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# LC-MS Method for the Simultaneous Quantitation of the Anti-inflammatory Constituents in Oregano (*Origanum* Species)

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Oregano (*Origanum* spp.), a popular herb in western and Middle Eastern cuisine, was reported to show anti-inflammatory activities in vitro and in vivo but without any information as to the compounds responsible, whether the plants were authenticated or only contained true *Origanum* spp. Using a wide range of botanically authenticated oregano, we were able to show that oregano had anti-inflammatory activity and then using biodirected-guided fractionation, identified the anti-inflammatory agents in oregano as rosmarinic acid, oleanolic acid, and ursolic acid. In this study, we successfully developed an LC-MS (SIM mode) method to achieve coquantitation of these three organic acids with the application of a unique tandem column system. The detection of rosmarinic acid was optimal under negative ion mode of SIM, whereas oleanolic acid and ursolic acid were sensitive to positive ion mode. The simultaneous quantitation was attained by setting two time segments in one run to facilitate the ESI polarity switch. For the investigated analytes romarinic acid, oleanolic acid, and ursolic acid, good linearities ($r^2 > 0.999$) were obtained for each calibration curve. Validation for this method showed a precision (relative standard deviation) ranging from 4.84 to 6.41%, and the recoveries varied from 92.2 to 100.8% for the three analytes. A quantitative survey of these anti-inflammatory constituents in different oregano species (*O. vulgare* ssp. *hirtum*, *O. vulgare*, and *O. syriacum*) and chemotypes within the species varied significantly in their accumulation of rosmarinic, oleanolic, and ursolic acids. Significant variation in chemical composition between species and within a species was found.

**KEYWORDS: Oregano; LC-MS; anti-inflammation; rosmarinic acid; oleanolic acid; ursolic acid**

## INTRODUCTION

Anti-inflammatory drugs are among the most frequently prescribed class of drugs in the world (*1*). The withdrawal of several novel prescription nonsteroidal anti-inflammatory drugs (NSAIDS) from the market due to potential health risk has elevated the safety concern for all such drugs and medicines, particularly those used in the treatment of chronic conditions (*2*, *3*). Meanwhile, natural dietary botanicals with anti-inflammatory activities are gaining increased interest both for their potential novel chemistry and therapeutic activities and for their ability to be made available as dietary supplements, where they are perceived to be of lower cost and less toxic while still providing the targeted health-promoting applications. Oregano (*Origanum* spp.), an aromatic plant in the Lamiaceae family and popular as a fresh or dried culinary herb and food seasoning, has been used since ancient times. Oregano is also a rich source of aromatic volatile compounds, most notably carvacrol, and the essential oil is used in a variety of flavoring and industrial applications. Greek oregano (*O. vulgare* ssp. *hirtum*) represents the most popular oregano species (*4*). The chemical components in oregano volatile oils have long been the focus of plant biologists and food scientists interested in its aromatic flavoring, antioxidative, antibacterial, and antiseptic properties. The genus, as other genera in the Lamiaceae family, is well-recognized for its great diversity in aroma and flavor due to the myriad of chemotypes available and from which many new varieties have been bred (*5*−*7*). Earlier work by Calapouzo was one of the first to report that oregano in the market also referred to a particular flavor and aroma since commercial oregano when examined included aromatic plants from many plant genera and over 30 species (*8*). Recently, the water-soluble extract of oregano was reported to inhibit COX-2 secretion showing anti-inflammatory activity in human epithelial carcinoma cells (*9*). Yoshino et al. found that oregano extract exhibited anti-inflammatory activities in mouse models of stress-induced gastritis and contact hypersensitivity (*10*). Moreover, the effect of methanol and aqueous methanol extract of *O. vulgare* ssp. *hirtum* on soybean lipoxygenase was noted, revealing a promising potential of oregano for anti-inflammatory efficacy (*11*). However, these anti-inflammatory studies were all performed on oregano crude

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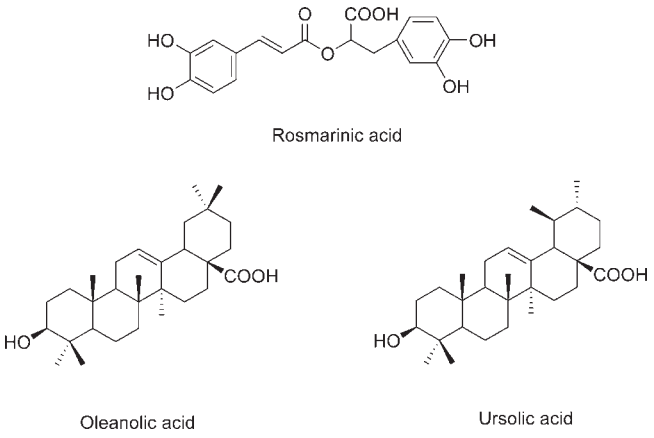


**Figure 1.** Chemical structures of the anti-inflammatory compounds in oregano.

extracts without any information as to what compounds may be responsible. No research has brought these into a molecular level, to isolate and identify those compound(s) responsible for the anti-inflammatory activity in authenticated oregano. We therefore aimed to first structurally elucidate all of these compounds in oregano coupled with bioactivity-guided isolation. These anti-inflammatory constituents, identified as rosmarinic acid, oleanolic acid, and ursolic acid (**Figure 1**), were then subjected to the LPS-induced nitrite production assay and Western blotting of LPS-induced iNOS and COX-2 protein levels in murine cells, and all showed stronger or comparable anti-inflammatory activities compared to the control indomethacin, a recognized anti-inflammatory agent (*12*).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid typically found in Lamiaceae plants such as basil (*Ocimum* spp.), rosemary (*Rosmarinus* spp.), thyme (*Thymus* spp.), and mint (*Mentha* spp.) (*13*). This phenolic compound may function as an antioxidant, scavenging superoxide, hydroxyl radicals and inhibiting oxidation of low-density lipoproteins (*14*, *15*). The anti-inflammatory properties were described by the inhibition of lipoxygenases and cyclooxygenases (*16*, *17*). Oleanolic acid and its isomer, ursolic acid, are triterpenoids, which exist in the plant kingdom as free acids or in the conjugated form known as triterpenoid saponins (*18*). The compound oleanolic acid has been patented in Japan as a health-promoting additive to drinks and marketed in China as a safe nonprescription drug for the treatment of liver disorders (*19*). Many triterpenoids possess anti-inflammatory effects, and oleanolic acid and ursolic acid are among the most notable bioactive triterpenoids (*20*, *21*). The anti-inflammatory mechanism of oleanolic acid and ursolic acid is postulated as simultaneously affecting multiple targets in one or more signaling pathways (*22*−*29*).

