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Novel Antioxidant Compounds from the Aqueous Extract of the Roots of *Decalepis hamiltonii* (Wight and Arn.) and Their Inhibitory Effect on Low-Density Lipoprotein Oxidation

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Roots of *Decalepis hamiltonii* are consumed as pickles and as a health drink in southern India for their health benefits. The antioxidant properties of the root extracts have been shown previously; this paper reports the isolation of antioxidant compounds from the aqueous extract of the roots of *D. hamiltonii*. Five novel antioxidant compounds were isolated and characterized by NMR and MS. The compounds exhibited free radical scavenging activity in vitro and inhibited low-density lipoprotein oxidation. This study demonstrates that the root extract of *D. hamiltonii* is a cocktail of several antioxidant compounds with health implications.

KEYWORDS: ***Decalepis hamiltonii*; antioxidant molecules; free radical scavenging; LDL oxidation; metal ion chelating activity**

INTRODUCTION

Free radicals are implicated in the etiology of several diseases such as atherosclerosis, neurodegenerative disorders, some forms of cancer, and aging (*1*). Antioxidants prevent the damage to macromolecules and cells by interfering with the free radicals. Currently there is a great deal of interest in newer bioactive molecules from nature with health-promoting potential. Natural products containing antioxidants from plants often called nutraceuticals are believed to modulate oxidative stress and prevent or delay degenerative disorders (*2*). Several natural compounds that possess antioxidant activity have been reported from plant sources and are commercially promoted as nutraceuticals. Phenolic antioxidants occur widely in the plant kingdom and act as free radical terminators or metal chelators (*3*). Examples of common plant phenolic antioxidants include cinnamic acid derivatives, coumarins, flavonoids, polyfunctional organic acids, and tocopherols. Natural antioxidant phytochemicals are also of interest in the prevention of atherosclerosis, which is thought to involve oxidative stress (*4*). Oxidation of low-density lipoprotein (LDL) is a critical event in the process of atherogenesis. Protection of LDL from oxidation is considered to be an effective strategy to prevent or slow the progression of atherosclerosis. Many plant-derived antioxidants are known to inhibit LDL oxidation *in vitro* (*5*).

Decalepis hamiltonii (Wight and Arn.) (family: Asclepiadaceae) grows as a climbing shrub in the forests of peninsular India. Its tubers are consumed as pickles and juice for its alleged health-promoting properties. The roots are used in folk medicine and as a substitute for *Hemidesmus indicus* in ayurvedic preparations of ancient Indian medicine (*6*). We have shown that the roots of *D. hamiltonii* possess potent antioxidant properties and hypothesized that it may be associated with their alleged health benefits (*7*). Earlier work indicates that the roots contain aldehydes, inositols, saponins, amyrins, lupeols, and volatile flavor compounds such as 2-hydroxy-4-methoxybenzaldehyde, vanillin, 2-phenylethyl alcohol, benzaldehyde, and others (*8*). Our recent work has shown that the methanolic extract of the roots of *D. hamiltonii* is a cocktail of antioxidants (*9*). In this paper, we describe the isolation and characterization of novel antioxidant constituents from the aqueous extract of *D. hamiltonii* roots and evaluation of their antioxidative properties including inhibition of LDL oxidation.

MATERIALS AND METHODS

Chemicals. Human LDL, butylated hydroxyanisole (BHA), nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), thiobarbituric acid (TBA), bovine serum albumin (BSA), quercetin, tetraethoxypropane, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nicotinamide adenine dinucleotide reduced (NADH), trichloroacetic acid (TCA), deoxyribose, ascorbic acid, and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All of the reagents were of analytical grade.

Preparation of the Root Powder and Extraction. Tuberous roots of *D.* hamiltonii (10 kg) were procured from the local suppliers. The roots were washed with water, followed by crushing with a roller to separate the inner woody core from the outer fleshy layer. The fleshy portion was collected, dried at 40 °C in a hot air oven, and then finely powdered. The powder (1.9 kg) was used for extraction.

