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Antioxidant Properties and Phytochemical Characteristics of Extracts from *Lactuca indica*

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Lactuca indica (Compositae) is an edible wild vegetable, used as a folk medicine in anti-inflammatory, antibacterial, and other medications in Asia. This is the first scientific evaluation of the chemopreventive therapeutic properties of *L. indica* using five antioxidation assay systems. An extract from *L. indica* was found to possess significant free radical scavenging activity, effectively protecting ϕx174 supercoiled DNA against strand cleavage and reducing oxidative stress in human promyelocytic leukemia HL-60 cells. Moreover, extracts of *L. indica* almost totally inhibited nitric oxide production and the mRNA expression of inducible nitric oxide synthase, at a dosage of 100 μg/mL, in LPS-stimulated macrophage RAW264.7 cells. Bioactivity-guided chromatographic fractionation and metabolite profiling coupled with spectroscopic analyses revealed that the six phenolic compounds, that is, protocatechulic acid (**1**), methyl *p*-hydroxybenzoate (**2**), caffeic acid (**3**), 3,5-dicaffeoylquinic acid (**4**), luteolin 7-*O*-β-glucopyranoside (**5**), and quercetin 3-*O*-β-glucopyranoside (**6**), are the major antioxidative constituents in the *L. indica* extract.

KEYWORDS: ***Lactuca indica*; antioxidant activity; inducible nitric oxide synthase (iNOS); phenolic compounds**

INTRODUCTION

Chemotherapy has long been a cornerstone of cancer therapy. Dietary supplements with antioxidant properties are considered as being able to enhance the anticancer effects of chemotherapy, reducing or preventing certain chemotherapy-induced side effects (*1*). Reactive oxygen species (ROS) are essential for life because they are involved in cell signaling and are used by phagocytes for their bactericidal action. However, nonessential production of ROS, for example, oxidative stress, is suggested to be strongly associated with the aging process and certain degenerative diseases including various cancers, cognitive dysfunctions, and coronary heart disease (*2*). Nitrogen oxide and its metabolite, peroxynitrite, are considered to be mutagenic because they can cause deamination of DNA and inactivation of DNA repair enzymes (*3*, *4*) and are predominant effectors of neurodegeneration (*5*). Inducible nitric oxide synthase (iNOS) catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO). Therefore, aberrant or improper up-regulation of iNOS is often considered to be implicated in the oncogenesis and pathogenesis of cancer. Compounds that can selectively inhibit undesirable expression of iNOS may serve as potential cancer chemopreventive candidates.

Complementary and alternative medicine (CAM) is being recognized as an important strategy for the development of new pharmaceuticals or nutraceuticals, as recently advocated by C. E. Koop in *Science* (*6*). A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical and drug research. According to a conservative estimation, 300,000−400,000 plant species grow on Earth; however, only a small percentage have had their phytochemistry and biological function investigated (*7*). Currently, we are systematically screening and evaluating various medicinal Compositae plant species for their potential chemopreventive bioactivities. *Lactuca indica* (Compositae) is an edible wild lettuce widely distributed in Asia and has been popularly used as a folk medicine. The whole plant of *L. indica*, prepared by using boiling water or an ethanolic solution, has been taken orally or topically administered in various medications for its anti-inflammatory and antibacterial activities and to treat intestinal disorders (*8*). However, limited scientifically proven information is available on the bioactivities, pharmacological functions, and specific clinical efficacies of this plant.

In the present study, we show that an *L. indica* extract possesses the ability to scavenge free radicals and reduce oxidative stress in human promyelocytic leukemia HL-60 and macrophage cell lines. Phytochemical studies of crude plant extracts and bio-organically fractionated metabolites of *L. indica* revealed that several phenolic compounds greatly contribute to the antioxidant activities of this plant. These compounds are reported for the first time from this plant. The evidence from this study suggests that *L. indica* could be used as a food supplement for human health care.

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MATERIALS AND METHODS

Plant Materials and Chemicals. *L. indica* plants were collected from Yang-Ming mountain, Taipei, Taiwan (May 2001), and a voucher specimen (LI001) was deposited at the Institute of BioAgricultural Sciences, Academia Sinica. Chemicals and reagents including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), trichloroacetic acid (TCA), quercetin, ascorbic acid, and ϕx174 RF1 DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose was obtained from Bio-Rad (Hercules, CA), and Dulbecco's modified essential medium and RPMI 1640 were from Gibco BRL (Grand Island, NY). All other chemicals and solvents used in this study were of reagent or HPLC grade.

