-Jun N-terminal kinase, tumor necrosis factor-α

Introduction

Epidermal defense against the intrusion of harmful substances from the environment, including chemicals and radiation, acts as an important barrier to prevent skin injury (1,2). Keratinocytes are the principal cells of the epidermis. In addition to their barrier-serving role, these cells are involved in immune responses within the skin; however, unperturbed keratinocytes exhibit deficient or absent production of inflammatory mediators (2). Under environmental or chemical stimuli (for example, UV irradiation or ambient air pollution), activated keratinocytes express numerous inflammation-associated cytokines including TNF-α, interleukin (IL)-1β, IL-8, and inducible nitric oxide synthase (iNOS), which may result in the abnormal expression and dysregulated action of inflammatory mediators (3,4). Activated keratinocytes may induce strong infiltration of inflammatory cells in the epidermis, which is associated with the underlying pathogenesis of inflammatory skin diseases, including psoriasis, atopic dermatitis (AD) and allergic contact dermatitis (5).

Increased expression of intercellular adhesion molecule-1 (ICAM-1) may induce leukocyte and keratinocyte interaction and is considered an important initiator in numerous types of inflammatory skin diseases (6). It has been reported that tumor necrosis factor-α (TNF-α)-induced ICAM-1 expression is a principal mediator of increased lymphocyte infiltration into inflamed areas in the skin. In addition, keratinocytes are activated by TNF-α, eliciting an inflammatory response with the release of proinflammatory cytokines and chemokines, including interleukin (IL)-1β, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1), in addition to

ICAM-1 (7-9). Additionally, TNF-α-induced expression of inflammatory mediators within keratinocytes simultaneously activates inflammatory signaling pathways, nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), resulting in aggravated inflammation (10).

Phosphorylation of inhibitor of NF-κB due to inflammatory stimuli induces the translocation of the transcription factor NF-κB to the nucleus and activates the transcription of proinflammatory genes (11,12). Extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 MAPK are members of the MAPK family (12). Activation of MAPKs has also been associated with the production of inflammatory mediators and the regulation of numerous inflammation-associated genes (9,12). The role of inflammation is to protect against harmful stimuli; however, long-term and excessive inflammation may cause severe tissue damage, which may lead to the development of inflammation-associated diseases, including diabetes, cancer and AD (13). Therefore, inhibiting the activation of the NF-κB and MAPK signaling pathways may be important for controlling and ameliorating the development of various skin diseases. On the contrary, heme oxygenase-1 (HO-1) has been reported to possess anti-inflammatory activity and is regulated by cytokine and chemokine interactions in AD; HO-1 has been suggested to alleviate inflammation (14,15).

To develop therapeutic drugs for the treatment of dermatitis, an initial step may include the identification of effective anti-inflammatory agents to abrogate and suppress inflammatory mediators in keratinocytes. Spilanthol [(2E, 6Z, 8E)-N-isobutylamide-2,6,8-decatrienamide] is a bioactive compound detected in *Acmella oleracea (Spilanthes acmella)* and other *Acmella* species, including *A. brachyglossa* and *A. ciliata* (16). *A. acmella* exerts a variety of biological properties, including antipyretic (17,18), anti-inflammatory (19-21), analgesic (22-24) and antimicrobial activities (22-26). Spilanthol inhibits lipopolysaccharide-induced inflammatory responses in murine RAW 264.7 macrophages via inactivation of the NF-κB signaling pathway (19). Leaf extracts of *S. acmella* have exhibited immunomodulatory activity, which may be beneficial for the treatment of rheumatism (27); spilanthol enhances immune activities in influenza and respiratory infections (28). In addition, spilanthol is absorbed by human skin (29); however, whether the compound is able to modulate inflammatory responses is unclear, and the associated mechanism underlying the effects of spilanthol within keratinocytes requires further investigation.

The present study investigated the biological activities and modulatory effects of spilanthol on TNF-α-induced HaCaT cells. Spilanthol was observed to inhibit TNF-α-induced ICAM-1 expression, which may be associated with protection against injuries induced by cytokines, including IL-6, IL-8, MCP-1 and ICAM-1 in the present study. In addition, the anti-inflammatory effects of spilanthol may be mediated by enhancing HO-1 expression and inhibiting the pJNK signaling pathway in HaCaT cells.