We have earlier reported that the aqueous extract of *D. hamiltonii* shows high antioxidant activity among the different solvent extracts (*7*). The aqueous extract was prepared by homogenizing the root powder (200 g) in warm water (50 °C) and allowing the mixture to stand for

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24 h; the mixture was filtered with Whatman paper no. 1, and the filtrate was lyophilized and weighed (34.75 g).

Isolation. The lyophilized aqueous extract (34.75 g) was re-extracted with methanol, and the supernatant showing antioxidant activity was concentrated under reduced pressure; it was subjected to fractionation by silica gel (60−120 mesh) column chromatography using a glass column (length = 43 cm, diameter = 3 cm) and eluted with chloroform followed by a stepwise gradient of chloroform, ethyl acetate, and methanol. Eighteen fractions of 300 mL each were collected, concentrated, and assayed for antioxidant activity by reactive oxygen species (ROS) and DPPH radical scavenging assays. On the basis of the activity and thin-layer chromatography (TLC) profile, the active fractions were pooled into two major fractions, I and II.

Fraction I was loaded onto a silica gel column (length = 32 cm, diameter = 2 cm) and eluted with a stepwise gradient of chloroform and ethyl acetate. The active subfractions collected were further purified by preparative TLC (solvent system of chloroform/methanol/acetic acid, 85:14:1), and purity was confirmed by reversed phase high-performance liquid chromatography (RP-HPLC). This yielded the pure compound DHA I.

Fraction II was concentrated under reduced pressure and fractionated on a silica gel column (length = 32 cm, diameter = 2 cm) and eluted with a stepwise gradient of ethyl acetate and methanol. Twenty fractions of 50 mL each were collected, concentrated, and assayed for antioxidant activity (ROS/DPPH radical scavenging). Purity of the fractions was monitored by TLC, and spots were detected by exposure to iodine vapors. The active fractions were pooled, concentrated, and subjected to another round of silica column chromatography as described above. Among the fractions collected, three were active—subfractions II A, II B, and II C, which were further subjected to preparative TLC using the solvent systems chloroform/methanol/acetic acid in the ratios of 80:19:1, 70:29:1, and 60:39:1, respectively. Subfraction II A showed four spots, of which one was active and pure, which was further confirmed by RP-HPLC (DHA II). Subfraction II B showed five spots, two of which were active and pure as confirmed by RP-HPLC (DHA III and DHA IV). Subfraction II C showed three spots, of which one was active but not pure; it was further purified on an LH-20 column (length = 20 cm, diameter = 1.5 cm) and eluted with methanol, which yielded a pure active compound as confirmed by RP-HPLC (DHA V) (**Scheme 1**).

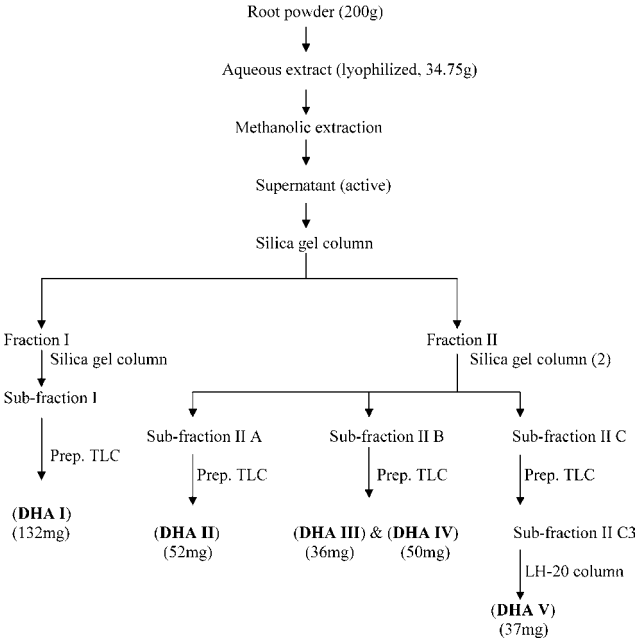
High-Performance Liquid Chromatography. RP-HPLC was done using a Shimadzu LC-8A system equipped with a Rheodyne 7725i injection valve fitted with a 20 μL sample port and a C_{18} column (length = 25 cm, i.d. = 2.12 mm, 5 μm particle size, from Supelco, Sigma-Aldrich). Purified fractions were eluted with an isocratic solvent mixture comprising 0.1% TFA in water/methanol (70:30) with a flow rate of 1 mL/min and monitored with UV detection at 216 nm.