Few papers have described the simultaneous quantitation of oleanolic acid and ursolic acid (*30*, *31*), because these two organic acids are position isomers; the only difference between the two is a single methyl group on ring E (Me-30 versus Me-29), which makes their separation for analytical purposes challenging. The selectivity of MS detection is based on the molecular ion and/or the fragments of analytes, whereas collision-induced dissociation of MS detection provides identical molecular ion and fragmentation patterns for these two isomers, so that the MS separation under selected ion monitoring (SIM) or multiple reaction monitoring (MRM) mode cannot be achieved unless they are baseline separated on the HPLC column. Rosmarinic acid differs largely from these two triterpenoid acids relative to chemical polarity, and to date no HPLC method has been reported for the quantification of these three organic acids together in a single run. Therefore, the present work aimed primarily to develop an analytical method to simultaneously quantitate and evaluate these anti-inflammatory organic acids, which was finally achieved by using a tandem column system coupled with MS detector.

## MATERIALS AND METHODS

**Materials.** The solvents methanol (HPLC grade), hexane, ethyl acetate (EtOAc), and acetone (HPLC grade) used for extraction and chromatography were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HPLC-grade water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA) and used for the preparation of all solutions. The standard compound rosmarinic acid and HPLC buffers formic acid and ammonium hydroxide were procured from Sigma Chemical Co. (St. Louis, MO). The Syrian oregano (*O. syriacum*) varieties (SO1−SO5 and SO7) were purchased from Lebanon as dry aerial parts and directly used for HPLC analysis. GO2 (*O. vulgare* ssp. *hirtum*), SO6 (*O. syriacum*), and seeds of a Cuban oregano (*Plectranthus amboinicus*) variety were procured, as a control check, from Richters Herbs (Goodwood, ON, Canada) and propagated in greenhouses of the Department of Plant Biology and Pathology, School of Environmental and Biological Sciences, Rutgers University. The rest of the Greek oregano (*O. vulgare* ssp. *hirtum*) and European oregano (*O. vulgare*) varieties were procured as part of our ongoing germplasm collection at Rutgers University and vegetatively transplanted into the Rutgers Snyder Research and Extension Farm, Pittstown, NJ, with parent plants being maintained and grown in the Rutgers greenhouse. This larger germplasm and breeding collection had been growing under organic conditions as perennial crops with the plots being maintained using drip irrigation and grown on raised beds, harvested once/year at full flowering. The above-ground biomass was manually collected from all live plants at full flowering and placed into a large-scale forced-air dryer at 40 °C for 2 weeks before any analytical study. All oregano varieties were microscopically authenticated by Dr. James Simon of our research team and deposited in Rutgers' botanical products library for future reference.

**Isolation and Identification of Oleanolic Acid and Ursolic Acid.** The standard compounds oleanolic acid and ursolic acid were isolated from oregano samples (*O. vulgare* ssp. *hirtum*) and used for the preparation of calibration standards. The dried oregano leaves (100 g) were extracted three times with ethanol and concentrated to dryness under reduced pressure. The residue was loaded to a silica gel (130−270 mesh) column and eluted by hexane/EtOAc (1:1), EtOAc, EtOAc/acetone (1:1), and acetone in sequence. A total of 16 fractions were collected, and a second fraction containing oleanolic acid and ursolic acid was further subjected to a preparative HPLC separation. The compounds oleanolic acid and ursolic acid were then purified using a Varian C18 preparative column (250 × 41.4 mm, 8 $\mu$m) eluted with methanol/water (8:2). $^{1}$H NMR and $^{13}$C NMR spectra were obtained on a 200 MHz instrument (Varian Inc., Palo Alto, CA).

**Cell Culture.** RAW 264.7 cells, derived from murine macrophages, were procured from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 mg/mL streptomycin. When the RAW 264.7 cells reached a density of (2−3) × $10^6$ cells/mL, they were activated by incubation in the medium containing *Escherichia coli* lipopolysaccharide (LPS, 100 ng/mL). Various concentrations of test compounds dissolved in dimethyl sulfoxide (DMSO) were combined together with LPS. Briefly, the cells were cultured in 100 mm tissue culture dishes and incubated with 100 ng/mL of LPS for 12 h (*32*). The cells were treated with 0.05% DMSO without LPS as vehicle control. The cells were then harvested and plated in a 24-well plate and treated with LPS only or with different concentrations of test compounds for a further 12 h.

**Cytotoxicity Assay.** The RAW 264.7 cells were cultivated at a density of 2 × $10^5$ cells in a six-well plate. The test compounds were added to the medium 18 h after inoculation. The cells were harvested after 18 h. Viability was determined by trypan blue exclusion and microscopy examination.

**Nitrite Assay.** The RAW 264.7 cells were treated with various compounds and LPS or LPS only. At the end of incubation time, 100 $\mu$L of the culture medium was collected for nitrite assay (*32*). The amount of nitrite, an indicator of NO synthesis, was measured using the Griess reaction. Briefly,

supernatants (100 μL) were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with the ELISA reader (Thermo Labsystems, Multiskan Ascent, Finland). The values are expressed as means ± standard error of triplicate tests.

**Western Blotting.** The cells were treated with test compounds (10 and 20 μg/mL) for 24 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2, and β-actin protein was detected by Western blot using specific antibodies (*33*). Quantification of iNOS and COX-2 protein expression was performed by densitometric analysis of the immunoblot. Briefly, the total proteins, isolated from cells after treatment with test compounds for 24 h, were extracted via addition of 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of proteins) were mixed with 5-fold sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and then subjected to 12% SDS−polyacrylamide minigels for electrophoresis at a constant current of 20 mA. The proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl and then immunoblotted with primary antibodies including iNOS, β-actin, and COX-2 (Transduction Laboratories, Lexington, KY). The blots were rinsed three times with PBST buffer followed by incubation with 1:5000 dilution of the horseradish peroxide (HRP)-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA). The transferred proteins were visualized with an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

**Preparation of Calibration Standards for HPLC Analysis.** The stock solution was prepared by dissolving ∼25 mg of each standard, rosmarinic acid, oleanolic acid. and ursolic acid, in 45 mL of methanol in a 50 mL volumetric flask. After sonication for 20 min, the flask was allowed to cool to room temperature and filled to volume with the diluent. Calibration curves were established on 15 data points by diluting the stock solution to cover the expected concentration range for rosmarinic acid, oleanolic acid, and ursolic acid across all of the oregano samples. The linearity ranges of the calibration curves were found to be 390 ng/mL−100 μg/mL for rosmarinic acid, 49 ng/mL−25 μg/mL for oleanolic acid, and 24 ng/mL−25 μg/mL for ursolic acid.