Materials and methods

Materials and antibodies. Spilanthol (ChromaDex, Inc., Irvine, CA, USA; Fig. 1A) was prepared as a 100 mM stock solution in dimethyl sulfoxide (DMSO) with a final DMSO concentration of ≤0.1% in the culture medium. ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary antibodies against β-actin, cyclooxygenase-2 (COX-2), HO-1 and ICAM-1 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and JNK, ERK, p38, phosphorylated (p)-JNK, p-ERK, and p-p38 antibodies were purchased from EMD Millipore (Billerica, MA, USA). Calcein-AM was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RNA was isolated using TRIzol reagent obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell line and treatment. HaCaT cells (human keratinocyte cell line) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Biological Industries, Ltd., Beit-Haemek, Israel), antibiotics (1% penicillin and streptomycin) and 2 mM glutamine; cells were incubated in a humidified incubator containing 5% CO_2 at 37˚C. THP-1 cells (a human monocytic cell line) were purchased from the Bioresource Collection and Research Center and incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum and 2 mM L-glutamine added.

Cell viability assay. An MTT assay (Sigma-Aldrich; Merck KGaA) was performed to assess cell viability. HaCaT cells (10^6 cells/well) were seeded onto 96-well plates and incubated with spilanthol at concentrations between 3 and 200 μM for 1 h and then TNF-α (10 ng/ml) was added and the cells co-cultured for 24 h in a humidified incubator containing 5% CO_2 at 37˚C. MTT solution (5 mg/ml) was added to each well for 2 h at 37˚C. Following removal of the MTT solution, DMSO (0.5 ml) was added to dissolve the blue formazan crystals. Using a microplate reader (Gene5, Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA), the absorbance was measured at 570 nm. A subsequent analysis was conducted with 0-150 μM spilanthol. Cells without TNF-α and spilanthol treatment served as negative control and cells treated with TNF-α as a positive control.

ELISA for the analysis of proinflammatory cytokines, chemokines and ICAM-1 production. HaCaT cells (10^6 cells/ml) were pretreated with spilanthol (10-150 μM) for 1 h in a humidified incubator containing 5% CO_2 at 37˚C, and TNF-α (10 ng/ml) was added, followed by incubation in a humidified incubator containing 5% CO_2 at 37˚C for 24 h. Supernatants were centrifuged (19,000 x g at 4˚C for 5 min) and collected for assaying the expression levels of IL-8, IL-6, MCP-1 and ICAM-1. The present study employed specific ELISA kits (cat. nos. DY208, DY206, DY279 and DY720, respectively; R&D Systems, Inc.) and the optical density was spectrophotometrically measured at 450 nm with a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.).

Preparation of total proteins. HaCaT cells (10^6 cells/ml) were seeded onto 6-well plates and pretreated with spilanthol (10, 30, 100 and 150 μM) for 1 h in a humidified incubator containing 5% CO_2 at 37˚C, and then stimulated with TNF-α (10 ng/ml)

for 24 h in a humidified incubator containing 5% CO_2 at 37˚C to evaluate total proteins or for 30 min to detect phosphorylated proteins. Cell lysates were collected after centrifuged at 19,000 x g for 15 min duration 4˚C, and the proteins were extracted in 300 μl protein lysis buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% NP40, 150 mM NaCl and 0.1% SDS] containing a protease inhibitor cocktail and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). Proteins were extracted and a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used for quantification.

Western blot analysis. Equal amounts of protein (20-30 μg) were obtained from HaCaT cells as mentioned above and underwent separation via 10% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore). Subsequently, the membranes were blocked with 5% BSA and incubated overnight with primary antibodies at 4˚C, including COX-2 (1:500; cat. no. sc-1746; Santa Cruz Biotechnology, Inc.); HO-1 (1:500; cat. no. sc-10789; Santa Cruz Biotechnology, Inc.); ERK1/2 (1:2,000; cat. no. ABS44; Millipore), p38 (1:500; cat. no. ABS29; EMD Millipore,), JNK1/2 (1:1,000; cat. no. 06-748 EMD Millipore), phosphorylated-ERK 1/2 (1:1,000; cat. no. P27361; EMD Millipore), phosphorylated-p38 (1:1,000; cat. no. 09-272; EMD Millipore), and phosphorylated-JNK1 (1:500; cat. no. 07-175; EMD Millipore); ICAM-1 (1:1,000; cat. no. GTX100450; Sigma-Aldrich; Merck KGaA) and β-actin (1:500; cat. no. MAB1501; Sigma-Aldrich; Merck KGaA).