UV and Infrared Spectrometry. Sample (0.1 mg/mL) dissolved in methanol was used for recording the UV spectrum at 200−800 nm in a Shimadzu UV−vis spectrophotometer. IR spectra of the compounds dissolved in DMSO (10 mg/mL) were recorded on a Perkin-Elmer FT-IR spectrometer (spectrum 2000) at 400−4000 cm^{-1}.

Liquid Chromatography−Mass Spectrometry (LC-MS). The LC system consisted of a Hitachi L-6000 pump (Hitachi, Tokyo, Japan), a Rheodyne model 7125 injector with a 25 mL loop, and a 4.6 i.d. 325.0 mm Devosil C_{30} UG-5 column (Nomura Chemical, Seto, Japan). LC was performed using an aqueous solution containing 1 vol % acetonitrile, 20 mM ammonium heptafluorobutyrate, and 10 mM ammonium formate (pH 4.0) as the mobile phase at a flow rate of 0.4 mL/min at 15 °C. The column was connected to the ion interface of the mass spectrometer through a fused-silica capillary without splitting. LC-MS spectra were recorded on a TSQ 700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an ESI source with an ICIS II data system in the positive ion mode. ESI was effected by a spray voltage of 14.8 kV, and the heated capillary temperature was maintained at 250 °C. Nitrogen served as the sheath gas at an operating pressure of 60 psi and as the auxiliary gas at a flow rate of 3 L/min. The dwell time was set at 500 ms/Da.

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz ^{13}C). Proton and carbon 90° pulse widths were 12.25 and 10.5 μs, respectively. About 40 mg of the sample dissolved in DMSO-d_6 was used for recording the spectra at 25 °C. Chemical shift values were expressed in parts per million relative to the internal tetramethylsilane standard.

Scheme 1. Purification Scheme for the Isolation of the Antioxidant Compounds from the Aqueous Extract of the Roots of *D. hamiltonii*



Optical Rotation. Optical rotation of the compounds DHA III and DHA V were recorded, using a known concentration of the compound dissolved in DMSO (50 mg/mL), on a Perkin-Elmer polarimeter.

Quantification of the Compounds in the Crude Extract. The isolated compounds in the aqueous extract were quantified by RP-HPLC with a C_{18} column, and the mobile phase used was a solvent mixture comprising 0.1% TFA in water/methanol (70:30) with a flow rate of 1 mL/min and monitored with a UV detector at 216 nm. On the basis of the retention time of the compounds and the corresponding peak area, the quantity of each compound in the extract was calculated (using calibration curve of individual compounds).

Antioxidant Activity. The antioxidant activity of the compounds was assayed by in vitro methods and compared with that of the standard antioxidants quercetin and BHA.

DPPH Radical Scavenging Assay. DPPH radical scavenging activity was assayed according to the method of Shon et al. (*10*). Briefly, 1 mL of DPPH solution (0.1 mM, in 95% ethanol) was mixed with different concentrations of the isolated compounds, shaken, and incubated for 20 min at room temperature, and the absorbance was read at 517 nm against a blank. The radical scavenging activity was measured as the decrease in the absorbance.

