JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Anti-Inflammatory Effects of Supercritical Carbon Dioxide Extract and Its Isolated Carnosic Acid from *Rosmarinus officinalis* Leaves

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ABSTRACT: Rosemary (*Rosmarinus officinalis*) leaves possess a variety of bioactivities. Previous studies have shown that the extract of rosemary leaves from supercritical fluid extraction inhibits the expression of inflammatory mediators with apparent dose-dependent responses. In this study, three different extraction conditions (5000 psi at 40, 60, and 80 °C) of supercritical carbon dioxide (SC-CO_2) toward the extraction of antioxidants from rosemary were investigated. Furthermore, simultaneous comparison of the anti-inflammatory properties between rosemary extract prepared from SC-CO_2 under optimal conditions (5,000 psi and 80 °C) and its purified carnosic acid (CA) using lipopolysaccharide (LPS)-treated murine RAW 264.7 macrophage cells was also presented. Results showed that the yield of 3.92% and total phenolics of 213.5 mg/g extract obtained from the most effective extraction conditions showed a high inhibitory effect on lipid peroxidation (IC_{50} 33.4 μg/mL). Both the SC-CO_2 extract and CA markedly suppressed the LPS-induced production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α), as well as the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), phosphorylated inhibitor-kappaB (P-IκB), and nuclear factor-kappaB (NF-κB)/p65 in a dose-dependent manner. The five major compounds of verbenone, cirsimaritin, salvigenin, carnosol, and CA existing in the SC-CO_2 extract were isolated by semipreparative HPLC and identified by HPLC-MS/MS analysis. CA was the most abundant recorded compound and the most important photochemical with an anti-inflammatory effect with an IC_{50} of 22.5 μM or 7.47 μg/mL presented to the best inhibitory activity on NO production better than that of the 14.50 μg/mL dosage prepared from the SC-CO_2 extract. Nevertheless, the effective inhibition of LPS-induced NF-κB signaling in RAW 264.7 cells from the SC-CO_2 extract extends the potential application of nutraceutical formulation for the prevention of inflammatory diseases.

KEYWORDS: Rosemary, supercritical carbon dioxide extraction, carnosic acid, anti-inflammatory activity, murine macrophage cell line RAW 264.7, HPLC-MS/MS

INTRODUCTION

Rosmarinus officinalis L. (rosemary) is known as a culinary as well as medicinal herb with biological activities.[1] Several studies have reported that the presence of a high percentage of phenolic diterpene antioxidants (PDAs) significantly contributes to the biological activities of rosemary.[2] Carnosic acid (CA) and carnosol are the principal phenolic compounds responsible for the antioxidant, anti-inflammatory, and antiproliferative effects of fresh rosemary leaves,[3] and CA is approximately ten times more effective than carnosol.[4]

Technologies using traditional solvents and supercritical fluids to obtain PDAs from rosemary have been described.[5] The extraction efficiency of PDAs from rosemary leaves has varied greatly with different methods. Among available methods, supercritical fluid extraction (SFE) has been the object of studies aimed at optimizing the extraction conditions for PDAs. In studies of rosemary extraction, supercritical carbon dioxide (SC-CO_2) has been reported to have the highest and most reproducible recovery of CA (35.7 ± 1.6 mg/g) compared with the conventional solvent extraction methods.[6]

In the course of screening antioxidant activities of the extracts from 12 different rosemary accessions, the concentration of CA has been demonstrated as a relevant indicator of antioxidant activity.[7] However, CA is a rather unstable compound and can be readily converted to carnosol by air oxidation.[8] Therefore, development of an efficient extraction method with minimal air exposition is necessary. In a previous report,[9] rosemary leaves were extracted by SFE under CO_2 pressure between 300 and 350 bar (that is, 4351−5079 psi). Ramírez et al.[10] extracted PDAs from rosemary leaves by SFE followed by supercritical fluid chromatography (SFC) separation on packed capillary columns. To determine the ideal conditions for the maximum yield of extract, we applied nine different conditions that combine three levels of CO_2 pressures (3000, 4000, and 5000 psi) with three temperatures at 40, 60, and 80 °C, respectively.[11] According to the extraction yield and PDAs content of the extracts, the ideal

Received: December 16, 2010
Accepted: February 15, 2011
Revised: February 15, 2011
Published: March 04, 2011

extraction condition was 5000 psi at 80 °C. The results also indicate that the antioxidant activity of rosemary extracts was proportional to the content of CA.

Inflammation is considered to play a crucial role in the etiology of atherosclerosis and carcinogenesis.[12,13] Carnosic acid (CA) has been shown to possess anti-inflammatory activity; however, the physiological application of CA is limited because of the costly purification procedure. To increase the prospective application of rosemary extract, this study aimed to compare the inflammatory capacity of rosemary extract with that of isolated CA.

Relative to the anti-inflammatory effects of rosemary components, phenolic diterpenes CA and carnosol are of vital importance in modulation actions. Carnosic acid (CA) activates the peroxisome proliferator-activated receptor gamma in human polymorphonuclear leukocytes (PMNL), implying an anti-inflammatory potential on the level of gene regulation.[14] Accordingly, although carnosol decreases LPS-induced iNOS mRNA and down-regulates the inhibitor κB (IκB) kinase (IKK) activity on the mouse macrophage RAW 264.7 cell line,[15] this study indicated that CA would play a pivotal role in anti-inflammatory properties owing to its higher content existing in the supercritical fluid extract from our previous report.[16] Especially, tests indicated that CA is significantly more effective than other naturally occurring antioxidants, as well as certain synthetic antioxidants, such as BHA and BHT.[17] However, the physiological application of CA is limited because of the costly purification procedure. Moreover, the cellular and molecular mechanisms underlying the anti-inflammatory effects of rosemary SC-CO_2 extract and its isolated major active compound CA are currently not well-defined. To increase the prospective application of rosemary extract, this study aimed to compare the inflammatory capacity of rosemary extract with that of isolated CA.

MATERIALS AND METHODS

Plant Material. The rosemary plants (*Rosmarinus officinalis*) used in this study were grown in the experimental fields of Taichung District Agricultural Research and Extension Station, Taichung, Taiwan. Fresh rosemary leaves were collected in April 2006 and were frozen in −80 °C after harvesting. After 24-h lyophilization, the leaves were ground to a fine powder (20 mesh) in a comminuting mill (Retsch Ultra Centrifugal Mill and Sieving Machine, Type ZMI, Haan, Germany). The powder was stored in the dark at −80 °C until use.

Preparation of SC-CO_2 Extract and CA. Supercritical carbon dioxide (SC-CO_2) extract preparation was performed according to our previous report[11] with some modifications. In brief, ground, dried rosemary leaves (5.0 g) were placed into the extraction vessel (10 mL) of the supercritical fluid extraction apparatus (ISCO Model SFX 2-10, Lincoln, NE, USA). Extractions with supercritical CO_2 (flow rate, 1.0 mL/min) were operated independently at 5000 psi (relative to 345 bar) in combination with temperatures at 40, 60, and 80 °C, respectively, for 30 min of static and followed by another 90 min of dynamic extractions. Extracted constituents were collected in a 20 mL vial that was prefilled with a trapping solvent (10 mL of acetone) and maintained at 4 °C during the extraction step. The sample was evaporated to dryness in a Bucchi rotating evaporator under reduced pressure at 40 °C. Isolation of CA from prepared SC-CO_2 extracts was performed using a semipreparative HPLC apparatus equipped with a Hitachi (Tokyo, Japan) L-7100 dual pump connected to a Luna C18 (2) (250 × 10.00 mm i.d.; particle size, 5 μm, Phenomenex, Torrance, CA, USA) column and a L-7455 photodiode-array detector (PAD) monitored at 278 nm (scanning range 210−400 nm) and collected in a fraction collector (Isco Retriever 500, Lincoln, NE, USA). The mobile phase with a flow rate of 4.0 mL/min consisted of two solvents: A and B. Solvent A was 1% (v/v) acetic acid in HPLC grade water, and solvent B was 1% acetic acid in acetonitrile. Gradient elution consisted of solvents A and B (60: 40, v/v) during the initial 5 min; solvent B was then linearly programmed from 40 to 100% within 10 min and remained at this percentage for the next 15 min and was then returned to the initial condition for 5 min. The eluted fraction containing only the pure compound was dried under a vacuum to yield a residue, which was further purified by crystallization to obtain a powder form. The five purified compounds were then weighed and identified again by comparing UV and ESI-MS spectra with our previous report[11] and the published data.[18] If authentic compounds were unavailable for the identification works, ^{1}H NMR spectroscopic analysis was further applied. The purity was determined to be higher than 95% by HPLC analysis.