The membrane was washed with TBS with Tween-20 [TBST; 150 mM NaCl, 10 mM Tris (pH 8.0) and 0.1% Tween-20], and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The bound antibodies, including goat anti-rabbit IgG-HRP (1:10,000; cat. no. A2315; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG (1:10,000; cat. no. A90-116P; Bethyl Laboratories, Inc., Montgomery, TX, USA) on the membranes were washed with TBST and incubated in Luminol/Enhancer Solution (EMD Millipore) according to the manufacturer's protocols for detection of the bands; quantification of protein expression was conducted using the BioSpectrum 600 automated system (UVP, LLC, Phoenix, AZ, USA) and its included software.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression. ICAM-1 mRNA expression levels were determined by RT-qPCR, with β-actin as the internal control. TRIzol solution (Thermo Fisher Scientific, Inc.) was used to extract total RNA, and reverse transcribed to acquire cDNA using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). qPCR using SYBR Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was performed using a spectrofluorometric thermal cycler (iCycler; Bio-Rad Laboratories, Inc.) to quantitative the expression of specific genes. The cycling conditions were as follows: Samples preincubated at 95˚C for 10 min. Next, the PCR was performed as 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, followed by analysis using TaqMan real-time quantitative PCR (Applied Biosystems; Thermo Fisher Scientific, Inc.). The specific primers used were as follows: ICAM-1, forward 5'-AGACGCAGAGGACCTTAA-3' and reverse 5'-CACACTTCACAGTTACTTGG-3'; β-actin, forward 5'-AAGACCTCTATGCCAACACAGT-3' and reverse 5'-AGCCAGAGCAGTAATCTCCTTC-3' (30). The specific genes were determined by comparing the average of gene cycle quantification (Cq), measured for each experiment and repeated three times, as described previously (31). The comparison Cq method was used for the specific genes determined by relative cDNA expressions ($2^{-\Delta\Delta Cq}$). $2^{-\Delta\Delta Cq}$ was the discrepancy between specific gene and housekeeping genes β-actin for each sample.

Cell-cell adhesion assay. To perform the cell-cell adhesion assay, HaCaT cells (10^6 cells/ml) were pretreated with spilanthol (10, 30, 100 and 150 μM) for 1 h in a humidified incubator containing 5% CO_2 at 37˚C and subsequently incubated with TNF-α (10 ng/ml) for 24 h in a humidified incubator containing 5% CO_2 at 37˚C. The control groups was treated with TNF-α alone. THP-1 cells (10^6 cells/ml) labeled with calcein-AM were co-cultured with HaCaT cells for 1 h in DMEM medium in a humidified incubator containing 5% CO_2 at 37˚C. Cells were washed with PBS and the extent of adhesion of THP-1 cells to HaCaT cells was observed under a fluorescence microscope (3 per view; magnification, x200; Olympus Corporation, Tokyo, Japan) with excitation and emission wavelengths of 490 and 515 nm, respectively. All experiments were repeated three times.

Statistical analysis. Data are presented as the mean ± standard deviation of at least three independent experiments. Data were analyzed with one-way analysis of variance and Dunnett's post hoc test using SPSS statistical software package version 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of proinflammatory cytokine and chemokine production by pretreatment with spilanthol in TNF-α-treated HaCaT cells. An MTT assay was used to evaluate the cytotoxicity of HaCaT cells against spilanthol. The present study reported that spilanthol did not significantly affect cell viability at concentrations ≤200 μM (Fig. 1B). Therefore, the present study conducted a subsequent analysis with 0-150 μM spilanthol. Spilanthol at 3 μM did not significantly affect cell viability and it had no effects in pretesting and was thus excluded from subsequent experimentation. Furthermore, the inhibitory effects of spilanthol on TNF-α-induced proinflammatory cytokine and chemokine production were investigated. HaCaT cells were pretreated with various concentrations of spilanthol and stimulated with TNF-α for 24 h. Pretreatment with spilanthol was associated with significantly decreased expression levels of IL-6, IL-8 and MCP-1 in TNF-α-stimulated HaCaT cells compared with TNF-α alone (Fig. 1C-E). Spilanthol concentrations ≥10 μM significantly inhibited IL-6 and MCP-1 expression levels; however, concentrations ≥30 μM significantly inhibited IL-8 production. The results of the present study suggested that spilanthol can inhibit proinflammatory cytokine (IL-6) and chemokines (IL-8 and MCP-1) expression levels, thus preventing inflammation.

