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Antioxidant Properties of Various Solvent Extracts of Total Phenolic Constituents from Three Different Agroclimatic Origins of Drumstick Tree (*Moringa oleifera* Lam.) Leaves

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Water, aqueous methanol, and aqueous ethanol extracts of freeze-dried leaves of *Moringa oleifera* Lam. from different agroclimatic regions were examined for radical scavenging capacities and antioxidant activities. All leaf extracts were capable of scavenging peroxyl and superoxyl radicals. Similar scavenging activities for different solvent extracts of each collection were found for the stable 1,1-diphenyl 2-picrylhydrazyl (DPPH•) radical. Among the three different moringa samples, both methanol and ethanol extracts of Indian origins showed the highest antioxidant activities, 65.1 and 66.8%, respectively, in the β-carotene−linoleic acid system. Nonetheless, increasing concentration of all the extracts had significantly ($P < 0.05$) increased reducing power, which may in part be responsible for their antioxidant activity. The major bioactive compounds of phenolics were found to be flavonoid groups such as quercetin and kaempferol. On the basis of the results obtained, moringa leaves are found to be a potential source of natural antioxidants due to their marked antioxidant activity. This is the first report on the antioxidant properties of the extracts from freeze-dried moringa leaves. Overall, both methanol (80%) and ethanol (70%) were found to be the best solvents for the extraction of antioxidant compounds from moringa leaves.

KEYWORDS: ***Moringa oleifera*** **leaves; flavonoids; quercetin; kaempferol; antioxidant activity; lipid peroxidation; reducing power; free radicals**

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INTRODUCTION

Lipid peroxidation in fats and fatty foods not only brings about chemical spoilage in foods but also produces free radicals or active oxygens such as peroxyl and hydroxyl radicals, which are purportedly associated with carcinogenesis, mutagenesis, and aging (*1*, *2*). The most widely used synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected to cause some safety concerns. Therefore, these antioxidants have been restricted recently, although they have marked antioxidant activity, because such materials may cause liver swelling and influence liver enzyme activities (*3*, *4*). α-Tocopherol, a natural antioxidant, is an effective antioxidant for lipid-containing foods but has limited usage (*5*). The fact that various antioxidants occur naturally in plants has been proven (*6*−*8*). Therefore, identification and development of safer, natural antioxidants is more beneficial. There is also a considerable amount of evidence revealing an association between individuals who have a diet rich in fresh fruits and vegetables and the decreased risk of cardiovascular diseases and certain forms of cancer (*9*, *10*), and it is generally assumed that these dietary elements, responsible for the protective effects, are antioxidant nutrients. However, more recent work has highlighted the additional role of the dietary polyphenolic components of the higher plants (*11*−*13*), which may act as antioxidants or as an agent of other mechanisms that contribute to their anticarcinogenic or cardioprotective actions. Several studies have shown that increased dietary intake of natural phenolic antioxidants correlates with reduced coronary heart diseases (*14*, *15*). Recent epidemiological studies have suggested that diets rich in phenolic compounds are associated with a longer life expectancy (*16*) and have also been found to exhibit many health-related properties because of their antioxidant activities. These properties include anticancer, antiviral, and anti-inflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation (*17*).

For some time it has been recognized that several classes of flavonoids show antioxidant activity toward a variety of readily oxidizable compounds. Flavonoids exist widely in the plant kingdom and are especially common in leaves, flowering tissues, and pollens (*18*). Plant flavonoids are an important part of the diet because of their effects on human nutrition (*19*). These phytochemicals can modulate lipid peroxidation involved in atherogenesis, thrombosis, and carcinogenesis, and their known properties include free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (phospholipase A_2, cyclooxygenase, lipoxygenase), and anti-inflammatory action (*19*). Evidence also suggests that the pharmaco-

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logical effects of flavonoids are correlated with their antioxidant activities. Superoxide radicals have been observed to kill cells, inactivate enzymes, and degrade DNA, cell membrane, and polysaccharide (*20*). Therefore, the study of antioxidant scavenging effects on $O_2^{\bullet-}$ is also one of the most important ways to clarify the mechanism of the antioxidant activity and therefore has aroused a growing interest among researchers.

Moringa oleifera Lam. (drumstick tree, horseradish tree) is indigenous to northwestern India and is often cultivated in hedges and home yards. The tree is valued mainly for the tender pods, which are esteemed as a vegetable (*21*). Flowers and young leaves are also eaten as vegetables. A paste of the leaves is used as an external application for wounds. Moreover, the leaves are a rich source of essential amino acids such as methionine, cystine, tryptophan, and lysine with a high content of proteins (*22*). Decoctions and extracts made from these leaves are also variously employed in native medicine (*23*). Recently Pal et al. (*24*) have reported that the methanol fraction of moringa leaf extract possesses antiulcer activity against induced gastric lesions in rats. On the other hand, pressed juice of the fresh leaves shows strong antibacterial activity against *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, and *Bacillus subtilis*. Nonetheless, the flowers of *M. oleifera* are considered to possess medicinal value as a stimulant, aphrodisiac, diuretic, and cholagogue, and they have been also reported to contain flavonoid pigments such as quercetin, kaempferol, rhamnetin, isoquercitrin, and kaempferitrin (*25*). Ghasi et al. (*26*) have found that administration of the crude leaf extract of *M. oleifera* along with a high-fat diet decreased the high-fat diet-induced increases in serum, liver, and kidney cholesterol levels by 14.4, 6.4, and 11.1%, respectively, in Wistar rats. Recently, Estrella et al. (*27*) reported that *M. oleifera* leaves increase breast milk production day from the third postpartum day to the fifth among mothers who delivered preterm infants. In most parts of The Philippines women consume moringa leaves mixed in chicken or shellfish soups to enhance breast milk production. In southern India, village people use the fresh leaves to prepare cow and buffalo ghee from butter fat. It has been found that there is a significant increase in the shelf life of ghee and that moringa leaves can be a good source of natural antioxidants. Such enhancement of the shelf life of ghee may be due to various types of antioxidant compounds such as ascorbic acid, carotenoids, and phenolic substances, which are present in moringa leaves. Accordingly, there is a strong need for effective antioxidants from natural sources as alternative to prevent deterioration of foods. Naturally occurring materials may provide advantages over synthetic compounds because they may be safer for humans. Moreover, the main sources of natural antioxidants are spices and herbs. Such materials have been used throughout history not only for flavoring but also for their preservative properties. On the other hand, it is also important that food phytochemicals are not consumed in isolated and purified form but in combination with other phytochemicals and food components. Only then could the consumption of such nutraceuticals of plant origins serve a vital role as dietary disease-preventive food components (*28*). However, no efforts have been made to evaluate the naturally available antioxidant principles from the abundantly available moringa leaves throughout the tropics. Therefore, the present study has been conducted to investigate the antioxidant activity of various solvent extracts using different in vitro models and to identify the major antioxidative components of moringa leaves.

MATERIALS AND METHODS

Materials. *M. oleifera* Lam. leaves were collected from India, Nicaragua, and Niger in December 2000. Soon after collection, the leaves were freeze-dried and stored at −18 °C. Linoleic acid, α-tocopherol, BHA, BHT, thiobarbituric acid (TBA), dihydronicotinamide adenine dinucleotide phosphate (NADPH), adenosine 5′-phosphate (ADP), egg lecithin, Trolox, riboflavin, methionine, ammonium thiocyanate, chloroform, Tween 40, and DPPH• stable free radical were purchased from Sigma Chemical Co. (St. Louis, MO). Ascorbic acid, 2,6-dichlorophenolindophenol, metaphosphoric acid, sodium acetate, ferric chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, rutin, potassium chloride, and H_2O_2 were purchased from E. Merck Co. (Darmstadt, Germany). β-Carotene was procured from Fluka Chemie GmbH, Deisenhofen, Germany). Standards of flavonoid aglycons and phenolic acids for HPLC analysis were obtained from different manufacturers: (+)-catechin, (−)-epicatechin, ferulic acid, ellagic acid, myricetin, quercetin, apigenin, kaempferol (Sigma); butein (A-APIN Chemicals Ltd., Abingdon, U.K.), and rhamnetin and isorhamnetin (Extrasynthese, Genay, France). All standards were prepared as stock solutions at 5 mg/50 mL in methanol, except for butein and apigenin (5 mg/50 mL in DMF/methanol, 1:6, v/v) and rhamnetin and isorhamnetin (5 mg/50 mL in DMF/methanol, 1:10, v/v). Stock solutions of the standards were stored in darkness at −18 °C. Standard solutions remained stable over 5 weeks.

Determination of Percentage Dry Weight. Freeze-dried leaves and extracts were accurately weighed and dried to constant weight in an oven at 105 °C for 24 h.

Different Solvent Extractions. Subsamples of freeze-dried powder (5.0 g each) of *M. oleifera* leaf samples from the three different locations were subjected to extraction with 300 mL of water, 80% methanol, or 70% ethanol for 3 h at a controlled temperature. The apparatus consisted of a round-bottom flask with an attached reflux condenser. Each extract was filtered, the solvent was evaporated in vacuo at under 50 °C, and the residue was freeze-dried and stored in a desiccator in the dark until further analysis.

