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Anti-inflammatory Effect of Spilanthol from *Spilanthes acmella* on Murine Macrophage by Down-Regulating LPS-Induced Inflammatory Mediators

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Spilanthes acmella (Paracress), a common spice, has been administered as a traditional folk medicine for years to cure toothaches, stammering, and stomatitis. Previous studies have demonstrated its diuretic, antibacterial, and anti-inflammatory activities. However, the active compounds contributing to the anti-inflammatory effect have seldom been addressed. This study isolates the active compound, spilanthol, by a bioactivity-guided approach and indicates significant anti-inflammatory activity on lipopolysaccharide-activated murine macrophage model, RAW 264.7. The anti-inflammatory mechanism of paracress is also investigated. Extracts of *S. acmella* are obtained by extraction with 85% ethanol, followed by liquid partition against hexane, chloroform, ethyl acetate, and butanol. The ethyl acetate extract exhibits a stronger free radical scavenging capacity than other fractions do, as determined by DPPH and ABTS radical scavenging assays. The chloroform extract significantly inhibits nitric oxide production ($p < 0.01$) and is selected for further fractionation to yield the active compound, spilanthol. The diminished levels of LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) mRNA and protein expression support the postulation that spilanthol inhibits proinflammatory mediator production at the transcriptional and translational levels. Additionally, the LPS-stimulated IL-1β, IL-6, and TNF-α productions are dose-dependently reduced by spilanthol. The LPS-induced phosphorylation of cytoplasmic inhibitor-κB and the nuclear NF-κB DNA binding activity are both restrained by spilanthol. Results of this study suggest that spilanthol, isolated from *S. acmella*, attenuates the LPS-induced inflammatory responses in murine RAW 264.7 macrophages partly due to the inactivation of NF-κB, which negatively regulates the production of proinflammatory mediators.

KEYWORDS: ***Spilanthes acmella*; spilanthol; inducible nitric oxide synthase; cyclooxygenase 2; nuclear transcription factor-κB; proinflammatory cytokine**

INTRODUCTION

Inflammatory responses, initiated by the invasion of pathogens or by tissue injury caused by free radicals, are a series of vascular and cellular reactions. The processes concomitantly come with nonspecific cellular responses, and generate chemical messages through two mediators, cell-derived and plasma-derived. Most cell-derived mediators, such as arachidonic acid derivatives (prostaglandins and leukotrines), cytokines, lymphokines, monokines, platelet activating factors, histamine, and the kinin system, are produced by white blood cells. The plasma-derived mediators predominate in plasma, including complement system and interferons. Among the cellular responses, the activation of macrophages is essential to the initiation and continuation of the defensive reactions. Once they are stimulated by pathogens, macrophages yield nitric oxide (NO), prostaglandin E_2 (PGE_2), TNF-α, interleukins (IL)-1, -6, and -12, and other proinflammatory cytokines to enhance the defense capacity (*1–3*).

Chronic exposure to high levels of NO frequently results in inflammatory diseases, including atherosclerosis, bowel disease, rheumatoid arthritis, glomerulonephritis, and septic shock (*4–7*). Most NO is produced from three nitric oxide synthases (NOS) in mammalian cells. Constitutively expressed endothelial and neuronal NOS catalyze the formation of a minority of NO and are responsible for cardiovascular and nerve physiological functions (*8, 9*). The third NOS, the inducible (iNOS) form, catalyzes the formation of the majority of NO and is present in neutrophils, macrophages, smooth muscle cells, and endothelial cells. Like iNOS, cyclooxygenase 2 (COX-2, or prostaglandin

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endoperoxide synthase-2) can also be induced during inflammation to catalyze the formation of prostaglandin. Both iNOS and COX-2 are involved in carcinogenesis (*10–12*).

Previous studies have postulated that transcription factors of the nuclear factor-κB family (NF-κB) trigger the transcription of iNOS and COX-2 and several other proinflammatory mediators, such as IL-6, IL-1, and TNF-α (*13, 14*). This family plays a significant role in cell signaling, such as in the expression of genes that encode chemokines (e.g., IL-8, MIP-1α, MCP1, RANTES, and eotaxin), adhesion molecules (such as ICAM, VCAM, and E-selectin), growth factors, acute phase proteins, and immune receptors. This family is also a critical point of convergence of many signal transduction pathways, including innate and adaptive immune responses, development, cell migration, and apoptosis (*15–17*).

NF-κB represents a group of structurally related and evolutionarily conserved proteins related to the proto-oncogene c-Rel. Five members of this group are present in mammals, namely, Rel (cRel), RelA (p65), RelB, NF-κB1 (p50, precursor p105), and NF-κB2 (p52, precursor p100). The p50/p65 heterodimer of NF-κB is the most abundant in cells as an activator for transcription. Inactive NF-κB is cytoplasmically localized because of its non-covalent binding to inhibitor proteins, such as IκBs. Signal cascades triggered by external stimuli such as bacterial endotoxin (e.g., lipopolysaccharide, LPS), oxidative stress, proinflammatory cytokines, and UV irradiation (*14*), cause NF-κB activation through the phosphorylation of IκB by cytoplasmic IκB kinase (IKK) complexes (IKKα and IKK*β*) and a regulatory subunit, IKK*γ* (NEMO).