Determination of Total Phenolic Content. The total phenolic content from the SC-CO_2 extract was determined according to the method of Zielinski and Kozlowska.[19] A 0.10 mL aliquot of the extract solution (in methanol) was mixed with 0.5 mL of a 0.2 M Folin−Ciocalteu reagent (Fluka, Buchs, Switzerland) and 0.4 mL of a 7.5% sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min and then measured at 760 nm with a spectrophotometer (Thermo Biomate 5, Thermo Electron Corporation, San Jose, CA, USA).

HPLC and LC/MS/MS analysis. Quantitative analysis of the SC-CO_2 extract was performed by the Finnigan Surveyor module separation system equipped with a photodiode-array (PDA) detector (Thermo Electron Co., MA, USA). An analytical column [150 × 2.0 mm i.d., 3 μm Luna C18 (2), Phenomenex Co., USA] was operated at a flow rate of 0.2 mL/min for the analyses of the SC-CO_2 extract and the collected compounds. The elution solvent system was performed by gradient elution using two solvents: solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate during the elution process was set at 0.2 mL/min. The entire course of programmed gradient elution was conducted as follows: 0−3 min, isocratic with 23% B; 3−35 min, with 23−75% B; 35−40 min, isocratic with 75% B; 40−45 min, with 75−95% B; followed by 5 min of isocratic 95% B; and returning to initial conditions for 10 min. The absorption spectra of eluted compounds were scanned within 190 to 600 nm using the in-line PDA detector monitored at 278 nm. The compounds having been eluted and separated were further identified with a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., MA, USA) with an ESI interface coupled to the Surveyor HPLC system. Briefly, in the negative ion mode, the instrument was operated under the following optimized conditions: spray needle voltage, 3.5 kV; tube lens offset, −60 V; ion transfer capillary temperature, 280 °C; voltages of the capillary column, −14 V; nitrogen sheath gas flow rate, 50 arbitrary units; and auxiliary gas flow rate, 10 arbitrary units. In the positive ion mode, the parameters were set as follows: spray needle, 3.5 kV; tube lens offset, +20 V; capillary temperature, 200 °C; and voltages of the capillary column, 3 V. The product-ion spectra (MS^2) were scanned from 80−400 m/z with the collision energy set at 38 V. The main compounds present in rosemary extracts were quantified regarding pure standard CA and reported in (mg/g) of the dry basis of the extract with the CA calibration curve ($y = 48989x + 66683$, $r^2 = 0.9969$) according to our previous report.[11] Full-scan mass spectra were obtained in the range of 140−700 m/z, with three microscans and 200 ms of maximum ion-injection time. All the data were processed with the Xcalibur 2.0 data system (Thermo Electron Co.).

Lipid Peroxidation. The reaction mixture contained 250 μL of linoleic acid (20 mM in ethanol, Sigma-Aldrich, St. Louis, MO), 250 μL of Tris-/HCl (100 mM, pH 7.5), 50 μL of $FeCl_2 \cdot 4\,H_2O$ (4 mM), and 50 μL of a varying concentration of the SC-CO_2 extract or CA. Linoleic acid peroxidation was initiated by the addition of 50 μL of ascorbic acid

(2 mM, Sigma-Aldrich), incubated for 30 min at 37 °C and terminated by the addition of 250 μL of trichloroacetic acid (5.5%). The mixture was added to 225 μL of thiobarbituric acid (1% in 50 mM NaOH, Sigma-Aldrich), followed by heating for 10 min. The mixtures were centrifuged at 3500*g* for 10 min, and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant (200 μL) was read at 532 nm. Inhibition of linoleic acid peroxidation (%) was calculated as $(Ac - As)/(Ac - An) \times 100$, where *Ac* is the absorbance of control (without extract), *As* is the absorbance of sample test, and *An* is the absorbance of blank (without extract and $FeCl_2 \cdot 4H_2O$).

Cell Culture. The mouse BALB/c macrophage cell line RAW 264.7, obtained from Biosource Collection and Research Center of Food Industry Research and Development Institute (Hsinchu City, Taiwan), was cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, HyClone, Logan, UT) supplemented with 10% fetal bovine serum, 0.22% sodium bicarbonate, 100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. When 50% confluence was attained, the cells were challenged by lipopolysaccharide (500 ng/mL) with or without the treatment of the SC-CO_2 extract or CA for 24 h. The medium was collected for cytokine assays. Cells were collected by centrifugation and washed twice with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM sodium fluoride (NaF), 0.1% SDS, 1.0 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), 0.1 μM aprotinin, 1% NP40, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate] incubated at 4 °C for 20 min. Cell debris was removed by centrifugation (10,000*g* at 4 °C for 10 min), followed by quick freezing of the supernatants. The concentration of cell lysates was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Determination of Cell Viability. Cell viability was measured by MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich) assay according to the method of Mosmann et al.[20] After the cells were seeded in a 24-well plate (3×10^5/well) for 24 h, the cells were challenged with LPS with or without the treatment of the SC-CO_2 extract or CA for another 24 h. Fifty microliters of MTT reagent (0.5 mg/mL) was then added to each well and incubated in the CO_2 incubator. Two hours later, acid−isopropanol (100 μL of 0.04 N HCl in isopropanol) containing 10% Triton X-100 was added to each well and mixed thoroughly to dissolve the purple crystals. The plates were read at 570 nm. The wells without cells were used as blanks. Cell viability was expressed as the percentage of the control group (without the treatment of LPS and test samples): viability (%) = $(A_{treat} - A_{blank}/A_{control} - A_{blank}) \times 100\%$, where A_{treat} is the absorbance of the extract-treated group at 570 nm, $A_{control}$ is the absorbance of the control group at 570 nm, and A_{bland} is the absorbance of the blank group at 570 nm.

Nitrite Assay. The aliquots of a supernatant culture medium (50 μL) of the cells treated with the extract, and different compounds were mixed with a Griess reagent (Sigma-Aldrich) in an equal volume of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (in deionized water) and 1% sulfanilamide solution (in 5% H_3PO_4). The mixed sample was incubated at 37 °C for 30 min. Absorbance at 550 nm was measured, and concentrations were calculated using a sodium nitrite standard curve.[15]

Determination of Tumor Necrosis Factor-α (TNF-α). The cells (3×10^5/well) were seeded into 24-well culture plates and incubated for 24 h, then treated with LPS (500 ng/mL) and SC-CO_2 extract or CA at the indicated concentrations for 6 and 24 h. The supernatant (10,000*g* at 4 °C for 10 min) was used for cytokine TNF-α analysis using ELISA kits commercially available from *e*Bioscience (San Diego, CA, USA). Briefly, 96-well plates were coated with 100 μL of capture antibody (in 1× assay buffer), and incubated overnight at 4 °C. After washing and blocking with PBST buffer, the diluted supernatant was added to each well, and the plates were maintained for 2 h at RT. The plates were washed, and the biotin-conjugated detecting mouse antibody was added to each well and incubated at room temperature for 1 h. The plates were washed and further incubated with avidin-HRP for 30 min before detection using the 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich) solution. Absorbance was measured at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amount of TNF-α was calculated from the linear portion of the standard curve.

