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Supercritical Fluid Extracts of Rosemary Leaves Exhibit Potent Anti-Inflammation and Anti-Tumor Effects

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Supercritical fluid SF-CO_2 treatment of *Rosemarinus officinalis* L. fresh leaves under optimum conditions (80 °C at 5,000 psi) yielded 5.3% of extract supercritical fluid extraction (SFE)-80, in which five major active principles were identified by liquid chromatography/mass spectrometry (LC/MS), *viz.*, rosmarinic acid, carnosol, 12-methoxycarnosic acid, carnosic acid, and methyl carnosate. Total phenolic content was 155.8 mg/gallic acid equivalent (GAE)/g in SFE-80, which showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging of 81.86% at 0.01 mg/ml. When treated in RAW 264.7, apparent dose-dependent NO inhibition occurred at dosages of 1.56 to 6.25 μg/ml, and more drastically at 12.5 and 25 μg/ml. At 0.5 to 5.0 μg/ml, SFE-80 exhibited dose-dependent viability suppression and significant tumor necrosis factor alpha (TNF-α) production in Hep 3B, whereas no effect was found in Chang liver cells. Furthermore, no effect was observed in RAW 264.7 at dosages of 3.13 to 25 μg/ml, indicating that SFE-80 exhibited a noncytotoxic character. Conclusively, rosemary can be considered an herbal anti-inflammatory and anti-tumor agent.

Key words: *Rosmarinus officinalis* L.; super-critical fluid extraction; anti-inflammation; anti-tumor

Rosemarinus officinalis L. (family Lamiaceae), commonly called Rosemary, is a woody perennial herb with fragrant evergreen needle-like leaves that are often used in cooking. It is native to the Mediterranean region and is now widely spread in European countries. It has been found to act both as a stimulant and as a mild analgesic, and has been in folk use to treat headaches, epilepsy, poor circulation, and many ailments for which stimulants are prescribed. Its extracts have been incorporated into drugs and cosmetics, and used for flavors and fragrance in foods.[1)]

Leaves of *R. officinalis* possess a variety of bioactivities, including antioxidant,[2)] antitumor,[3)] anti-inflammatory,[1,4)] and anti-HIV.[1)] The relevant main constituents are composed of a vast number of polyphenolics, including carnosic acid, carnosol, rosemarinic acid, ursolic acid, *etc.*[2,5–8)] Among these, carnosic acid is the most potent antioxidant constituent, but its drawback is that it is thermal-, oxidation-, and photo-labile. Recently, reports indicated that carnosol is active in anti-inflammation[9)] and is an active antimetastatic against malignant melanocytes.[10)] It is as an effective suppressor of inducible nitric oxidide synthase through modulation of the nuclear factor NF-κB.[9)] In Japan, patented documents in regard to *R. officinalis* and the derivatives of carnosic acid are accumulating, especially with respect to its effects on the synthesis of nerve growth factor (NGF).[11,12)]

In recent years, much effort has been dedicated to the development of herbal therapeutics. Extension of herbal

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESI, slectrospray ionization; FBS, fetal bovine serum; GAE, gallic acid equivalent; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; *i*NO, inducible nitric oxide; LC/MS, liquid chromatography/mass spectrometry; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide; NED, N-(1-naphthyl) ethylenediamine dihydrochloride; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; NPP, *p*-nitrophenyl-phosphate; NO, nitric oxide; PBS, phosphate buffer saline; SFE, supercritical fluid extraction; SWE, subcritical water extraction; TAA, thioacetamide; TNF-α, tumor necrosis factor alpha

applications is expected to modulate activation of extracellular signaling protein tumor necrosis factor (TNF), a major mediator of apoptosis and inflammatory responses.[13] Inflammatory response and oxidative damage are two major factors inducing cardiovascular[14] and neurodegenerative diseases, such as Alzheimer's disease,[15] while herbal polyphenolics are capable of reducing such hazards.

An increasing number of lipid extraction methods are being superseded by supercritical fluid extraction (SFE) methods, which are rapid, automatable, and selective, and avoid the use of large amounts of solvent. Supercritical fluid CO_2 (SF-CO_2) is the most commonly used supercritical fluid because it has modest critical conditions, including the fact that it is readily separated from solutes, poses no environmental problems, and is nonflammable and inexpensive.[16,17] The intrinsic features of SFE are ideal for extraction of natural herbal products from plant materials.[18] SFE is especially indicated for thermo-labile compounds, since extraction is carried out at low temperatures. It provides cleaner plant extracts because the degradation of certain compounds by lengthy exposure to high temperatures and oxygen is avoided; moreover, chlorophylls are insoluble in SF-CO_2. The constituents are more concentrated and nontoxic in the extraction solvent of SF-CO_2.[19] In most cases, SFE is more efficient than conventional extraction methods; changing the extraction temperature and/or pressure allows different classes of compounds to be fractionated.[19] It has been reported that SF-CO_2 provided the highest recovery of carnosic acid (35.7 mg/g), the lowest relative standard deviation (4.4%), and the cleanest extract from rosemary leaves. Among the liquid solvent studies, only acetone provided comparable results, but it requires decoloration with active carbon prior to HPLC analysis.

The aim of the present study was to survey the optimal conditions for extraction of rosemary leaves, and to determine whether the extracts exert antioxidative, anti-inflammatory, or/and anti-tumor bioactivities.