Upon activation, IκB is phosphorylated by IKK complexes and then degraded through an ubiquitin−proteasome pathway. Therefore, NF-κB discharges from the NF-κB/IκB complexes and translocates into the nucleus, where it binds to the cis-acting κB enhancer element of target genes and activates the transcription of target genes, including its own inhibitor, IκB, the inactive form of NF-κB, proinflammatory mediators, cytokines, chemokines, and adhesion molecules (*17*). The newly formed nuclear NF-κB/IκB complexes are exported to the cytoplasm to replenish the cytoplasmic pool of inactive NF-κB/IκB complexes, which are primed for another round of activation.

Secretion of the inflammatory mediators against invading pathogens is essential for innate immunity and promotes the actions of a specific immune system. The mediators are required in tissue repair (*18*) and are critical to the survival of the host. However, excessive production of macrophage-derived mediators may lead to collateral damage to normal cells (*19*), which may even be lethal in cases of systemic exposure (*20*). Therefore, the control of the magnitude of the released mediators is important in anti-inflammatory drug development. Anti-inflammatory agents from plants have been discovered, and many of them are regarded as NF-κB related (*12*).

Spilanthes acmella (Paracress), of the family Compositae, is used as a common spice and an annual herb with natural analgesic effect (*21*). The flowers and leaves of *S. acmella* have a pungent taste and cause tingling and numbness (*22, 23*). They are applied as a traditional folk medicine for toothaches, stammering, stomatitis, and throat complaints (*22*). *S. acmella* has been reported to have therapeutic actions in diuretic (*24*), antiviral, antiseptic, and anti-inflammation activities, enhancing wound healing, antifeedant (*23*), and antibacterial properties (*25, 26*). Compounds such as *N*-isobutylamides have been isolated from the flowers of *S. acmella* (*23*).

Spilanthol, an olefinic alkamide with an isobutyl side chain (**Figure 1**) isolated from *S. acmella*, is the main constituent in flowers of*S. acmella* (*27*) and demonstrates activities in antiseptic, saliva, and immune stimulation (*28*). It is effective against blood parasites and, indeed, is a poison to most invertebrates while remaining harmless to warm-blooded creatures (*29*). Although several compounds of *S. acmella* have been identified (*23*), the compounds that contribute to anti-inflammatory effect and the mechanisms by which they work have seldom been addressed.

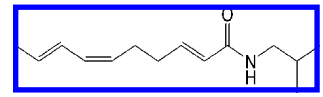

Figure 1. Structure of spilanthol.

This study isolated spilanthol, an anti-inflammatory active compound of *S. acmella*, in a bioactivity-guided manner. A murine macrophage cell line, RAW 264.7 cells, was used to study the bioactivity. The production of inflammatory mediators, NO and proinflammatroy cytokines, was determined. Additionally, the inhibitory effect on iNOS and COX-2 expression was detected at the transcriptional and translational levels. *S. acmella* was initially extracted by 85% ethanol and then sequentially portioned against hexane, chloroform, ethyl acetate, and butanol. Chloroform extracts, which inhibited NO production more potently than other extracts, were used for further separation to yield spilanthol. Analytical results revealed that spilanthol, isolated from chloroform extracts, efficiently down-regulated the production of inflammatory mediators IL-1*β*, IL-6, and TNF-α and attenuated the expression of COX-2 and iNOS. The inhibitory effect could be partly due to the reduced activation of NF-κB. Moreover, the extracts of *S. acmella* demonstrated radical scavenging activity, which could reduce cell and tissue damage caused by free radicals.

MATERIALS AND METHODS

Extraction and Isolation. Dried flowers of *S. acmella* L. var. *oleracea* Clarke were obtained from a local market in Puli, Taiwan. The voucher specimens were deposited at the Chi Nan University. The isolation of bioactive compounds was performed by a bioactivity-guided fractionation approach. The lyophilized flowers were crushed and extracted with 85% ethanol overnight at room temperature to yield ethanol extract. The residue was extracted with hexane and followed by chloroform, ethyl acetate, and butanol to yield the hexane extract, chloroform extract, ethyl acetate extract, and butanol extract, respectively. These extracts were bioassayed for the inhibitory effect on nitric oxide production on LPS-stimulated macrophages, RAW 264.7 cells. The chloroform extract exhibited the highest activity among all extracts and was therefore used for further study. Fractionation of the chloroform extract was carried out by silica gel chromatography using hexane with increasing levels of ethyl acetate and finally with ethyl acetate/methanol as the eluting solvents. Nine fractions (A−I) were collected and subjected to bioassay. Fraction C was selected due to its potent inhibitory activity on NO production. Spilanthol was purified chromatographically from fraction C and identified by comparison of its spectral data (^{1}H, ^{13}C NMR and mass spectrum) with published values (*22*).

Cell Culture. Mouse macrophage cell line RAW 264.7 cells were purchased from the Culture Collection and Research Center (CCRC), Taiwan, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 1% nonessential amino acid, 1 mM pyruvate, 100 units/mL penicillin, and 100 *μ*g/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

DPPH Radical Scavenging Activity. The radical scavenging activity was assayed by the DPPH radical method, as described previously (*30, 31*). A 0.5 mL aliquot of 80% ethanol dissolved extract was mixed with 0.25 mL of ethanolic 0.5 mM DPPH radical solution and 0.5 mL of

100 mM acetate buffer (pH 5.5). The decrease in absorbance of DPPH radicals at 517 nm was measured by a Beckman UV–vis spectrophotometer. All tests were performed in triplicate. The ability to scavenge DPPH radicals was calculated from the formula $(A_{control} - A_{test}) \times 100/A_{control}$, where $A_{control}$ denotes the absorbance of the control (DPPH radical solution without the test sample) and A_{test} denotes the absorbance of the test sample. The scavenging activity of the tested samples was expressed as the median effective concentration for radical scavenging activity (EC_{50}), that is, tested extracts (milligrams) required for a 50% decrease in absorbance of DPPH radicals, and expressed relative to ascorbic acid (Vit C) in terms of Vit C equivalents.