Quantitative Determination of Ascorbic acid. Ascorbic acid was quantitatively determined according to the 2,6-dichlorophenolindophenol dye method described by Jones and Hughes (*29*). The ascorbic acid of both freeze-dried and powdered samples of raw and different solvent extracts of leaves (1 g) was extracted by grinding with a small amount of sand and 6% metaphosphoric acid (v/v). The extract was made up accurately to a suitable volume (15 mL), mixed, and centrifuged at 3000*g* for 15 min at room temperature (24 °C). Five milliliters of the supernatant was titrated against standard 2,6-dichlorophenolindophenol dye, of which 1 mL = 0.2 mg of ascorbic acid; the dye has been previously standardized by titration against a 0.02% standard solution of ascorbic acid in 6% metaphosphoric acid. Results are presented on a dry matter basis.

Extraction and Estimation of Total Phenolics. Powdered freeze-dried (0.5 g) and different solvent extracts of respective leaf samples (0.1 g) were extracted with 40 mL of 80% acetone (80 mL of acetone plus 20 mL of distilled water) under a sonication bath for 25 min. The solution was centrifuged at 3800*g* for 10 min and the supernatant saved. The residue was re-extracted as above; both supernatants were combined and made up to 100 mL with 80% acetone. The total phenolic substances were estimated according to the Folin−Ciocalteu method using gallic acid as standard (*30*).

Extraction of Total Flavonoids. Powdered freeze-dried leaf (1 g) samples were extracted in an apparatus containing a round-bottom flask and reflux condenser with 100 mL of 80% methanol for 3 h, and the extract was filtered. Finally the volume of the extract was made up to 100 mL with 80% methanol, whereas the freeze-dried materials (0.2 g) of different solvent extracts of the respective leaf samples were dissolved in 20 mL of 80% methanol, extracted by using a magnetic stirrer for 2 h at room temperature and centrifuged at 3000*g* for 15 min. These extracts were used for the estimation of total flavonoids.

Estimation of Total Flavonoids. A known volume of extract (0.10 mL) was placed in a 10 mL volumetric flask, and distilled water was added to make 4 mL. To this was added 0.3 mL of $NaNO_2$ (5 g 100

mL^{-1}) and mixed well. Three milliliters of $AlCl_3$ solution (1:100) was added 5 min later. After 6 min, 2 mL of 1 M NaOH solution was added, and the total volume was made up to 10 mL with distilled water. The solution was mixed well again, and the absorbance was measured against a blank at 510 nm with a UV−visible spectrophotometer (Hitachi, U-2000) (*31*). Rutin (E. Merck) was used as the standard for the calibration curve. The flavonoid content was calculated using the following linear equation based on the calibration curve:

$$Y = 0.0014X - 0.0033, \qquad r = 0.9997$$

Y is the absorbance, and X is the flavonoid content in $\mu g\ g^{-1}$.

Separation of Flavonoids in Moringa by HPLC. Extraction and Hydrolysis. The extraction method employed for dried leaf samples was the method of Hertog et al. (*32*). The freeze-dried samples of fresh leaves (0.5 g) and different solvent extracts (0.1 g) were weighed into a 100 mL Erlenmeyer flask and then dispersed in 40 mL of 62.5% aqueous methanol containing 2 g L^{-1} of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA). The mixture was then ultrasonicated for 5 min. To this extract was added 10 mL of 6 M HCl with careful mixing. The extraction solution thus obtained consisted of 1.2 M HCl in 50% aqueous methanol (v/v). The sample was bubbled with nitrogen for 40−60 s, after which the flask was sealed tightly. Hydrolysis was carried out in a shaking water bath at 90 °C for 2 h. After hydrolysis, the extract was allowed to cool and filtered, subsequently made up to 100 mL with methanol, and sonicated for 5 min. Approximately 2 mL was filtered through a 0.2 μm membrane filter prior to injection in HPLC.

Conditions of HPLC and Quantification of Flavonoids. Chromatographic separation of flavonoids was done by a Thermo Hypersil (Runcorn, U.K.) ODS (4.6 × 125 mm, 3 μm) column with a C-18 guard column (Bondapak C18, 4.6 × 10 mm, 10 μm). Both columns were placed in a column oven set at 35 °C. The HPLC system consisted of a Merck Hitachi (Hitachi, Tokyo, Japan) Lachrom Pump L-7100, Lachrom autosampler L-7200, diode array detector (DAD) L-7450, and interface D-7000. Wavelengths used for identification of catechins and identification and quantification of other flavonoids and phenolic acids, with the diode array detector, were 270 nm for ellagic acid; 280 nm for (+)-catechin and (−)-epicatechin; 340 nm for ferulic acid and apigenin; and 370 nm for myricetin, quercetin, butein, kaempferol, isorhamnetin, and rhamnetin. Gradient elution was employed for flavonoids other than catechins with a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (solution A), and acetonitrile (solution B) as follows: isocratic elution 95% A/5% B, 0−5 min; linear gradient from 95% A/5% B to 50% A/50% B, 5−55 min; isocratic elution 50% A/50% B, 55−65 min; linear gradient from 50% A/50% B to 95% A/5% B, 65−67 min; post-time 6 min before next injection. Catechins were quantified using isocratic elution: 86% solution A and 14% solution B (*33*).

The flow rate of the mobile phase was 0.7 mL/min, and the injection volumes were 10 μL of the standards and sample extracts. All flavonoids were quantified using the external standard method. Quantification was based on peak area. Calibration curves of the standards were made by diluting stock standards in methanol to yield 2−10 μg/mL (catechins) and 2−20 μg/mL (other flavonoids and phenolic acids). The samples were prepared and analyzed in triplicate.

Determination of Antioxidant Activity. Antioxidant Activity Determination in Linoleic Acid. The antioxidant activity of sample extracts was determined according to the method of Osawa and Namiki (*5*). Sample extracts (5 mg) of each sample were added to a solution mixture of linoleic acid (0.13 mL), 99.8% ethanol (10 mL), and 0.2 M sodium phosphate buffer (pH 7.0, 10 mL). The total volume was adjusted to 25 mL with distilled water. The solution was incubated at 40 °C, and the degree of oxidation was measured according to the thiocyanate method (*34*), with 10 mL of ethanol (75%), 0.2 mL of an aqueous solution of ammonium thiocyanate (30%), a 0.2 mL sample solution, and a 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl) being added sequentially. After 3 min of stirring, the absorption values of mixtures measured at 500 nm were taken as peroxide contents. A control was performed with linoleic acid but without the extracts. Synthetic antioxidants, BHT, BHA, and ascorbic acid were used as a positive control. The maximum peroxidation level observed at 336 h in the sample that contained no antioxidant component was used as a test point. The percent inhibition of linoleic acid peroxidation, 100 − [(Abs increase of sample at 336 h/Abs increase of control at 336 h) × 100], was calculated to express antioxidative activity.

Antioxidant Activity of Various Solvent Extracts Using the *β*-Carotene—Linoleic Acid System. Two milliliters of a solution of β-carotene in chloroform (1 mg mL^{-1}) was pipetted into a flask containing 40 mg of linoleic acid and 400 mg of Tween 40. The chloroform was removed by rotary vacuum evaporator at 45 °C for 4 min, and 100 mL of distilled water was added slowly to the semisolid residue with vigorous agitation to form an emulsion. A 5 mL aliquot of the emulsion was added to a tube containing 0.2 mL of the antioxidant solution at 500 mg L^{-1}, and the absorbance was measured at 470 nm, immediately, against a blank, consisting of the emulsion without β-carotene (*35*). The tubes were placed in a water bath at 50 °C, and the absorbance measurements were conducted again at 15 min intervals up to 120 min. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of β-carotene using the following formula: $AA = [1 - (A_0 - A_t)/(A'_0 - A'_t)] \times 100$, where A_0 and A'_0 are the absorbance of values measured at zero time of the incubation for test sample and control, respectively and A_t and A'_t are the absorbances measured in the test sample and control, respectively, after incubation for 120 min.

Determination of Reducing Power. The reducing power of different solvent extracts was determined according to the method of Oyaizu (*36*) as described by Yen et al. (*37*). Lyophilized extract (2.5−15.0 mg) in 1 mL of methanol was mixed with a phosphate buffer (5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%); the mixture was incubated at 50 °C for 20 min. A portion (5.0 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 650*g* for 10 min. The upper layer of the solution (5.0 mL) was mixed with distilled water (5.0 mL) and ferric chloride (1.0 mL, 0.1%), and then the absorbance was read spectrophotometrically at 700 nm. A higher absorbance of the reaction mixture indicated greater reducing power.