Materials and Methods

Chemicals. 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were from Gibco (Langley, OK). Acetic acid, acetonitrile, methanol, *p*-nitrophenyl-phosphate, and NaOH were from Wako Pure Chemical (Tokyo, Japan). All other chemicals used in this study were from authentic sources and were of the highest grade and purity. Authentic carnosic acid, 12-methoxycarnosic acid, carnosol, rosmarinic acid, methyl carnosate, and gallic acid were also from Sigma Chemical (St. Louis, MO).

Plant materials. R. officinalis L., grown on the local Taichung Transform Farm at the District Agricultural Research and Development Station, Chang-Hua County, Taiwan, was harvested with fresh leaves in January, debranched immediately after harvest, frozen at −80 °C and lyophilized. The dehydrated leaves were then pulverized to a fine powder (#20 mesh) with a comminuted mill (Retsch Ultra Centrifugal Mill and Sieving Machine, Type ZM1, Haan, Germany). The powder thus obtained was stored at −20 °C until use. Light exposure was avoided during storage.

Supercritical fluid CO_2 extraction (SFE). Pulverized desiccated rosemary leaves (5.0 g) were placed in the extraction vessel (10 ml) of the Supercritical Fluid Extraction (SFE) apparatus (ISCO Model SFX 2-10, Lincoln, NE). Extractions with 10% co-solvent of ethanol in supercritical fluid CO_2 (flow rate, 1.0 ml/min) were arbitrarily operated at 5,000 psi (= 345 bar) in combination with different temperatures, 40, 60, and 80 °C respectively, for 1 h statically, followed by an additional 1 h with dynamic extraction. The SFEs were collected into a 20 ml vial that was pre-filled with a trapping solvent, acetone (10 ml), and maintained at 4 °C during the extraction. Extracts were combined and evaporated to dryness on a rotary evaporator at 40 °C under reduced pressure. The concentrated SFE were then weighed and stored at −20 °C. In further analyses, the SFEs were re-dissolved in ethanol to various concentrations, as indicated.

Liquid solvent extraction procedure. Following the method described by Tena and Valcárcel (1997), 3 ml of a liquid solvent (acetone or ethanol) was added to the sample (1.3 g of ground and sieved fresh rosemary leaves); the extraction was sonicated in an ultrasonic bath for 5 min and then centrifuged at 3,500 g for 5 min. The solvent supernatant was transferred to a test tube and the extraction procedure was repeated twice. In order for samples to be applied to HPLC analysis, the highly colored extracts obtained were combined and bleached by adding 0.3 g of active carbon. The mixture was centrifuged and the bleached supernatant was transferred to a volumetric flask and made up to 10 ml. An aliquot of the solvent extracts was directly injected into the HPLC column.

LC/MS analysis. HPLC analyses were carried out using a Finnigan Surveyor Modular HPLC system (Thermo Electron, Akron, USA), which was equipped with an analytical column Luna 3 μ C18(2) 150 × 2.0 mm and a guard column. In addition, a Security Guard C18 (ODS) 4 × 3.0 mm ID (Phenomenex, Torrance, CA) at a flow rate of 0.2 ml/min was used. The mobile phases A (acetonitrile containing 0.1% formic acid) and B (double distilled deionized water containing 0.1% formic acid) were run according to a programmed protocol. Briefly, from 0 to 5 min, the elution was run with 95% B;

from 5 to 35 min, with 95–40% B; from 35 to 55 min, with 40% to 5% B in a linear gradient manner; from 55 to 60 min, 5% B was used; while from 60 to 65 min, an isocratic 95% B was retained. The photodiode array (PDA) detector was operated at wavelengths between 220 and 400 nm. This system was coupled to a Finnigan LCQ Advantage MAX ion trap mass spectrometer, which was operated in electrospray ionization (ESI) mode. An aliquot of sample SFE-80 (20 µl) was directly introduced into the column through the Rheodyne (Model 7725i) injection valve, where nitrogen was used as the nebulizing and drying gas. ESI source and negative ionisation mode were used at different fragment voltages. The normal operation conditions in each part were spray needle voltage, 5 kV; ion transfer capillary temperature, 300 °C; nitrogen sheath gas, 40; and auxiliary gas, 5 (arbitrary units). An ion trap containing helium damping gas was introduced following the manufacturer's protocols. The mass spectra were acquired in an m/z range of 100 to 1,000 with 5 microscans and a maximum ion injection time of 200 ms. The SIM analysis was a narrow scan event that monitored the m/z value of the selected ion within a range of 1.0 Th centered on the peak for the molecular ions, by which analysis of flavonoid and diterpenoid molecular ions by MS/MS in negative ELS mode was proceeded. MS/MS fragment spectra were produced using normalized collision energies with an increment of 5% from 10 to 30%, and also with wideband activation off.

Total phenolic content. Total phenolics were determined according to Thoss *et al.*[20] An aliquot of SFE (0.10 ml) was mixed with Folin-Ciocalteu reagent (Kanto Chemicals, Tokyo; 0.8 ml, 0.2 M) and sodium carbonate solution (0.5 ml, 10%). After it was mixed thoroughly, the mixture was allowed to stand at room temperature for 1 h, and absorbance was taken at 760 nm with a spectrophotometer. A calibration curve was established using gallic acid as the reference standard. The total polyphenolic content was expressed as mg gallic acid equivalent per gram of extract (GAE/g).