Plant Extract Preparation. Fresh whole plant materials of *L. indica* (3 kg, dry weight ~300 g) were extracted twice by boiling for 2 h with 30 L of distilled water. After filtration with cheesecloth, the filtrates were collected and concentrated by lyophilization, yielding 58 g of crude hot water extracts (yield = 16%, w/dry weight of plant materials). Total crude extract (hot water extracts) was further divided into ethyl acetate (EtOAc) and water fractions by a liquid−liquid partition procedure.

Free Radical Scavenging Activity. The scavenging activity of DPPH radicals by *L. indica* extracts was measured according to the method reported by our group (*9*, *10*). Assays were performed in 3 mL reaction mixtures containing 2.0 mL of 0.1 mM DPPH−ethanol solution, 0.9 mL of 50 mM Tris-HCl buffer (pH 7.4), and 0.1 mL of deionized H_2O (as control) or test plant extracts. After 30 min of incubation at room temperature, absorbance (517 nm) of the reaction mixtures was taken. The inhibitory effect of DPPH was calculated according to the following formula:

$$\text{inhibition (\%)} = [(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})/\text{absorbance}_{\text{control}}] \times 100$$

IC_{50} represents the level at which 50% of the radicals were scavenged by test samples.

Superoxide Scavenging Activity. Superoxide anion was generated by xanthine−xanthine oxidase and detected by using the NBT reduction method (*11*, *12*). Reagents in this study were prepared in 50 mM KH_2PO_4−KOH buffer (pH 7.4). The reaction mixture contained 20 μL of 15 mM Na_2EDTA (pH 7.4), 50 μL of 0.6 mM NBT, 30 μL of 3 mM hypoxanthine, 50 μL of xanthine oxidase solution (1 unit in 10 mL of buffer), and 150 μL of various concentrations of *L. indica* plant extracts or 150 μL of KH_2PO_4−KOH buffer (as a control). The reaction was initiated by the addition of xanthine oxidase at 25 °C, and absorbance values (405 nm) were recorded every 20 s for 5 min using an ELISA reader (Labsystems Multiskan MS). Results were expressed as percentage of inhibition relative to the control, given by

$$[(\text{rate of control} - \text{rate of sample reaction})/\text{rate of control}] \times 100\%$$

Analysis of Hydroxyl Radical-Induced DNA Strand Scission. This assay was performed according to the method of Keum et al. (*13*). The reaction mixture (30 μL) contained 0.3 μg of ϕx174 RF1 DNA, 30 mM H_2O_2, and 10 mM Tris-EDTA buffer (pH 8.0), in the presence or absence of plant extracts. Various amounts of *L. indica* extracts dissolved in 10 μL of ethanol were added to the reaction mixture prior to the addition of H_2O_2. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 30 cm with a 12 W UV lamp (Spectroline, Spectronics Co., Lexington, KY) at room temperature for 10 min. Reactions were terminated by the addition of a loading buffer (0.02% bromophenol blue tracking dye and 40% sucrose). The reactions were then analyzed by 0.8% submarine agarose gel electrophoresis. The gels were stained with ethidium bromide, destained with water, and photographed on a transilluminator.

Reduction of Oxidative Stress in HL-60 Cells. In this study the effect of extracts from *L. indica* on reducing oxidative stress in human promyelocytic leukemia HL-60 cells was evaluated, on the basis of the method reported by Simizu et al. (*14*). HL-60 cells (1×10^6) were obtained from American Type Culture Collection (ATCC) and cultured at 37 °C in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 100 units/mL penicillin and streptomycin in a 5% CO_2 incubator as recommended by ATCC. Cell suspensions (1.5 mL), at a concentration of 3×10^5 cells/well, were seeded in six-well plates (Falcon, Somerville, NJ) and incubated with varying dosages of *L. indica* extracts at 37 °C for 40 min. Cells were then co-incubated at 37 °C with 25 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in the absence or presence of 25 μM H_2O_2 in darkness for 20 min. After incubation, cells were collected and washed once with ice-cold phosphate-buffered saline (PBS) buffer, resuspended in 1 mL of fresh PBS buffer on ice, in the dark, and then subjected to flow cytometry analysis (Coulter EPICS XL flow cytometer; Beckman/Coulter, Fullerton, CA). The percent reduction of H_2O_2-induced DCFH-DA fluorescence was calculated using the formula

$$\text{reduction (\%)} = 100 - [100 \times (I_{\text{extract} + H_2O_2} - I_{\text{control}})/(I_{H_2O_2} - I_{\text{control}})]$$

where $I_{\text{extract} + H_2O_2}$, $I_{H_2O_2}$, and I_{control} are the mean of DCF fluorescence values in cells treated with extract plus H_2O_2, in cells treated with H_2O_2 alone, and in untreated cells, respectively.