**Analytical Instruments.** Chromatographic analysis was performed on a Waters 2695 HPLC system (Waters Corp., Milford, MA) equipped with an autosampler, a quaternary pump system, a thermostated column compartment, a degasser, and Millenium 3.2 software. Separation was achieved by using a tandem column system: a Synergi 50 × 4.6 mm, i.d. = 4 μm, Polar-RP column (Phenomenex Inc., Torrance, CA) and downstream a Microsorb 100 × 4.6 mm, i.d. = 3 μm, C18 column (Varian Inc., Palo Alto, CA). The mass spectrometer used in this research was a triple-stage quadrupole Quattro II (Micromass Co., Altrincham, U.K.) equipped with the orthogonal Z-spray electrospray ionization (ESI) interface and the acquisition data processor Masslynx 3.4 software.

**Sample Preparation.** All dried oregano samples were first finely ground with a coffee grinder. About 25 mg of powder was accurately weighed from each sample and placed into a 50 mL volumetric flask, and ∼45 mL of methanol was added. Each sample was sonicated for 20 min, allowed to cool to room temperature, and then filled to volume with the diluent. The extract was transferred to a centrifuge tube and centrifuged at 12000 rpm for 2 min to obtain a clear solution and filtered through a 0.45 μm filter for HPLC analysis. The recoveries were validated by spiking each sample with known quantities of the standard compounds, rosmarinic acid, oleanolic acid, and ursolic acid, to approximately 100, 75, and 50% of the expected values in the oregano samples and then extracting according to the same extraction method described above.

**Mass Spectrometry Conditions.** The ESI source of the Quattro II was operated with nitrogen serving as the nebulizing gas (10 L/h) and curtain gas (500 L/h). The source temperature was set at 120 °C, and the desolvation temperature was held at 350 °C. Full-scan mass spectra were obtained with a scan time of 2 s and an interscan delay of 0.1 s, operating in negative ion mode for rosmarinic acid and positive ion mode for oleanolic acid and ursolic acid. For quantitation, a selection of $m/z$ values corresponding to rosmarinic acid ($m/z$ 359, $[M - H]^-$), oleanolic acid ($m/z$ 479, $[M + Na]^+$), and ursolic acid ($m/z$ 479, $[M + Na]^+$), were monitored by using the instrument in the SIM mode with a dwell time of 1 s and an interchannel delay of 0.03 s. The mass spectrometer was set for two time segments: 0−10 min for the detection of rosmarinic acid, where the SIM was carried out in the negative ion mode with the capillary voltage at 3.0 V, cone voltage at 40 V, and extractor voltage at 5 V; and 10−40 min for the detection of oleanolic acid and ursolic acid, and the instrument was set in positive mode with the capillary voltage, cone voltage, and extractor voltage at 3.2, 45, and 8 V, respectively.

**HPLC Analysis.** The mobile phase for chromatographic separation consisted of solvent A (5 mM ammonium formate in water, pH 7.4, adjusted with ammonium hydroxide) and solvent B (5 mM ammonium formate in 90% methanol, pH 7.4) under an isocratic condition (13.5% solvent A and 86.5% solvent B) at a flow rate of 0.8 mL/min. One-fifth of the total effluent was split and injected into the electrospray LC-MS interface. The column compartment temperature was kept at 25 °C, and the injection volume was 10 μL. Calibration curves were plotted using a $1/x$-weighted quadratic model for the regressing of peak area versus analyte concentration, resulting in equations of $y = 2.1655x + 1157.9$ ($r^2 = 0.999$) for rosmarinic acid, $y = 183.14x - 14038$ ($r^2 = 1$) for oleanolic acid, and $y = 270.22x + 22627$ ($r^2 = 0.999$) for ursolic acid. All samples were run in duplicate.

## RESULTS AND DISCUSSION

**Identification of Rosmarinic Acid, Oleanolic Acid, and Ursolic Acid in Oregano by LC-MS and NMR Techniques.** To facilitate the bioactive compound identification, we utilized a bioactivity-guided fractionation approach whereby a LPS-induced nitrite assay and the Western blotting of LPS-induced INOS and COX-2 protein levels in murine cells were employed as the guide to evaluate the anti-inflammatory effect of each fraction during the isolation process. The fractions continuing to show activity were carried through further isolation until the pure compound was obtained. Rosmarinic acid is a phenolic compound found in many culinary herbs within the Lamiaceae family, and several papers have reported its presence in *Origanum* spp. (*34*, *35*). In this study, full-scan LC-MS spectra under negative ion mode provided a pseudo molecular ion at $m/z$ 359 ($[M - H]^-$) and fragmentation ions at $m/z$ 197 ([salvianic acid A − H]$^-$), $m/z$ 179 ([M − H − caffeic acid]$^-$), and $m/z$ 161 ([M − H − salvianic acid A]$^-$) for rosmarinic acid (**Figure 2**). The comparison of the retention time with the commercial reference compound on HPLC further supported the identification of rosmarinic acid in our oregano samples. Oleanolic acid and ursolic acid have been isolated from more than 120 plant species (*18*), but never before have been reported in *Origanum* spp. Oleanolic acid and ursolic acid both produce identical molecular ions and fragmentations on mass spectra (**Figure 2**). The ion peaks at $m/z$ 479 were the sodium adduct molecular ions $[M + Na]^+$ of oleanolic acid and ursolic acid, and the peaks appearing at $m/z$ 511 were designated $[M + MeOH + Na]^+$. Dehydration ($m/z$ 439) and decarboxylation ($m/z$ 411) products were observed as MS fragments. The fragmentation ions at $m/z$ 191 and 203 were due to retro-Diels−Alder (RDA) reactions, the characteristic MS fragmentation of $\Delta^{12}$-unsaturated triterpenoids. The structures of oleanolic acid and ursolic acid were further elucidated by NMR data at the stage of isolated pure compounds (**Table 1**) and compared with the literature (*36*−*39*).