Determination of the Scavenging of Superoxide Radicals. Superoxide radicals were generated according to a modified method of Beauchamp and Fridovich (*38*) as described by Zhishen et al. (*31*). All solutions were prepared in 0.05 M phosphate buffer, pH 7.8. The photoinduced reactions were performed in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached ∼4000 lx. The total volume of the reactant was 5 mL, and the concentrations of riboflavin, methionine, and nitro blue tetrazolium (NBT) were 3×10^{-6}, 1×10^{-2}, and 1×10^{-4} mol L^{-1}, respectively. The reactant was illuminated at 25 °C for 25 min. The photochemically reduced riboflavins generated $O_2^{\bullet-}$**,** which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. Absorbance (A) was measured at 560 nm. Leaf extracts were added to the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting the NBT reduction. Absorbance (A_1) was measured, and the decrease in $O_2^{\bullet-}$ was represented by $A - A_1$. The degree of scavenging was calculated with the following equation:

$$\text{scavenging (\%)} = (A - A_1/A) \times 100$$

Liposomes Preparation and Determination of Antioxidation Potential. Liposomes were prepared according to the method of Tsuda et al. (*39*) as described by Yen and Chuang (*8*). Egg lecithin (5 g) was dispersed in 500 mL of sodium phosphate buffer (20 mM, pH 7.4) and sonicated in a Vibra-Cell sonicator (Bioblack Scientifica) for 30 min under N_2 atmosphere in an ice-cold water bath. Subsequently, small vesicles were obtained. Substance concentrations (1−5 mg/mL) of the extracts were tested for lipid peroxidation activities as follows: The extract (0.5 mL) was mixed with liposomes (2.0 mL), 25 mM $FeCl_3$ (0.1 mL), 25 mM H_2O_2 (0.1 mL), 25 mM ascorbic acid (0.1 mL), and 0.2 M phosphate buffer (1.2 mL, pH 7.4). The reaction mixture was incubated at 37 °C for 4 h with occasional shaking. At the end of the incubation, 1 mL of BHA (20 mg/mL in methanol) was added to the mixture to terminate the oxidation reaction. The extent of oxidation of

Table 1. Total Phenolics, Flavonoids, and Ascorbic Acid Contents of Freeze-Dried Leaf Samples and Various Solvent Extracts of *M. oleifera* from Different Agroclimatic Zones (Grams per 100 g, Dry Matter Basis)[a]

A. Freeze-Dried Leaf Samples

component	Nicaragua	India	Niger
total phenolics[b]	4.25 ± 0.14	2.94 ± 0.17	3.66 ± 0.21
total flavonoids[c]	4.43 ± 0.21	2.10 ± 0.18	3.81 ± 0.25
ascorbic acid	0.92 ± 0.03	0.84 ± 0.04	0.68 ± 0.05

B. Solvent Extracts

component	Nicaragua			India			Niger		
	A[d]	B[d]	C[d]	A	B	C	A	B	C
total phenolics[b]	7.43 ± 0.09	12.33 ± 0.46	11.04 ± 0.42	5.25 ± 0.34	8.87 ± 0.07	8.11 ± 0.06	6.83 ± 0.30	9.76 ± 0.34	8.78 ± 0.31
total flavonoids[c]	10.83 ± 0.15	14.07 ± 0.83	10.14 ± 0.47	3.26 ± 0.24	5.76 ± 0.10	5.92 ± 0.16	7.32 ± 0.46	10.19 ± 0.18	9.01 ± 0.12
ascorbic acid	0.055 ± 0.01	0.036 ± 0.01	0.051 ± 0.01	0.043 ± 0.01	0.048 ± 0.01	0.020 ± 0.02	0.050 ± 0.01	0.040 ± 0.01	0.045 ± 0.01
yield % of extract[e]	31.7 ± 2.4	28.2 ± 1.3	28.5 ± 2.7	34.3 ± 1.9	25.6 ± 2.0	27.7 ± 2.1	37.7 ± 1.1	24.5 ± 2.2	28.1 ± 0.8

[a] Values are means of triplicate determination ± standard deviation. [b] As equivalent to gallic acid. [c] As equivalent to rutin. [d] A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts. [e] Yield percent = [solvent extracts wt (g)/sample wt (g)] × 100.

liposomes was subsequently determined by measuring the thiobarbituric acid reactive substances (TBARS). The reaction mixture was added with 1 mL each of 1% TBA and 10% HCl, and it was then heated in a water bath at 100 °C for 30 min. After the mixture had been cooled in an ice bath for 15 min, 5 mL of chloroform was added, and the mixture was centrifuged at 3000*g* for 20 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm. The inhibition percent of the TBARS formation of the sample was calculated as $100 \times (A_0 - A_t)/(A_0 - A_1)$, where A_0, A_1, and A_t are the absorbance values for the control, for the blank, and for the test sample added, respectively. α-Tocopherol was assayed for comparison of the results.

Preparation of Liver Microsomes and Assaying the Oxidation Induced by Enzymatic System. By using the method of Hanna et al. (*40*), microsomes were prepared. Microsomes were isolated from male rats weighing ~240 g as follows. The liver was sliced and homogenized in 4 volumes of 10 mM potassium phosphate buffer, pH 7.4, containing 1.15% KCl. The homogenate was centrifuged at 600*g* for 10 min at room temperature, the resulting supernatant was recentrifuged at 4 °C and 9000*g* for 10 min, and the final supernatant was ultracentrifuged at 4 °C and 100000*g* for 45 min. The resulting pellet was resuspended in the homogenization buffer. Liver microsomal oxidation was performed according to a modified method of Tsuda et al. (*39*) as outlined by Yen and Chuang (*8*). The extract (0.2 mL, 0.05−0.5 mg mL^{-1}) was mixed with liver microsomes (0.2 mL, 1.5 mg of protein mL^{-1}). The mixtures were incubated in the presence or absence of a reduced NADP (NADPH)-generating system (0.2 mL, 1 mM NADPH; 0.2 mL, 1 mM ADP and 0.2 mL, 0.25 mM $FeCl_3$) in a phosphate buffer (0.2 M, pH 7.4). The total final volume was 1 mL. The reaction mixtures were incubated at 37 °C for 1 h. The extent of microsome oxidation was measured by means of the TBARS measurement as described by Yen and Chuang (*8*).

Determination of Antioxidant Activity with the DPPH• Radical Scavenging Method. The antioxidant activity of different solvent extracts of *M. oleifera* leaves from different agroclimatic origins, BHA, BHT, ascorbic acid, and quercetin was measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical, DPPH•, method (*41*) as modified by Sánchez-Moreno et al. (*42*). A methanolic solution (0.1 mL) of sample extract at various concentrations was added to 3.9 mL (0.025 g L^{-1}) of DPPH• solution. The decrease in absorbance at 515 nm was determined continuously at every 1 min with a Hitachi UV−visible model U-2000 spectrophotometer until the reaction reached the plateau. The decrease in the absorbance depends on the concentration of the antioxidant and the radical, the molecular structure of the antioxidant, and its kinetic behavior (*41*, *42*). The remaining concentration of DPPH• in the reaction medium was calculated from a calibration curve obtained with DPPH• at 515 nm. The percentage of the remaining DPPH• ($DPPH^{\bullet}_R$) was calculated as follows:

$$\%\ DPPH^{\bullet}_R = [(DPPH^{\bullet})_T/(DPPH^{\bullet})_{T=0}] \times 100$$

where $DPPH^{\bullet}_T$ was the concentration of DPPH• at the time of steady state and $DPPH^{\bullet}_{T=0}$ was the concentration of DPPH• at time zero (initial concentration).

The percentage of the remaining DPPH• against the extract/standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH• by 50% (EC_{50}). On the basis of the parameter EC_{50}, the result was expressed in terms of grams of dry matter of leaf extract/standard equivalent per gram of DPPH• in the reaction medium.

Statistical Analysis. The data were subjected to a one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple-range test ($P < 0.05$) using Statistica for Windows H'97, version 5.1 (Statsoft Inc., Tulsa, OK). Values expressed are means of three replicate determinations ± standard deviation.

RESULTS AND DISCUSSION

Total Phenolics, Flavonoids, and Ascorbic Acid Composition. Total phenolics, flavonoids, and ascorbic acid contents of freeze-dried *M. oleifera* leaf samples from different agroclimatic zones are given in **Table 1**. Among the samples collected from different countries, leaf samples collected from Nicaragua seem to contain a higher concentration of total phenolics (4.25%) than samples from India (2.94%) and Niger (3.66%). However, these values are lower than the values reported earlier in moringa leaves from Nicaragua (*22*) and other green vegetables (*43*). On the other hand, they are considered to be more potent, and the nutraceutical phenolic constituents such as total flavonoids levels found in the different moringa leaf samples are comparable to that of the values reported in mulberry leaves (*31*). Although fresh moringa leaves contain an appreciable amount (~1400 mg/100 g; dry matter basis) of ascorbic acid (vitamin C), the concentrations present in the freeze-dried samples from Nicaragua (920 mg/100 g), India (836 mg/100 g), and Niger (678 mg/100 g) appear to be 20−50% lower. On the basis of the HPLC data, the following flavonoid aglycon compounds such quercetin and kaempferol (**Figures 1** and **2** and **Table 3**) have been identified and quantified. Interestingly, all of the freeze-dried leaf samples contain both quercetin and kaempferol in concentration ranges between 633.5 and 926 mg 100 g^{-1} and between 104.7 and 225.4 mg 100 g^{-1}, respectviely. Similarly, such higher concentrations of potentially anticarcinogenic flavonoids, quercetin and kaempferol, have also been reported in various common vegetables and fruits (*44*, *45*).