Nitric Oxide (NO) Inhibition Assay. NO inhibition activities of hot water extracts of *L. indica* were performed according to the method reported by Hwang et al. (*15*) with minor modifications. RAW264.7 cells, a murine macrophage cell line, were obtained from ATCC and cultured at 37 °C in Dulbecco's modified essential medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator, as recommended by ATCC. Briefly, RAW264.7 cells grown on a 100 nm culture dish were seeded in 96-well plates at a density of 2×10^5 cells/well. Adhered cells were then incubated for 24 h with or without 1 μg/mL of lipopolysaccharide (LPS), in the absence or presence of *L. indica* extracts. Nitrite concentration was measured, using the supernatant of RAW264.7 cells by the Griess reaction (*16*). One hundred microliter cell culture supernatants were reacted with 100 μL of Griess reagent [1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and the absorbance was recorded using an ELISA reader at 540 nm (Labsystems Multiskan MS). $NaNO_2$ was used as an external calibration. Data were expressed at the total μM nitrite produced by 2×10^5 RAW264.7 cells for 24 h.

RT-PCR Analysis. RAW264.7 cells grown on a 100 mm culture dish were incubated for 6 h with or without various concentrations of *L. indica* extracts and 1 μg/mL LPS. After three washings with ice-cold calcium and magnesium-free PBS, the cells were harvested. Total RNA was isolated using TRIzol reagent (Life Technologics, St. Paul, MN). Total RNA (1 μg) was used to synthesize first-stranded cDNA, using the Titan one-tube RT-PCR system (Roche), according to the manufacture's instruction. Amplification of the inducible nitric oxide synthase (iNOS) gene used the following primers: 5′-CAG AAG CAG AAT GTG ACC ATC-3′ (sense) and 5′-CTT CTG GTC GAT GTC ATG AGC-3′ (antisense) (*15*). The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control in a similar procedure using the following primers: 5′-CCA TCA ATG ACC CCT TCA TTG ACC (sense) and 5′-GAA GGC CAT GCC AGT GAG CTT CC-3′ (antisense). The PCR amplification conditions were as follows: 94 °C for 2 min for 1 cycle, then 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles. The amplified PCR products were analyzed on 1% agarose gels and visualized by staining with ethidium bromide.

Compound Isolation and Index Compound Quantification. The hot water extracts of *L. indica* (30 g in 300 mL of water) were twice further extracted with equal volumes of ethyl acetate (EtOAc) to give EtOAc (6 g) and water (21 g) soluble fractions. The EtOAc fraction was further separated by semipreparative high-performance liquid chromatography (HPLC) using a Waters HPLC system equipped with a Waters 600 controller, a Waters Delta 600 pump, and a 2487 Duel λ absorbance detector. A 5 μm C-18 column (250 mm × 10 mm, Merck, Darmdstadt, Germany) was employed in this study with two solvents systems, 20% MeOH (A) and 100% MeOH (B). The gradient elution profile was as follows: 0−20 min, 100−20% A to B (linear gradient); 20−35 min, 20−80% A to B (linear gradient); 35−50 min, 80−100% A to B (linear gradient); the flow rate was 3.5 mL/min, and the detector

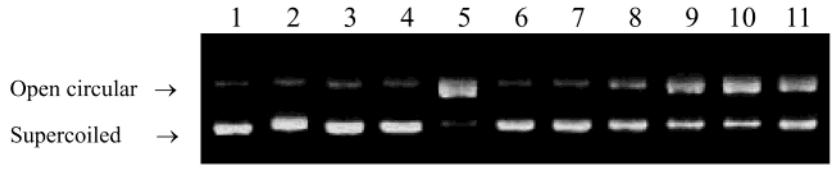

Figure 1. Inhibitive effect of *L. indica* extracts on hydroxyl radical-induced DNA strand scission: lane 1, native supercoiled DNA without any treatment; lane 2, DNA treated with UV irradiation alone; lanes 3 and 4, DNA samples treated with 10000 and 1000 μg/mL *L. indica* extract alone; lane 5, DNA treated with H_2O_2 + UV irradiation; lanes 6–11, DNA samples treated with H_2O_2 + UV irradiation in the presence of 10000, 5000, 1000, 500, 100, and 10 μg/mL *L. indica* extracts, respectively. Results were analyzed by 0.8% agarose gel electrophoresis.