**Anti-inflammatory Bioassays on Rosmarinic Acid, Oleanolic Acid, and Ursolic Acid.** Stimulation of RAW 264.7 cells with LPS results in nitric oxide (NO) accumulation in the media. The nitrite assay measures the amount of nitrite generation, an indicator of NO synthesis. Rosmarinic acid, at a concentration range of 10−100 μg/mL, dose-dependently suppressed NO generation

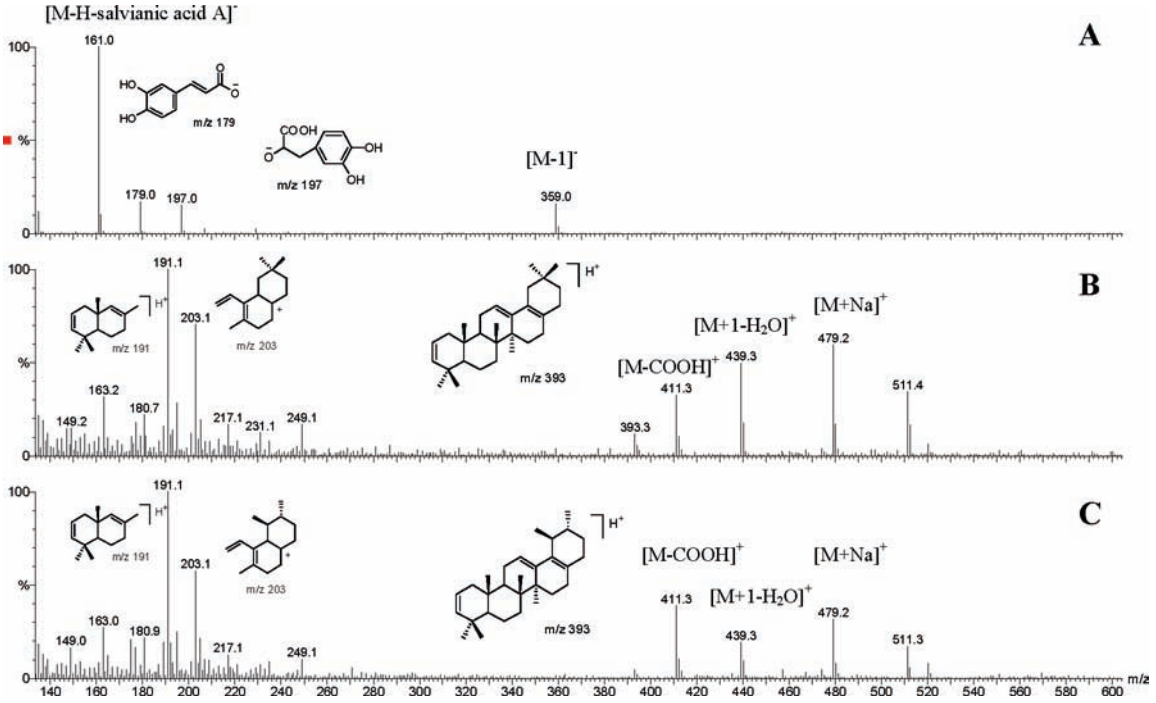


**Figure 2.** Negative (**A**, rosmarinic acid) and positive (**B**, oleanolic acid; **C**, ursolic acid) ESI mass spectra, obtained from the LC-MS TIC of oregano extract.

**Table 1.** $^{13}C$ and $^{1}H$ NMR Assignments for Oleanolic Acid and Ursolic Acid (in Pyridine-$d_5$)

| | oleanolic acid | | ursolic acid | |
|---|---|---|---|---|
| position | $\delta^{13}C$ | $\delta^{1}H$ | $\delta^{13}C$ | $\delta^{1}H$ |
| 1 | 37.8 | | 38.3 | |
| 2 | 27.0 | | 27.1 | |
| 3 | 77.0 | 3.44 m | 77.1 | 3.46 m |
| 4 | 38.3 | | 38.0 | |
| 5 | 54.7 | 0.83 br s | 54.8 | 0.83 br s |
| 6 | 17.7 | | 17.7 | |
| 7 | 32.1 | | 32.5 | |
| 8 | 38.7 | | 38.9 | |
| 9 | 47.0 | | 47.0 | |
| 10 | 36.3 | | 36.2 | |
| 11 | 22.6 | | 22.6 | |
| 12 | 122.9 | 5.49 br s | 124.6 | 5.49 br s |
| 13 | 143.6 | | 138.2 | |
| 14 | 41.1 | | 41.5 | |
| 15 | 27.6 | | 27.6 | |
| 16 | 22.7 | | 23.9 | |
| 17 | 45.5 | | 47.0 | |
| 18 | 40.9 | | 52.5 | 2.64 d ($J$ = 11.2 Hz) |
| 19 | 45.4 | | 38.3 | |
| 20 | 29.8 | | 38.4 | |
| 21 | 33.1 | | 30.0 | |
| 22 | 32.1 | | 36.4 | |
| 23 | 27.2 | 1.27 s | 27.8 | 1.24 s |
| 24 | 15.4 | 1.02 s | 15.5 | 1.02 s |
| 25 | 14.4 | 0.88 s | 14.6 | 0.89 s |
| 26 | 16.3 | 1.02 s | 16.4 | 1.05 s |
| 27 | 25.0 | 1.23 s | 22.9 | 1.22 s |
| 28 | 179.3 | | 178.9 | |
| 29 | 32.2 | 0.93 s | 16.5 | 1.00 d ($J$ = 7.2 Hz) |
| 30 | 22.6 | 1.00 s | 20.4 | 0.94 d ($J$ = 5.6 Hz) |

by 19−100% (**Figure 3**). Similar results were obtained for ursolic acid and oleanolic acid, indicating their inhibition of NO production by > 50% at the 20 $\mu$g/mL concentration level. Inhibition of nitrite production was not toxic, as determined by the trypan blue

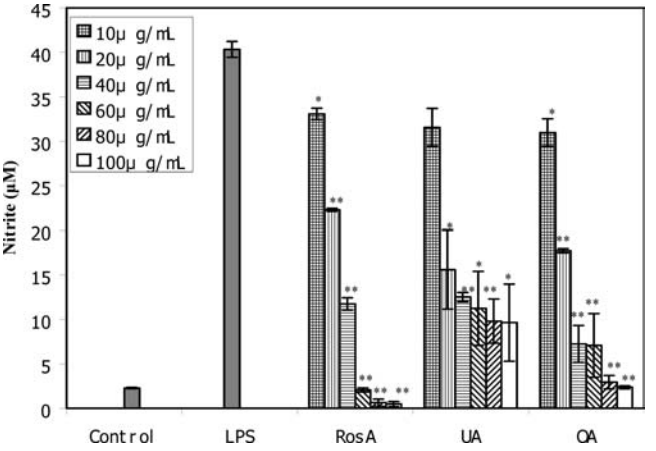


**Figure 3.** Anti-inflammatory effect of compounds rosmarinic acid (RosA), ursolic acid (UA), and oleanolic acid (OA) on LPS-induced nitrite production in RAW 264.7 macrophages, at different concentration levels. The values are expressed as means ± standard error of triplicate tests. *, $P < 0.05$, and **, $P < 0.01$, indicate statistically significant differences from the LPS-treated group.

exclusion assay. COX-2 and iNOS protein expression of rosmarinic acid, ursolic acid, and oleanolic acid was then observed from Western blotting (**Figure 4**). The reduced expression of iNOS protein by these compounds was consistent with reductions on the nitrite production assay. In addition, these three compounds also suppressed COX-2 protein expression on Western blotting, showing comparable anti-inflammatory activities contrasting to indomethacin, a recognized COX inhibitor.