Effects of spilanthol on TNF-α-induced ICAM-1 protein and mRNA expression. In the present study, the effects of spilanthol

on ICAM-1 expression in TNF-α-induced HaCaT cells were investigated. Spilanthol significantly reduced ICAM-1 protein expression levels compared with treatment with TNF-α alone, as determined by ELISA and western blotting (Fig. 2A and B). In addition, the mRNA expression levels of ICAM-1 were significantly decreased (Fig. 2C) compared with treatment with TNF-α alone, as determined by RT-qPCR. Collectively, these results suggested that spilanthol significantly suppressed ICAM-1 in TNF-α-induced HaCaT cells.

Spilanthol inhibits monocyte adhesion to TNF-α-activated HaCaT cells. A recent study reported that increased monocyte adhesion to human keratinocytes may be associated with increased ICAM-1 expression (31). In the present study, it was demonstrated that spilanthol significantly decreased ICAM-1 expression levels in inflammatory HaCaT cells (Fig. 2). Thus, the present study investigated whether spilanthol may suppress TNF-α-induced monocyte adhesion. The results revealed that THP-1 cells adhered to TNF-α-stimulated HaCaT cells; however, the extent of monocyte adhesion significantly decreased in a dose-dependent manner in response to treatment with spilanthol, compared with in HaCaT cells treated with TNF-α alone (Fig. 3). Fluorescence density is highest in Fig. 3A-b indicating that TNF-α induced THP-1 adhesion to HaCat cells compared with negative control (Fig. 3A-a). SP-treatment decreased THP-1 adhesion to HaCat cells compared with positive control (Fig. 3A-b).

Spilanthol induces HO-1 expression and inactivates the pJNK signaling pathway to inhibit COX-2 protein expression in TNF-α-induced HaCaT cells. To improve understanding as to how spilanthol reduces inflammations, the expression levels of inflammation-associated proteins were analyzed. A recent study suggested that the induction of HO-1 inhibits inflammation in HaCaT cells (32). It was observed that spilanthol concentrations ≥100 μM significantly increased the expression levels of HO-1 expression in HaCaT cells compared with TNF-α alone ($P<0.01$; Fig. 4A). A previous study reported that COX-2 expression is associated with inflammation in human keratinocytes (33). Thus, the present study investigated the effects of spilanthol on TNF-α-activated HaCaT cells, and it was indicated that pretreatment with ≥30 μM spilanthol significantly suppressed COX-2 protein expression compared with TNF-α-stimulated control cells (Fig. 4B). The activation of the MAPK signaling pathway is closely associated with the expression of proinflammatory mediators in keratinocytes (34); whether spilanthol affects the MAPK signaling pathway in TNF-α-activated HaCaT cells was analyzed in the present study. Treatment with ≥30 μM spilanthol significantly decreased the phosphorylation of JNK compared with in TNF-α-activated HaCaT control cells (Fig. 4C). These results suggested that spilanthol may induce the expression of HO-1 expression via inactivation of pJNK to potentially inhibit the expression of COX-2 in TNF-α-activated HaCaT cells; however, spilanthol did not suppress the phosphorylation of ERK and P38 MAPK (data not shown).

Discussion

Epidermal keratinocytes are subjected to exposure to harmful factors, including ultraviolet light, which may induce keratinocyte activation and the release of inflammatory mediators (1). ICAM-1 is expressed on activated keratinocytes to attract more inflammatory cells to infiltrate the inflamed skin (2-4). Numerous reports have described the importance of ICAM-1 expression in keratinocytes in skin inflammation-associated diseases (4,5), which has led to a focus on the regulation of this particular adhesion molecule in drug development for the treatment of dermatitis. The present study reported that pretreatment with spilanthol significantly suppressed ICAM-1 protein and mRNA expression, as detected by ELISA and western blotting, and RT-PCR analyses, respectively. In addition, treatment with spilanthol reduced the extent of adhesion between HaCaT and THP-1 cells; this may have been mediated by reductions in the secretion and expression of ICAM-1 by HaCaT cells. Inflammatory keratinocytes could have released proteins into culture medium and excess ICAM-1 cleaved to soluble ICAM, and moved into the supernatant. Thus, ELISA could detect specific protein (soluble ICAM) in the supernatant. Soluble ICAM-1 is a molecule meriting exploration in the future. Additionally, the present study reported that spilanthol affected the extent of monocyte adhesion to keratinocytes, suggesting that spilanthol may attenuate the inflammatory response via the inhibition of ICAM-1 expression in TNF-α-induced keratinocytes.