Table 1. Free Radical (DPPH) and Superoxide Anion Scavenging Activities of *L. indica* Extract[a]

	DPPH radical IC_{50} (μg/mL)	superoxide anion IC_{50} (μg/mL)
hot water extract	12.2 ± 0.02	16.0 ± 0.03
EtOAc subfraction	6.1 ± 0.01	*b*
water subfraction	19.4 ± 0.02	*b*
quercetin[c]	2.3 ± 0.01	3.4 ± 0.02
ascorbic acid[c]	1.5 ± 0.01	3.2 ± 0.02

[a] Each assay was performed in triplicate. [b] Not determined. [c] Quercetin and ascorbic acid were used as reference compounds in this experiment.

wavelength was set at 254 nm. The major compounds in the EtOAc fraction were obtained at retention times of 21.2 min (**1**), 22.7 min (**2**), 24.0 min (**3**), 25.3 min (**4**), 26.8 min (**5**), and 27.3 min (**6**). The structures of compounds **1**−**6** were then elucidated using spectroscopic analyses. UV spectra of test compounds were recorded with a Jasco V-550 spectrometer and IR spectra obtained from a Bio-Rad FTS-40 spectrophotometer. Electron impact mass spectrometry (EIMS) and high-resolution electron impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-958 mass spectrometer, and NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C).

The individual peak area corresponding to quercetin 3-*O*-β-glucopyranoside (**6**), serving as the index compound, in the HPLC profile of the EtOAc fraction was determined at the observed maximal absorbance of OD_{254}. A standard calibration curve (peak area vs concentration) of the candidate index compound (**6**) was obtained at a range of compound concentrations, 5, 10, 50, and 100 μg/mL. Quantification of the index compound in the EtOAc fraction was then performed by HPLC analysis. The peak area of the candidate compound in the chromatogram of the EtOAc fraction (with known loading concentration) was then defined, and its content in the extract was calculated on the basis of the quantity calibrated from the standard calibration curve.

RESULTS

Radical Scavenging Activities of Extracts from *L. indica*. To evaluate the radical scavenging activities of *L. indica* extracts, two different assay systems, namely, the DPPH and NBT assays, were performed. The results are shown in **Table 1**. The IC_{50} values (50% inhibition concentration) of the total hot water extract from *L. indica* for scavenging DPPH radicals and superoxide anions were 12.2 ± 0.02 and 16.0 ± 0.03 μg/mL, respectively. Two well-known antioxidants, quercetin and ascorbic acid, were used as references in this study. The IC_{50} values of quercetin and ascorbic acid were 2.3 ± 0.01 and 1.5 ± 0.01 μg/mL in the DPPH assay and 3.4 ± 0.02 and 3.2 ± 0.02 μg/mL in the NBT test, respectively.

Inhibition of Hydroxyl Radical-Induced DNA Strand Scission. The protective effect of *L. indica* extract on ϕx174 RF1 DNA cleavage by hydroxyl radicals, generated from UV-irradiated H_2O_2, was evaluated. **Figure 1** shows supercoiled DNA irradiated with UV (lane 2) or incubated with different concentrations of extracts (lane 3 and 4) exhibiting no DNA cleavage relative to the native form of DNA (lane 1). However, treating the native DNA with both H_2O_2 and UV caused >90% conversion of the DNA from a supercoiled DNA form to an open circular form (lane 5). Extracts of *L. indica* were able to reduce the DNA strand scissions in a dose-dependent manner (lanes 6−11). A detectable effect on the prevention of DNA cleavage was found at the extract dosages between 10 and 500 μg/mL (lanes 9−11), with significant protection observed at 1000−10000 μg/mL of *L. indica* extract. At a dose of 5000 μg/mL (lane 7) the supercoiled ϕx174 RF1DNA was virtually totally protected, in comparison to native DNA (lane 1) and DNA attached by hydroxyl radicals (lane 5).