**Analytical Method Optimization.** Separation of organic acids can be difficult due to the retention time shift, peak broadening, and inability to achieve adequate resolution on the chromatogram. The challenge of coquantitation for this research resides in the chemical similarity of oleanolic acid and ursolic acid and the drastic difference of polarity between rosmarinic acid and the two triterpenoid acids. Most commercially available C18 columns

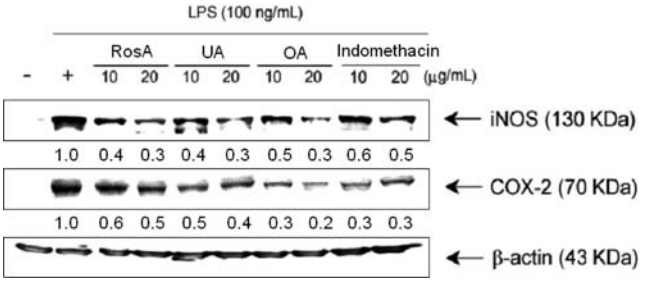


**Figure 4.** Effects of compounds rosmarinic acid (RosA), oleanolic acid (OA), ursolic acid (UA), and indomethacin on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. The values below the figure represent the relative intensity in protein expression of the bands normalized to $\beta$-actin.

were found to lack sufficient resolution to fully separate oleanolic acid and ursolic acid on chromatograms, and some of them even coeluted these two constituents. We found an older type of ODS column (Microsorb C18) provided improved peak resolution between these two position isomers, probably due to the silanol interactions present on the stationary phase. Most acidic buffers provided poor resolution for the separation of these two position isomers, whereas basic buffers caused peak tailing. Ammonium formate as the buffer in mobile phase at slightly basic condition (pH 7.4) was finally found to be optimal to achieve sharp peaks, a baseline separation of oleanolic acid and ursolic acid, and ideal retention time stability. Additionally, the use of ammonium formate enhanced the signal response and improved the sensitivity for MS detection. Finally, the coquantitation of rosmarinic acid with oleanolic acid and ursolic acid was achieved by employing a tandem column system using two different stationary phases: first, a Synergi Polar-RP column from Phenomenex (50 × 4.6 mm) and, downstream, a Microsorb ODS column from Varian (100 × 4.6 mm). The Polar-RP column is an ether-linked phenyl phase with polar end-capping, used to increase retention times of highly polar compounds and offer selective retention on aromatic compounds by $\pi-\pi$ interactions between the aromatic rings of the analyte and the phenyl functional group of the Synergy Polar-RP. Methanol, a protic solvent with pronounced hydrogen-bonding ability, was found to be superior for chromatographic resolution compared to acetonitrile when combining with water as the mobile phase. Also, aromatic selectivity is further enhanced by the presence of methanol in the mobile phase on the Synergy Polar-RP column due to the ability of methanol to facilitate $\pi-\pi$ interactions between the aromatic rings of rosmarinic acid and the phenol functional group of the stationary phase. In contrast, the $\pi$ electrons of the "CN" bond in acetonitrile are presumed to compete for the phenyl binding sites on the stationary phase (*40*).

Although rosmarinic acid, oleanolic acid, and ursolic acid are all organic acids, they perform differently under electrospray ionization. Rosmarinic acid is more sensitive for ionization under negative mode, whereas the optimal ionization condition for oleanolic acid and ursolic acid is under positive ion mode. The polarity switch is attained by creating two time segments in a single run where the polarity mode changed from negative (0−10 min) to positive (10−40 min).

**Validation of the LC-MS Method.** The precision of this method was validated by carrying out six replicate determinations of a single oregano sample on the same day (intra-assay) and six analytical batches on three different days (interassay). The relative standard deviations (RSDs) of intra-assay were 6.16, 5.37, and 4.84% for rosmarinic acid, oleanolic acid, and ursolic acid, respectively. Interassay RSDs were 6.22, 6.41, and 5.31% for those same analytes. The recoveries of this method were evaluated by the addition of known concentrations of the standards, rosmarinic acid, oleanolic acid, and ursolic acid, at three concentration levels, approximately 100, 75, and 50% of the expected values in the oregano sample GO12 (*O. vulgare* ssp. *hirtum*). No significant difference was found among the recoveries at different concentration levels, with RSDs being 3.0, 3.4, and 2.0% for 100, 75, and 50% spiked levels, respectively (**Table 2**). The mean recoveries were calculated as 98.2% for rosmarinic acid, 97.4% for oleanolic acid, and 94.2% for ursolic acid. These validation studies showed that our newly developed analytical method was reliable, precise, and sensitive for the simultaneous quantitation of rosmarinic acid, oleanolic acid, and ursolic acid in oregano samples.

**Table 2.** Recoveries of Rosmarinic Acid, Oleanolic Acid, and Ursolic Acid at Different Added Levels

| analyte | concentration (g/kg) | added (g/kg) | detected (g/kg) | recovery (%) | mean (%) | RSD (%) |
|---|---|---|---|---|---|---|
| rosmarinic acid | 48.63 | 44.40 | 93.36 | 100.7 | | |
| | | 33.30 | 80.24 | 94.9 | 98.2 | 3.0 |
| | | 22.20 | 70.57 | 98.8 | | |
| oleanolic acid | 2.50 | 2.07 | 4.59 | 100.8 | | |
| | | 1.55 | 4.01 | 97.2 | 97.4 | 3.4 |
| | | 1.04 | 3.47 | 94.1 | | |
| ursolic acid | 8.48 | 9.32 | 17.30 | 94.6 | | |
| | | 6.99 | 14.92 | 92.2 | 94.3 | 2.0 |
| | | 4.66 | 12.95 | 95.9 | | |