Spilanthol possesses antioxidant and antinociceptive properties and is of particular importance as a potential anti-inflammatory agent (16), which is mediated via the inactivation of NF-κB; spilanthol negatively regulates the production of proinflammatory mediators, including IL-1β, IL-6, TNF-α and COX-2 (19). Flavonoid extracts from *S. acmella* inhibit the secretion of IL-1β and TNF-α, reducing infection-associated inflammation, and suppress COX-2 expression, reducing fever (16,19). These findings suggest that *S. acmella* extracts have analgesic and antipyretic effects (17,18). In addition, spilanthol exerts anti-inflammatory effects in *in vitro* and *in vivo* models (16-19); however, the associated anti-inflammatory mechanisms in keratinocytes require further investigation. In the present study, spilanthol was observed to significantly suppress the expression of pro-inflammatory cytokines, including IL-6, IL-8 and MCP-1 production. Additionally, spilanthol effectively suppressed COX-2 protein expression in keratinocytes, indicating that spilanthol may inhibit inflammatory responses and prevent the inflammatory loop within TNF-α-induced keratinocytes.

Of note, HO-1 expression has been reported to exert an anti-inflammatory response in keratinocytes, and attenuates AD-like lesions via its protective effects against inflammatory skin diseases (7,9,15). HO-1 expression is regulated by inflammation-associated cytokines and chemokines associated with the development of AD (15). The present study observed that spilanthol induced HO-1 protein expression, and decreased cytokine and chemokine expression levels, indicating that HO-1 expression may be associated with the underlying anti-inflammatory mechanism of spilanthol within keratinocytes.

Furthermore, previous studies demonstrated that flavonoid and immunosuppressive drugs (rapamycin and mycophenolic acid) modulated the expression of ICAM-1, which is regulated by NF-κB activation within TNF-α-induced HaCaT cells (7,9,10). The modulatory activity of spilanthol on the signaling of NF-κB