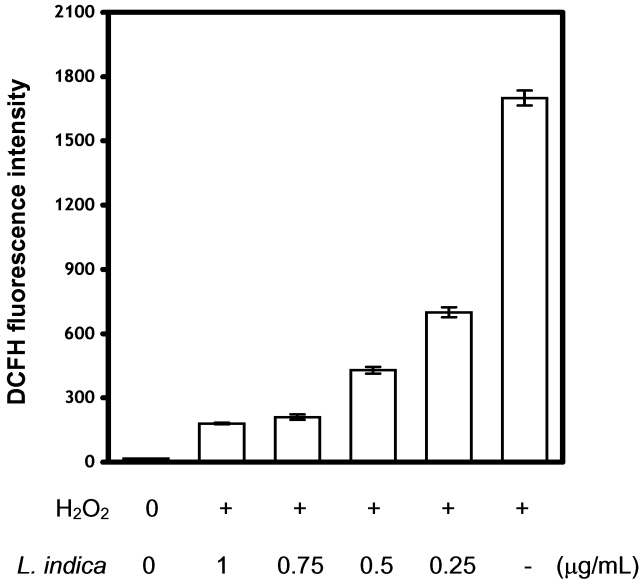

Figure 2. Reductive effect upon oxidative stress in human HL-60 cells treated with various concentrations of hot water extract of *L. indica* as indicated.

***L. indica* Extract Reduces Oxidative Stress in Mammalian Cell Lines.** The effect of *L. indica* extracts on oxidative stress in HL-60 cells, induced by the addition H_2O_2, was investigated using flow cytometry analysis using DCFH-DA, a free radical-sensitive indicator. When DCFH-DA is oxidized by reactive oxygen species (ROS), for example, H_2O_2, it is converted to 2′,7′-dichlorofluorescein (DCF) and emits green fluorescence. The fluorescence intensity, related to the H_2O_2 concentration in the culture medium, can thus be measured quantitatively by flow cytometric analysis. **Figure 2** reveals the effectiveness of hot water extracts of *L. indica* in reducing oxidative stress in HL-60 cells. A 106-fold increase in fluorescence intensity in human promyelocytic leukemia HL-60 cells, relative to the untreated cells, was detected when the cells were treated with 25 μM H_2O_2. Reductions of approximately 58 and 90% in the total increased DCF fluorescence intensity of test cells were observed in cells co-incubated with 250 and 1000 μg/mL of *L.*

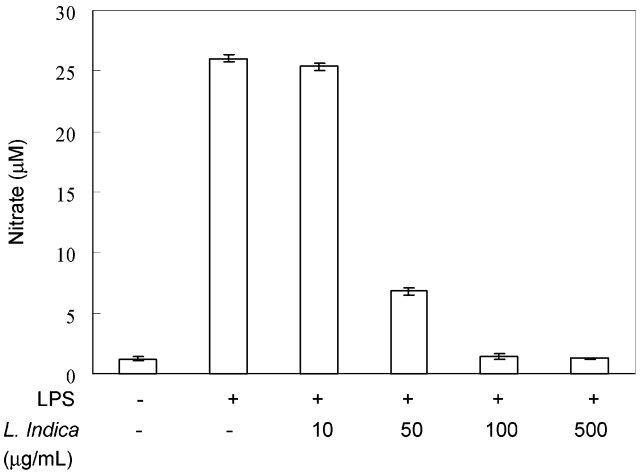

Figure 3. Inhibitory effect of *L. indica* extracts at 10, 50, 100, and 500 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells.

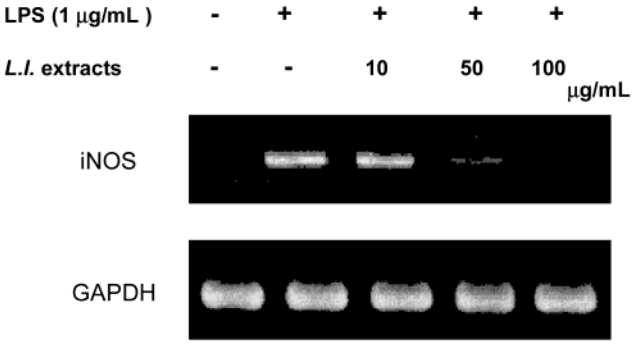

Figure 4. Effect of *L. indica* on the expression of iNOS mRNA in LPS-stimulated RAW264.7. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for the experiment.

indica plant extract, respectively. These results suggest that the damage from oxidative stress in test cells could be greatly alleviated by *L. indica* extracts.