Superoxide Radical Scavenging Assay. Superoxide radical scavenging ability of the isolated compounds was measured according to the method of Nishikimi et al. (*11*). The reaction mixture containing varying concentrations of the isolated compounds, PMS (0.1 mM), NADH (1 mM), and NBT (1 mM) in phosphate buffer (0.1 M, pH 7.4), was incubated at room temperature for 5 min and the color due to NBT reduction was read at 560 nm against a blank. The scavenging efficiency was calculated as in the case of DPPH radical scavenging assay.

Hydroxyl Radical Scavenging Assay. Reaction mixture containing different concentrations of the isolated compounds was incubated with deoxyribose (10 mM), H_2O_2 (10 mM), $FeCl_3$ (5 mM), EDTA (1 mM), and ascorbic acid (5 mM) in potassium phosphate buffer (50 mM, pH 7.4) for 60 min at 37 °C (*12*). This was followed by the addition of TCA (5% w/v) and TBA (0.2% w/v) and boiling in a water bath for 15 min. The absorbance was measured at 535 nm against a reagent blank, and inhibition of the oxidation of deoxyribose was calculated against a control.

Nitric Oxide Radical Scavenging Assay. Nitric oxide was generated from sodium nitroprusside in aqueous solution at physiological pH (*13*)

Table 1. Spectral Characterization of the Compounds Isolated from the Aqueous Extract of the Roots of *D. hamiltonii*

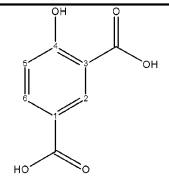
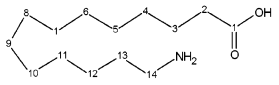
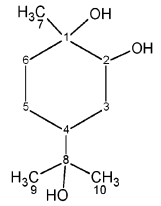
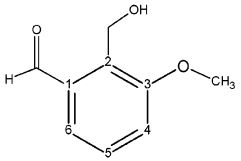
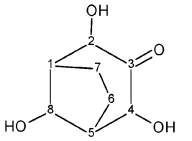
Compound (Mass)	Optical rotation $[\alpha]^{25}_D$	UV λmax (nm)	IR (Stretching)	NMR	Structure
DHA I. 4-hydroxy isophthalic acid (182.13)	-	221,253.5 & 291	3424 cm^{-1} (OH) 1645 cm^{-1} (C=O) 2253 cm^{-1} (CH) 1564 cm^{-1} (C=C)	**^{1}H-NMR:** δ 7.35 (d, 1H, J=2.0, H-2), 7.25 (dd, 1H, J=7.70, 2.0, H-6), 6.72 (d, 1H, J=7.95, H-5). **^{13}C-NMR:** δ 171.58 (C_1-COOH), 166.83 (C-4), 164.88 (C_3-COOH), 135.91 (C-2), 131.72 (C-6), 125.66 (C-1), 119.85 (C-5), 111.19 (C-3).	
DHA II. 14- aminotetradecanoic acid (243.38)	-	212	3427 cm^{-1} (NH) 1658 cm^{-1} (C=O) 2995 cm^{-1} (CH)	**^{1}H-NMR:** δ 3.70 (m, 2H, NH_2), 2.50 (t, 2H, J=6.08, CH_2-2), 2.05 (t, 2H, J=6.6, CH_2-14), 1.51 (m, 2H, CH_2-13), 1.31 (m, 2H, CH_2-3), 1.06 -1.24 (m, 18H, CH_2- 4, 5, 6, 7, 8, 9,10, 11, 12). **^{13}C-NMR:** δ 180.66 (COOH), 42.60 (C-14), 34.55 (C-13), 34.22 (C-2), 29.99 (C-11), 29.80 (C-6,7), 29.63 (C-10), 29.52 (C-5), 29.48 (C-9), 29.32 (C-8), 29.17 (C-4), 27.40 (C-12), 24.76 (C-3).	
DHA III. 4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol (188.26)	-1.4	205.5	3624 cm^{-1} (OH) 2870 cm^{-1} (CH)	**^{1}H-NMR:** δ 4.61, 3.44, 3.32 (s, 1H, 3 X OH), 3.45 (m, 1H, CH-2), 1.60 (m, 2H, CH_2-5), 1.59 (m, 2H, CH_2-6), 1.58 (m, 1H, CH-4), 0.97 (s, 3H, CH_3-7), 0.81 (s, 3H, CH_3-10), 0.77 (s, 3H, CH_3-9). **^{13}C-NMR:** δ 80.05 (C-2), 77.15 (C-8), 72.15 (C-1), 46.32 (C-4), 37.20 (C-6), 31.51 (C-3), 27.35 (C-5), 26.49 (C-9), 23.49 (C-7).	
DHA IV. 2-hydroxymethyl-3-methoxybenzaldehyde (166.17)	-	219.5 & 278.5	3479 cm^{-1} (OH) 1483 cm^{-1} (C=C) 1589 cm^{-1} (C=O) 3004 cm^{-1} (CH)	**^{1}H-NMR:** δ 8.47 (s, 1H, CHO), 6.92 (dd, 1H, J=7.6, 2.2, CH-6), 6.75 (m, 1H, CH-5), 6.73 (dd, 1H, J=8.2, 2.1, CH-4), 4.48 (s, 2H, CH_2OH), 3.77 (s, 3H, OCH_3). **^{13}C-NMR:** δ 195.53 (CHO), 157.44 (C-3), 138.91 (C-1), 130.90 (C-5), 128.84 (C-6), 124.73 (C-2), 119.79 (C-4), 56.38 (OCH_3), 53.64 (CH_2OH).	
DHA V. 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one (172.17)	-30.8	221.8	3435 cm^{-1} (OH) 2996 cm^{-1} (CH) 1724 cm^{-1} (C=O)	**^{1}H-NMR:** δ 3.92 (m, 2H, CH-2, 4), 3.75 (m, 1H, CH-8), 3.25 (s, 3H, 3 X OH), 1.92 (m, 2H, CH-1, 5), 1.89 (m, 4H, CH_2-6, 7). **^{13}C-NMR:** δ 207.60 (C-3), 76.70 (C-8), 70.02, (C-2, 4), 43.88 (C-1, 5), 24.21 (C-6, 7).	