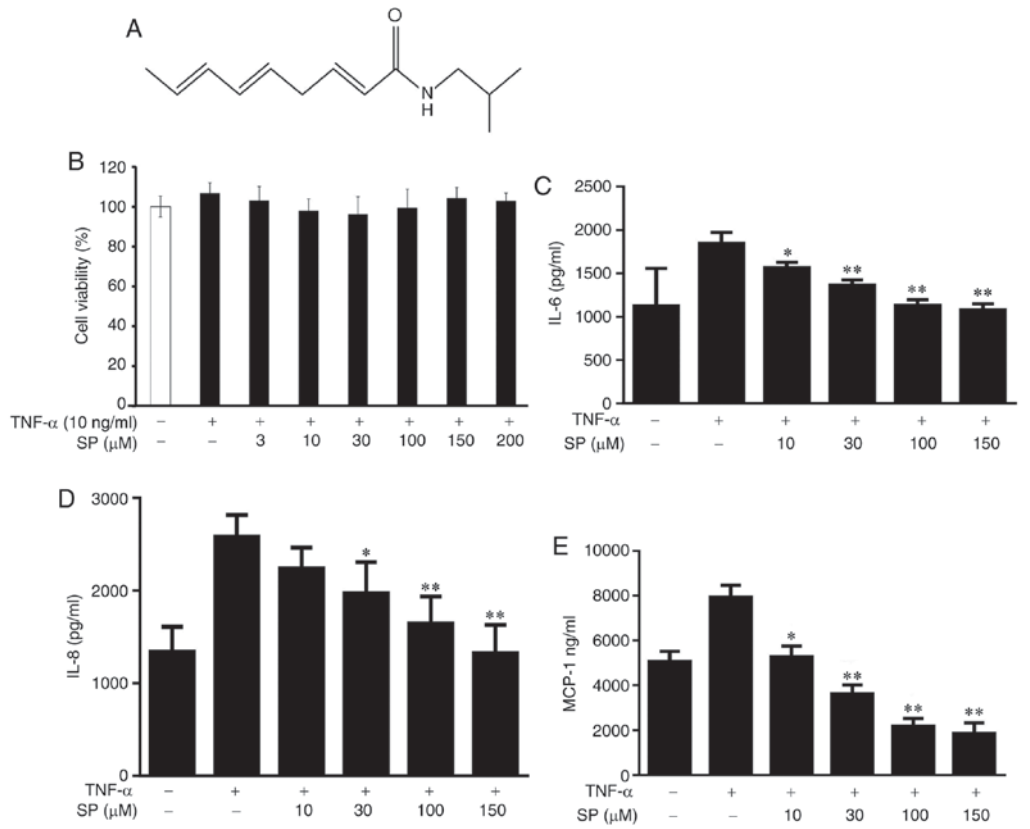

Figure 1. SP inhibition of proinflammatory cytokine and chemokine production in TNF-α-stimulated HaCaT cells. (A) Structure of SP. (B) Cytotoxicity of SP-treated HaCaT cells. SP inhibited TNF-α-induced cytokine and chemokine expression: (C) IL-6, (D) IL-8 and (E) MCP-1. HaCaT cells (10^6 cells/well) were pretreated with the indicated concentrations of SP (3-150 μM) for 1 h and subsequently stimulated with TNF-α (10 ng/ml) for 24 h. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. TNF-α-treated group. IL, interleukin; MCP-1, monocyte chemoattractant protein-1; SP, spilanthol; TNF-α, tumor necrosis factor-α.

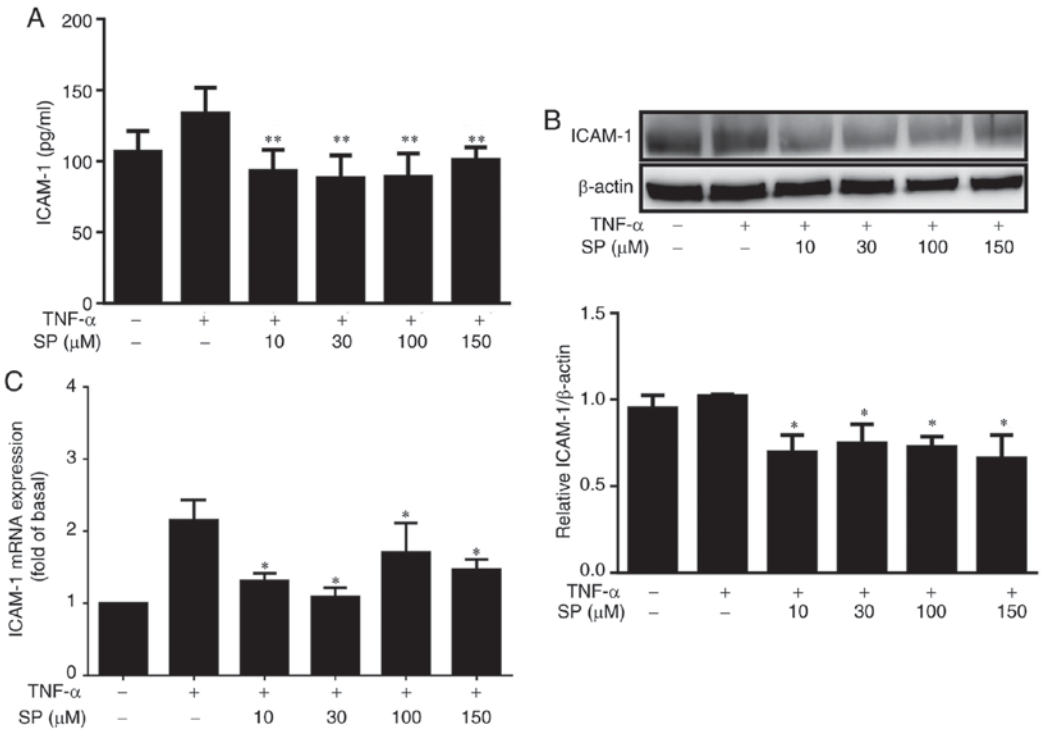

Figure 2. SP effects on ICAM-1 production and expression in TNF-α-induced HaCaT cells. (A) SP inhibited ICAM-1 expression levels as determined by (A) ELISA and (B) western blotting. HaCaT cells (10^6 cells/ml) were pretreated with 10-150 μM SP for 1 h and subsequently stimulated with TNF-α (10 ng/ml) for 24 h. (C) SP suppressed ICAM-1 gene expression. HaCaT cells (10^6 cells/ml) were pretreated with the indicated doses of SP for 1 h and subsequently exposed to TNF-α (10 ng/ml) for 1 h. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. TNF-α-treated group. ICAM-1, intercellular adhesion molecule 1; SP, spilanthol; TNF-α, tumor necrosis factor-α.