It is known that activation of iNOS with proinflammatory agents such as LPS can significantly increase NO production in macrophages (*17*, *18*). In this study, the LPS-stimulated RAW264.7 cell assay was employed to evaluate the NO inhibition activity of *L. indica* extracts. **Figure 3** shows the inhibitory effect of *L. indica* plant extracts on NO production in LPS-stimulated RAW264.7 cells. The NO levels in test cells with and without LPS stimulation were 1.16 ± 0.08 and 25.98 ± 1.04 μM, respectively. After LPS-treated cells were co-incubated with *L. indica* extracts, NO production was significantly inhibited. The NO levels were detected as 25.38 ± 1.2, 6.86 ± 0.03, 1.43 ± 0.08, and 1.25 ± 0.02 μM in the presence of 10, 50, 100, and 500 μg/mL of *L. indica* extracts, respectively. In fact, >94% of NO, in LPS-stimulated RAW264.7 cells, was diminished after treatment with 100−500 μg/mL of *L. indica* extract.

Effect of *L. indica* Extracts on iNOS mRNA Expression. iNOS is known to be involved in the synthesis of NO. To evaluate potential mechanisms for the inhibition of NO production, we further investigated the effect of the *L. indica* extracts on the expression of iNOS mRNA in LPS-stimulated RAW264.7 cells, using RT-PCR analysis. As shown in **Figure 4**, unstimulated RAW264.7 cells expressed very low or undetectable levels of iNOS; in contrast, iNOS mRNA expression was drastically induced after the test cells were incubated with 1 μg/mL LPS for 6 h. *L. indica* extracts effectively suppressed LPS-induced iNOS mRNA expression in a dose-dependent manner. Extracts of *L. indica* at 50 μg/mL inhibited ∼75% of LPS-induced iNOS expression; total inhibition was observed at a dosage of 100 μg/mL. iNOS mRNA was not detected in non-LPS-stimulated RAW264.7 cells in the presence of *L. indica* extracts (100 μg/mL), and the test cells were healthy and viable, as determined by MTT colorimetric assay, as described elsewhere (*19*) (data not shown).

Extract Fractionation and Compound Identification. The hot water extract of *L. indica* was further divided into EtOAc and water fractions through liquid−liquid partition, and the antioxidant activities of these subfractions were evaluated using DPPH assays. **Table 1** revealed that the EtOAc fraction exhibited the highest antioxidant activity among the tested extracts. The IC_{50} values for scavenging DPPH radicals were 6.1 ± 0.01 (EtOAc fraction), 12.2 ± 0.02 (total crude extract), and 19.4 ± 0.02 μg/mL (water fraction). These results indicated that the free radical scavenging activity of the *L. indica* extracts could be effectively enriched by further partition of the total crude extract into an EtOAc fraction. The EtOAc faction was therefore further investigated for its phytochemical characteristics. **Figure 5a** presents the HPLC chromatogram of the EtOAc fraction; six phenolic compounds (**1**−**6**) were isolated and characterized. According to the mass and NMR analyses, compounds **1**−**6** were identified as protocatechulic acid (**1**) (*20*), methyl *p*-hydroxybenzoate (**2**) (*21*), caffeic acid (**3**) (*20*), 3,5-dicaffeoylquinic acid (**4**) (*22*), luteolin 7-*O*-*β*-glucopyranoside (**5**) (*20*), and quercetin 3-*O*-*β*-glucopyranoside (**6**) (*9*). **Figure 5b** shows the standard calibration curves (peak area vs concentrations) of quercetin 3-*O*-*β*-glucopyranoside, ranging from 5 to 100 μg/mL. The linear regression equation was $y = 1.4309x + 0.9379$ and revealed a good linearity ($R^2 = 0.9997$). According to HPLC analysis, each gram of hot water *L. indica* extract contains 27.3 mg of quercetin 3-*O*-*β*-glucopyranoside (**6**) equivalent to 0.57% (w/w) whole dry plant tissue of *L. indica*.

DISCUSSION

The potential chemopreventive therapeutic properties of *L. indica* extracts were evaluated in this study. We have used in vitro biochemical and cellular as well as molecular biological assays to effectively define the bioactivities of plant extracts of *L. indica* against various antioxidative stresses. The antioxidant activity of *L. indica* extracts was first tested as its capacity to scavenge free radicals of DPPH, which has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources (*9*, *10*, *23*). According to the results obtained by Matthäus (*24*), the IC_{50} values for DPPH radical scavenging in various oilseed byproducts were between 150 and 1160 μg/mL. Similar IC_{50} values for the DPPH radical have been found for crude extracts from other plant species, for example, *Cynara scolymus*, *Hierochloe odorata*, and *Sesamum indicum* (*25*−*28*). In comparison, the hot water extract of *L. indica* exhibited a significantly higher ability to inhibit DPPH radicals than the plant extracts mentioned previously, as the IC_{50} value of *L. indica* extract was determined at 12.2 ± 0.02 μg/mL. Moreover, an EtOAc fraction derived from *L. indica* extract exhibited a better free radical inhibition activity (IC_{50} value = 6.1 ± 0.01 μg/mL), suggesting that a number of compounds with potent antioxidant activity have been effectively enriched in the EtOAc fraction.