Free radical scavenging capability. The scavenging effect of each extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assayed according to the procedure described by Shimada *et al.*[21] An aliquot (4 ml) of methanol solution prepared from different extract concentrations (0.1 to 5.0 mg/ml) was added to 1 ml of freshly prepared methanolic solution containing a final DPPH (Sigma Chemical, St. Louis, MO) radical concentration of 0.2 mM. After it stood for 30 min in the dark, the absorbance of the mixture was measured at 517 nm against an ethanol control with a Thermo Biomate 5 spectrophotometer (Thermo Electron Corporation, San Jose, CA). Measurements were performed in triplicate and the results were treated statistically. The percent scavenging capability was calculated according to the following equation:

$$\% \text{ Scavenging capability} = [(A_{517\,nm} \text{ of control}) - (A_{517\,nm} \text{ of sample}) / (A_{517\,nm} \text{ of control})] \times 100$$

Cells. Human hepatoma cells (Hep 3B) and human normal liver cells (Chang liver) were obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). For determination of the viability of Hep 3B and Chang liver cells, the method described by Su *et al.*[22] was followed. In brief, cells (Hep 3B and Chang liver cells) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37 °C under 5% CO_2. The cells were seeded and allowed to attach to 24-well plates, and then cultivated for 24 h with supplementation with SFE-80 (20 µl/well to give final concentrations of 0, 0.01, 0.10, 0.50, and 1.00 and µg/ml). Prior to determining the number of viable cells, cells in each well were washed with 200 µl of phosphate-buffered saline (PBS) (pH 7.4). After PBS was withdrawn and replenished with 100 µl of substrate solution containing 0.1 M sodium acetate, 0.1% Triton X-100, and 10 mM *p*-nitrophenyl-phosphate, the cells were incubated at 37 °C for 2 h, and then the reaction was terminated by the addition of 10 µl of 1 N NaOH into each well. The contents of the wells were subjected to absorbance determination at 410 nm with an ELISA reader.

Mouse macrophage cell line RAW 264.7 was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). The cells were cultivated in a DMEM growth medium consisting of 10% FBS and 2 mM glutamine in an incubator at 37 °C under an atmosphere of 5% CO_2.

MTT assay for cell viability. Following Mosmann,[23] cells (5×10^5/ml) were seeded onto a 24 well-plate and cultivated in DMEM medium for 24 h, supplemented with SFE-80 at various concentrations as indicated, and incubation was continued for an additional 24 h. To each well, MTT (0.5 mg/ml) was added, and the reaction was allowed to proceed for 1.5 h. The supernatant was sucked off. The precipitated formazan crystals were dissolved in a preset volume of DMSO, and the absorbance was read at 570 nm with an ELISA reader.

TNF-α production in Hep 3B cells. Cells (Hep 3B and Chang liver cells) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37 °C under 5% CO_2. The cells were seeded and cultivated for 24 h to allow them to attach to 24-well plates, and then they were cultivated for an additional 24 h with supplementation with SFE-80 (20 µl/well to give a final concentration of 1.0 µg/ml). To determine whether the Rosemary SFE-stimulated human hepatoma (Hep 3B) and human

immortalized liver cells (Chang liver) would express certain known differentiation-inducing factors, the culture medium was measured for production of TNF-α. A commercial solid-phase enzyme linked immunoassay (EIA) assay kit (Cistron Biotechnology, Pine Brook, NJ) was used for TNF-α assay.

Nitric oxide (NO) expression in RAW264.7 cells. RAW264.7 cells (5×10^5/ml) were seeded onto a 24-well plate and cultivated in DMEM medium for 24 h, supplemented with SFE-80 at various concentrations as indicated, and incubation was continued for an additional 24 h. Aliquots of supernatant (50 µl) were added with sulfanilamide (50 µl), and the reaction proceeded for 10 min, then (N-(1-naphthyl) ethylenediamine dihydrochloride, NED) (50 µl). The reaction mixture was left to stand for 10 min to facilitate the reaction. The absorbance of the water soluble purplish red product was read at 550 nm with an ELISA reader. A calibration curve was established with standard nitrite expressed in µM.

Statistical methods. The statistical significance of the differences among the samples tested was analyzed with Statistical Analysis System (1998) software. As for statistical treatment of the percent yields of SFE-80 from Rosemary leaves, the content of the main constituents in the Rosemary leaves, the scavenging capability of Rosemary SFE on 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radicals, the effects of Rosemary extracts on Hep 3B viability as compared with authentic carnosic acid, and the effects of Rosemary extracts on the viability of Chang liver cells, analysis of variance was performed by one-way ANOVA followed by Duncan's multiple range tests. To compare the effects of Rosemary SFE-80 on nitric oxide expression, the cell viability of RAW 264.7 cells, and cell viability and induction of cytokine TNF-α in Hep-3B and Chang liver cells, Student's t test was performed. Differences were considered to be significant at a level of $p < 0.05$. Results are presented as mean ± S.D. within triplicate samples.

Results

SFE for R. officinalis *was economically optimum at 80 °C and 5,000 psi*

As compared at an operational pressure of 5,000 psi, the percent yield at different temperatures was 2.04, 2.23, and 5.30% respectively at operational temperatures of 40 °C (SFE-40), 60 °C (SFE-60), and 80 °C (SFE-80) (Table 1). Although in a preliminary test (not shown), we found that the highest DPPH free radical scavenging activity resided in SFE-40, its lowest percent yield (2.04%) limited us to further the experiments. Accordingly, the extract of SFE-80 having the most economically optimum percent yield (5.3%) was used in the entire study (Table 1).