**Quantitative Survey of Rosmarinic Acid, Oleanolic Acid, and Ursolic Acid Contents in Different Oregano Varieties.** Different sources, species, and varieties of oregano were compared for the accumulation of these three anti-inflammatory constituents. Several oregano sources were commercially purchased as dry aerial parts directly for analysis; others were vegetatively transplanted to our field research station, with parent plants being maintained and grown in greenhouses. This part of the analysis was not to definitively compare growing conditions or "sources" per se but to first ask whether chemical differences in these three bioactive acids were found among the many oreganos, and if so, then further comparison could be made to the subgroups of oregano from each source (e.g., within the commercial products or within the cultivated species and breeding lines). Most of the varieties belonged to *O. vulgare* ssp. *hirtum*, *O. vulgare*, and *O. syriacum*, which are among the major species of *Origanum* that enter into the global trade (*41*). On the basis of the LC-MS (SIM mode) method developed (**Figure 5**), nearly 30 different oregano sources were quantitatively analyzed for rosmarinic acid, oleanolic acid, and ursolic acid content (**Table 3**). Many *Origanum* varieties were found to be an extraordinarily rich source of the anti-inflammatory constituents, although the variation was large within the same species. Romarinic acid was the predominant compound in the varieties of *O. vulgare* ssp. *hirtum* and *O. vulgare*, ranging from 13.73 to 63.69 mg/g on a dry weight basis. The average levels of oleanolic acid and ursolic acid in these two species were 1.96 and 6.72 mg/g, as calculated from 22 different species and varieties. The sources of *O. syriacum* showed a distinct feature of a high content level of triterpenoid acids, with oleanolic acid averaging 9.40 mg/g in seven different sources and ursolic acid averaging 24.07 mg/g. Cuban oregano (*Plectranthus amboinicus*), a Caribbean native, having the same common name but belonging to another genus in the Lamiaceae family, was also collected and analyzed by using the same LC-MS method as a comparison for analytical purposes. Results showed that none of the three analytes were detected in the aerial part of Cuban oregano, demonstrating again that it is distinct from the true members of *Origanum*. Results of our data allow us to

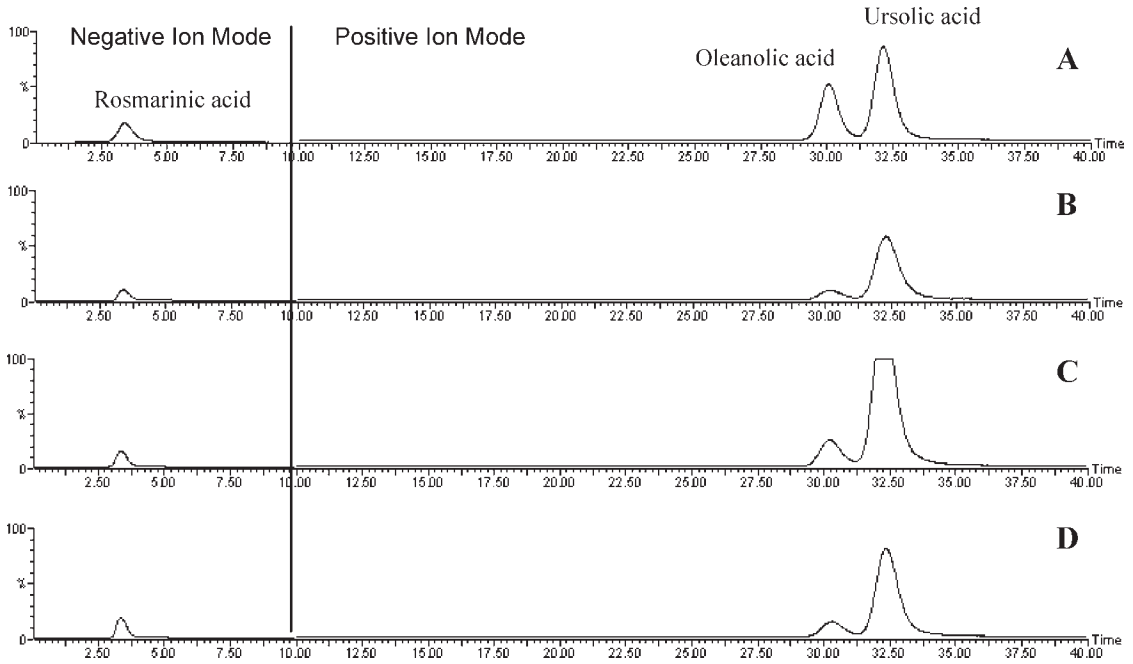


**Figure 5.** Selected ion monitoring chromatogram of (**A**) standards and representative chromatograms of (**B**) sample EO1 (*O. vulgare*), (**C**) sample SO6 (*O. syriacum*), and (**D**) sample GO2 (*O. vulgare* ssp. *hirtum*). The two time segments were set as 0−10 min at *m*/*z* 359 (rosmarinic acid) and 10−40 min at *m*/*z* 479 (oleanolic acid and ursolic acid).

**Table 3.** Contents of the Anti-inflammatory Compounds in Different *Origanum* Species and Varieties or Advanced Rutgers Selection Lines (Milligrams per Gram of Dry Matter)