The superoxide anion is one of the most representative free radicals. In cellular oxidation reactions, superoxide radicals have their initial effects magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents, for example,

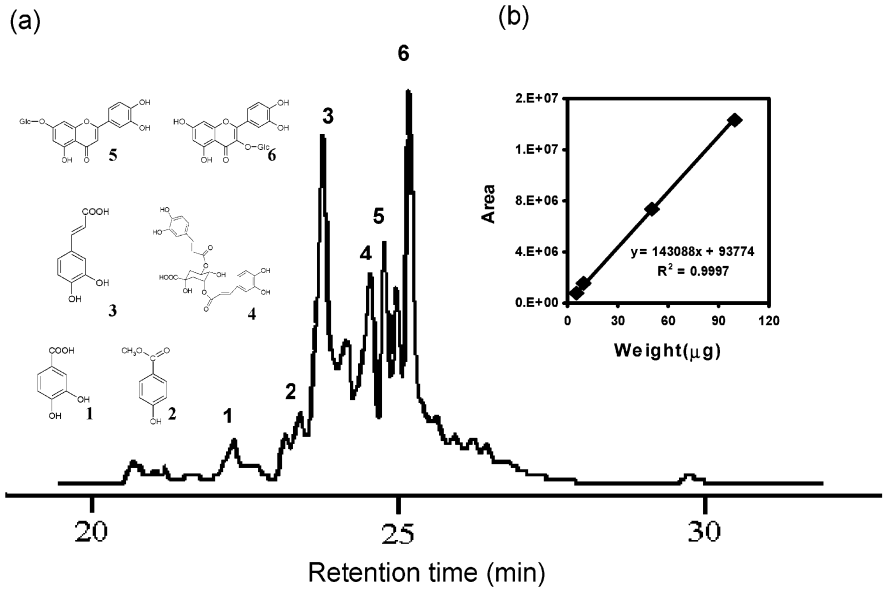

Figure 5. (a) HPLC chromatogram of the EtOAc fraction of the hot water extract of *L. indica*: **1**, protocatechulic acid; **2**, methyl *p*-hydroxybenzoate; **3**, caffeic acid; **4**, 3,5-dicaffeoylquinic acid; **5**, luteolin 7-*O*-β-glucopyranoside; **6**, quercetin 3-*O*-β-glucopyranoside. (b) Standard calibration curve of quercetin 3-*O*-β-glucopyranoside (**6**) content in the EtOAc fraction.

hydroxyl radicals (*29*). Hydroxyl radicals can attack DNA molecules to cause strand scission (*29*). In this study, we found that the hot water extract of *L. indica* could effectively, dose-dependently, inhibit NBT reduction, as induced by hypoxanthine−xanthine oxidase. The 50% inhibition of superoxide anions was determined at a dosage of 16.0 ± 0.03 μg/mL. Incubation of ϕx174 RF1 DNA with both H_2O_2 and UV converts mostly supercoiled DNA (>90%) to its open circular form (**Figure 1**, lane 5). Here we show that a hot water extract of *L. indica*, at a dosage of 5000 μg/mL, could dramatically inhibit DNA strand cleavage under the same UV/H_2O_2 condition. Previously, Keum et al. (*13*) had demonstrated that methanol extracts of heat-processed ginseng could completely prevent DNA strand scission at a dosage of 33000 μg/mL. An ~6-fold lower dosage was observed for *L. indica* extracts, with similar effect to that of the ginseng extract. Furthermore, *L. indica* extract can also significantly reduce the oxidative stress resulting from H_2O_2 or its product, hydroxy radicals, in human promyelocytic leukemia HL-60 cells. Approximately 90% of ROS derived from H_2O_2 in test HL-60 cells were reduced by treatment with 500−1000 μg/mL of *L. indica* extract. These results indicate that *L. indica* extract with a combination of compound mixtures can effectively inhibit free radical activities at chemical and cellular levels.