ABTS Radical Scavenging Assay. The ABTS radical cation used in the assay was generated from ABTS salt, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), in which 2.45 mM potassium persulfate ($K_2S_2O_8$) was reacted with 7 mM ABTS salt in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 16 h at room temperature in the dark (*32*). The resultant ABTS radical cation was diluted with 0.01 M PBS, pH 7.4, to obtain an absorbance of around 0.70 at 734 nm. The standard and sample extracts were diluted 100× with the ABTS solution to a total volume of 1 mL and allowed to react for 3 min. Control (without a standard or sample) was used as blank. Trolox, the water-soluble *R*-tocopherol (vitamin E) analogue, was adopted as a standard, and the results of the assay were expressed relative to Trolox in terms of Trolox equivalents.

Nitrite Measurement. The inhibitory effect of extracts and spilanthol on the production of nitric oxide (NO) was determined by using the Griess reaction to measure the level of nitrite, an indicator of NO synthesis. The supernatant of LPS-induced RAW 264.7 cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). Absorbance at 550 nm was measured, and concentrations were calculated against a sodium nitrite standard curve.

Cell Viability. The growth inhibitory effect of extracts or spilanthol on RAW 264.7 cells was evaluated by MTT assay. At the end of the culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5%) was incubated with cells for 4 h at 37 °C. The medium was aspirated, and the resulting purple formazan crystals, which were formed by mitochondrial-dependent reduction (*33*), were dissolved in DMSO. Absorbance at 550 nm was measured to quantify the reduction of MTT as cell viability.

Western Blotting. Confluent RAW 264.7 cells in a 10 cm Petri dish were incubated with either LPS (1 μg/mL, Sigma, St. Louis, MO) alone or cotreatment of LPS and spilanthol. After incubation, cells were washed with PBS and scraped into microcentrifuge tubes and then pelleted. Cells were resuspended in ice-cold PBS (pH 7.4) and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% NP-40, 0.5% deoxycholate, 0.1% SDS) with three cycles of freezing and thawing (each step for 5 min). For p-IκBα analysis, cytoplasmic extracts were prepared by NE-PER reagents (Pierce, Rockford, IL). Lysates (50 μg of protein/lane) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in blocking buffer (5% skim milk in TBST solution) for 1 h at room temperature. Membranes were then subsequently probed by mouse monoclonal anti-iNOS antibody (1:1,000 in 0.1% TBST solution, Santa Cruz Biochemicals, Santa Cruz, CA), mouse monoclonal anti-COX-2 antibody (1:1000 in 0.1% TBST solution, Santa Cruz Biochemicals), or mouse monoclonal antiphospho-IκBα antibody (1:1,000 in 0.1% TBST solution, Santa Cruz Biochemicals) overnight at 4 °C. Blots were washed with 0.5% TBST three times (5 min each) and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:2,000) for 1 h at 4 °C. Immunoreactive bands were visualized with an ECL detection system (Chemicon, Temecula, CA) after three washes in TBST solution (5 min each).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. The total cellular RNA was prepared from ~10^7 cells/sample according to the manufacturer's instructions provided for Trizol reagents (Invitrogen Corp.). The RT-PCR reaction for each sample was conducted with 500 ng of total RNA using Super Script III Reverse Transcriptase (Invitrogen) and oligo dT18 primers. PCR products were fractionated on 2% agarose gel (*34*). The primers used in this experiment were forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ for iNOS; forward primer 5′-CCGTGGTGAATGTATGAGCA-3′ and reverse primer 5′-CCTCGCTTCTGATCTGTCTT-3′ for COX-2; and forward primer 5′-GACGTGCCGCCTGGAGAAA-3′ and reverse primer 5′-GGGGGCCGAGTTGGGGATAG-3′ for GAPDH. Following reverse transcription, the PCR was performed with initial denaturation at 94 °C for 2 min, followed by 10 cycles of primary and 15 cycles of secondary amplification. The extension step was performed at 72 °C for 10 min.

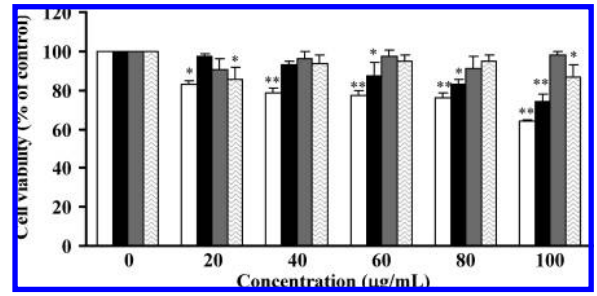

Figure 2. Effect of extracts on RAW 264.7 macrophage viability. Cultures were treated with various concentrations of extracts (white bars, hexane; black bars, chloroform; gray bars, ethyl acetate; patterned bars, butanol) at various concentrations for 24 h, and the cell viability was measured by MTT assay. Data are the mean ± SD of triplicates. The asterisk (*) indicates a significant difference from the control (no extract added) group (*, $p < 0.05$; **, $p < 0.01$).