Table 1. Percent Yields of SFE from Rosemary Leaves*

Temp. (°C)	% Yield
40	2.04 ± 0.53^a
60	2.23 ± 0.89^b
80	5.30 ± 0.61^c

*SFE conditions: Rosemary leaves were treated with supercritical fluid-CO_2 at 5,000 psi at 40, 60, and 80 °C. SFEs are indicated as SFE-40, SFE-60, and SFE-80.

Results are presented as mean ± S.D. (n = 3) for each experiment, and were statistically analyzed by one-way ANOVA followed by Duncan's multiple-range test. Rows with different superscripts (a–c) indicate significant differences at a confidence level of $p < 0.05$.

LC/MS identified major constituents in fresh rosemary leaves: rosmarinic acid, carnosol, carnosic acid, 12-methoxycarnosic acid, and methyl carnosate

HPLC analysis revealed eight major constituents in rosemary SFE-80. From peak 1 to peak 5 were respectively rosmarinic acid (t_R, 27.11 min, [M − H]-m/z, 359.1), carnosol (t_R, 45.91 min, [M − H]-m/z, 329.2), 12-methoxycarnosic acid (t_R, 46.82 min, [M − H]-m/z, 361.2), carnosic acid (t_R, 50.36 min, [M − H]-m/z, 331.1), and methyl carnosate (t_R, 53.31 min, [M − H]-m/z, 345.1). Other unknown constituents N1 to N3 are also indicated (Fig. 1).

In SFE-40, carnosic acid (36.7 mg/g) and carnosol (12.3 mg/g) were significantly more abundant than any other extract. The carnosic acid contents were 31.3, 30.4, 13.6, and 24.7 mg/g, and those of carnosol were 7.5, 8.2, 5.4, and 3.5 mg/g for extracts SFE-60, SFE-80, ethanolic, and acetonic respectively (Table 1). In addition, rosmarinic acid, the other bioactive constituent in rosemary leaves, was more enriched in extracts SFE-60 (3.3 mg/g) and SFE-80 (3.2 mg/g), and all were significantly different from each other. Considering the percent yield and the recovery of target carnosic acid, and concomitantly to search for the most economical extraction method, we used SFE-80 as a test sample through the entire experiment (Table 2).

SFE of R. officinalis *showed very high total polyphenolic content*

The total polyphenloic content was 158.88 mg GAE/g SFE-80 (data not shown), comparable to the 155.87 mg GAE/g in the acetonic extract of *Psidium gujava* L. leaves, as previously reported.[24)]

SFE of R. officinalis *showed better free radical scavenging than BHT or Trolox in a dose-dependent manner*

In testing free-radical scavenging capability on DPPH radicals, a dose-dependent capability was observed for all SFEs. The significant difference was found at 0.01 mg/ml, at which SFE-80 had the highest scavenging capability, of 81.86%, compared to 40.67 and 42.74% for SFE-60 and SFE-40 respectively (Table 3). At concentrations above 0.01 mg/ml, the three SFEs did not show any difference in this respect. However, SFE-

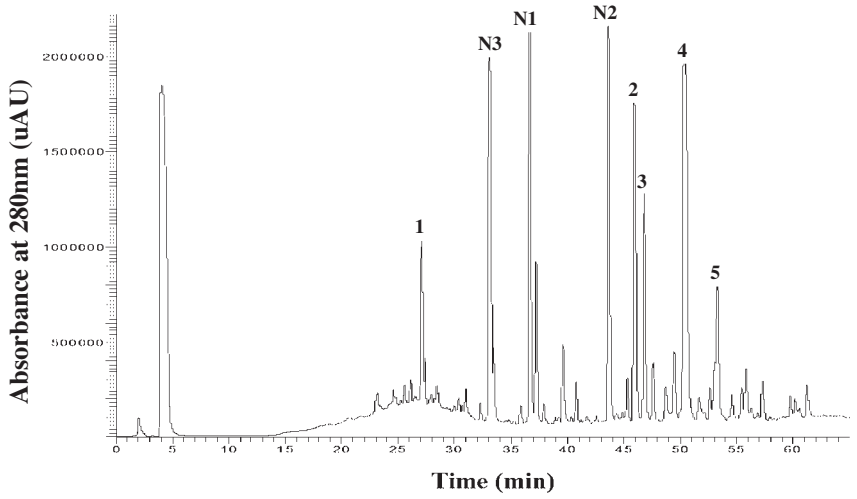

Fig. 1. Characteristic HPLC Chromatogram of SFE-80.
Peak 1, Rosmarinic acid; 2, carnosol; 3, 12-methoxycarnosic acid; 4, Carnosic acid; and 5, methyl carnosate. Three further unknown constituents N1, N2, and N3 are still undefined.

Table 2. Content of Main Constituents of Rosemary Leaves*

Extract	Main constituent (mg/g dry basis of extract)				
	Carnosic acid	Carnosol	Rosmarinic acid	12-methoxy-carnosic acid	Methyl carnosate
SFE-40	36.7 ± 1.56^{a}	12.3 ± 1.12^{a}	0.2 ± 0.11^{a}	0.6 ± 0.08^{a}	0.3 ± 0.25^{a}
SFE-60	31.3 ± 1.26^{b}	7.5 ± 0.77^{b}	3.3 ± 0.26^{b}	2.2 ± 0.27^{b}	3.1 ± 0.63^{b}
SFE-80	30.4 ± 1.77^{b}	8.2 ± 0.87^{c}	3.2 ± 0.17^{b}	2.5 ± 0.32^{c}	3.3 ± 0.23^{b}
EtOH	13.6 ± 1.07^{c}	5.4 ± 0.46^{d}	2.7 ± 0.02^{c}	0.6 ± 0.13^{d}	1.2 ± 0.27^{c}
Acetone	24.7 ± 1.25^{d}	3.5 ± 0.33^{e}	1.4 ± 0.06^{d}	0.3 ± 0.03^{e}	2.7 ± 0.58^{d}