Table 2. Relative Concentration of the Isolated Antioxidant Compounds in the Aqueous Extract of the Roots of *D. hamiltonii*[a]

compound	retention time (min)	quantity (mg/g of extract)
DHA I	7.61	4.63 ± 0.35
DHA II	7.88	2.16 ± 0.23
DHA III	1.58	1.29 ± 0.11
DHA IV	6.19	1.89 ± 0.17
DHA V	1.23	1.34 ± 0.12

[a] Quantitation of compounds was performed by RP-HPLC using a C_{18} column, eluted with methanol and water with 0.1% TFA (30:70).

and measured by the Griess reaction. Sodium nitroprusside (5 mM) was mixed with different concentrations of the isolated compounds in phosphate-buffered saline and incubated at 25 °C for 150 min followed by the addition of Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the reaction was read at 546 nm against a standard solution of potassium nitrite, treated in the same way with Griess reagent. The nitric oxide radical scavenging activity was measured as in the case of the DPPH radical scavenging assay.

Inhibition of Microsomal Lipid Peroxidation (LPO). Liver excised from adult male Wistar rats was homogenized (20% w/v) in 0.02 M Tris buffer (pH 7.4). Microsomes were isolated according to the calcium aggregation method (*14*). One hundred microliters (0.5 mg of protein) of the liver microsomal suspension was mixed with $FeSO_4$ (1 mM) and ascorbic acid (1 mM) with or without the isolated compounds in a total volume of 1 mL of 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 60 min. This was followed by the addition of 1 mL each of TCA (10%) and TBA (0.67%) and boiled in a water bath for 15 min. The absorbance of the supernatant was read at 535 nm, and the thiobarbituric acid reactive substances (TBARS) value of the supernatant was calculated using tetraethoxypropane as the standard (*15*). The TBARS value was taken as a measure of lipid peroxide generation.