Effect of the Solvent on the Yield of Total Soluble Solids, Polyphenolics, Flavonoids, and Ascorbic Acid. The various

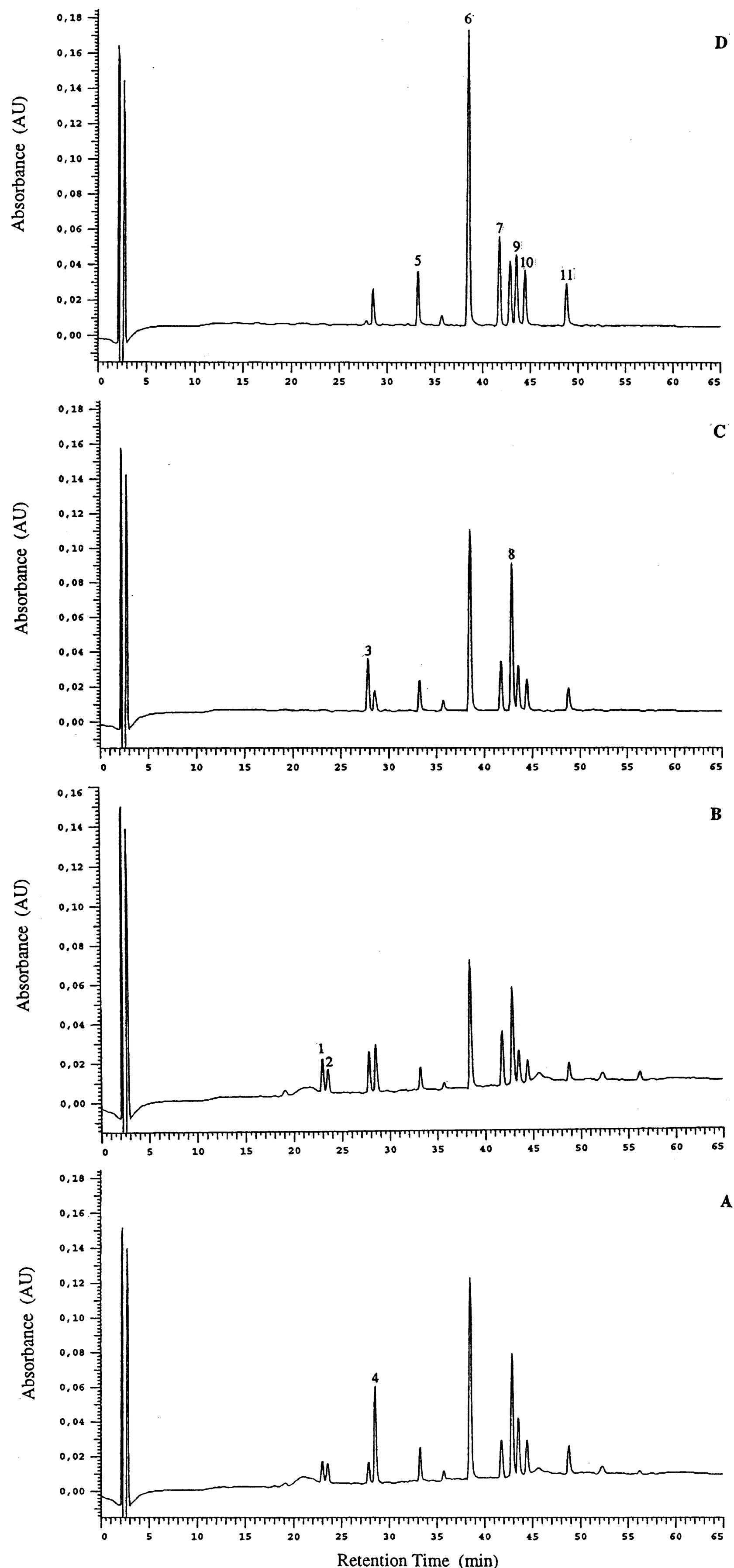


Figure 1. HPLC-DAD chromatograms of the flavonoid and phenolic acid mixture recorded at 270 (A), 280 (B), 340 (C), and 370 nm (D). Peaks: 1, (+)-catechin; 2, (−)-epicatechin; 3, ferulic acid; 4, ellagic acid; 5, myricetin; 6, quercetin; 7, butein; 8, apigenin; 9, kaempferol; 10, isorhamnetin; 11, rhamnetin.

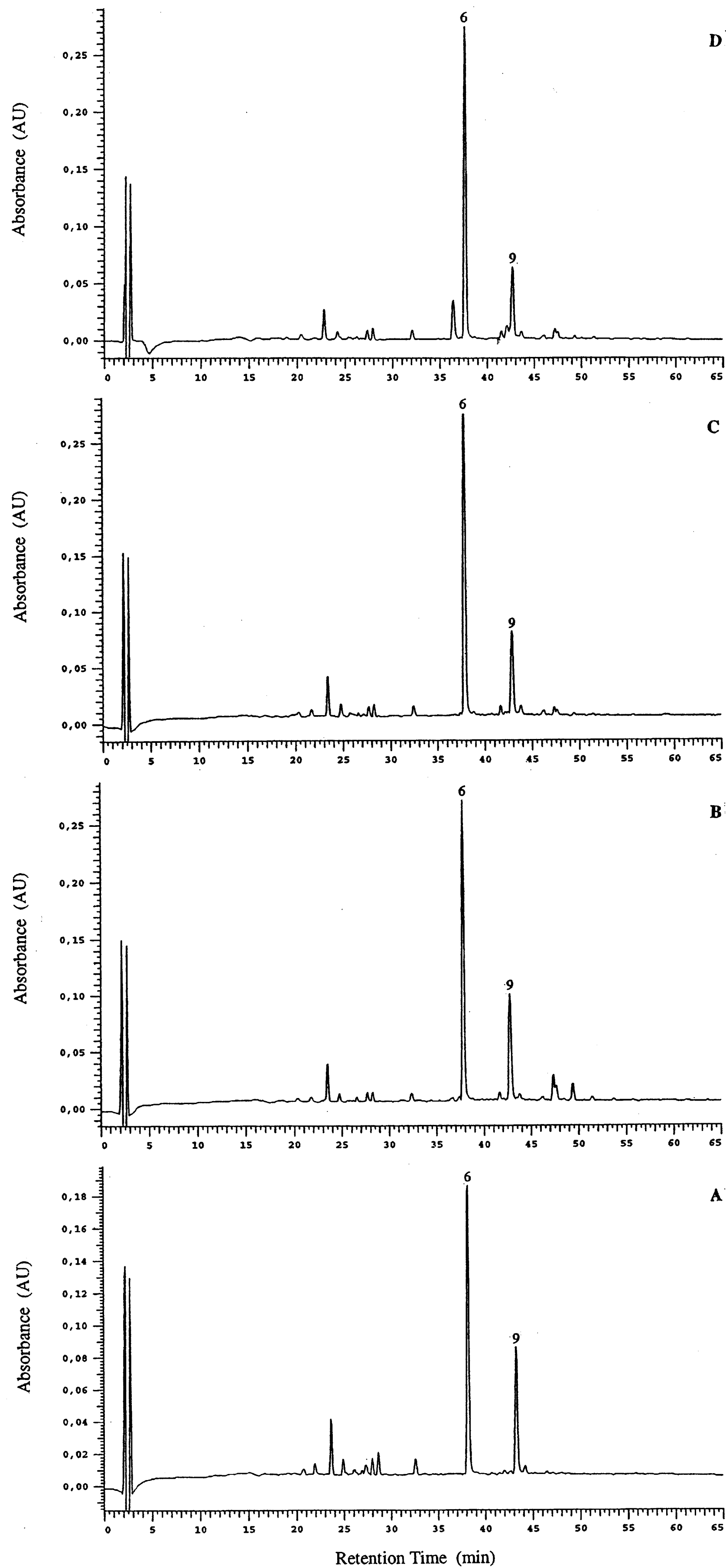


Figure 2. HPLC-DAD chromatograms of moringa leaves from Nicaragua recorded at 370 nm: raw leaves (A), water extracts (B), 80% methanol extracts (C), and 70% ethanol extracts (D). Flavonoid peaks identified in the chromatograms are the same as **Figure 1**.

Table 2. Reducing Power of Various Solvent Extracts of *M. oleifera* Leaves from Different Agroclimatic Origins[a]

extract amount (mg)	Nicaragua			India			Niger		
	A[b]	B[b]	C[b]	A	B	C	A	B	C
0.0	0.015g ± 0.002	0.012g ± 0.001	0.012g ± 0.003	0.014g ± 0.002	0.026g ± 0.004	0.010g ± 0.003	0.004g ± 0.001	0.004g ± 0.002	0.055g ± 0.006
2.5	0.41f ± 0.02	0.90f ± 0.03	0.72f ± 0.01	0.48f ± 0.01	0.59f ± 0.00	0.41f ± 0.02	0.51f ± 0.01	0.78f ± 0.02	0.56f ± 0.00
5.0	0.94e ± 0.00	1.53e ± 0.11	1.53e ± 0.02	0.88e ± 0.02	1.07e ± 0.01	0.90e ± 0.01	0.95e ± 0.03	1.37e ± 0.06	1.14e ± 0.09
7.5	1.33d ± 0.05	2.39d ± 0.01	1.92d ± 0.11	1.24d ± 0.02	1.50d ± 0.05	1.21d ± 0.02	1.24d ± 0.01	1.79d ± 0.09	1.51d ± 0.04
10.0	1.60c ± 0.03	3.03c ± 0.01	2.58c ± 0.02	1.54c ± 0.00	1.81c ± 0.06	1.38c ± 0.01	1.50c ± 0.02	2.55c ± 0.18	1.97c ± 0.05
12.5	2.14b ± 0.05	3.36b ± 0.06	3.33b ± 0.00	1.75b ± 0.00	2.03b ± 0.11	1.82b ± 0.01	2.04b ± 0.00	2.91b ± 0.08	2.64b ± 0.02
15.0	2.60a ± 0.02	3.65a ± 0.01	3.57a ± 0.04	1.94a ± 0.04	2.18a ± 0.01	2.63a ± 0.02	2.68a ± 0.01	3.30a ± 0.15	3.13a ± 0.11
ascorbic acid[c]	1.21 ± 0.03								