Western Blot Analysis. RAW 264.7 (1.2×10^6 cells/well) incubated in DMEM were seeded into 6-well culture plates for 24 h, then 500 ng/mL of LPS and different concentrations of the SC-CO_2 extract or CA were treated for 24 h. Cellular proteins were extracted from the control and SC-CO_2 extract or CA-treated RAW264.7 cells. Cells were collected by centrifugation and washed twice with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM sodium fluoride (NaF), 0.1% SDS, 1.0 mM PMSF, 0.1 μM aprotinin, 1% NP40, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate] incubated at 4 °C for 20 min. Cell debris was removed by centrifugation (10,000*g* at 4 °C for 10 min), followed by quick freezing of the supernatants. Total cellular protein extracts were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer instructions. An equal amount of proteins were denatured and separated by sodium dodecyl-sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) performed on 9% polyacrylamide gels and then transferred onto PVDF (polyvinylidene difluoride) membranes (Biotrace PVDF, Pall Gelman Laboratory, MI, USA) by the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween (TBST, 20 mM Tris-HCl, pH 8.3, 137 mM NaCl, and 0.1% Tween-20) for 1 h at 4 °C and then incubated sequentially with primary antibodies that recognize iNOS, β-actin, COX-2, IκB-α, and p65 (Santa Cruz Biotechnology, CA, USA) for 2 h, and horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing with PBST, goat antirabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) (1:1000 dilution in PBST) were incubated for1 h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL, PerkinElmer, Waltham, MA), and the relative expression of the protein bands was quantified by densitometry with ImageQuant TL software (GE Healthcare).

Statistical Analysis. Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). An analysis of variance (ANOVA) and the Student−Newman−Keuls multiple range test were used to determine significant difference among means ($\alpha = 0.05$).

RESULTS

Yield and Chemical Characterization of Rosemary Extracts. Supercritical fluids potentially have highly useful physical properties, such as low viscosity and high diffusivity into the sample matrix. Carbon dioxide (CO_2) is the most widely used solvent among the supercritical fluids because it is harmless, safe, nonexplosive, and readily available. Diverse extraction conditions of temperature (T) and pressure (P) were examined for the investigation of optimal extraction conditions for rosemary antioxidant extracts in the present study. As shown in Table 1, the highest extraction yield (3.92 ± 0.59%) was obtained at 5000 psi and 80 °C (S80), compared to the yield of 3.24 ± 0.18% by 5000 psi at 40 °C (S40) and the lowest yield of 3.07 ± 0.66% by 5000 psi at 60 °C (S60). Moreover, the total phenolics content in the three extracts was obtained at 5000 psi and 80 °C as the highest yield of 213.5 ± 39.3 mg/g dry basis of extract, in which

Table 1. Yield, Total Phenolics, and Contents in Rosemary Leaf Extract Obtained under Different Supercritical Fluid Extraction Conditions

			content (mg/g extract)[c]				
extract[a]	yield (%)	total phenolics[b]	verbenone	cirsimaritin	salvigenin	carnosol	carnosic acid
S40	3.24 ± 0.18 b	173.9 ± 54.8 c	49.05 ± 8.95 b	42.08 ± 8.12 c	32.37 ± 6.56 a	18.0 ± 5.5 c	104.9 ± 10.4 a
S60	3.07 ± 0.66 c	196.7 ± 61.6 b	76.09 ± 25.32 a	48.63 ± 7.25 b	26.84 ± 3.23 b	42.0 ± 2.7 a	110.5 ± 5.9 a
S80	3.92 ± 0.59 a	213.5 ± 39.3 a	48.51 ± 5.23 b	55.72 ± 9.02 a	35.78 ± 5.12 a	30.6 ± 1.7 b	107.7 ± 1.4 a

[a] S40, S60, and S80: SC-CO_2 extract at 5000 psi and 40, 60, and 80 °C, respectively. [b] Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE)/g extract. [c] Data are referred to the calibrated standard curve of carnosic acid from the HPLC analysis. Each value represents the mean ± SD. Values with different letters within a column are significantly different ($p < 0.05$).

the content of the major antioxidant CA from S80 extract was 107.7 ± 1.4 mg/g comparable to that of 110.5 ± 5.9 mg/g of the S60 extract (Table 1) without significant difference ($p = 0.46$). Considering that the highest yield and total phenolics existed in the S80 extract, we therefore recommend this sample for further studies in the preparation of pure compounds by semipreparative liquid chromatography. The analysis of chemical compounds in each extract was performed using an HPLC, and the highest yield sample obtained from 5000 psi and 80 °C conditions is demonstrated in Figure 1A. The HPLC chromatogram marked five major compounds of the aforementioned SC-CO_2 extract: **1**, verbenone; **2**, cirsimaritin; **3**, salvigenin; **4**, carnosol; and **5**, carnosic acid. The amounts of each compound in three different extracts are listed in Table 1. CA had the highest amount of those major compounds in each SC-CO_2 extract. The amounts of CA obtained from SFE at 40, 60, and 80 °C are substantially higher than those obtained by solvent extraction.[21] To characterize the contribution of five major compounds in the SC-CO_2 extract to the inhibition effect of NO production on the inflammatory response of macrophages, we purified each compound from the SC-CO_2 extract (5000 psi, 80 °C) by semipreparative liquid chromatography. The HPLC chromatogram from the mixture of each compound is shown in Figure 1B. Identification of the major five compounds was conducted by comparing retention times and MS spectra with those of available authentic standards. Three of them, verbenone, carnosol, and carnosic acid, were identified as they showed MS/MS characteristics identical to those of the standards. For the remaining two compounds (cirsimaritin and salvigenin) for which no standards were available, identification was based on MS spectra of positive ionization and CID fragment ions, and, furthermore, compared with those of published ^{1}H NMR spectra.[22,23] Data obtained from the positive or negative ESI-MS and ESI-MS-MS analyses, as well as UV−vis spectral analyses of the five compounds, are summarized in Table 2.

Inhibitory Effect on Lipid Peroxidation. Results from the extraction yield of rosemary leaves shown in Table 1 were further evaluated by the assays for inhibiting the lipid peroxidation effect using BHT as the reference compound (Table 3). Rosemary extract from SC-CO_2 extraction at a concentration of 3.2 μg/mL was almost completely ineffective, the least in inhibition effect being only 5.6 ± 2.3% by the extract at 5000 psi and 80 °C in contrast to that of BHT, 99.7 ± 0.4%, and CA, 53.8 ± 2.7% (Table 3). With the extract at 2000 μg/mL, the inhibition effect was observed reaching the most favorable at 94.9 ± 5.1% from a sample obtained at 5000 psi, 80 °C. Similar results have comparatively shown the reference compound BHT and isolated CA at corresponding levels with inhibition effects at 99.7 ± 0.8% and 99.7 ± 0.4%. All of the inhibition effects from the SC-CO_2 extract or CA, as well as BHT, demonstrated a dose-dependent manner. Considering the extraction yield from dried rosemary leaves and the contents of PDAs, the optimized conditions resulted in 5000 psi and 80 °C (Tables 1 and 2).

Research has shown that numerous antioxidants possess anti-inflammatory effects.[15] The effects of lipid peroxidation from SC-CO_2 extracts were consequently investigated for screening the optimal candidate for the following anti-inflammatory studies. As shown in Table 3, the IC_{50} from three extracts, S40, S60, and S80, are 40.8, 33.1, and 33.4 μg/mL, respectively. Although the S60 and S80 extracts presented an equal activity in antioxidant activity, S80 is suggested as the optimal candidate from the yield and total phenolics determinations (Table 1). It is interesting to note that both of the IC_{50} values of CA and BHT were under the aforementioned values and expressed as 14.0 and 14.8 μg/mL, respectively, and presented mostly as a similar activity when their test concentrations were over 400 μg/mL. However, there was a higher antioxidant activity in CA than BHT when the concentration was under 400 μg/mL.

Cell Viability. The rosemary extract and CA did not show cytotoxic effects when the treatment concentration was lower than 25 μg/mL and 40 μM, respectively (Figure 2). According to this result, a series of extract concentrations of 0 to 25 μg/mL and a series of CA concentrations of 0−40 μM were chosen for subsequent experiments.