Metal Ion Chelating Assay. The Fe^{2+} chelating ability of the isolated compounds was assayed by measuring the formation of ferrous iron−ferrozine complex (*16*). The reaction mixture containing $FeCl_2$ (2 mM) and ferrozine (5 mM) and the antioxidant compounds was adjusted to a total volume of 0.8 mL with methanol, shaken well, and incubated for 10 min at room temperature. The absorbance of the resultant color was read at 562 nm against a blank. EDTA was used as the positive control.

Inhibition of Human LDL Oxidation. Human LDL was diluted in phosphate-buffered saline (PBS) to 200 mg of protein/L and dialyzed overnight against PBS at 4 °C to remove the EDTA. LDL (100 μg of protein/mL) was oxidized with 10 μM $CuSO_4$ in the presence or absence of the isolated compounds in 10 mM PBS for 12 h followed by the addition of 1 mM EDTA to stop the reaction. After incubation, aliquots of the reaction mixture were used for measuring TBARS formation and relative electrophoretic mobility (REM). TBARS was measured by addition to 0.5 mL of the above aliquots of 1 mL each of 2.5% TCA and 1% TBA; the mixture was vortexed and boiled for 30 min. After cooling to room temperature, the samples were centrifuged and the fluorescence of the product formed was measured in a spectrofluorometer at 515 nm excitation and 553 nm emission wavelengths (*17*). The electrophoretic mobility of the native or oxidized LDL was determined by agarose gel electrophoresis on 0.7% agarose gel run at 85 V in the running buffer (40 mM Tris, 40 mM glacial acetic acid, and 1 mM EDTA) for 2 h. The lipoprotein bands were stained with Coomassie Brilliant Blue, and REM, defined as the ratio of the distances migrated from the origin by oxidized LDL versus native LDL, was calculated (*18*).

Protein estimation of the microsomes was done according to the method of Lowry et al. (*19*) using BSA as the standard.

Statistical Analysis. Data were expressed as mean ± standard error (SE) of three separate experiments, and the significance was determined by the analysis of variance ($p < 0.05$) using the computer program Excel and Statistica software.

RESULTS AND DISCUSSION

Identification and Quantification of the Compounds. In this study, we have shown that the aqueous extract of the roots of *D. hamiltonii* contains at least five antioxidant compounds. The compounds showing antioxidant activity were isolated and characterized by UV, IR spectral characteristics, and MS and NMR spectroscopic analysis. The compounds were identified and designated DHA I−V (**Table 1**): DHA I, 4-hydroxyisophthalic acid; DHA II, 14-aminotetradecanoic acid; DHA III, 4-(1-hydroxy-1-methylethyl)-1-methyl-1,2-cyclohexanediol; DHA IV, 2-(hydroxymethyl)-3-methoxybenzaldehyde; DHA V, 2,4,8-trihydroxybicyclo[3.2.1]octan-3-one.

The compounds DHA I−V from *D. hamiltonii* were found to be novel antioxidant molecules. Of the five antioxidant compounds, three (DHA II, III, and V) are nonphenolic, whereas the other two (DHA I and IV) are phenolic in nature. All five antioxidant compounds isolated were newly found in *D. hamiltonii*. However, Matsumura et al. (*20*) have isolated a compound of unknown biological activity from caraway (*Carvum carvi*), which is similar to the compound DHA III from *D. hamiltonii* reported in this study. Among the compounds isolated, DHA I was present in the highest concentration and DHA III in the lowest in the extract (**Table 2**). Our earlier work has shown that the roots of *D. hamiltonii* possess antioxidant properties (*11*). Recently we have identified the active principles in the methanolic extract of the roots of *D. hamiltonii*. Six antioxidant compounds were identified from the methanolic extract of *D. hamiltonii*, namely, 2-hydroxy-4-methoxybenzaldehyde, *p*-anisaldehyde, vanillin, borneol, salicylaldehyde, and decalepin, a novel polyphenolic glucopyranoside. The present study adds five more novel compounds that contribute to the antioxidant activity of the roots of *D. hamiltonii*.