| sample code[a] | rosmarinic acid[b] | oleanolic acid[b] | ursolic acid[b] |
|---|---|---|---|
| *O. vulgare* ssp. *hirtum* | | | |
| GO1 | 42.56 ± 1.38 | 1.56 ± 0.07 | 5.01 ± 0.11 |
| GO2 | 58.32 ± 0.47 | 3.70 ± 0.23 | 8.59 ± 0.36 |
| GO3 | 43.62 ± 3.05 | 1.48 ± 0.07 | 6.65 ± 0.33 |
| GO4 | 40.32 ± 0.38 | 1.47 ± 0.07 | 4.83 ± 0.11 |
| GO5 | 40.37 ± 3.39 | 2.18 ± 0.03 | 6.86 ± 0.31 |
| GO6 | 37.02 ± 1.24 | 1.90 ± 0.14 | 7.24 ± 0.51 |
| GO7 | 26.12 ± 1.87 | 2.36 ± 0.08 | 8.32 ± 0.48 |
| GO8 | 39.66 ± 0.76 | 2.05 ± 0.15 | 8.34 ± 0.43 |
| GO9 | 63.69 ± 4.54 | 1.07 ± 0.10 | 4.96 ± 0.42 |
| GO10 | 35.01 ± 1.81 | 1.49 ± 0.05 | 5.91 ± 0.46 |
| GO11 | 45.88 ± 0.19 | 1.65 ± 0.11 | 5.80 ± 0.19 |
| GO12 | 48.63 ± 1.81 | 2.50 ± 0.12 | 8.48 ± 0.26 |
| GO13 | 50.94 ± 1.96 | 1.55 ± 0.11 | 5.36 ± 0.21 |
| GO14 | 41.16 ± 1.69 | 1.43 ± 0.10 | 4.70 ± 0.17 |
| GO15 | 62.33 ± 5.19 | 2.26 ± 0.13 | 8.11 ± 0.30 |
| GO16 | 53.24 ± 0.32 | 1.57 ± 0.12 | 5.33 ± 0.19 |
| *O. vulgare* | | | |
| EO1 | 19.97 ± 0.99 | 2.89 ± 0.13 | 7.78 ± 0.56 |
| EO2 | 14.65 ± 0.79 | 1.99 ± 0.14 | 6.35 ± 0.12 |
| EO3 | 14.49 ± 1.29 | 1.82 ± 0.10 | 7.72 ± 0.23 |
| EO4 | 32.41 ± 2.10 | 1.75 ± 0.11 | 6.66 ± 0.21 |
| EO5 | 13.73 ± 1.17 | 2.96 ± 0.18 | 10.60 ± 0.35 |
| EO6 | 14.17 ± 0.93 | 1.47 ± 0.09 | 4.28 ± 0.13 |
| *O. syriacum* | | | |
| SO1 | 9.13 ± 0.18 | 9.84 ± 0.11 | 23.84 ± 0.74 |
| SO2 | 35.73 ± 3.42 | 9.87 ± 0.57 | 24.65 ± 0.87 |
| SO3 | 27.65 ± 1.21 | 11.03 ± 0.52 | 34.42 ± 0.91 |
| SO4 | 30.60 ± 1.13 | 12.72 ± 0.05 | 29.47 ± 0.71 |
| SO5 | 24.57 ± 0.30 | 8.30 ± 0.08 | 22.04 ± 0.38 |
| SO6 | 40.37 ± 2.03 | 6.99 ± 0.38 | 17.94 ± 0.21 |
| SO7 | 16.02 ± 0.27 | 7.08 ± 0.32 | 16.12 ± 0.21 |

[a] GO1−16 are the varieties and lines of Greek oregano (*O. vulgare* ssp. *hirtum*), EO1−6 are the varieties and lines of European oregano (*O. vulgare*), and SO1−7 are the varieties of Syrian oregano (*O. syriacum*). [b] Mean value ± SD in duplicate injections.

conclude that rosmarinic acid, oleanolic acid, and ursolic acid are the major nonvolatile second metabolites found in *Origanum* spp. These compounds accumulated in the aerial part, and the distribution could vary greatly by species and source. We also conclude that oregano can serve as a rich source of these bioactive anti-inflammatory compounds.

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## LITERATURE CITED

(1) Asero, R. Clinical management of adult patients with a history of nonsteroidal anti-inflammatory drug-induced urticaria/angioedema: update. *Allergy Asthma Clin. Immunol.* **2007**, *3*, 24–30.

(2) Goldkind, L.; Laine, L. A systematic review of NSAIDs withdrawn from the market due to hepatotoxicity: lessons learned from the bromfenac experience. *Pharmacoepidemiol. Drug Saf.* **2006**, *15*, 213–220.

(3) Singh, G.; Triadafilopoulos, G. Epidemiology of NSAID induced gastrointestinal complications. *J. Rheumatol.* **1999**, *26* (Suppl.), 18–24.

(4) Adam, K.; Sivropoulou, A.; Kokkini, S.; Lanaras, T.; Arsenakis, M. Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. *J. Agric. Food Chem.* **1998**, *46*, 1739–1745.

(5) Kulisic, T.; Radonic, A.; Katalinic, V.; Milos, M. Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chem.* **2004**, *85*, 633–640.

(6) Rodrigues, M. R. A.; Krause, L. C.; Caramao, E. B.; Dos Santos, J. G.; Dariva, C.; Vladimir de Oliveira, J. Chemical composition and extraction yield of the extract of *Origanum vulgare* obtained from sub- and supercritical $CO_2$. *J. Agric. Food Chem.* **2004**, *52*, 3042–3047.