[a] The reducing power of extracts was determined by absorbance at 700 nm with a spectrophotometer. Values are mean ± standard deviation of three replicate analyses. Means within a column with the different letters are significantly different ($P < 0.05$). [b] A, water extract; B, 80% methanol extract; C, 70% ethanol extract. [c] Amount of ascorbic acid was 0.4 mg.

solvent extraction yields of soluble substances, expressed as percent by weight of the leaves, and the total extractable polyphenolics expressed as gallic acid equivalents and total flavonoids expressed as rutin equivalents are closely dependent on the solvent, as shown in **Table 1**. Among the various solvent extracts of different moringa leaf samples, water, 80% methanol, and 70% ethanol extracts of samples from Nicaragua in total phenolics (7.43, 12.33, and 11.04%) and total flavonoids (10.83, 14.07, and 10.14%) contents were found to be higher than the samples from India and Niger when subjected to respective solvent extraction. Moreover, the concentrations of aglycons of quercetin and kaempferol in water, 80% methanol, and 70% ethanol extracts of all the leaf samples were 811−1412 and 278−627 mg 100 g^{-1}, 1040−2749 and 194−586 mg 100^{-1}, and 857−2545 and 154−647 mg 100 g^{-1}, respectively. Nonetheless, 80% methanol (v/v) was found to be the most efficient solvent to extract antioxidant-containing phenolic constituents including flavonoids, quercetin, and kaempferol from all three leaf samples. This is in agreement with the reports of Yen et al. (*7*) and Hertog et al. (*11*) that methanol is a widely used and effective solvent for extraction of antioxidants. In addition to the solvent efficiency on extractable phenolics, the differences in phenolic content within *M. oleifera* samples may be due to the growing location as well as stage of leaf development, genetic variability, and postharvest handling of the leaf samples. Therefore, more detailed studies on the effect of these variables are still necessary. The ascorbic acid level in the freeze-dried materials of different solvent extracts in all three leaf samples was found to be negligible (20−55 mg 100 g^{-1}).

Reducing Power of Different Solvent Extracts from Moringa Leaves. Yen and Duh (*46*) and Siddhuraju et al. (*47*) have reported that the reducing power of bioactive compounds was associated with antioxidant activity. Thus, it is necessary to determine the reducing power of phenolic constituents to elucidate the relationship between their antioxidant effect and their reducing power. The reducing power of water, 80% methanol, and 70% ethanol extracts of different moringa leaf samples is shown in **Table 2**. The reducing power of the extracts increased with an increase in concentration; however, when compared to both aqueous organic solvent extracts, water extracts appear to be less effective on reducing power. From the above findings the results are well correlated with the amount of phenolic constituents, which are present in the respective extracts. Thus, phenolics present in moringa leaf extracts are good electron donors and could terminate the radical chain reaction by converting free radicals to more stable products. The various crude solvent extract concentrations at 7.5 mg (containing 1.50−4.13 μg of ascorbic acid) exhibited a greater reducing power than did 0.4 mg of ascorbic acid, which is a potent reducing agent. Furthermore, the presence of a negligible concentration of ascorbic acid in the respective extracts contributed to the effectiveness of phenolics-induced reducing power. These results are in good agreement with the reports of the above-mentioned authors, who showed that the antioxidant properties were concomitant with the development of reducing power.

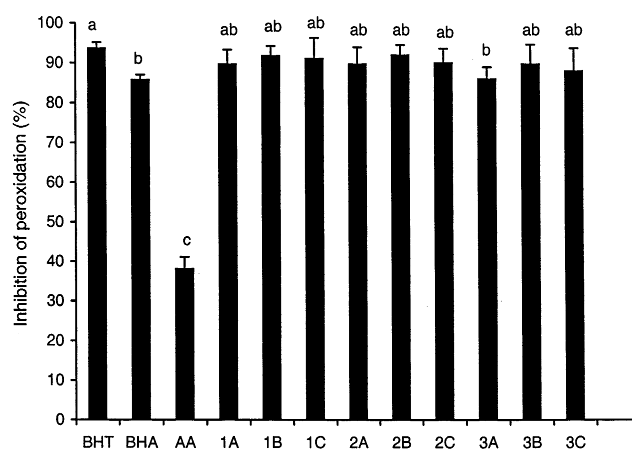


Figure 3. Antioxidant activities of various solvent extracts (0.2 mg/mL) of *M. oleifera* leaves from three different agroclimatic origins: 1, Nicaragua; 2, India; 3, Niger; A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; AA, ascorbic acid. Values are means of triplicate determination ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

Antioxidant Activity of Different Solvent Extracts in the Linoleic Acid Peroxidation System. From the results shown in **Figure 3**, the freeze-dried extracts of various solvent extractions from moringa leaves exhibited good antioxidant activity in the linoleic acid peroxidation system. At a concentration of 0.2 mg/mL all of the crude extracts of moringa leaves inhibited 89.7−92.0% peroxidation of linoleic acid after incubation for 336 h (14 days). This percentage was comparable to that of the values obtained for BHT (93.6%) and BHA (85.7%), and statistically no significant differences were noted at $P < 0.05$. However, the values observed in the water extract of the sample collected from Niger appeared to be significantly lower than those of the synthetic antioxidant, BHT. On the other hand, when compared to ascorbic acid, all of the leaf extracts were found to have significantly ($P < 0.05$) higher inhibition of peroxidation in the linoleic acid system. Similarly, Eberhardt

Table 3. HPLC Data on Quantitative Determination of Flavonoids, Quercetin, and Kaempferol and DPPH• Free Radical Scavening Activity of Total Phenolics of Moringa Leaf Samples from Three Different Agroclimatic Origins

	Nicaragua				India				Niger			
	raw[a]	A[a]	B[a]	C[a]	raw	A	B	C	raw	A	B	C
quercetin (mg 100 g^{-1} DM[b])	926 ± 102	1412 ± 115	2749 ± 42	2545 ± 90	634 ± 84	838 ± 59	1040 ± 83	957 ± 48	770 ± 120	811 ± 17	1071 ± 32	657 ± 76
kaempferol (mg 100 g^{-1} DM[b])	225 ± 23	627 ± 54	586 ± 28	647 ± 26	175 ± 35	322 ± 20	527 ± 15	476 ± 6	105 ± 19	278 ± 14	194 ± 12	154 ± 14
DPPH• EC_{50} (g DM g^{-1}DPPH•)[c,d]		2.00c ± 0.05	1.13ef ± 0.05	1.05f ± 0.10		3.07a ± 0.22	1.79c ± 0.11	1.94c ± 0.13		2.72b ± 0.13	1.28de ± 0.05	1.45d ± 0.10

[a] Raw, freeze-dried leaves; A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts. [b] DM, dry matter basis. [c] Amount of sample required to decrease by 50% of the initial DPPH• concentration. [d] Values followed by different letters are significantly different ($P < 0.05$).

et al. (*48*) recently reported that phytochemicals (flavonoids) in fresh apples, other than ascorbic acid, exhibit significantly higher total antioxidant activity. Depending on the conditions, ascorbic acid can act as an antioxidant, a pro-oxidant, a metal chelator, a reducing agent, or an oxygen scavenger. Moreover, the hydrophilic antioxidant, ascorbic acid, was less effective in an oil-in-water emulsion system, whereas the opposite trend was found for the hydrophobic antioxidants (phenolic acids, flavanones, flavonols, etc.). By moving to the water phase, the hydrophilic antioxidants become too diluted to adequately protect the oil at the oil−water interface. According to this mechanism, hydroperoxide formation and decomposition in emulsion systems are dependent on the effective concentrations of antioxidants in the oil and water phases and the interface (*49*), and thus the efficiency of hydrophilic antioxidants may be offset by their prooxidant activity, resulting from reduction of metal into the more active lower valence state. On the other hand, the efficiency of polyphenols as antioxidant compounds greatly depends on their chemical structure. Flavonoids are among the most potent plant antioxidants because they possess one or more of the following structural elements that are involved in the antioxidant/antiradical activity: an *o*-diphenol group in ring B, a 2−3 double bond conjugated with the 4-oxo function, and hydroxyl groups in positions 3 and 5 (*50*). Flavonoids are very effective scavengers of peroxyl radicals, and they are also chelators of metals and inhibit the Fenton and Haber−Weiss reactions, which are important sources of active oxygen radicals. In addition, flavonoids retain their free radical scavenging capacity after forming complexes with metal ions. In this context, on the basis of the above stated chemical reactivity, the presence of relative concentrations of various flavonoid groups such as quercetin, kaempferol, and other phenolic substances in the various solvent extracts from moringa leaves might have been involved in the inhibition of peroxidation. Similarly, the addition of phenolics preparations from *Mangifera indica* seed kernels was found to be more resistant against autoxidation of buffalo ghee, as is evident from the increase in the induction period and antioxygenic index (*51*). Velioglu et al. (*52*) have reported that the total phenolic content of various fruits and vegetables contained potential antioxidant activities against the linoleic acid peroxidation system.