*SFE conditions: Rosemary leaves were treated with supercritical fluid-CO_2 at 5,000 psi and 40, 60, and 80 °C. SFEs are indicated as SFE-40, SFE-60, and SFE-80. EtOH, ethanolic extract; acetone, acetonic extract of rosemary leaf powder.
Results are presented as mean ± S.D. (n = 3) for each experiment, and were statistically analyzed by one-way ANOVA followed by Duncan's multiple-range test. Rows with different superscripts (a–c) indicate significant differences at a confidence level of $p < 0.05$.

Table 3. Scavenging Capabilities of Different Rosemary Extracts on 1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radicals*

Sample (mg/ml)	% Scavenging capability				
	0.0001	0.001	0.01	0.10	1.00
SFE-40	13.12 ± 0.51^{a}	22.45 ± 0.41^{a}	42.74 ± 0.34^{a}	97.62 ± 1.02^{a}	98.20 ± 0.86^{a}
SFE-60	11.56 ± 0.26^{b}	19.32 ± 0.21^{b}	40.67 ± 0.30^{a}	95.23 ± 0.28^{a}	96.59 ± 0.68^{a}
SFE-80	16.48 ± 0.67^{c}	29.61 ± 0.32^{c}	81.86 ± 0.16^{b}	96.65 ± 0.28^{a}	97.17 ± 0.97^{a}
EtOH	2.11 ± 0.54^{d}	6.15 ± 0.55^{d}	11.18 ± 0.57^{c}	20.62 ± 1.75^{b}	45.43 ± 1.63^{b}
Acetone	7.12 ± 0.48^{e}	21.12 ± 1.78^{a}	48.12 ± 1.85^{d}	75.12 ± 2.65^{c}	89.12 ± 2.73^{c}
BHT	20.95 ± 0.10^{f}	31.10 ± 0.76^{c}	79.89 ± 0.94^{b}	95.32 ± 0.14^{a}	96.83 ± 0.08^{a}
Ascorbic acid	4.84 ± 0.06^{g}	8.78 ± 0.19^{e}	13.13 ± 0.37^{e}	41.75 ± 0.48^{d}	90.77 ± 0.60^{d}
Trolox	7.63 ± 0.07^{e}	12.63 ± 0.03^{f}	67.50 ± 0.11^{f}	94.41 ± 0.34^{a}	96.69 ± 0.04^{a}

*SFE conditions: Rosemary leaves were treated with supercritical fluid-CO_2 at 5,000 psi and 40, 60, and 80 °C. SFEs are indicated as SFE-40, SFE-60, and SFE-80. EtOH, ethanolic extract; acetone, acetonic extract of rosemary leaf powder.
Results are presented as mean ± S.D. (n = 3) for each experiment, and were statistically analyzed by one-way ANOVA followed by Duncan's multiple-range test. Rows with different superscripts (a–c) indicate significant differences at a confidence level of $p < 0.05$.

80 at all dosages had better effects than the common reference samples BHT, Trolox, and ascorbic acid. Hence the most economic utilization might be in a concentration range from 0.01 to 0.1 mg/ml of SFE-80. In subcritical water extraction (SWE) of rosemary leaves, the most active compounds had been identified to be carnosol, rosmanol, carnosic acid, methyl carnosate, and some flavonoids such as cirsimartin and genkwain. The antioxidant activity of the fractions obtained by SWE was comparable to those achieved by SFE of rosemary leaves.[25)]

SFE of R. officinalis *effectively suppressed NO production in RAW 264.7 cells*

NO production is a common phenomenon of the inflammation process. In the present investigation, NO

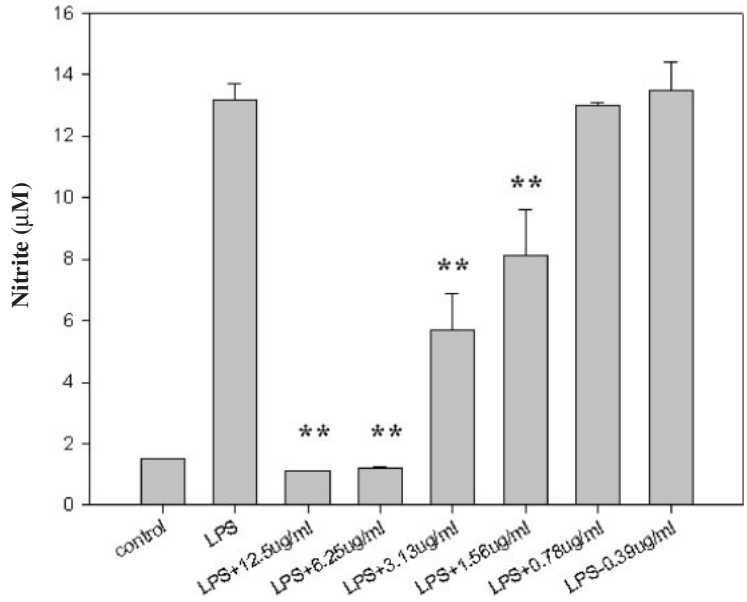

Fig. 2. Effect of Rosemary SFE-80 on the Expression of Nitric Oxide in RAW 264.7 Cells.