Cytokine Measurements. To assess the anti-inflammatory effect of spilanthol, levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6 were measured by ELISA kits (Biosource, Camarillo, CA). Assays were carried out according to the manufacturer's instructions.

Activated NF-kB p65 Enzyme-Linked Immunosorbent Assay. Cultures were treated with spilanthol for 10 h prior to the addition of 1 μg/mL LPS for 30 min. Nuclear proteins were prepared by NE-PER reagents (Pierce, Rockford, IL) and were frozen at −70 °C until use (*35*). The binding activity was measured by NF-κB p65 transcription factor assay colorimetric kit (Chemicon). The procedures were modified according to the manufacturer's instructions.

Statistical Analysis. Statistical analysis was performed using one-tailed Student's *t* test. Values at $p < 0.05$ are considered to be significant.

RESULTS

Effect of Extracts on Cytotoxicity. The cytotoxic effects of *S. acmella* extracts on macrophages, RAW 264.7, were determined by MTT assay. **Figure 2** indicates the cell viability at various concentrations of extracts. After 24 h of incubation, hexane and chloroform extracts (80 μg/mL) reduced cell viability to about 75 and 81% ($p < 0.05$), respectively, whereas ethyl acetate or butanol extracts did not significantly alter cell viability (91 and 93%, respectively). These results indicate that both hexane and chloroform extracts exhibited cytotoxic effects at high concentrations.

Inhibitory Effect of Extracts on LPS-Induced Nitric Oxide (NO) Production. Murine macrophage-like RAW 264.7 cells are commonly used for the investigation on the anti-inflammatory response (*36*). Macrophages produced NO to mediate inflammation when activated by LPS. LPS-induced (1 μg/mL) RAW 264.7 cells with or without treatment with the extracts were incubated for 24 h to study whether the extracts of *S. acmella* inhibited NO production (**Figure 3**). The results indicate that, at 80 μg/mL, hexane and chloroform extracts strongly suppressed the production of NO to 28 and 15%, respectively, whereas EA and butanol extracts reduced less NO production to 64 and 77%, respectively (**Figure 3**). The decline in NO

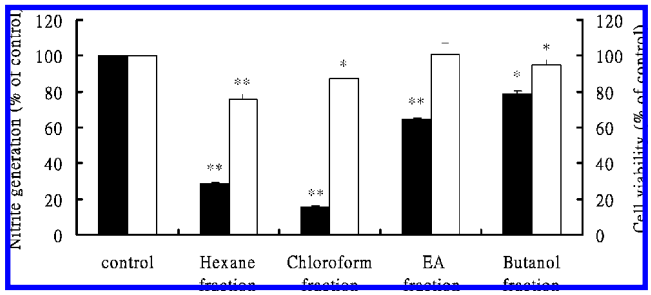

Figure 3. Effect of *Spilanthes acmella* extracts on LPS-induced nitrite production in RAW 264.7 macrophages. Cell viability (white bars) was determined by MTT assay. For nitrite assay (black bars), cultures were treated with 1 μg/mL LPS alone or with 80 μg/mL of extracts for 24 h. The concentration of nitrite in the supernatant of RAW 264.7 macrophages was measured by Griess reagents. Absorbance at 550 nm was measured, and concentrations were calculated against a sodium nitrite standard curve. Data are the mean ± SD of triplicates. The asterisk (*) indicates a significant difference from the control (LPS only) group (*, $p < 0.05$; **, $p < 0.01$).

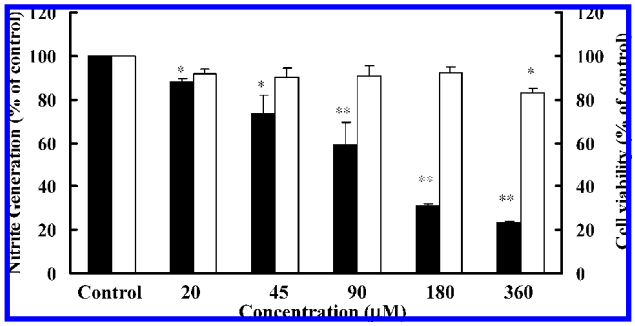

Figure 4. Dose-dependent effect of spilanthol on LPS-induced nitrite production in RAW 264.7 macrophages. Cultures were treated with or without LPS (1 μg/mL). For the groups treated with LPS, various concentrations of spilanthol (20, 45, 90, 180, 360 μM) were treated for 24 h. Cell viability (white bars) were determined by MTT assay. Nitrite assay (black bars) was described under Materials and Methods. Data are mean ± SD of triplicates. The asterisk (*) indicates a significant difference from the control (LPS only) group (*, $p < 0.05$; *, $p < 0.01$).

production may be attributed to the decrease of inducible NO synthase (iNOS) at mRNA or protein levels. Transcriptional and translational investigations on iNOS were then performed. Chloroform extracts were selected for further isolation of bioactive compounds owing to its significant inhibitory effect.

Inhibitory Effect of Spilanthol on LPS-Induced NO Production. To obtain bioactive compounds, chloroform extracts were fractionated by a bioactivity guided fractionation approach. As mentioned under Materials and Methods, *S. acmella* was first extracted with 85% ethanol, followed by sequential liquid partition with hexane, chloroform, ethyl acetate, and butanol. The potent NO production inhibitors, chloroform extracts, were further fractionated to yield spilanthol (**Figure 1**). As seen in **Figure 4**, spilanthol dose-dependently suppressed NO production without significant cytotoxicity (cell viability >90% at 180 μM). **Figure 5** presents the morphology of macrophages under spilanthol treatments. Cells were round without the stimulation of LPS (**Figure 5a**) and became irregularly shaped with accelerated spreading and formation of pseudopodia after being activated by LPS (**Figure 5b**). The addition of spilanthol reduced the degree of cell spreading and pseudopodia formation (45, 180 μM; **Figure 5c**,**d**), revealing the suppression of cell activation.