Scavenging Effect of Different Solvent Extracts from Various Moringa Leaf Samples on Superoxide Radical ($O_2^{\bullet -}$). The superoxide radicals were generated by illuminating a solution. The relative scavenging effect of total phenolics of different solvent extracts from three different moringa samples was analyzed, and the results are illustrated in **Figure 4**. All of the extracts had a scavenging activity on superoxide radicals in a dose-dependent manner (0.08−0.2 mg mL^{-1}), the highest scavenging ability being exhibited by the phenolics extracted from the Nicaraguan samples. When the scavenging effect is 50%, the concentration of different solvent extracts falls within the range of 0.08−0.2 mg/mL, except for the water extract of moringa leaf samples from India (>0.3 mg/mL). The scavenging effects of phenolics (flavonoids) such as rutin and quercetin on superoxide radicals have also been established by the ESR method (*53*). Nonetheless, the differences in content of these two flavonoids in the mulberry samples have also been reported to be responsible for their varied scavenging effects (*31*). As was discussed in the section on total peroxidation inhibition, the similar structural configurations of the flavonoids play a major role as potent antiperoxidative properties upon the compounds. The substantial concentration of flavonoids in all moringa leaves, quercetin and kaempferol, contain an ortho-

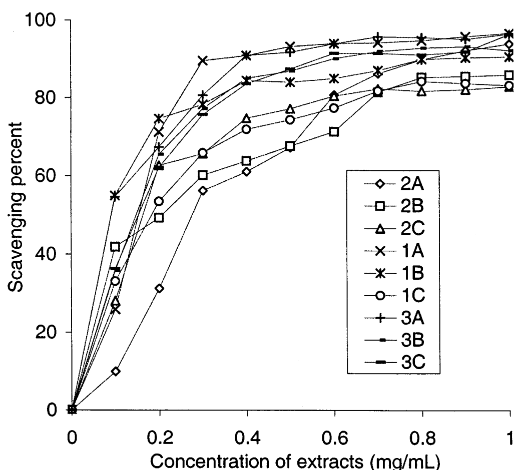
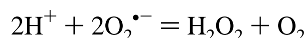


Figure 4. Scavenging effects of various solvent extracts of *M. oleifera* leaves originating from different agroclimatic regions on superoxide radicals: 1, Nicaragua; 2, India, 3, Niger; A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts.

position hydroxyl that provides active hydrogen to take part in the following reaction to scavenge $O_2^{\bullet -}$:



$$2H^+ + 2O_2^{\bullet -} = H_2O_2 + O_2$$

The superoxide dismutase participates in the above reaction by catalysis and as an antioxidant through the supply of hydrogen. Tajima used ESR to determine the scavenging effect of V_E on P_{450} and proved that the mechanism requires the supply of active hydrogen (*54*). This may confirm that the presence of a hydrogen-donating group of flavonoids from moringa leaf samples scavenges $O_2^{\bullet -}$ in a similar way. Due to their lower redox potentials ($0.23 < E_7 < 0.75$ V), flavonoids (Fl-OH) are thermodynamically able to reduce highly oxidizing free radicals with redox potentials in the range of 2.13−1.0 V, such as superoxide, peroxyl, hydroxyl, and alkoxyl radicals, by hydrogen atom donation (*55*).

Antioxidant Activity of Various Solvent Extracts of Moringa Leaves from Different Origins on DPPH• Radical Scavenging Capacity. The scavenging activity of crude phenolic extracts of freeze-dried moringa leaves from different origins was determined by the DPPH• assay, and the results are shown in **Table 3**. All of the solvent extracts of leaf samples had a very high radical scavenging activity. The order of the scavenging activity for the different solvent extracts, except the Nicaraguan samples, is 80% methanol > 70% ethanol > water. However, among the different samples, various solvent extracts of leaves collected from Nicaragua on DPPH• radical scavenging capacity (EC_{50} = 2.00, 1.13, and 1.05 g (DM) g^{-1} DPPH• for water, 80% methanol, and 70% ethanol extracts, respectively) were found to be significantly ($P < 0.05$) higher than the respective solvent extracts of the other two samples. In general, the higher polyphenols extraction yield corresponds with the higher antioxidant activity, probably due to the combined action of the present substances in variable concentrations and their high hydrogen atom donating abilities. Similarly, a linear correlation between DPPH• radical scavenging activity and polyphenolic extract has been reported as variable ranges in different vegetables and fruits (*56*, *57*). In addition, the additive or synergistic effects of polyphenols make the antioxidant activity of the crude extracts higher than that of the isolated compounds. On the other hand, the presence of glycoside in the flavonoids could also decrease the antioxidant activity (DPPH• radical scavenging ability) by affecting the donation of hydrogen (*58*).

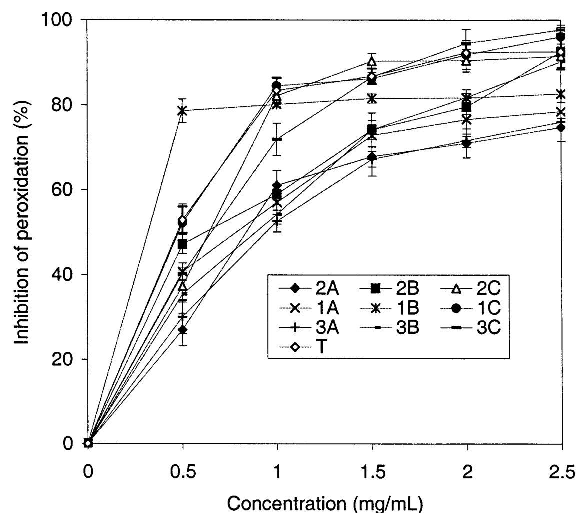


Figure 5. Effects of various solvent extracts of *M. oleifera* leaves from different agroclimatic origins on the lipid peroxidation of liposomes induced by Fe^{3+}/H_2O_2/ascorbic acid: A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts; 1, Nicaragua; 2, India; 3, Niger; T, α-tocopherol.

From the results of our study, the free radical scavenging ability of various solvent extracts of moringa has also been related to various antioxidants: 1 g of water, 80% methanol, and 70% ethanol extracts of moringa leaves have DPPH• activities equal to that of 20, 37, and 35 mg of quercetin; 124, 229, and 217 mg of BHT; and 35, 66, and 62 mg of BHA, respectively (EC_{50} values of quercetin, BHT, and BHA were 0.052 ± 0.002, 0.321 ± 0.011, and 0.092 ± 0.001 g g^{-1} DPPH•, respectively). Similarly, on the basis of the antiradical activity of the total phenolic constituents, 1 g of tropical fruit, guava peel and pulp, has been reported to be equivalent to that of 104.1 and 54.0 mg of *d*,*l*- α-tocopherol, respectively (*57*).

Effect of Moringa Leaf Extracts on Liposome Peroxidation Induced by Fe^{3+}/H_2O_2/Ascorbic Acid. The inhibition of different solvent extracts of total phenolics from moringa leaves on peroxidation of the cell membrane was evaluated using liposomes induced by Fe^{3+}/H_2O_2/ascorbic acid. As shown in **Figure 5**, the different extracts of moringa leaves had a better antioxidant activity. They exhibited ∼52.6−84.5% inhibition effect at a concentration of 1 mg/mL, which increased to 74.8−97.7% at a concentration of 2.5 mg/mL, comparable to the effect of α-tocopherol. However, among the various solvent extracts, ethanol extracts in the leaf samples from Nicaragua and Niger and methanol extracts of the Indian samples were found to have a higher peroxidation inhibition effect than the water extracts of the respective samples. These results are similar to the trend of inhibition of peroxidation in the linoleic acid system. Moreover, several authors have also reported that ethanol and methanol are more efficient solvents than water for the extraction of antioxidants including various groups of flavonoids from plant samples (*31*, *59*). Saija et al. (*60*) demonstrated that quercetin has a better antioxidant activity than rutin in a biomemebrane system. This might be due to its more lipophilic affinity and interaction rather than rutin in the bilayer membrane of phospholipid.

Effect of Moringa Leaves Extracts on Enzymatic Lipid Peroxidation of Microsomes Induced by NADPH/ADP/Fe^{3+}. Membrane lipids are particularly susceptible to oxidation, not only because of their high polyunsaturated fatty acid content but also because of their association in the cell membrane with

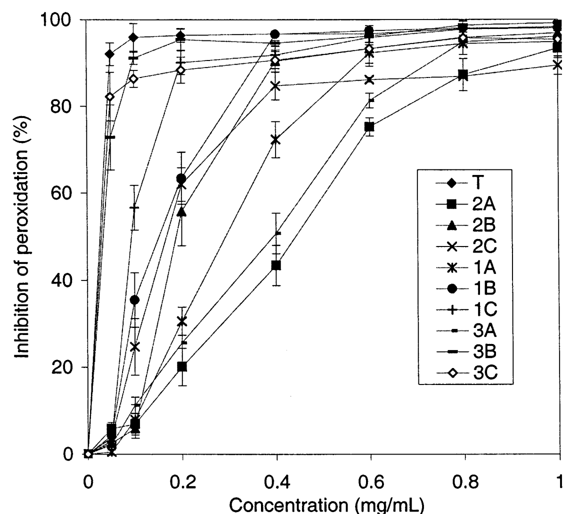


Figure 6. Effects of various solvent extracts of *M. oleifera* leaves from different agroclimatic origins on the enzymatic lipid peroxidation of microsomes induced by NADPH/ADP/Fe^{3+}: A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts; 1, Nicaragua; 2, India; 3, Niger; T, trolox.