TNF-α and NO Production. RAW 264.7 cells were stimulated with the bacterial endotoxin LPS for 6 or 24 h. After 6 h, the TNF-α level in the culture medium was significantly elevated up to 60 μg/mL, while after a longer time of LPS stimulation (24 h), TNF-α was increased obviously (96 μg/mL) (Figure 3) compared to cells treated with vehicle only. Supercritical fluid extract attenuated the LPS-induced TNF-α production when concentration was higher than 25 or 12.5 μg/mL for 6-h or 24-h culture, respectively. Carnosic acid (CA) did not decrease the LPS-induced TNF-α production until the treatment concentration reached 40 μM. The tendency of TNF-α production in 24-h culture was significantly higher than that of 6-h culture, and only the 24-h culture was adopted for further study. LPS induces a strong innate and adaptive response and stimulates the macrophages to release NO. Because the half-life of released NO is very short after the macrophages were activated by LPS, we detected nitrite production as a determination of nitric oxide (NO) content in the culture medium by the Griess assay. As shown in Figure 4, administration of LPS in normal macrophages (L) dramatically increased the NO production level by 35-fold compared to the control group (C) DMSO, which had no effect on NO production. The inhibition of NO production was dose-dependent for both CA and the extract (Figure 4), whose performances was

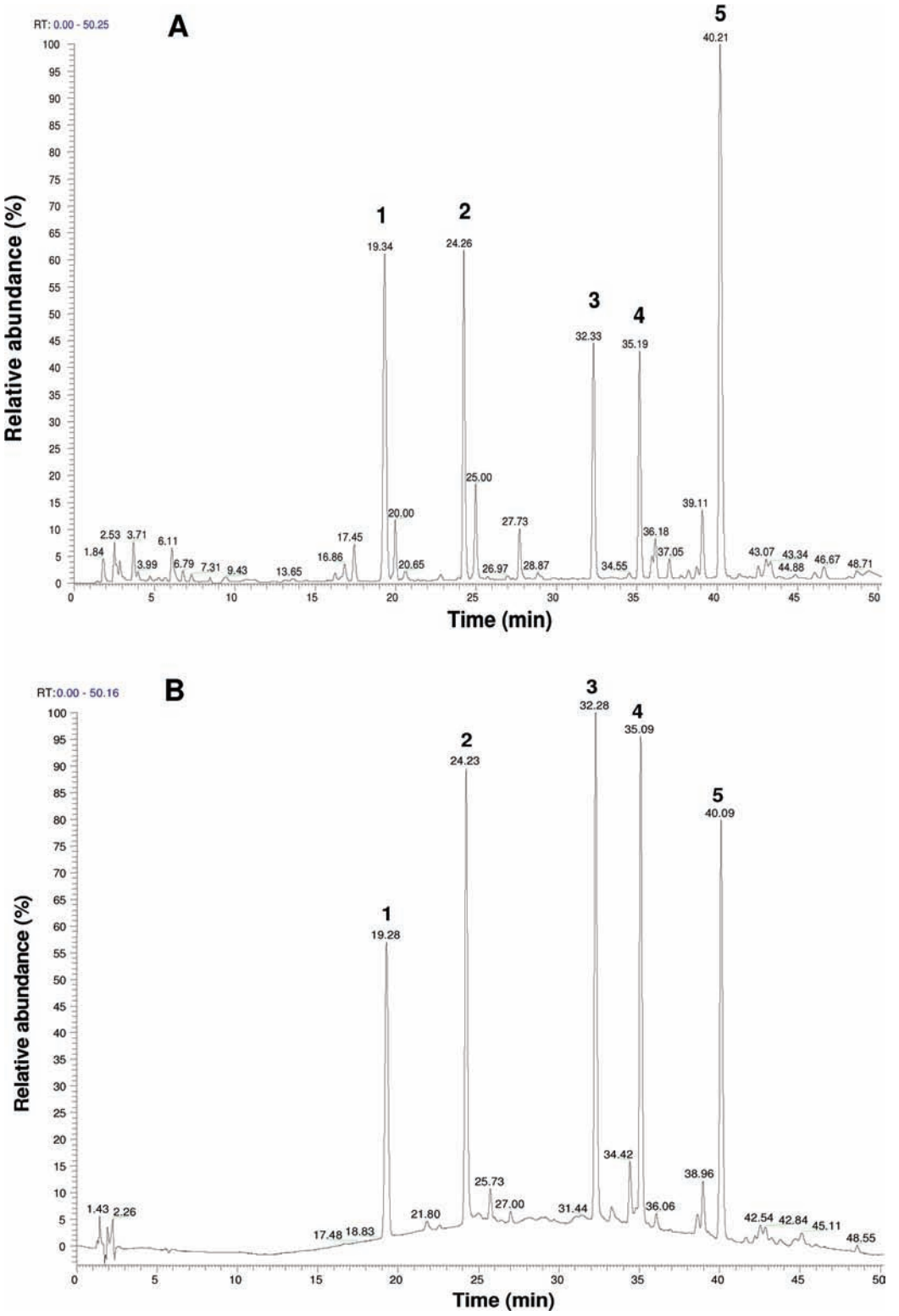

Figure 1. HPLC/UV chromatograms (278 nm) of rosemary supercritical carbon dioxide extract (A) and the five isolated and identified compounds corresponding to the HPLC-DAD-MS/MS analysis (Table 2) of peaks 1−5 (B). Peaks: **1**, verbenone; **2**, cirsimaritin; **3**, salvigenin; **4**, carnosol; and **5**, carnosic acid.

confirmed from the dosage used under the range of causing the loss of cell viability in RAW 264.7 cells (Figure 2). The inhibitory activity was observed when the concentration was as low as 3.13 μg/mL and 2.5 μM for the extract and CA, respectively.

Expression of iNOS and COX-2. Western blot analysis was performed to determine whether the inhibitory effects of the rosemary extract or CA on the pro-inflammatory mediators (NO and TNF-α) were related to the modulation of the expression of iNOS and COX-2. As shown in Figures 5 and 6, the addition of

Table 2. Chemical Characterization of the Main Compounds Detected in the Rosemary Supercritical Carbon Dioxide Extracts by Liquid Chromatography−Tandem Mass Spectrometry (LC-MS-MS) Analysis

peak no.	compounds	t_R (min)	UV−vis absorbance (nm)	$[M+H]^+$	$[M-H]^-$	MS-MS major fragments
1[a]	verbenone	19.28	259, 230, 338	150.9		108.9, 122.9, 132.9
2[b]	cirsimaritin	24.23	336, 277, 237	315.0		299.9, 282.0
3[b]	salvigenin	32.28	334, 278, 238	329.0		313.9, 296.1
4[a]	carnosol	35.09	236, 284		329.0	285.2
5[a]	carnosic acid	40.09	239, 284		331.1	287.3

[a] Identification confirmed with pure standard. [b] Identification was further confirmed by comparing the ^{1}H-NMR with previous reports.[22,23].

Table 3. Inhibitory Effects of SC-CO_2 of Rosemary and Carnosic Acid on Lipid Peroxidation

	inhibition (%)[a]				
amount (μg/mL)	S40	S60	S80	carnosic acid	BHT
3.2	10.3 ± 2.0 c	3.5 ± 0.7 d	5.6 ± 2.3 d	53.8 ± 2.7 b	99.7 ± 0.4 a
16.0	31.4 ± 8.6 bc	36.4 ± 5.6 bc	26.8 ± 4.8 c	70.6 ± 4.3 a	69.6 ± 3.3 a
80.0	56.1 ± 8.4 c	61.4 ± 6.4 bc	64.5 ± 5.7 bc	84.1 ± 4.2 a	80.8 ± 6.4 a
400.0	86.5 ± 7.1 bc	90.2 ± 7.4 abc	91.6 ± 6.5 ab	98.5 ± 1.8 a	97.4 ± 1.5 a
2000.0	92.6 ± 6.7 ab	93.4 ± 6.6 ab	94.9 ± 5.1 ab	99.7 ± 0.4 a	99.7 ± 0.8 a
IC_{50} (μg/mL)	40.8	33.1	33.4	14.0	14.8

[a] Each value represents the mean ± SD. Values with different letters in the same row are significantly different ($p < 0.05$). S40, S60, and S80: SC-CO_2 extract at 5000 psi and 40, 60, and 80 °C, respectively. Butylated hydroxytoluene (BHT) was used as a positive control.