Inhibitory Effect of Spilanthol on LPS-Induced iNOS and COX-2 Protein Production. Western blot analysis was performed on whole cell lysates to determine whether the inhibition of NO production resulted from reduced iNOS protein expression. **Figure 6** shows the dose-dependent inhibition of iNOS protein expression. The iNOS protein production was noticeably reduced at concentrations of up to 180 μM. These data indicate that the expression of iNOS protein declined as the level of spilanthol increased. In addition to iNOS, the inducible COX-2 was highly expressed during inflammation, catalyzing arachidonic acid to generate prostaglandin E_2 (PGE_2), a crucial mediator of inflammatory pain sensitization (*37*). As indicated in **Figure 6**, the expression of COX-2 protein was also suppressed by 180 μM spilanthol. The observed results likely arose from the reduced mRNA expression. The transcriptional level of iNOS and COX-2 was further studied.

Inhibitory Effect of Spilanthol on LPS-Induced iNOS and COX-2 mRNA Production. Figure 7 displays the dose-dependent inhibition of iNOS mRNA expression after treatment with spilanthol. The expression of iNOS mRNA showed 65, 71, and 88% inhibition at spilanthol concentrations of 45, 90, and 180 μM, respectively, after 12 h of incubation. Similar dose-dependent inhibition was also observed in COX-2 (**Figure 7**). The expression of the housekeeping gene, glycerol-3-phosphate dehydrogenase, was not influenced by the treatment. These results indicate that the administration of spilanthol restrained iNOS and COX-2 production at the transcriptional and translational levels (**Figures 6** and **7**).

Inhibitory Effects of Spilanthol on LPS-Induced Proinflammatory Cytokine Production. Proinflammatory cytokines are formed mostly by activated macrophages and are involved in the up-regulation of inflammatory reactions. The concentrations of IL-1β, IL-6, and TNF-α in the supernatant of the culture were determined to investigate whether spilanthol could reduce the production of proinflammatory cytokines. After being induced with 1 μg/mL LPS, macrophages produced substantial quantities of these cytokines in a time-dependent manner (IL-6, from 60.68 ± 5.24 pg/mL in 6 h to 221.68 ± 36.91 pg/mL in 24 h; TNF-α, from 16.19 ± 0.32 ng/mL in 2 h to 22.12 ± 0.8 ng/mL in 6 h; IL-1β, from 35.26 ± 0.25 pg/mL in 6 h to 66.14 ± 0.5 pg/mL in 24 h) (**Figure 8**).

The LPS-induced macrophages dose-dependently produced lower levels of proinflammatory cytokines after they were treated with spilanthol (90 and 180 μM) (**Figure 8**). The production of IL-6 and IL-1β of cotreated (LPS/spilanthol, 180 μM) cells appeared to be suppressed by 87 and 34%, respectively, compared to the LPS-treated only group (LPS-treated only group vs LPS/spilanthol-treated groups: IL-6, 221.68 vs 28.98 pg/mL; IL-1β, 66.14 vs 43.77 pg/mL) after 24 h of incubation. A similar inhibitory effect was observed on TNF-α. The concentration of TNF-α in the medium treated with 180 μM spilanthol was reduced in a dose-dependent manner, from 16.19 to 7.13 ng/mL after 2 h and from 22.12 to 17.96 ng/mL after 6 h of incubation. These results indicate that spilanthol substantially reduced the production of proinflammatory cytokines, IL-1β, IL-6, and TNF-α.

Effect of Spilanthol on LPS-Induced NF-κB Activation. The regulation of gene expression of several inflammatory mediators is associated with nuclear factor-κB (NF-κB). The NF-κB responsive genes include cytokines, inflammatory mediators, acute phase response proteins, and adhesion molecules. In this study, the administration of spilanthol markedly sup-