Antioxidant Activity. All of the isolated compounds showed antioxidant activity in the in *vitro assays*, and the antioxidant potency of the compounds varied with the in vitro assay.

DPPH Radical Scavenging Activity. Proton-radical scavenging action is an important attribute of antioxidants, which is measured by the DPPH radical scavenging assay. The DPPH radical scavenging property of the isolated compounds is shown in **Table 3**. All of the compounds showed high radical scavenging activity. Among the compounds, DHA I (4-hydroxyisophthalic acid) was the most potent radical scavenger.

Superoxide Radical Scavenging Activity. Superoxide anion is by itself a weak oxidant but gives rise to the highly reactive hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. **Table 3** shows inhibition of superoxide radical generation by the isolated compounds. The IC_{50} values for the isolated compounds ranged from 11.74 to 508.24 nmol/mL. Among the compounds, DHA I (4-hydroxyisophthalic acid) showed the highest scavenging activity and DHA III [4-(1-hydroxy-1-methylethyl)-1-methyl-1,2-cyclohexanediol] the lowest, in the following order: DHA I > DHA IV > DHA II > DHA V > DHA III.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical is an extremely reactive and damaging species capable of damaging biomolecules including DNA strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity. The hydroxyl radical scavenging potential of the isolated compounds is presented in **Table 3**. The order of potency based on the IC_{50} values is DHA I > DHA V > DHA IV > DHA II > DHA III. Because water-insoluble compounds cannot be tested by this method, BHA and quercetin activities were not checked for comparison.

Table 3. Free Radical Scavenging and Metal Chelating Activity of the Compounds Isolated from the Aqueous Extract of *D. hamiltonii.*

	free radical scavenging activity[a]					
compound	LOO•	DPPH	•OH	O_2•$^-$	•NO	metal chelating activity[a]
DHA I	2.15 ± 0.19	21.36 ± 26	27.56 ± 2.45	11.74 ± 1.23	187.73 ± 16.59	20.6 ± 1.96
DHA II	36.78 ± 2.98	1042 ± 9.98	323.15 ± 30.8	135.89 ± 12.69	159.56 ± 13.89	304.6 ± 28.65
DHA III	56.48 ± 4.25	2715 ± 29.17	565.12 ± 49.34	508.24 ± 49.21	582.31 ± 51.34	198.7 ± 17.34
DHA IV	5.14 ± 62	151 ± 14.7	214.53 ± 26.34	126.34 ± 13.58	174.23 ± 18.13	987.5 ± 71.64
DHA V	42.65 ± 3.81	1104.98 ± 99.49	192.3 ± 20.64	281.43 ± 29.67	291.43 ± 30.56	213.7 ± 18.99
BHA	1.45 ± 1.29	34.4 ± 4.12		0	0	2115.6 ± 164.35
quercetin	1.86 ± 0.92	8.61 ± 0.79		335.58 ± 31.24	415.41 ± 39.73	557.2 ± 43.28

[a] Expressed as IC_{50} (nmol/mL).

Nitric Oxide Radical Scavenging Activity. Under physiological conditions, nitric oxide (NO) plays an important role as a vasodilator and neurotransmitter and in the immune system as a defense against tumor cells and infectious agents. NO *per se*, as a reactive radical, directly damages normal tissues and can also react with superoxide anion radical to form the even stronger oxidant, peroxynitrite (*21*). Nitric oxide was scavenged by the isolated compounds with IC_{50} in the range of 159.56−582.31 nmol/mL (**Table 3**). DHA II (14-aminotetradecanoic acid) showed the highest scavenging activity and DHA III [4-(1-hydroxy-1-methylethyl)-1-methyl-1,2-cyclohexanediol], the lowest.