Macrophage RAW 264.7 cells (5×10^5/ml) were seeded onto a 24-well plate and cultivated in DMEM medium for 24 h and supplemented with the Rosemary SFE-80, and incubation was continued for an additional 24 h. NO production was analyzed with an ELISA reader. Student's *t*-test was performed for statistical analysis of the data obtained. Results are presented as mean ± S.D. of triplicates. The symbol ** indicates significant difference compared to the LPS control group ($p < 0.01$).

Table 4. Effects of Different Rosemary Extracts on Hep 3B Viability Compared with Carnosic Acid*

	% Viability				
Sample (μg/ml)	0.01	0.1	0.5	1.0	5.0
EtOH	90.27 ± 2.22^a	83.88 ± 2.37^a	63.63 ± 1.80^a	48.45 ± 1.37^a	6.39 ± 0.21^a
Acetone	93.71 ± 0.10^a	86.92 ± 0.03^a	72.69 ± 0.14^b	57.42 ± 0.20^b	21.55 ± 0.02^b
SFE-80	88.51 ± 0.05^b	78.62 ± 0.10^b	41.50 ± 0.11^c	31.48 ± 0.20^c	2.55 ± 0.20^c
Carnosic acid	83.05 ± 0.08^c	72.81 ± 1.12^c	32.53 ± 1.64^d	6.99 ± 0.15^d	2.50 ± 0.52^d

*SFE conditions: Rosemary leaves were treated with supercritical fluid-CO_2 at 5,000 psi and 40, 60, and 80 °C. SFEs are indicated as SFE-40, SFE-60, and SFE-80. EtOH, ethanolic extract; acetone, acetonic extract of rosemary leaf powder.

Results are presented as mean ± S.D. (n = 3) for each experiment, and were statistically analyzed by one-way ANOVA followed by Duncan's multiple-range test. Rows with different superscripts (a–c) indicate significant differences at a confidence level of $p < 0.05$.

production was effectively retarded by SFE-80, although the minimum effective dosage started from 0.78 μg/ml (13.0 μM of NO), and apparent suppression of NO expression was seen to begin only at 1.56 μg/ml, in a dose-dependent manner within 1.56 to 6.25 μg/ml. As can be found in Fig. 2, NO production was 13.0, 8.1, 5.5, 1.2, and 1.0 μM at dosages of 0.78, 1.56, 3.13, 6.25, and 12.5 μg/ml respectively, compared to the LPS control of 13.3 μM (Fig. 2). These results suggest that a potential anti-inflammatory effect is exerted by SFE-80.

SFE of R. officinalis *may possess potent viability-suppressing effect on Hep 3B cells*

When tested for anti-hepatoma bioactivity on Hep 3B cells, an inversely dose-dependent cytotoxic effect was found for all extracts, including the ethanolic-, the acetonic-, and the SFE-80. At concentrations from 0.01 to 5.0 μg/ml, cell viability decreased from 90.27 to 16.39%, from 93.71 to 21.55%, and from 88.51 to 12.55% for the ethanolic extract, the acetonic extract, and SFE-80 respectively, whereas carnosic acid showed a more prominent result, from 83.05 to 2.50% (Table 4). SFE-80 exhibited better effects than ethanolic or acetonic extracts at all concentrations, yet a more prominent effect at concentrations 0.5–1.0 μg/ml was shown by SFE-80. In the contrast, at dosages over 0.1 μg/ml, the cytotoxic effect of carnosic acid apparently prevailed (Table 4). As a reference, the-three extracts appeared to be far less toxic to normal Chang lever cells, and can even be deemed non-toxic (Table 5). For comparison, at moderate concentrations ($\leq$ 1.0 μg/ml), SFE-80 was more toxic to Hep 3B cells than the ethanolic and acetonic extracts (Table 4).

SFE of R. officinalis *induced significant production of TNF-α in Hep 3B cells*

TNF-α has been found to transduce death signals in many previous studies. In the present investigation, Chang liver cells and Hep 3B cells were incubated with Rosemary extract SFE-80 (1 μg/ml) for 48 h. Production of TNF-α was apparently induced more significantly in

Table 5. Effects of Different Rosemary Extracts on Viability of Chang Liver Cells*

Extract (μg/ml)	% Viability				
	0.01	0.1	0.5	1.0	5.0
EtOH	80.88 ± 5.62^{a}	76.11 ± 0.50^{a}	68.77 ± 2.98^{a}	65.77 ± 1.38^{a}	62.77 ± 3.86^{a}
Acetone	74.88 ± 3.02^{b}	71.00 ± 2.08^{b}	65.33 ± 2.08^{a}	63.22 ± 1.83^{a}	58.22 ± 4.28^{b}
SFE-80	87.66 ± 1.76^{c}	81.44 ± 0.19^{c}	73.88 ± 1.67^{c}	70.00 ± 1.20^{b}	64.33 ± 2.60^{a}

*SFE conditions: Rosemary leaves were treated with supercritical fluid-CO_2 at 5,000 psi and 40, 60, and 80 °C, respectively. SFEs are indicated as SFE-40, SFE-60, and SFE-80.
EtOH, ethanolic extract; acetone, acetonic extract of rosemary leaf powder.
Results are presented as mean ± S.D. (n = 3) for each experiment, and were statistically analyzed by one-way ANOVA followed by Duncan's multiple-range test. Rows with different superscripts (a–c) indicate significant differences at a confidence level of $p < 0.05$.