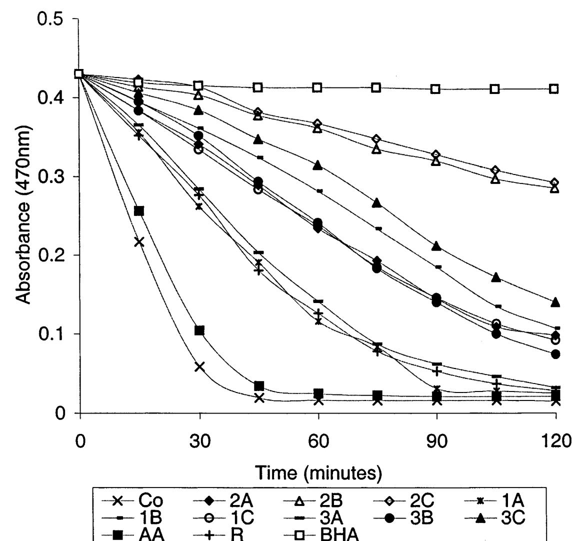


Figure 7. Antioxidant activity of various solvent extracts of *M. oleifera* leaves from different agroclimatic origins at 1 mg/mL in the β-carotene/linoleic acid system: 1, Nicaragua; 2, India; 3, Niger; A, water extracts; B, 80% methanol extracts; C, 70% ethanol extracts; AA, ascorbic acid; R, rutin; BHA, butylated hydroxyanisole; Co, control.

enzymatic and nonenzymatic systems capable of generating free radical species. Microsomes are a heterogeneous mixture of vesicles derived from both endoplasmic reticulum and plasma membranes and are used as an in vitro test system to assess the lipid peroxidation effect of a wide range of drugs and dietary components. NADPH cytochrome P_{450} reductase is involved in NADPH-induced (i.e., enzymatically induced) microsomal lipid peroxidation.

All extracts of moringa leaves dose-dependently inhibited the enzymatic lipid peroxidation of microsomal lipids (**Figure 6**). At the concentration of 0.4 mg/mL, ethanolic and methanolic extracts of all the samples were found to have ∼80−90% inhibitory effects, values that are well comparable to that of Trolox. Hanna et al. (*40*) reported that alcoholic and aqueous extracts of MA-631 (a complex herbal mixture) also inhibited enzyme-induced rat liver microsomal lipid peroxidation in a concentration-dependent manner. Similarly, the phenolic constituents of leaf extracts from du-zhong (*Eucommia ulmoides*) were found to have 93.9% inhibition on rat liver microsomal peroxidation system (*61*) at a concentration of 0.2 mg/mL. Moreover, the antioxidant activities in rat liver microsomal peroxidation induced by enzyme and the Fe^{3+}/H_2O_2/ascorbic acid induced system on liposomes were similar. Therefore, we suggest that extracts of moringa leaves are more effective scavengers on free radicals (peroxyl and hydroxyl radicals) generated either enzymatically or by the Fenton reaction system. In addition, the presence of a relatively low concentration of phenolics in the aqueous extracts showed less of an effect on inhibition of the lipid peroxidation at the above specified concentration level, and at this concentration level the free radical blocking ability was poor.

Antioxidant Activity Assessment of Moringa Leaf Extracts Using the β-Carotene—Linoleic Acid Method. Figure 7 shows the decrease in absorbance of β-carotene in the presence of different solvent extracts of moringa leaves with the coupled oxidation of β-carotene and linoleic acid. Different bleaching rates were demonstrated for the antioxidants, with BHT, ascorbic acid, and rutin. When compared to both 80% methanol and 70% ethanol extracts of all the moringa leaves, ascorbic acid and rutin were found to oxidize β-carotene rapidly, and the trend was similar to that of the control sample without the addition of an antioxidant. Among the respective solvent extracts of various moringa leaf samples, water (20%), 80% methanol (65.1%), and 70% ethanol (66.8%) extracts of Indian origins exhibited the highest antioxidant activity. However, these values were found to be lower than the values observed for BHA (95.5%). Even though the water extracts of moringa leaf samples from Nicaragua and Niger oxidized the β-carotene at 120 min at levels similar to that of the control, rutin, and ascorbic acid, the initial oxidation rate was slow up to 75 min. Such a slower rate of oxidation ability on β-carotene might be due to the presence of the relative concentration and the synergistic effect of compounds such as quercetin, kaempferol, and other phenolics. On the other hand, the presence of a relatively higher concentration of phenolics, particularly flavonoids, in the ethanol and methanol extracts proved to not only have more antioxidant power but also to be a potent antioxidant extractant. Similarly, flavonoids such as quercetin, epicatechins, and procyanidine oligomers and other phenolic constituents of vegetables, fruits, and medicinal plants were reported as potent antioxidants in the β-carotene−linoleic acid bleaching system (*52*, *62*). As has been discussed for the peroxidation inhibition of antioxidant extracts on the linoleic acid system, in addition to the hydroxylation at the B-ring, methylation, and a 2−3 double bond at the C-ring of various flavonoids, the hydrophilic and hydrophobic nature of such compounds could also play a vital role in the inhibition of oxidation/antiradical scavenging capacity in the above system (*49*).

In this study, the antioxidant properties of moringa leaf extract have been demonstrated in the following ways: (1) It reduces potassium ferricyanide. (2) It scavenges superoxide radicals. (3) It prevents the peroxidation of lipid membranes in liposomes. (4) It inhibits the oxidation of microsomes in rat liver. (5) It inhibits the peroxidation of linoleic acid and prevents the bleaching of carotene in carotene/linoleic acid mixtures. (6) It can donate hydrogen and scavenge radicals in the DPPH• method. The polyphenolic components extracted from moringa leaves, except those extracted with water, were found to be potent antioxidants comparable in activity with the widely used synthetic antioxidants BHT and BHA. Although moringa leaves contain substantial concentrations of the natural antioxidant,

ascorbic acid, its antioxidant activity was found to be very low when compared to that of the phenolic compounds. The phenolic compounds, flavonoids, have been identified as quercetin and kaempferol. Additional work is necessary to determine whether this unique mixture of plant phenolics results in a synergistic antioxidant activity. Nonetheless, a preliminary screening test of water extracts of moringa leaves appears to reveal greater activity in cancer preventative effect, which was assayed by the differentiating activity against human promyelocytic leukemia cells (HL-60) (data not shown), and a further detailed research investigation is in progress. Furthermore, research investigations on the quantitative and qualitative evaluation of other phenolic constituents and flavonoids are urgently needed. An assessment of the toxicity and function of these extracts in food systems is also required. For example, moringa leaves are used by indigenous people during the preparation of buffalo and cow butter ghee. The effects that such leaf antioxidants and the addition of NaCl have on the antioxidant activity should be investigated, as should the extent to which they can be used to increase the shelf life of liquid ghee.

By combining the knowledge of indigenous people with laboratory assessments of in vitro antioxidant activity and suitable in vivo experiments, it can be shown that nutritionally enriched moringa leaves can serve as a food source and that their extracts, particularly those containing dietary polyphenolic substances, may have potential as "nutraceuticals" for improving the health of the human population.

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LITERATURE CITED

(1) Yagi, K. Lipid peroxides and human disease. *Chem. Phys. Lipids* **1987**, *45*, 337−341.

(2) Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of aging. *Nature* **2000**, *408*, 239−247.

(3) Martin, A. D.; Gilbert, D. Enzyme changes accompanying liver enlargement in rats treated with 3-*tert*-butyl-4-hydroxyanisole. *Biochem. J.* **1968**, *106*, 22P−23P.

(4) Halladay, S. C.; Ryerson, B. A.; Smith, C. R.; Brown, J. P.; Parkinson, T. M. Comparison of effects of dietary administration of butylated hydroxytoluene or a polymeric antioxidant on the hepatic and intestinal cytochrome P-540 mixed-function-oxygenase system of rats. *Food Cosmet. Toxicol.* **1980**, *18*, 569−574.

(5) Osawa, T.; Namiki, M. A novel type of antioxidant isolated from leaf wax of eucalyptus leaves. *Agric. Biol. Chem.* **1981**, *45*, 735−739.

(6) Ramarathnam, N.; Osawa, T.; Namiki, M.; Kawakishi, S. Chemical studies on novel rice hull antioxidants. I. Isolation, fractionation, and partial characterization. *J. Agric. Food Chem.* **1988**, *36*, 732−737.

(7) Yen, G. C.; Wu, S. C.; Duh, P. D. Extraction and identification of antioxidant components from the leaves of mulberry (*Morus alba* L.). *J. Agric. Food Chem.* **1996**, *44*, 1687−1690.

(8) Yen, G. C.; Chuang, D. Y. Antioxidant properties of water extracts from *Cassia tora* L. in relation to the degree of roasting. *J. Agric. Food Chem.* **2000**, *48*, 2760−2765.