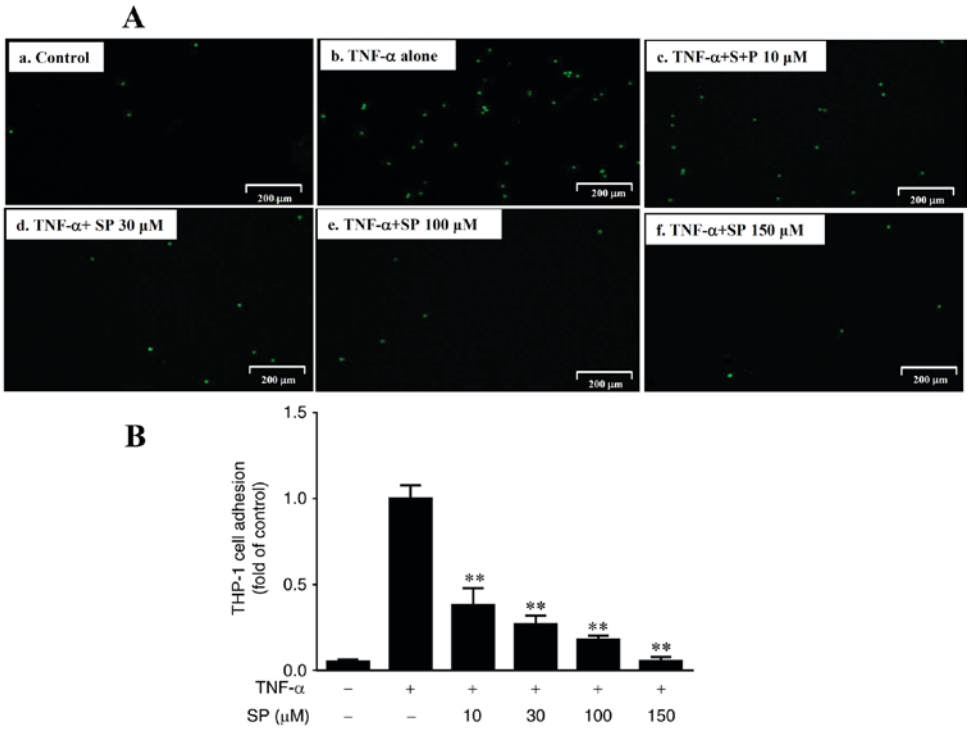

Figure 3. SP suppresses the monocyte adhesive ability of TNF-α-activated HaCaT cells. Effects of SP on monocyte adhesiveness in TNF-α-induced HaCaT cells were investigated; THP-1 cells were incubated with: (A-a) Untreated HaCaT cells; (A-b) TNF-α-treated HaCaT cells; (A-c) HaCaT cells treated with 10 μM SP; (A-d) HaCaT cells treated with 30 μM SP; (A-e) HaCaT cells treated with 100 μM SP; and (A-f) HaCaT cells treated with 150 μM SP, respectively. Scale bar=200 μm. (B) A fluorescence plate reader was used to quantify calcein-AM fluorescence. Data are presented as the mean ± standard deviation. **P<0.01 vs. TNF-α-treated group. SP, spilanthol; TNF-α, tumor necrosis factor-α.