Table 6. Effect of SFE-80 on Cell Viability and Production of TNF-α in Hep-3B and Chang Liver Cells*

Rosemary SCE treatment	Cell number (10^5/ml)	TNF-α (pg/ml)
Chang liver cell (untreated)	23.6 ± 1.2	ND
Chang liver cell (+SFE-80)	22.8 ± 1.4	27.6 ± 3.0
Hep 3B (untreated)	25.6 ± 1.6	11.4 ± 3.0
Hep 3B (+SFE-80)	$7.90 \pm 1.1^{\dagger\ddagger}$	$1{,}576 \pm 75.6^{\dagger\ddagger}$

*Cells (5×10^5/ml) were seeded into 24-well plates and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C under 5% CO_2 for 24 h, and then supplemented with 1 μg/ml of SFE-80 and incubated for an additional 24 h. The culture medium was measured for production of TNF-α.
Results are presented as mean ± S.D. (n = 3). Student's *t*-test was performed for statistical analysis of data. The symbols † and ‡ indicate highly significant difference compared to the untreated Hep 3B and treated Chang liver cells respectively ($p < 0.001$).

Hep 3B DMEM cultures (1,576 pg/ml) than in Chang liver cells (27.6 pg/ml), compared to the 11.4 pg/ml of the untreated Hep B3 control cells, and was not detected (ND) in untreated Chang liver cells (Table 6).

Viability of RAW 264.7 cells was suppressed only at higher doses of SFE-80

The results indicate that the viability of RAW 264.7 cells was almost unaffected by SFE-80 at dosages ranging from 3.13 μg/ml to 12.5 μg/ml. At 25.0 μg/ml, cell viability remained at 95%. Suppression was 68% and 12%, which was observed only at higher dosages of 50 and 100 μg/ml respectively, indicating the non-cytotoxic nature of rosemary SFE-80 towards normal cells (Fig. 3).

Discussion

Higher temperature cost can be compensated by higher yield

Based on equivalent biological effects, SFE-CO_2 extraction was seen to be optimum at 80 °C at 5,000 psi (Table 1). The yield (5.30%) was over 2-fold those obtained either at 40 (2.04%) or 60 °C (2.23%), indicating that operational costs at higher temperatures can be compensated by the yield obtained at 80 °C. As is well known, the transformation of carnosic acid to carnosol is a very fast reaction in the presence of oxygen molecules,[26] while such an SFE technique has the advantage of time-saving extraction. Carnosic acid has been reported to possess very potent antioxidant bioactivity, also. Fast operation to gain higher yield might be a better policy for the production of rosemary extract preparations. In rosemary SFE-80, we identified five constituents: rosmarinic acid, 12-methoxy carnosic acid, carnosol, carnosic acid, and methyl carnosate. The similar analysis by Almela *et al.*,[8] combining HPLC with diode array (DAD) and electrospray ion (ESI) trap-MS, identified several active compounds in rosemary extract: rosmarinic acid, carnosic acid, seven of their terpene-type metablolites, and seven flavones.

Scavenging capabilities are comparable under all conditions

The scavenging capability at dosages from 0.1 to 1.00 mg/ml were comparable under the three temperature conditions, *viz.*, for SFE-40, SFE-60, and SFE-80, and in comparison with Trolox, which has been used as a standard scavenger (Table 3). As can be seen, we found scavenging capabilities of 97.62, 95.23, and 95.32% at 0.01 mg/ml, and 98.20, 96.59, and 96.83% at 1.00 mg/ml respectively for the three operational temperatures (Table 3). All exceeded 95% of scavenging capability, indicating that successful recovery of the active constituents from rosemary even at 80 °C at 5,000 psi is achievable. In contrast, ascorbic acid was found to be far inferior in this respect (Table 3).

The data in Table 2 are, however, inconsistent with those shown in Table 3. Although carnosic acid has been reported to be a potent antioxidant constituent of rosemary and appeared more abundant in SFE-40 (36.7 mg/g) than in SFE-60 (31.3 mg/g) or SFE-80 (30.4 mg/g) on a dry basis (Table 2), the free-radical scavenging effect (Table 2) was not consistently in parallel to their actual content of carnosic acid (Table 2). Obviously, other phenolics contributed to this bioactivity. The varying solubility resulting from different combined proportion of compositions shown in Fig. 1 might exhibit differently in the resultant bioactivity. Speculatively, other than rosmarinic acid, 12-methoxy carnosic acid, methyl carnosate, and some other important phenolics such as epirosmanol, rosmanol, and 7-O-

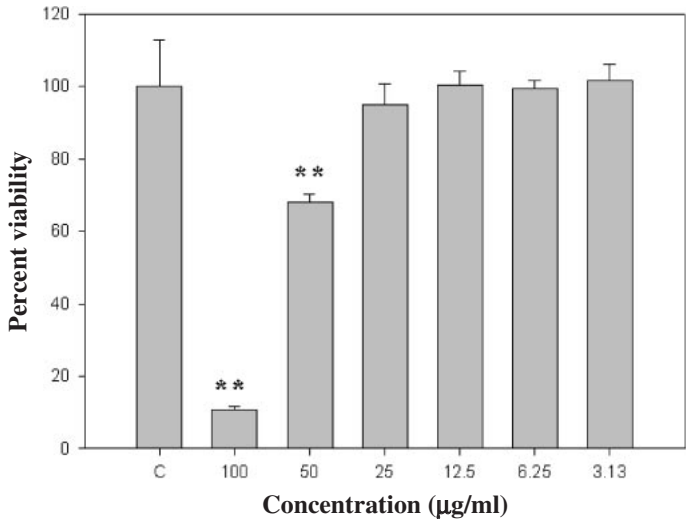

Fig. 3. Effect of Rosemary SFE-80 on the Cell Viability of RAW 264.7 Cells.
Macrophage RAW264.7 cells (5×10^5/ml) were seeded onto a 24-well plate and cultivated in DMEM medium for 24 h and supplemented with the Rosemary SFE-80, and incubation was continued for an additional 24 h. Cell viability was analyzed by MTT assay. Student's *t*-test was performed for statistical analysis of the data obtained. Results are presented as mean ± S.D. of triplicates. The symbol ∗∗ indicates significant difference compared to the control group ($p < 0.01$).