(9) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavonols as scavenger of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *2*, 339−346.

(10) Hertog, M. G. L.; Sweetnam, P. M.; Fehily, A. M.; Elwood, P. C.; Kromhout, D. Antioxidant flavonols and ischemic heart disease in a welsh population of men: the Caerphilly study. *Am. J. Clin. Nutr.* **1997**, *65*, 1489−1494.

(11) Hertog, M. G. L.; Hollman, P. C. H.; van de Putte, B. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J. Agric. Food Chem.* **1993**, *41*, 1242−1246.

(12) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317−333.

(13) Saura-Calixto, F. Antioxidant dietary fiber product: a new concept and a potential food ingredient. *J. Agric. Food Chem.* **1998**, *46*, 4303−4306.

(14) Stampfer, M. J.; Henneekens, C. H.; Manson, J. E.; Colditz, G. A.; Rosner, B.; Willet, W. C. Vitamin E consumption and the risk of coronary disease in women. *New Engl. J. Med.* **1993**, *328*, 1444−1449.

(15) Middleton, E.; Kandaswamy, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673−751.

(16) Hertog, M. G. L.; Hollman, P. C. H. Potential health effects of the dietary flavonoid quercetin. *Eur. J. Clin. Nutr.* **1996**, *50*, 63−66.

(17) Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Rio, J. A. D. Use and properties of citrus flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505−4515.

(18) Larson, R. A. The antioxidants of higher plants. *Phytochemistry* **1988**, *27*, 969−978.

(19) Frankel, E. N. The antioxidant and potential nutritional properties of plant extracts. In *COST Action 916. Bioactive Plant Cell Wall Components in Nutrition and Health. Bioactive Compounds in Plant Foods: Health Effects and Perspectives for the Food Industry*, European Scientific Conference, Tenerife, Canary Islands, Spain, April 26−28, 2001; European Communities: Luxembourg, 2002; pp 63−71.

(20) Fridovich, I. The biology of oxygen radicals. *Science* **1978**, *201*, 875−880.

(21) Ramachandran, C.; Peter, K. V.; Gopalakrishnan, P. K. Drumstick (*Moringa oleifera*): a multipurpose Indian vegetable. *Econ. Bot.* **1980**, *34*, 276−283.

(22) Makkar, H. P. S.; Becker, K. Nutrients and antiquality factors in different morphological parts of the *Moringa oleifera* tree. *J. Agric. Sci. (Cambridge)* **1997**, *128*, 311−322.

(23) Morton, J. A. The horseradish tree, *Moringa pterygosperma* (Moringaceae)—a boon to arid lands? *Econ. Bot.* **1991**, *45*, 318−333.

(24) Pal, S.; Mukherjee, K.; Saha, B. P. Studies on the antiulcer activity of *Moringa oleifera* leaf extract on gastric ulcer models in rats. *Phytother. Res.* **1995**, *9*, 463−465.

(25) Nair, A. G. R.; Subramanian, S. S. Pigments of the flowers of *Moringa pterygosperma*. *Curr. Sci.* **1962**, *31*, 155−156.

(26) Ghasi, S.; Nwobodo, E.; Ofili, J. O. Hypocholesterolemic effects of crude extract of leaf of *Moringa oleifera* Lam in high-fat diet fed wistar rats. *J. Pharmacol.* **2000**, *69*, 21−25.

(27) Estrella, M. C. P.; Mantaring, J. B. V.; David, G. Z. A double-blind, randomised controlled trial on the use of malunggay (*Moringa oleifera*) for augmentation of the volume of breastmilk among non-nursing mothers of preterm infants. *Philippine J. Pediat.* **2000**, *49*, 3−6.

(28) Dillard, C. J.; German, J. B. Phytochemicals: nutraceuticals and human health. review. *J. Sci. Food Agric.* **2000**, *80*, 1744−1756.

(29) Jones, E.; Hughes, R. E. Foliar ascorbic acid in some angiosperms. *Phytochemistry* **1983**, *22*, 2493−2499.

(30) Makkar, H. P. S.; Becker, K.; Abel, H.; Pawelzik, E. Nutrient contents, rumen protein degradability and antinutritional factors in some colour-and white-flowering cultivars of *Vicia faba* beans. *J. Sci. Food Agric*. **1997**, *75*, 511−520.

(31) Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555−559.

(32) Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B. Dietary antioxidant flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. *J. Agric. Food Chem.* **1992**, *40*, 2379−2383.

(33) Mattila, P.; Astola, J.; Kumpulainen, J. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *J. Agric. Food Chem.* **2000**, *48*, 5834−5841.
(34) Mitsuda, H.; Yasumoto, K.; Iwami, K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to shokuryo* **1966**, *19*, 210−214.
(35) Taga, M. S.; Miller, E. E.; Pratt, D. E. Chia seeds as a source of natural lipid antioxidants. *J. Am. Oil Chem. Soc.* **1984**, *61*, 928−931.
(36) Oyaizu, M. Studies on products of browning reaction: antioxidative activity of products browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, *44*, 307−315.
(37) Yen, G. C.; Duh, P. D.; Chuang, D. Y. Antioxidant activity of anthroquinones and anthrone. *Food Chem.* **2000**, *70*, 437−441.
(38) Beauchamp, C.; Fridovich, I. Superoxide dimutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **1971**, *44*, 276−287.
(39) Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S. W.; Kawakishi, S.; Osawa, T. Antioxidant activity of the anthocyanin pigments cyanidin 3-*O*-*β*-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 248−251.
(40) Hanna, A. N.; Sharma, H. M.; Kauffman, E. M.; Newman, H. A. I. *In vitro* and *in vivo* inhibition of microsomal lipid peroxidation by MA-631. *Pharmacol. Biochem. Behav.* **1994**, *39*, 505−510.
(41) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **1995**, *28*, 25−30.
(42) Sánchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270−276.
(43) Vinson, J. A.; Hao, Y.; Su, X.; Zubik, L. Phenol antioxidant quantity and quality in foods: vegetables. *J. Agric. Food Chem.* **1998**, *46*, 3630−3634.
(44) Hertog, M. G. L.; Hollman, P. C. H.; Venema, D. P. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *J. Agric. Food Chem.* **1992**, *40*, 1591−1598.
(45) Trichopoulou, A.; Vasilopoulou, E.; Hollman, P.; Chamalides, C.; Foufa, E.; Kaloudis, T.; Kromhout, D.; Miskaki, P.; Petrochilou, I.; Poulima, E.; Stafilakis, K.; Theophilou, D. Nutritional composition and flavonoid content of edible wild greens and green pies: a potential rich source of antioxidant nutrients in the Mediterranean diet. *Food Chem.* **2000**, *70*, 319−323.
(46) Yen, G. C.; Duh, P. D.; Tsai, C. L. Relationship between antioxidant activity and maturity of peanut hulls. *J. Agric. Food Chem.* **1993**, *41*, 67−70.
(47) Siddhuraju, P.; Mohan, P. S.; Becker, K. Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chem*. **2002**, *79*, 61−67.
(48) Eberhardt, M. V.; Lee, C. Y.; Liu, R. H. Antioxidant activity of fresh apples. *Nature* **2000**, *405*, 903−904.
(49) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925−1941.
(50) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(51) Puravankara, D.; Boghra, V.; Sharma, R. S. Effect of antioxidant principles isolated from mango (*Mangifera indica* L) seed kernels on oxidative stability of buffalo ghee (butter-fat). *J. Sci. Food Agric.* **2000**, *80*, 522−526.
(52) Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113−4117.
(53) Chen, Y. T.; Li, X. J.; Zao, B. L.; Zheng, R. L.; Xin, W. J. ESR study of the scavenging effect of natural products of rutin etc on superoxide and hydroxyl radical. *Acta Biophys. Sin.* **1989**, *5*, 235−240.
(54) Tajima, K.; Sakamoto, M.; Okada, K. Reaction of biological phenolic antioxidants with superoxide generated by cytochrome P_{450} model system. *Biochem. Biophys. Res. Commun.* **1983**, *115*, 1002−1008.
(55) Pietta, P. G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035−1042.
(56) Robards, K.; Prenzeler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401−436.
(57) Jiménez-Escrig, A.; Rincón, M.; Pulido, R.; Saura-Calixto, F. Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. *J. Agric. Food Chem.* **2001**, *49*, 5489−5493.
(58) Von Gadow, A.; Joubert, E.; Hansmann, C. F. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Asalathus linearis*), α-tocopherol, BHT, and BHA. *J. Agric. Food Chem.* **1997**, *45*, 632−638.
(59) Moure, A.; Franco, D.; Sineiro, J.; Dominguez, H.; Nunez, M. J.; Lema, J. M. Evaluation of extracts from *Gevuina avellana* hulls as antioxidants. *J. Agric. Food Chem.* **2000**, *48*, 3890−3897.
(60) Saija, A.; Scalese, M.; Lanza, M.; Marzullo, D.; Bonina, F.; Castelli, F. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radical Biol. Med.* **1995**, *19*, 481−486.
(61) Yen, G. C.; Hsieh, C. L. Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) towards various lipid peroxidation models *in vitro*. *J. Agric. Food Chem.* **1998**, *46*, 3952−3957.
(62) Lu, Y.; Foo, L. Y. Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chem.* **2000**, *68*, 81−85.

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