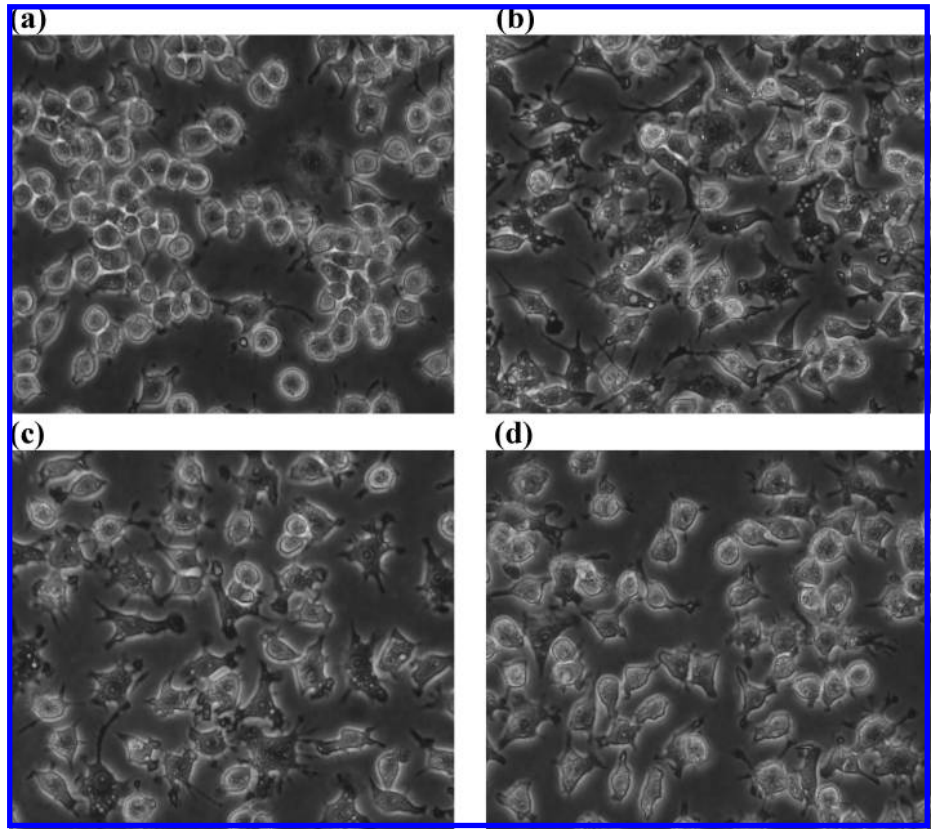

Figure 5. Effect of spilanthol on the activation of murine macrophage cell line, RAW 264.7: (**a**) control; (**b**) LPS-treated only; (**c**) LPS-treated with spilanthol at 45 μM; (**d**) 180 μM. The respective phase contrast microscopic images of control were taken after 24 h of incubation. The images are in the magnitude of 400×.

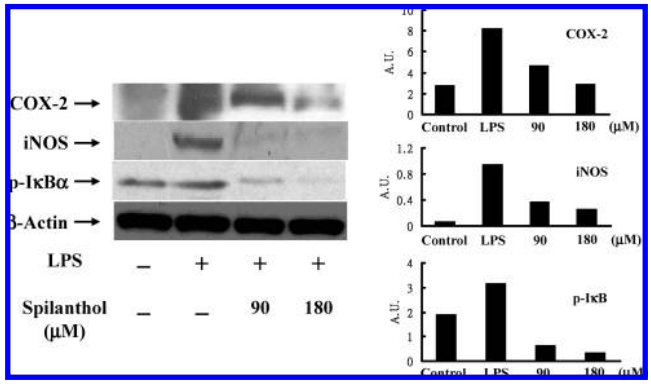

Figure 6. Effects of spilanthol treatment on iNOS and COX-2 protein expression by LPS stimulated RAW 264.7 cells and the cytosolic phosphorylation of IκBα. Cultures were treated with or without LPS (1 μg/mL). For the groups treated with LPS, spilanthol concentrations of 90 or 180 μM were treated. Cells were collected at the indicated times (iNOS, 12 h; COX-2, 14 h; p-IκBα, 30 min). The proteins were isolated for Western blotting assay as described under Materials and Methods.

pressed the production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α), iNOS protein, and its transcripts. Therefore, the level of NF-κB activation needed to be examined. The phosphorylation of inhibitor-κB was essential for the activation of NF-κB. The cytoplasmic phospho-IκBα (pIκBα), a subunit of I-κB, was determined to monitor the activity. As revealed in **Figure 6**, the level of LPS-stimulated pIκBα decreased with increasing spilanthol content. These results show that the reduced levels of phosphorylated I-κB could increase the amount of NF-κB/IκB complexes in the cytoplasm, in turn limiting the translocation of NF-κB to the nucleus to transcribe inflammatory mediators.

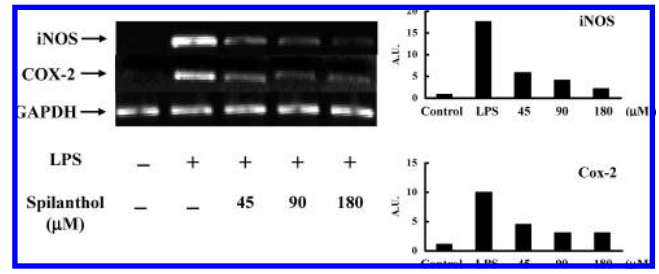

Figure 7. RT-PCR for iNOS and COX-2 mRNA in the presence and absence of spilanthol. Cultures were treated with or without LPS (1 μg/mL). For the groups treated with LPS, spilanthol concentrations of 45, 90, and 180 μM were treated. Cells were collected at the indicated times (iNOS, 12 h; COX-2, 12 h); the RNA was isolated for RT-PCR assay as described under Materials and Methods.

The binding activity of nuclear protein RelA (p65), a member of NF-κB, to the consensus sequence, 5′-GGGACTTTCC-3′, was also measured. As shown in **Figure 9**, treatment with 90 and 180 μM spilanthol reduced binding activity to 57 and 38%, respectively, revealing that the p65 nuclear translocation was impeded.