methylrosmanol (epirosmanol methyl ether),[27] unidentified compounds (N1, N2, and N3 in Fig. 1) acted synergistically to exhibit a final integrated scavenging capability.

Anti-inflammatory activity can be attributed to carnosic acid and carnosol

The bioassay-oriented experimentation performed by Altinier *et al.*[4] led to the identification of triterpenes, ursolic acid, oleanolic acid, and micrometric acid as the main anti-inflammatory principles when used externally, while carnosol, a major phenolic diterpenoid component of *Rosmarinus officinalis*, exhibits potent antioxdant and anti-inflammatory activities. As SFE possesses a higher content of carnosic acid and carnosol (Table 2), better anti-inflammatory activity thus can be expected.

As often reported, activators of peroxisome proliferator-activated receptor gamma (PPARγ) can show beneficial anti-inflammatory and anti-tumor effects.[28] In addition to anti-inflammatory and anti-tumor effects, Rau *et al.*[28] demonstrated carnosol and carnosic acid to be the principle constituents of rosemary, and showed that the hypoglycemic effect of rosemary can be attributed to PPARγ activation.

Inhibitory effect of SFE-80 on NO production is not due to cellular toxicity

In chronic inflammation, cytokines induce production of nitric oxide (NO), which is converted to DNA-damaging, carcinogenic peroxynitrite and nitrite. Whereas when SFE-80 was tested on its NO suppressing capability, suppression of NO expression was seen to begin at 1.56 μg/ml, in a dose-dependent manner at 3.13 and 6.25 μg/ml, and a maximum was attained at 25 μg/ml of SFE-80 (Fig. 2). The percent inhibition trend was 13.0, 8.1, 5.5, 1.2, and 1.0 μM at concentrations dosages of 0.78, 1.56, 3.13, 6.25, and 12.5 μg/ml respectively, compared to the LPS control at 13.3 μM (Fig. 2). Since carnosol has been found to be anti-inflammatory and cancer-preventive, cell viability and NO production assays verified that the inhibition was not due to general cellular toxicity (Figs. 2 and 3).[29]

Viability of tumor Hep 3B cells can be prominently effected by SFE-80 at low dosages

Galisteo *et al.*[30] found that an ethanol extract of *R. tomentosus* protected thioacetamide (TAA)-induced cirrhosis, preventing most of the histological changes and pathological functionality alterations.

In the present study, SFE-80 exhibited better effects than ethanolic or acetonic extracts, especially at concentrations of 0.5 to 1.0 μg/ml. Considering the possible toxicity, we performed a bank test on cell viability suppressing capability. We found that normal Chang liver cells were not affected much at lower dosages, ranging from 0.01–1.0 μg/ml. More significant suppression of cell viability was observed only at 5.0 μg/ml. In this regard, the acetonic extract showed higher toxicity (Table 5). SFE-80 exerted less toxicity on normal Chang liver cells, whereas it effectively suppressed the proliferation of hepatic tumor cells Hep 3B. As can be seen in Table 6, TNF-α production was 27.6 and 1576 pg/ml respectively for SFE-80 induced Chang liver cells and Hep 3B cells. These results indicate that the highly effective anti-tumor potential of Rosemary can be attributed to up-regulation of TNF-α. TNF-α has been shown to mediate apoptosis *via* the Fas associated death domain.[31] Co-treatment with TNF-α, TRAIL, and anti-Fas agonist antibody synergistically induced apoptotic cascades in a breast cancer cell line, which require the

involvement of p21.[32] Furthermore, it was reported that xenobiotic can induce TNF-α expression through the p38 MAPK signaling pathway.[33]

Normal cell viability can be suppressed only at high dosages of SFE-80

Almost 95.0% of RAW 264.7 cells remained viable even at a concentration of 25 μg/ml SFE-80. More prominent suppression was observed only at 50 μg/ml (viability 68%) and 100 μg/ml (viability 12%). The results evidenced the nontoxic characteristics of SFE-80 (Fig. 3).

Conclusion

The optimum operation conditions for supercritical fluid CO_2 extraction (SFE) of active constituents from *R. officinalis* L. are 80 °C and 5,000 psi. Five major active principles, including carnosic acid and carnosol, may be present in SFE-80, which possesses non-toxic potent anti-tumor bioactivity at dosages of 0.5 to 5.0 μg/ml, exhibiting inversely related dose-dependent cell viabilities in Hep 3B. In addition, the upregulation of TNF-α induced by SFE-80 at 1 μg/ml can be very large (1,576 pg/ml) in Hep 3B as compared with Chang liver cells (27.6 pg/ml). The anti-inflammatory effect is also significant and showed good dose-dependent responses, since NO production can be greatly reduced by SFE-80. In conclusion, Rosemary SFE-80 can be effective when used as an adjuvant anti-hepatoma and anti-inflammatory therapy.

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