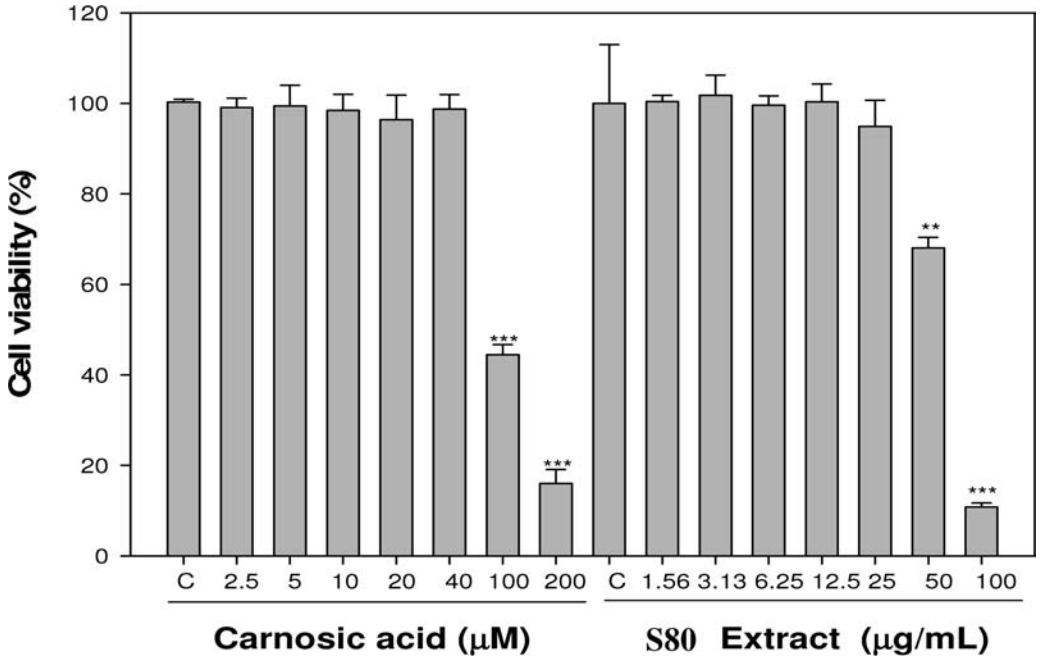

Figure 2. Effects of various concentrations of carnosic acid (A) and rosemary SC-CO_2 extract (B) on the viability of murine macrophage RAW 264.7 cells. Each bar represents the mean ± SD of three individual experiments and is expressed as a ratio to the control group (C). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group (C).

LPS (0.5 μg/mL) for 24 h resulted in a significant increase in expression of iNOS and COX-2. Co-treatment with the SC-CO_2 extract or CA significantly down-regulated the expression of both pro-inflammatory proteins. The detection of β-actin was also performed as the loading control and noted as a consistent band. The results indicate that the inhibitory effects of the SC-CO_2 extract or CA on LPS-induced NO and TNF-α production was caused by the suppression of iNOS and COX-2 expression.

Inhibitory Effects on LPS-Induced Degradation and Phosphorylation of IκB-α and Nuclear Translocation of the NF-κB/p65 Subunit. Since p65 is the major component of NF-κB activated by LPS in the macrophage, the levels of p65 in the nuclear extract were determined by a Western blot analysis. RAW 264.7 cells were incubated with LPS in the presence or absence of the rosemary extract or CA for 24 h. The expression of NF-κB/p65 in the nucleus was markedly increased upon exposure to LPS

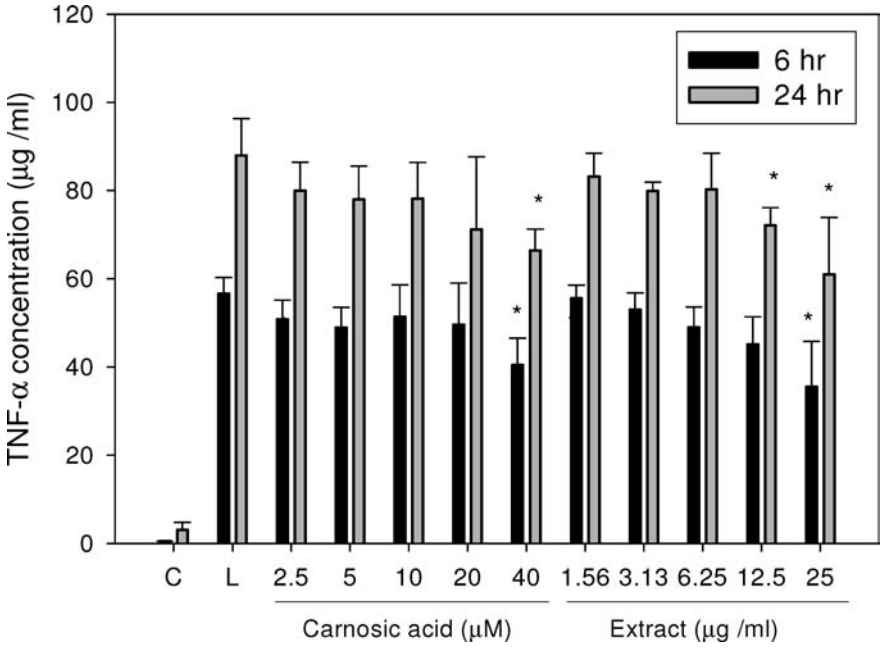

Figure 3. Effects of carnosic acid and rosemary SC-CO_2 extract on LPS-induced TNF-α expression in murine macrophage RAW 264.7 cells. Each bar represents the means ± SD of triplicate experiments. $^*p < 0.05$ as compared with the LPS group (L). C, cells were not stimulated by LPS. L, cells were stimulated by LPS but not treated with the extract or carnosic acid.

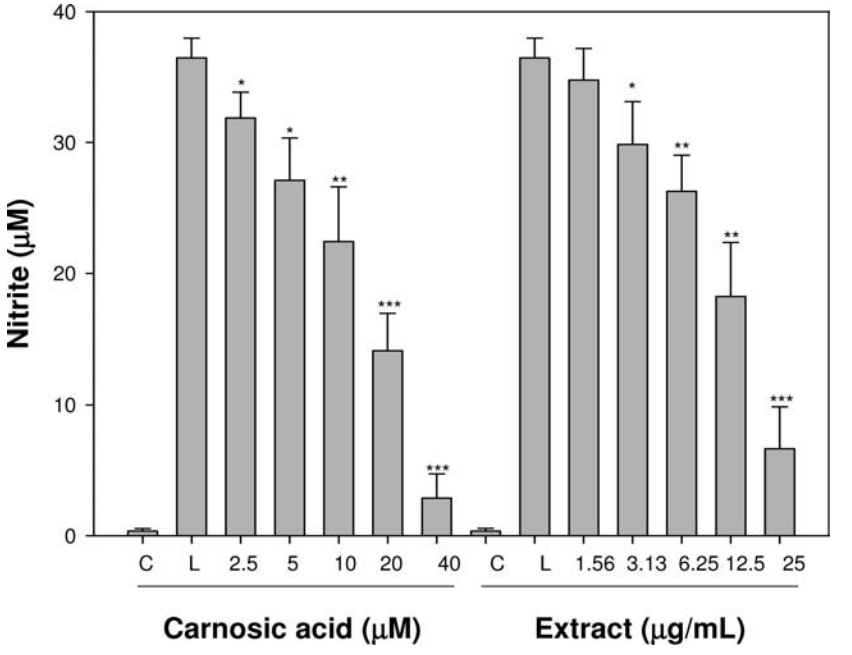

Figure 4. Effects of carnosic acid (A) and rosemary SC-CO_2 extract (B) on LPS-induced NO production in murine macrophage RAW 264.7 cells. The macrophage cells were treated with 500 ng/mL LPS only or cotreated with different concentrations of tested compounds or extract for 24 h. Each bar represents the means ± SD of triplicate experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the LPS group (L).