Radical Scavenging Activity of Extracts. The attack of free radicals on cellular structures may result in cellular injury, eventually leading to cell death. Chronic exposure to a high level of free radicals is usually associated with oxidative stress, neuronal injury, and elevated inflammatory response (*38–40*). The scavenging effects of different extracts were examined by α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity assay and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. The EA extract had the highest radical scavenging capacity, with EC_{50} values of 1.38 μmol of Vit C equiv/mg of dried extract and 3.32 μmol

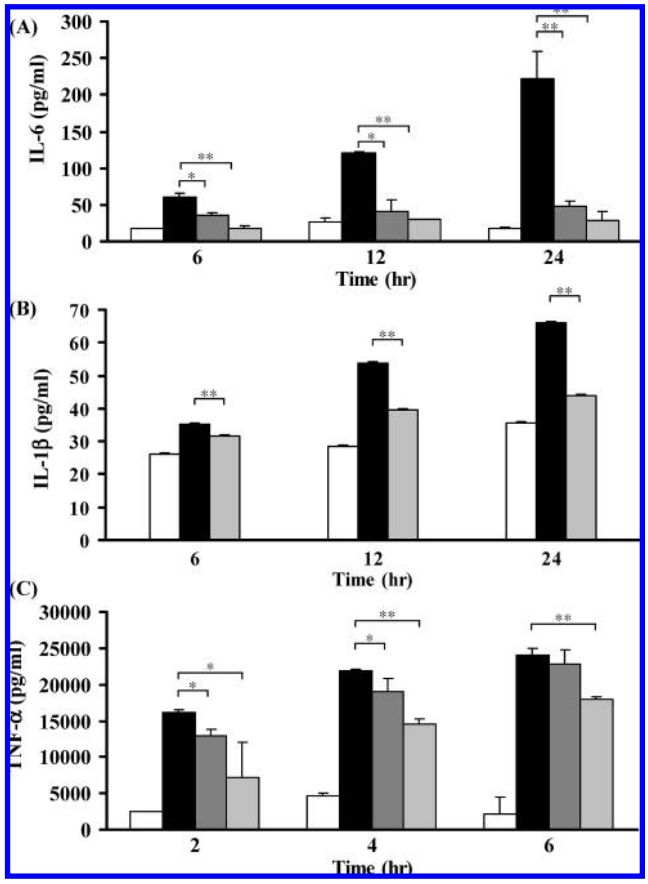

Figure 8. Effects of spilanthol on the production of proinflammatory cytokines of LPS-stimulated RAW 264.7 cells. Cultures were treated with (black bars) or without (white bars) LPS (1 μg/mL). For the groups treated with LPS, spilanthol concentrations of 90 (dark gray bars) or 180 (light gray bars) μM were used. Data are mean ± SD of triplicates. The asterisk (∗) indicates a significant difference from the LPS only group (∗, $p < 0.05$; ∗∗, $p < 0.01$).

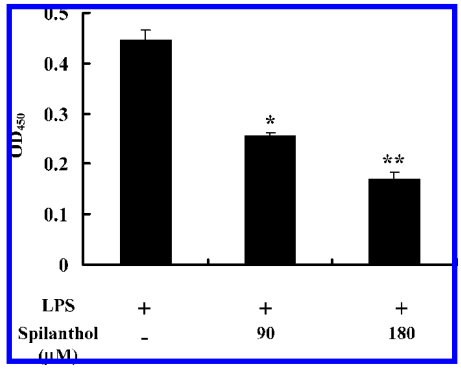

Figure 9. NFκB−DNA binding activity of spilanthol treatment on LPS-stimulated RAW 264.7 cells. Cultures were treated with different concentrations of spilanthol for 10 h before the addition of LPS (1 μg/mL) for 30 min. Data are mean ± SD of triplicates. The asterisk (∗) indicates a significant difference from the LPS only group (∗, $p < 0.05$).

of Trolox equiv/mg of dried extract, as determined by DPPH and ABTS assays, respectively. Chloroform and butanol extracts exhibited similar radical scavenging abilities according to both assays. These results suggested that these extracts, particularly the EA fraction, served as free radical scavengers and were likely to lower the inflammatory responses caused by free radicals.

DISCUSSION

S. acmella has long been used as a spice for appetizers and as a folk medicine for toothache, as an antiseptic, and for other therapeutic activities. Several components of this herb, such as spilanthol, (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, (2*E*,7*Z*)-*N*-isobutyl-2,7- tridecadiene-10,12-diynamide, (7*Z*)-*N*-isobutyl-7-tridecene-10,12-diynamide, and undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide, have been isolated and characterized (*23, 41*). However, the bioactive compounds involved in anti-inflammation are not yet revealed. This study has isolated the anti-inflammatory bioactive compound, spilanthol, demonstrating significant suppression effects on inflammatory responses. This is the first report to identify the anti-inflammatory component, spilanthol, of *S. acmella*.

Spilanthol dose-dependently inhibits the production of NO, due to inhibition of iNOS protein expression and the respective alterations of iNOS gene transcription. Overexpression of iNOS is generally accompanied by inflammatory disorders, in which inflammatory cytokines like IL-1 and TNF-α are responsible for various acute and chronic responses to inflammatory diseases (*42*). Proinflammatory cytokines are important mediators of inflammatory responses. IL-1β, IL-6, and TNF-α serve as endogenous pyrogens that cause fever by initiating metabolic changes in the hypothalamic thermoregulatory center during inflammation. These cytokines up-regulate the inflammatory responses and stimulate the production of acute phase reactants. This study indicates that spilanthol markedly reduces the production of inflammatory cytokines (IL-1β, IL-6, TNF-α) in LPS-treated murine RAW 264.7 macrophages (**Figure 8**), which could possibly ameliorate the inflammation. Additionally, spilanthol also inhibits the expression of COX-2, reducing the progression of inflammatory responses. These suppression effects are partly due to the inactivation of NF-κB. Results reveal that spilanthol dose-dependently inhibits the phosphorylation of IκB in cytoplasm (**Figure 6**), resulting in the possible accumulation of NF-κB/IκB complex in cytoplasm. Unsurprisingly, the p65 translocation was impeded by spilanthol (**Figure 9**), indicating that the NF-κB activation was limited. The production of proinflammatory cytokines (**Figure 8**) and the expressions of other inflammatory mediators, such as NO (**Figure 4**), iNOS, and COX-2 (**Figures 6** and **7**), were thus reduced.