(7) Velluti, A.; Sanchis, V.; Ramos, A. J.; Marin, S. Effect of essential oils of cinnamon, clove, lemon grass, oregano and palmarosa on growth of and fumonisin B1 production by *Fusarium verticillioides* in maize. *J. Sci. Food Agric.* **2004**, *84*, 1141–1146.
(8) Calpouzos, L. Botanical aspects of oregano. *J. Econ. Bot.* **1954**, *8*, 222–233.
(9) Lemay, M. Anti-inflammatory phytochemicals: *in vitro* and *ex vivo* evaluation. In *Phytochemicals*; Meskin, M. S., Bidlack, W. R., Randolph, R. K., Eds.; CRC Press: Boca Raton, FL, 2006; pp 41−60.
(10) Yoshino, K.; Higashi, N.; Koga, K. Antioxidant and antiinflammatory activities of oregano extract. *J. Health Sci.* **2006**, *52*, 169–173.
(11) Koukoulitsa, C.; Zika, C.; Hadjipavlou-Litina, D.; Demopoulos, V. J.; Skaltsa, H. Inhibitory effect of polar oregano extracts on aldose reductase and soybean lipoxygenase *in vitro*. *Phytother. Res.* **2006**, *20*, 605–606.
(12) Ismaili, H.; Tortora, S.; Sosa, S.; Fkih-Tetouani, S.; Ilidrissi, A.; Della Loggia, R.; Tubaro, A.; Aquino, R. Topical anti-inflammatory activity of *Thymus willdenowii*. *J. Pharm. Pharmacol.* **2001**, *53*, 1645–1652.
(13) Petersen, M.; Simmonds, M. S. J. Rosmarinic acid. *Phytochemistry* **2003**, *62*, 121–125.
(14) Nakamura, Y.; Ohto, Y.; Murakami, A.; Ohigashi, H. Superoxide scavenging activity of rosmarinic acid from *Perilla frutescens* Britton var. *acuta* F. *viridis*. *J. Agric. Food Chem.* **1998**, *46*, 4545–4550.
(15) Fuhrman, B.; Volkova, N.; Rosenblat, M.; Aviram, M. Lycopene synergistically inhibits LDL oxidation in combination with vitamin E, glabridin, rosmarinic acid, carnosic acid, or garlic acid. *Antioxid. Redox Signal.* **2000**, *2*, 491–506.
(16) Parnham, M. J.; Kesselring, K. Rosmarinic acid. *Drugs Future* **1985**, *10*, 756–757.
(17) Yamamoto, H.; Sakakibara, J.; Nagatsu, A.; Sekiya, K. Inhibitors of arachidonate lipoxygenase from defatted perilla seed. *J. Agric. Food Chem.* **1998**, *46*, 862–865.
(18) Liu, J. Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.* **1995**, *49*, 57–68.
(19) Chen, L.; Zhang, Y.; Kong, X.; Peng, S.; Tian, J. Synthesis and biological evaluation of nitric oxide-releasing derivatives of oleanolic acid as inhibitors of HepG2 cell apoptosis. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2979–2982.
(20) Price, K. R.; Johnson, I. T.; Fenwick, G. R. The chemistry and biological significance of saponins in foods and feeding stuffs. *Crit. Rev. Food Sci. Nutr.* **1987**, *26*, 27–135.
(21) Mahato, S. B.; Sarkar, S. K.; Poddar, G. Triterpenoid saponins. *Phytochemistry* **1988**, *27*, 3037–3067.
(22) Dai, Y.; Hang, B. Q.; Li, P. Z.; Tan, L. W. Effects of oleanolic acid on immune system and type I allergic reaction. *Acta Pharmacol. Sin.* **1989**, *10*, 381–384.
(23) Tsuruga, T.; Chun, Y. T.; Ebizuka, Y.; Sankawa, U. Biologically active constituents of *Melaleuca leucadendron*: inhibitors of induced histamine release from rat mast cells. *Chem. Pharmacol. Bull.* **1991**, *39*, 3276–3278.
(24) Simon, A.; Najid, A.; Chulia, A. J.; Delage, C.; Rigaud, M. Inhibition of lipoxygenase activity and HL60 leukemic cell proliferation by ursolic acid isolated from heather flowers (*Calluna vulgaris*). *Biochim. Biophys. Acta* **1992**, *1125*, 68–72.
(25) Najid, A.; Simon, A.; Cook, J.; Chable-Rabinovitch, H.; Delage, C.; Chulia, A. J.; Rigaud, M. Characterization of ursolic acid as a lipoxygenase and cyclooxygenase inhibitor using macrophages, platelets and differentiated HL 60 leukemic cells. *FEBS J.* **1992**, *299*, 213–217.
(26) Zhou, C.; Sun, X.; Liu, W.; Shi, H.; Gao, H.; Miao, Y. Effects of oleanolic acid on the immune complex allergic reaction and inflammation. *J. Clin. Pharmacol. Sci.* **1993**, *2*, 69–79.
(27) Ying, Q. L.; Rinehart, A. R.; Simon, S. R.; Cheronis, J. C. Inhibition of human leucocyte elastase by ursolic acid. *Biochem. J.* **1991**, *277*, 521–526.
(28) Dai, Y.; Hang, B. Q.; Tan, L. W. Anti-inflammatory effect of oleanolic acid. *Chin. J. Pharmacol. Toxicol.* **1989**, *3*, 96–99.
(29) Kapil, A.; Shanna, S. Anti-complement activity of oleanolic acid: an inhibitor of C3 tonvertase of the classical complement pathway. *J. Pharm. Pharmacol.* **1994**, *46*, 922–923.
(30) Chen, J. H.; Xia, Z. H.; Tan, R. X. High-performance liquid chromatographic analysis of bioactive triterpenes in *Perilla frutescens*. *J. Pharm. Biomed. Anal.* **2003**, *32*, 1175–1179.
(31) Altinier, G.; Sosa, S.; Aquino, R. P.; Mencherini, T.; Della Loggia, R.; Tubaro, A. Characterization of topical antiinflammatory compounds in *Rosmarinus officinalis* L. *J. Agric. Food Chem.* **2007**, *55*, 1718–1723.
(32) Kim, H.; Lee, H. S.; Chang, K. T.; Ko, T. H.; Baek, K. J.; Kwon, N. S. Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor-$\kappa$B. *J. Immunol.* **1995**, *154*, 4741–4748.
(33) Pan, M. H.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. H.; Lin, J. K. Suppression of lipopolysaccharide-induced nuclear factor-$\kappa$B activity by theaflavin-3,30-digallate from black tea and other polyphenols through down-regulation of I$\kappa$B kinase activity in macrophages. *Biochem. Pharmacol.* **2000**, *59*, 357–367.
(34) Exarchou, V.; Nenadis, N.; Tsimidou, M.; Gerothanassis, I. P.; Troganis, A.; Boskou, D. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. *J. Agric. Food Chem.* **2002**, *50*, 5294–5299.
(35) Hideyuki, M.; Hideyuki, C.; Chikako, A.; Midori, A.; Teruhiko, Y.; Junya, M. DPPH radical scavengers from dried leaves of oregano (*Origanum vulgare*). *Biosci.,Biotechnol.,Biochem.* **2003**, *67*, 2311–2316.
(36) Seto, H.; Otake, N.; Luo, S. Q.; Jin, H. F. Studies on chemical constituents of *Bupleurum* genus. Part I. A new triterpenoid glycoside from *Bupleurum chinense* DC. *Agric. Biol. Chem.* **1986**, *50*, 939–942.
(37) Ahmad, V. U.; Noorwala, M.; Mohammad, F. V.; Sener, B. A new triterpene glycoside from the roots of *Symphytum officinale*. *J. Nat. Prod.* **1993**, *56*, 329–334.
(38) Huang, P.; Wang, L.; Lin, C. New triterpenoids of *Mallotus repandus*. *J. Nat. Prod.* **1999**, *62*, 891–892.
(39) Alves, J. S.; De Castro, J. C. M.; Freire, M. O.; Leitao da-Cunha, E. V.; Barbosa-Filho, J. M.; De Silva, M. S. Complete assignment of the $^{1}$H and $^{13}$C NMR spectra of four triterpenes of the ursane, artane, lupane and friedelane groups. *Magn. Reson. Chem.* **2000**, *38*, 201–206.
(40) Yang, M.; Fazio, S.; Munch, D.; Drumm, P. Impact of methanol and acetonitrile on separations based on $\pi-\pi$ interactions with a reversed-phase phenyl column. *J. Chromatogr., A* **2005**, *1097*, 124–129.
(41) Dogan, S.; Arslan, O.; Ozen, F. Polyphenol oxidase activity of oregano at different stages. *Food Chem.* **2005**, *91*, 341–345.

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