alone by 0.8-fold, but the extract or CA inhibited LPS-mediated nuclear translocation of NF-κB p65 in a dose-dependent manner (Figure 7). Furthermore, we examined the level of IκB-α because of the translocation of the NF-κB to the nucleus relative to the proceeding of phosphorylation, ubiquitination, and proteolytic degradation of IκB-α.[24] As shown in Figure 8, a challenge with LPS increased the phosphorylation of IκB-α (P-IκB-α), but the coincubation with either CA or the SC-CO_2 extract decreased the phosphorylation of IκB-α in a dose-dependent manner. The expression of IκB-α was opposite to the result of IκB phosphorylation. Taken together, these results suggested that CA or the SC-CO_2 extract might block LPS-induced nuclear translocation of p65 by the suppression of IκB-α phosphorylation and degradation.

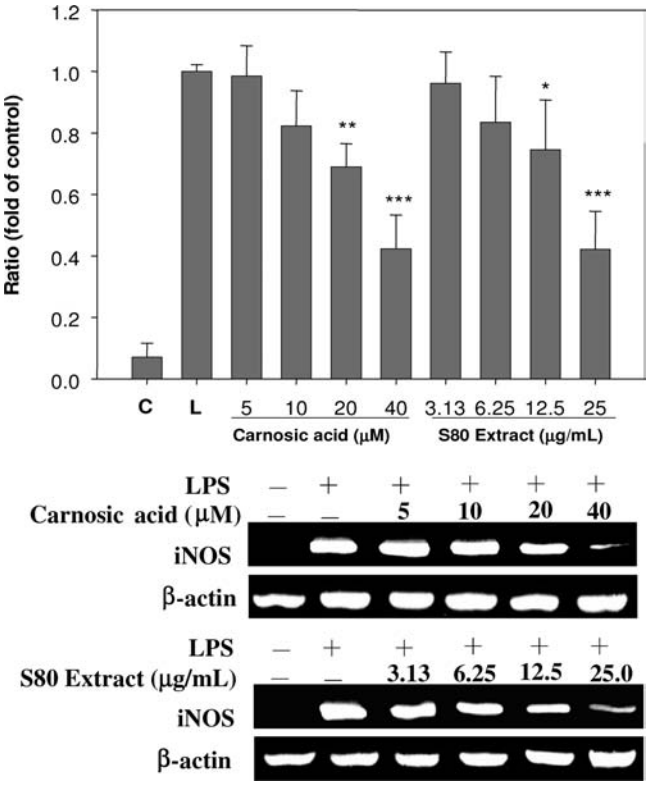

Figure 5. Inhibition of LPS-induced iNOS expression at the translation level in activated murine macrophage RAW 264.7 cotreated with carnosic acid or rosemary SC-CO_2 extract for 24 h. C, cells were not stimulated by LPS. L, cells were stimulated by LPS but not treated with extract or carnosic acid. The ratio of iNOS to β-actin expression observed in the L group is set as 1. S80 extract, the extraction performed at 5000 psi, 80 °C; iNOS, inducible NO synthase. Each bar represents the mean ± SD of three individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the LPS group (L).

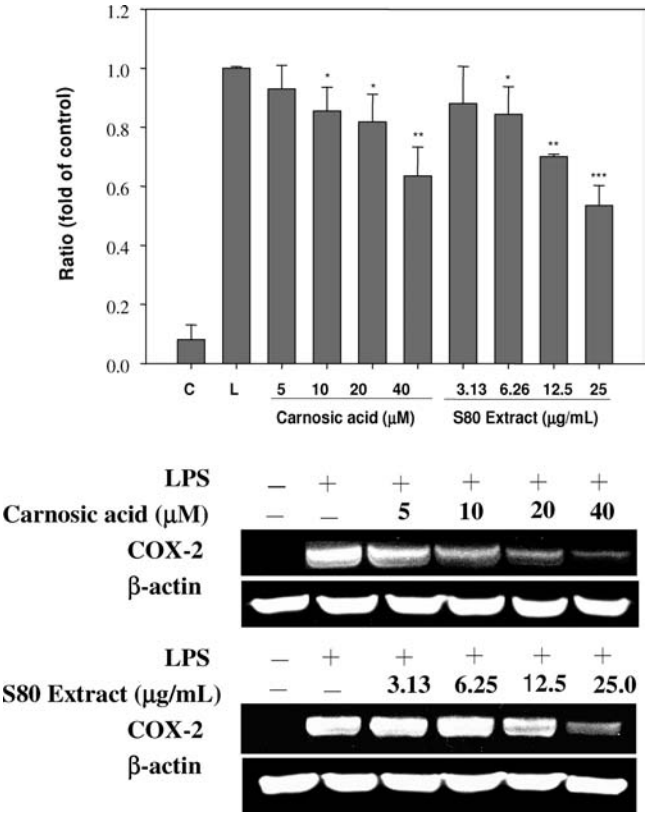

Figure 6. Inhibition of LPS-induced COX-2 expression at the translation level in activated murine macrophage RAW 264.7 cells cotreated with carnosic acid and rosemary SC-CO_2 extract for 24 h. C, cells were not stimulated by LPS. L, cells were stimulated by LPS but not treated with the extract or carnosic acid. The ratio of COX-2 to β-actin expression observed in the L group is set as 1. S80 extract, the extraction performed at 5000 psi, 80 °C; COX, cyclooxygenase. Each bar represents the mean ± SD of three individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the LPS group (L).

DISSCUSSION

In previous studies of rosemary extraction, SC-CO_2 has been found to be an efficient extraction technology with the highest and most reproducible recovery of CA compared to the common solvent extractions that use acetone, methanol, hexane, or dichloromethane.[6] In the present study, the initial trials concentrated on the selected conditions at 5000 psi of pressure according to our previous study,[11] and three different temperatures of 40, 60, and 80 °C for the comparison of extraction efficiency were conducted to isolate the major phytochemicals from the complicated components of extracts. The extraction yield produced, the inhibition activity on lipid peroxidation of extracts, and their total phenolic content (table 1) resulted in the most effective conditions at 5000 psi and 80 °C. Owing to the fact that supercritical carbon dioxide extraction does not obtain pigments, resins, or most of the hydrophilic substances, the yield of the SC-CO_2 extract in this study was lower than that obtained by organic solvents (e.g., acetone, data not shown), but the composition was less complicated than that of solvent extracts as shown in Figure 1A. Therefore, five separated compounds were presented in the SC-CO_2 extract in sufficient amounts to be identified and quantified (Table 2), although numerous phenolics, flavonoids, and diterpenes have been reported in the rosemary extract.[2,3,5−7,9,16,21]

Numerous reports in related literature have described the analytical endeavors regarding rosemary extracts, focusing on the leaf parts of the plant, by HPLC and LC-MS.[7,25,26] In this work, attention was directed toward the analytical determination of components present in the SC-CO_2 extract using LC-MS-MS. The separated compounds were identified according to pure standards or tentatively identified by analyzing their mass spectra detected in both positive and negative ionization modes; and, if necessary, the ^{1}H NMR spectroscopic method was further used (Table 2). In the positive ESI-MS analysis, mass spectra data have indicated that polymethoxylated flavones $[M+H]^+$ primarily lose a methyl group to form $[M+H\text{-}CH_3]^+$, further dehydrate to $[M+H\text{-}CH_3\text{-}H_2O]^+$ and then lose a carbonyl group. This suggestion can be verified by the MS spectra of cirsimaritin (Table 2), which has been presented by Hasrat et al.[27] The protonated ion at m/z 315 was subjected to low energy collision-induced dissociation, and the CID spectrum was characterized by a relatively abundant ion at m/z 300 (70%), which is due to the loss of a $CH_3^{\bullet}$ radical and is typical of a methylated flavone. Moreover, an intense ion at m/z 282 and a less intense ion at m/z 254 correspond to the combined loss of $CH_3^{\bullet}$ and H_2O and of $CH_3^{\bullet}$, H_2O, and CO, respectively.[27,28] Another compound, verbenone, was initially determined to be a moderate amount of the SC-CO_2 extract in this study. Verbenone has been