The metabolite profile and six major phenolic constituents serving as candidate indexing compounds in the active EtOAc fraction of *L. indica* (**Figure 5**) were characterized. The primary goal of this study was to aid quality control measures for the preparation of phytocompounds and plant extract(s), before or in parallel with bioactivity assays. Moreover, none of these phenolic compounds have been previously reported from *L. indica.* They all possess a highly significant free radical-scavenging activity (IC_{50} value = 0.1−5 μg/mL), as individually investigated in this study and elsewhere (*9*, *30*−*32*), suggesting these compounds may play an important role(s) in the observed antioxidant activity of *L. indica*.

The majority of naturally occurring phenolics retain antioxidative and anti-inflammatory properties that appear to contribute to their chemopreventive or chemoprotective activity. Caffeic acid (**4**) displays a very low IC_{50} value for inhibiting DPPH radicals (~0.2 μg/mL) (*30*). It is believed that caffeic acid contributes greatly to the antioxidant activity of *L. indica* extract. Recently, Huang and Yen (*33*) also demonstrated that the phenolic acids (e.g., caffeic acid and 3,5-dicaffeoylquinic acid) could be important antioxidant constituents in *Mesona procumbens*, a popular herbal drink and folk medicine in the Orient for treating various diseases. The bioavailability of caffeic acid and the modification of plasma antioxidant status following red wine intake have been evaluated by Simonetti and coworkers (*34*). Their results indicate that caffeic acid is bioavailable and may be correlated with the antioxidant potential of plasma.

Quercetin metabolites in plasma are also correlated with their antioxidant properties (*35*). We have previously demonstrated that flavonoid glycosides in plant extracts can be effectively converted into their flavonoid aglycons via an in vitro acid−hydrolytic treatment, with the total antioxidant activity being increased drastically in the acid−hydrolytic extract (*9*). This observation is in good agreement with the studies of metabolism and bioavailability of flavonoids and their glycosides in rats and humans (*35*, *36*). In these in vivo studies most of the flavonoid glycosides were found to convert into their aglycons after consumption. Several medicinal properties of flavonoids, for instance, antioxidant activity, anti-inflammatory activity, and the ability to lower the risk of coronary heart disease, have been reported (*37*−*39*). Therefore, we propose here the dietary value and potential benefits of *L. indica* extract on the basis of the phytochemical characteristics of the extract. After consumption of the hot water extract of *L. indica*, as prepared by using a method similar to that reported in this study, the constituent compounds in the extract, for example, caffeic acid and flavonoid glycosides, can retain good antioxidant activities in vivo, which could then contribute to its chemopreventive properties.

Activation of macrophages plays a critical role in the inflammatory process by releasing a variety inflammatory mediators (*40*), such as NO, which can be produced by iNOS. iNOS is one of the important enzyme mediators that mediates inflammatory processes. Improper expression of iNOS has been associated with the pathophysiology of certain types of human

cancers as well as inflammatory disorders (*41*). In the present study an LPS-stimulated RAW264.7 macrophage assay system was employed to evaluate the effects of *L. indica* extract on NO radicals. NO production was detected in LPS-treated RAW264.7 cells at 25.98 μM; a >22-fold increase in NO concentration from 1.16 μM NO at a basal level in test cells was found. However, ~74% of NO induced by LPS in macrophage cells can be effectively inhibited in the presence of 50 μg/mL *L. indica* extract. At 100 μg/mL of extract, the NO concentration in the cells was reduced to a basal level (**Figure 3**). We further determined whether inhibition of NO product was directly related to the ability of *L. indica* to inhibit expression of iNOS. We found that iNOS mRNA expression in macrophage cells could be dose-dependently inhibited after treatment with the hot water extract of *L. indica* (**Figure 4**). At a dose of 50 μg/mL, *L. indica* extract inhibited ~75% of iNOS mRNA expression and almost completely inhibited iNOS gene expression in the presence of 100 μg/mL. The abilities of *L. indica* extract to inhibit NO production and iNOS expression were in good consistency, indicating the NO production was inhibited through the inhibition of iNOS expression. The modulation of NO production by inhibiting iNOS expression is suggested to be of potential therapeutic value in relation to inflammation and septic shock (*41*). Therefore, the similar inhibition actions on NO and iNOS observed for *L. indica* extract may be correlatable to the anti-inflammatory and antibacterial bioefficacies of this herb. Further epidemiological studies on this plant, for nutraceutical or pharmaceutical applications, are warranted.

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