Inhibition of Lipid Peroxidation. Lipid peroxidation of the microsomal membrane was inhibited by all of the isolated compounds (**Table 3**). On the basis of the IC_{50} values, the inhibition of LPO was in the following order: DHA I > DHA IV > DHA II > DHA V > DHA III.

Metal Ion Chelating Activity. The metal ion chelating capacity plays a significant role in the antioxidant mechanism because it prevents oxyradical generation and the consequent oxidative damage (*22*). The isolated compounds from *D. hamiltonii* showed metal chelating activity, which was concentration dependent. The order of metal chelating activity was DHA I > DHA III > DHA V > DHA II > DHA IV (**Table 3**).

Inhibition of Human LDL Oxidation. Oxidation of LDL is considered to be an essential step in the pathogenesis of atherosclerosis. LDL oxidation is characterized by alterations in structure and biological properties of lipids and apolipoprotein B (apo B), and evidence suggests that some endogenous and exogenous antioxidant compounds could have beneficial effects in the prevention of atherosclerosis. It has been suggested that the protection of LDL by antioxidants in a copper-induced oxidation of LDL could be due to both metal chelating and radical scavenging action (*23*). All of the isolated compounds showed protection against copper-induced LDL oxidation as measured by REM and TBARS formation. At equimolar concentration, the protective effect of the compounds was in the following order: DHA I > DHA V > DHA IV > DHA II > DHA III (**Table 4**). In our study, a close correlation was observed between REM and TBARS value of LDL oxidation. The metal chelating activity of the antioxidant compounds from *D. hamiltonii* could be partly responsible for inhibition of LDL oxidation in addition to their free radical scavenging activity. These non-nutrient phytochemicals may have a protective effect on the susceptibility of LDL to oxidative modification and therefore in the prevention of atherosclerosis. Studies of antioxidant supplementation in animal models of atherosclerosis have shown reduction in the disease (*24*).

The present study along with our earlier work (*9*) show that the root extracts of *D. hamiltonii* contain at least a dozen distinct antioxidant constituents that scavenge free radicals, chelate metal ions, and prevent LDL oxidation. The root extracts of *D. hamiltonii* are, therefore, a cocktail of antioxidants and provide a scientific basis for their alleged health-promoting properties. Isolation of the antioxidant compounds confirms our earlier hypothesis that *D. hamiltonii* crude extract could be a source of new bioactive antioxidant nutraceuticals (*7*). All of the compounds from *D. hamiltonii* presented in this study are novel antioxidant compounds reported for the first time. Our study opens up avenues for exploiting newer sources such as the roots of *D. hamiltonii* for the preparation and use of novel bioactive molecules with health implications in the prevention and amelioration of degenerative diseases.

Table 4. Inhibition of LDL Oxidation by the Compounds Isolated from the Aqueous Extract of *D. hamiltonii*

treatment[a]	TBARS (nmol of MDA/mg of protein)	REM
LDL	3.56 ± 0.29	1.0
LDL + $CuSO_4$	38.24 ± 2.98	2.48 ± 0.13
LDL + $CuSO_4$ + DMSO	37.65 ± 3.02	2.46 ± 0.12
LDL + $CuSO_4$ + DHA I	11.52 ± 1.23	1.21 ± 0.06
LDL + $CuSO_4$ + DHA II	19.28 ± 1.86	1.53 ± 0.08
LDL + $CuSO_4$ + DHA III	29.87 ± 3.14	1.98 ± 0.1
LDL + $CuSO_4$ + DHA IV	17.53 ± 1.67	1.41 ± 0.7
LDL + $CuSO_4$ + DHA V	12.86 ± 1.31	1.3 ± 0.04
LDL + $CuSO_4$ + BHA	26.94 ± 2.73	1.61 ± 0.06
LDL + $CuSO_4$ + quercetin	16.27 ± 1.63	1.4 ± 0.05

[a] The antioxidant compounds (DHA I–V) and the standard antioxidants were used at equimolar concentrations (5 μM).

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