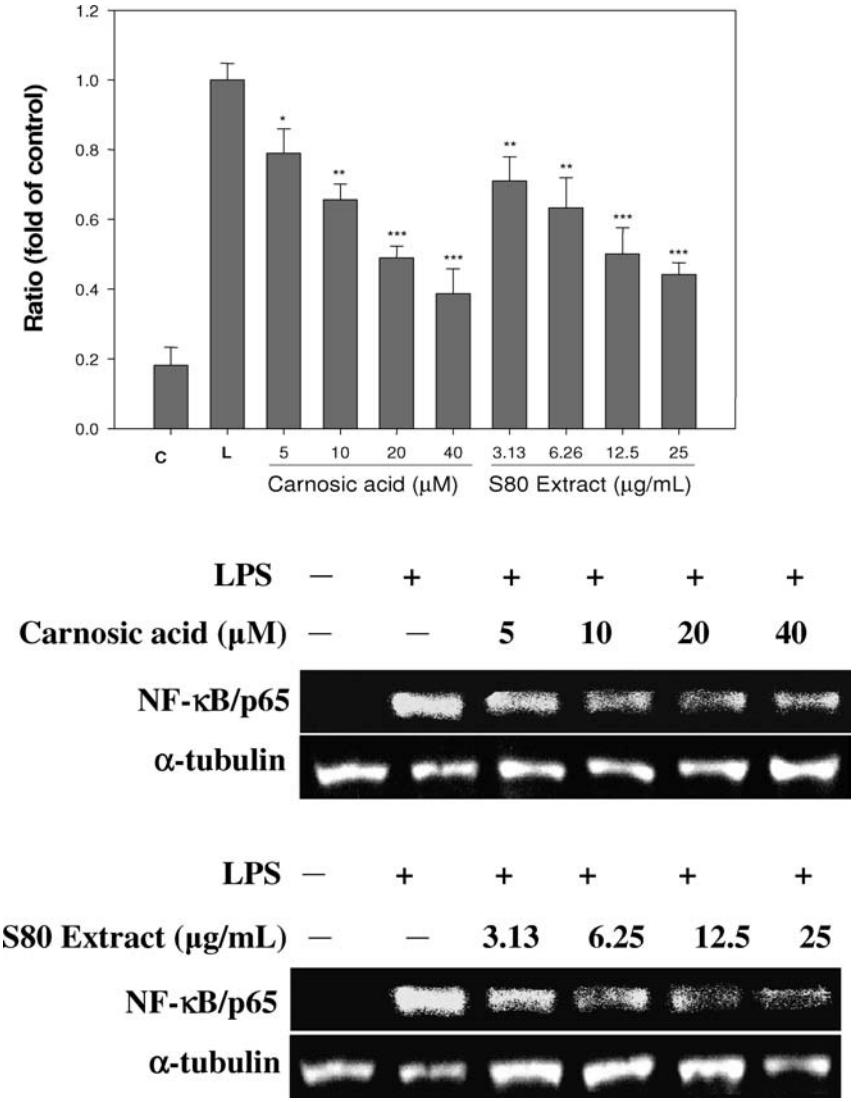

Figure 7. Expression of NF-κB/p65 in murine macrophage RAW 264.7 cells stimulated by LPS (0.5 μg/mL) and cotreated with carnosic acid or SC-CO_2 extract for 24 h. C, cells were not stimulated by LPS. L, cells were stimulated by LPS but not treated with extract or carnosic acid. The ratio of NF-κB/p65 to α-tubulin expression observed in the C group is set as 1. NF-κB, nuclear factor-kappaB; S80 extract, the extraction performed at 5000 psi and 80 °C. Each bar represents the mean ± SD of five individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the LPS group (L).

discovered in essential oil-rich fractions obtained by supercritical CO_2 extraction from rosemary[29] and can be separated from the different fractions of extraction pressure.[30] It has been reported that the oil displays a potential acetylcholinesterase inhibitory capacity and may enhance some improvements in symptoms of cognitive decline.[31]

Two general types of assays are widely used for different antioxidant studies. One is an assay associated with lipid peroxidation; another is associated with electron or radical scavenging.[32] The assay of lipid peroxidation, such as the thiobarbituric acid (TBA) assay for detecting oxidative degradation of lipids, has been widely used in natural antioxidants study. In this work, different extraction conditions in the SC-CO_2 extraction were studied. All extracts obtained by SC-CO_2 extraction showed potential valuable antioxidant activity demonstrated by the low values of IC_{50} (less than 40.8 μg/mL in all cases), as shown in Table 3, in which, CA has been described by several reports as the most active compound among rosemary extracts;[2,33] therefore, the inhibition activity in lipid peroxidation from different concentrations of CA was determined using the TBA assay. We determined that CA provided the most favorable antioxidant activity results against the oxidation of linoleic acid with the IC_{50} value of 14.0 μg/mL of even higher quality than that of BHT 14.8 μg/mL. Antioxidative activity of salvigenin assayed using the ferric thiocyanate method displays a higher activity than α-tocopherol and a lower activity than BHA.[34]

The CA concentrations 2.5, 5, 10, 20, and 40 μM used in evaluating anti-inflammatory capacity correspond to 0.83, 1.66, 3.32, 6.64, and 13.30 μg/mL, respectively, comparable to the concentrations of the supercritical CO_2 extract used (1.56, 3.13, 6.25, 12.5, and 25 μg/mL, respectively). Although the amount of CA contained in the extract is lower than the corresponding concentration of isolated CA, the synergistic interactions of phenolic compounds in rosemary extract may contribute to the anti-inflammatory effect. For example, 40 μM (13.28 μg/mL) CA and 25 μg/mL rosemary extract (containing 7.1 μg/mL of