Many natural compounds or herbal extracts have been reported to exhibit anti-inflammatory activities (*43–45*), generally involving the activation of NF-κB or activator protein 1 (AP-1). Flavonoids such as resveratrol, epigallocatechin gallate, and quercetin have been reported as potential inhibitors through the NF-κB pathway (*46–49*). Alkaloids also demonstrate anti-inflammatory via cell-dependent mechanisms. Berberine, an aprotoberberine alkaloid that exists in *Hydrastis canadensis* (golden seal), *Phellodendron amurense*, *Coptis chinensis*, *Berberis vulgaris* (barberry), *Berberis aquifolium* (Oregon grape), and *Berberis aristata* (tree turmeric) (*50*), suppresses IL-1β and TNF-α in human lung cells by inhibiting IκB phosphorylation and degradation (*51*). It also suppresses LPS-induced COX-2, TNF-α and iNOS productions in lung macrophage (*52*) and also inhibits TPA-mediated inflammation and cyclooxygenase-2 (COX-2) expression through the regulation of AP-1 (*53*).

Anti-inflammatory effects are observed in structurally diverse alkaloids. Sanguinarine, a quaternary benzo[*c*]phenanthridine alkaloids found in *Sanguinaria canadensis* (*54*), acts as a potent inhibitor of NF-κB activation, IκBα phosphorylation, and degradation (*55*). Sanguinarine has antibacterial, antifungal, antiviral, antineoplastic, and anti-inflammation properties (*56*)

and has been applied in dental care products, including toothpaste and mouthwash (*57*). Tylophorine, a phenanthroindolizidine alkaloid, exhibits potent suppression on NO production without significant cytotoxicity to the LPS/IFNγ-stimulated RAW 264.7 cells (*58*). It inhibits iNOS and COX-2 expression in the LPS/IFNγ-stimulated RAW 264.7 cells through AP-1 due to the enhanced phosphorylation of Akt, which decreases the expression and phosphorylation levels of *c*-Jun protein, and therefore results in the subsequent inhibition of AP-1 activity. Synthetic alkaloids, such as 1-(β-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, reduce not only nitric oxide production (IC_{50} = 23.5 μM) but also iNOS mRNA expression of RAW 264.7 cells, in a concentration-dependent manner (*59*). Apart from heterocyclic alkaloids, alkamides also demonstrate an anti-inflammatory effect. For instance, capsaicin inhibits NF-κB activation in response to different agents, including TNF-α (*60*) and obesity-induced inflammation (*61*). Like capsaicin, spilanthol has an NF-κB inactivation effect, but with different chemical structure characteristics. Spilanthol has a double-bond conjugated to an amide carbonyl group and a conjugated diene group with *Z*,*E* or *E*,*Z* configuration, whereas capsaicin has a nonenamide and a methyl group. On the amine moiety, spilanthol possesses an isobutylamino group, whereas capsaicin has an *N*-vanillyl group.

Hinz et al. recently reported that isobutylamides from *Echinacea angustifolia* roots inhibit COX-2 activity in human neuroglioma cells (*62*). They found that certain isobutylamides, including undeca-2*Z*-ene-8,10-diynoic acid isobutylamide, dodeca-2*E*-ene-8,10-diynoic acid isobutylamide, and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylisobutylamide, significantly inhibit COX-2-dependent PGE_2 formation by interfering with COX-2 activity rather than at the transcriptional or translational levels. However, alkamides, such as anandamide, have been reported to dose-dependently attenuate the TNF-α-induced ICAM-1 and VCAM-1 expression and NF-κB activation in human coronary artery endothelial cells (HCECs), and the adhesion of monocytes to HCECs in a CB(1)- and CB(2)-dependent manner (*63*). Therefore, the experimental system (e.g., cell model) critically determines the outcome of investigations as well as the particular structures of various alkamides.

The mechanism by which alkamides inactivate NF-κB is not clearly known and remains to be determined. These compounds might be considered to play a role in the redox-sensitive steps in the NF-κB activation signal transduction pathways (*64*) or at the transcriptional level because the transcription of NF-κB is regulated by NAD-dependent histone deacetylases, which are susceptible to antioxidant modulation (*65*). Additionally, the LPS-activated p38 pathway might also be a target of alkamides.

In conclusion, the anti-iflammatory component of *S. acmella*, spilathol, was isolated by a bioactivity-guided approach and found to exerted an inhibitory effect on NF-κB activation through restrained IκB phosphorylation and degradation, leading to the reduced downstream inflammation mediator expression, including iNOS, COX-2, IL-1β, IL-6, and TNF-α. Moreover, the extracts that scavenged free radicals might also contribute to the anti-inflammatory effect of *S. acmella*. These findings suggest that spilanthol can be a useful inhibitor of inflammatory mediators and is potentially applicable for COX-2 selective nonsteroidal anti-inflammatory drugs (NSAIDs).

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