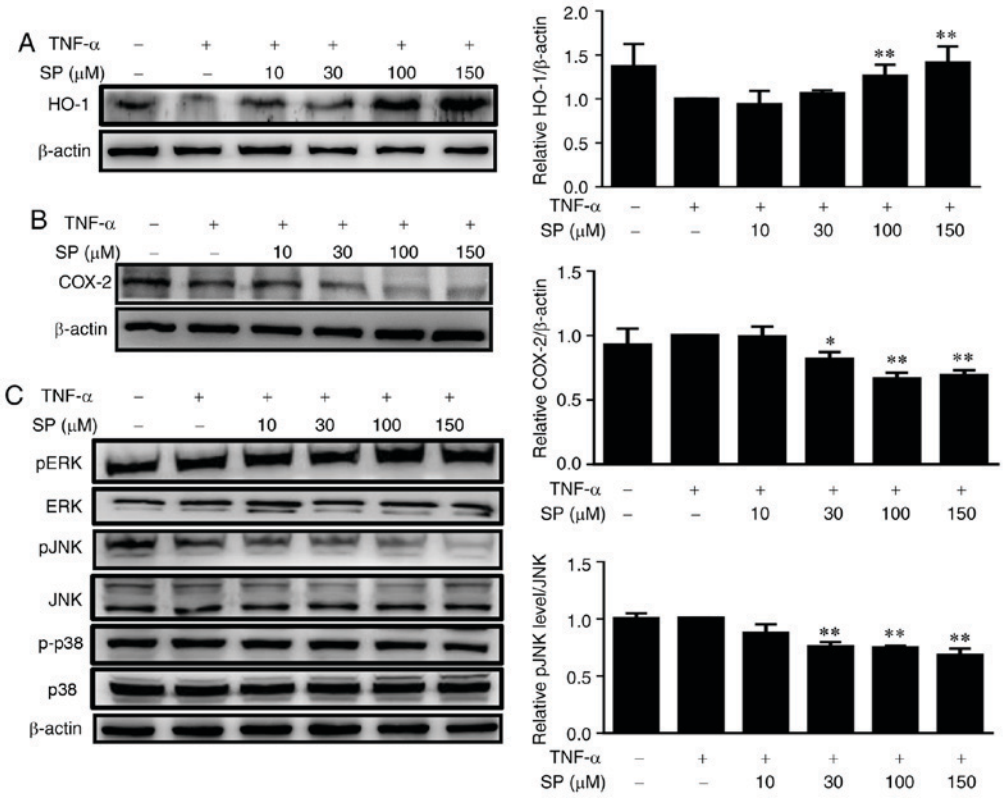

Figure 4. SP induces HO-1 expression and inactivates the pJNK signaling pathway to inhibit COX-2 protein expression in TNF-α-induced HaCaT cells. (A) SP induced HO-1 expression. (B) SP inhibited COX-2 protein expression. (C) SP inactivated the pJNK signaling pathway. HaCaT cells (10^6 cells/ml) were incubated in the absence or presence of 10-150 μM SP for 1 h and subsequently exposed to TNF-α (10 ng/ml) for 24 h (total proteins) or 30 min (phosphorylated proteins), followed by western blot analysis. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. TNF-α-treated group. COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; p, phosphorylated; SP, spilanthol; TNF-α, tumor necrosis factor-α.

activation was evaluated in TNF-α-induced HaCaT cells, and no effect on the NF-κB signaling pathway detected (data not show). Collectively, these results indicated that spilanthol may inhibit the upregulation of proinflammatory cytokines induced by TNF-α, and suppress ICAM-1 expression via spilanthol-induced HO-1 expression in keratinocytes.

The p38/MAPK, ERK and JNK signaling pathways are important in the mediation of cellular inflammation, and these MAPK signal transduction pathways may be induced by TNF-α (2,12). Furthermore, MAPK signaling has been associated with the expression of pro-inflammatory mediators and skin inflammation (9). In particular, JNK signaling serves a critical role in inflammatory responses; TNF-α may induce the phosphorylation of JNK (35). The present study indicated that TNF-α induced the activation of JNK signaling via phosphorylation; however, pretreatment with spilanthol reduced the extent of this activation. The findings of the present study suggested that the TNF-α-induced inflammatory response may be mediated by JNK signaling in HaCaT cells and that spilanthol may ameliorate the inflammatory response by inhibiting JNK phosphorylation. In addition, the JNK-MAPK signaling pathway is also involved in ultraviolet B-induced keratinocyte inflammation (36). Therefore, the JNK-MAPK signaling pathway may be considered as a therapeutic target in treatment of conditions associated with inflammation. Collectively, these data suggested that spilanthol attenuated the production of IL-6, IL-8 and MCP-1 induced by TNF-α, in addition to JNK activation, in HaCaT cells.

In conclusion, to the best of our knowledge, the present study is the first to report that spilanthol may exert an inhibitory effect on the production of inflammation-associated mediators, including IL-6, IL-8, MCP-1, ICAM-1 and COX-2, and may decrease the extent of monocyte adhesion to TNF-α-induced HaCaT cells. In addition, the anti-inflammatory activity of spilanthol may be associated with enhanced HO-1 expression levels and the inhibition of pJNK-MAPK signaling. The present study proposed that spilanthol may possess therapeutic potential for the treatment of inflammatory skin diseases.

Acknowledgements

The authors would like to thank the Graduate Institute of Health Industry Technology of Chang Gung University of Science and Technology and Dr Wen-Chung Huang for their support in using bioinformatics tools.

Funding

The present study was supported by grants from the Chang Gung Memorial Hospital (grant nos. CMRPF1F0122 and CMRPF1G0041) and the Ministry of Science and Technology in Taiwan (grant no. MOST 105-2320-B-255-004), and in part by a grant from the Chang Gung University of Science and Technology (grant no. EZRPF3FG0061).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

CHH, LC and SW designed and performed the experiments. SH and CYH performed analysis and interpretation of data. SW drafted the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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