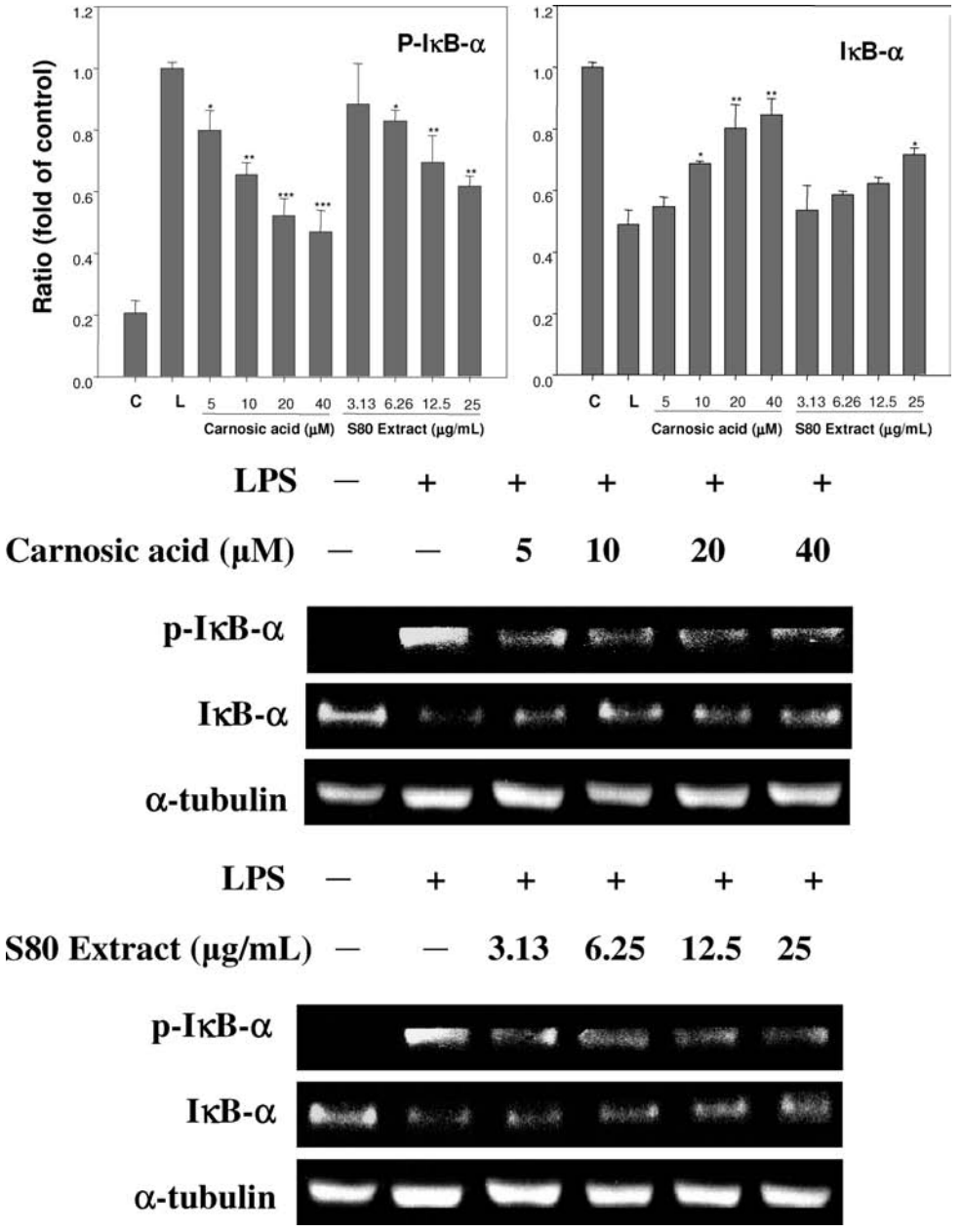

Figure 8. Cytosolic IκB-α degradation in murine macrophage RAW 264.7 cells stimulated by LPS (0.5 μg/mL) and cotreated with carnosic acid or SC-CO_2 extract for 24 h. C, cells were not stimulated by LPS. L, cells were stimulated by LPS but not treated with extract or carnosic acid. The ratio of IκB-α or pIκB-α to α-tubulin expression observed in the L group is set as 1. IκB, inhibitor of nuclear factor kappaB; pIκB, phosphorylated IκB-α; S80 extract, the extraction performed at 5000 psi and 80 °C. Each bar represents the mean ± SD of five individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the LPS group (L).

CA) showed a similar inhibitory effect on the expression of iNOS (Figure 5). In addition to the two diterpenes CA and carnosol, two flavonoids, cirsimaritin and salvigenin, were suggested to be involved in the anti-inflammatory activity of the SC-CO_2 extract on the inhibition effect of NO production with IC_{50} values of 32.8 and 39.3 μg/mL (data not shown), respectively. Previously, the inhibitory activity on the proliferation and activation of T cells in vitro has been investigated from the purified cirsimaritin and has exhibited only a modest effect.[35] Low levels of NO in the neurons and endothelium are essential for the maintenance of physiological functions; however, it has been reported that overproduction of NO contributes to the pathology of inflammatory diseases.[36] Among the 10 flavonoids and 5 phenolic compounds isolated from the leaves of rosemary, only CA and carnosol significantly inhibited the production of nitrite.[3] In this study, 50% inhibition (IC_{50}) on NO production by the SC-CO_2 extract is 14.50 μg/mL, while an identical effect was observed at 22.5 μM, 7.47 μg/mL CA (Figure 4). Although the amount of CA (4.3 μg/mL) in 14.50 μg/mL extract is less than that of 7.47 μg/mL CA, the inhibitory effect of the SC-CO_2 extract on NO production is comparable to that of isolated CA. We suggested that certain of components in the SC-CO_2 extract might contribute the synergistic inhibitory effect on NO production, especially from the effect of carnosol (IC_{50} 36.2 μM or 11.9 μg/mL, data

not shown). However, the IC_{50} value is different from the previous report with an IC_{50} of 9.4 μM in the inhibition of NO production.[15]

TNF-α is one of the major cytokines involved in the initiation of inflammation.[37] The production of TNF-α by immune cells is increased by the presence of stimuli, such as lipopolysaccharide. Carnosic acid (CA) at the concentration of 40 μM (equal to 13.28 μg/mL) and 25 μg/mL extract showed a significant inhibitory effect on the production of TNF-α (Figure 3). Twenty-five micrograms of SC-CO_2 extract contains only 7.12 μg of CA; therefore, the comparable inhibitory effect of the extract may result from the synergistic reaction of various antioxidants in SFE.

Nitric oxide is synthesized from L-arginine by nitric oxide synthase in various tissues.[36] Three NOSs have been identified: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are constitutively expressed, but iNOS is expressed mainly in macrophages following exposure to cytokines or microbial products, acting as a proinflammatory protein. Cyclooxygenase-2 is another crucial protein involved in the progression of inflammation. Cyclooxygenase catalyzes the conversion of fatty acids to eicosanoids. COX-1 is constitutively expressed in many cell types, whereas the expression of COX-2 is restrictively observed after the stimulation of cells with proinflammatory cytokines.[38] In this study, the CA and SC-CO_2 extract have anti-inflammatory activities in LPS-stimulated macrophages by inhibiting the expression of iNOS and COX-2 resulting in the inhibition of NO (Figure 4) and TNF-α production (Figure 3). The inhibition of iNOS and COX-2 expression was observed when the CA concentration was only 10 μM (equal to 3.32 μg/mL) (Figures 5 and 6). A similar effect was observed when extract concentration was 6.25 μg/mL, containing only 1.78 μg/mL CA. Down-regulation of iNOS and COX-2 expression by CA and extract are both dose-dependent.

NF-κB is considered essential for the enhanced expression of iNOS and COX-2. It is a transcription factor and is tightly regulated by the interaction with inhibitory IκB proteins. Thus, in most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm.[24] Signals that induce NF-κB activity cause the phosphorylation and ubiquitination of IκB, leading to the translocation of NF-κB dimers p50 and p65 to the nucleus.[24] In the nucleus, it binds to DNA, allowing the transcription of iNOS mRNA and is subsequently accompanied by the increased production of NO.[39] In our study, the SC-CO_2 extract (3.13 μg/mL) and CA (5 μM, 1.66 μg/mL) significantly suppressed NF-κB/P65 nuclear translocation from the analysis of nuclear extracts of RAW 264.7 cells after being activated by LPS (Figure 7). Both CA and the rosemary extract decreased the LPS-induced phosphorylation of IκB-α in a dose-dependent manner. The 50% inhibition of I-κB phosphorylation was observed at about 20 μM of CA, but not shown in the concentrations of extract applied. Consistent with the results of p-IκB-α, the expression of NF-κB p65 was inhibited by CA and rosemary extract at a dose-dependent manner. Therefore, the inhibition of IκB-α phosphorylation, as observed with the SC-CO_2 extract or CA (Figure 8), would inhibit the generation of NF-κB dependent cytokine (Figure 3) and the expression of iNOS (Figure 5) and COX-2 (Figure 6).

According to the current study, the anti-inflammatory capacity of the SC-CO_2 extract is comparable with that of CA. Inhibition of TNF-α-induced NF-κB signaling extends the potential therapeutic application of a nutraceutical formulation comprising rosemary extract for the prevention of inflammatory diseases.

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Funding Sources

This work was supported by grants from the National Science Council, ROC (Grant no. NSC 94-2313-B-241-004) and Show Chwan Memorial Hospital, Changhua, Taiwan.

ACKNOWLEDGMENT

We are grateful to Dr. Long-Zen Chang of the Taichung District Agricultural Research and Extension Station, Taiwan for supplying the rosemary plant used in this study.

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