JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation of Antioxidant Compounds from the Methanolic Extract of the Roots of *Decalepis hamiltonii* (Wight and Arn.)

R. HARISH,[†] S. DIVAKAR,[‡] ANUP SRIVASTAVA,[†] AND T. SHIVANANDAPPA*,[†]

Departments of Food Protectants and Infestatin Control and Fermentation Technology & Bioengineering, Central Food Technological Research Institute, Mysore-570 020, India

The tuberous roots of *Decalepis hamiltonii* are consumed as pickles and beverages and are believed to possess health-promoting properties. We have investigated the antioxidant potential of the roots. The methanolic extract of the root showed a high antioxidant activity. The methanolic extract was fractionated on a silica gel column, which showed three major fractions with good antioxidant activity. The active fractions were further subjected to preparative thin layer and silica gel column chromatography, which yielded six pure compounds. The purified compounds were characterized by MS, ^{1}H NMR, ^{13}C NMR, and two-dimensional NMR spectroscopic techniques and identified as 2-hydroxy-4-methoxybenzaldehyde, *p*-anisaldehyde, vanillin, borneol, salicylaldehyde, and bis-2,3,4,6-galloyl-α/β-D-glucopyranoside. The latter compound, named decalepin, is a new antioxidant molecule from the plant kingdom. The purified compounds showed antioxidant activities in in vitro assays such as inhibition of lipid peroxidation, hydroxyl radical, superoxide anion, and 1,1-diphenyl-2picrylhydrazyl radical scavenging. This is the first report of the antioxidant constituents of the roots of *Decalepis hamiltonii*.

KEYWORDS: ***Decalepis hamiltonii*; antioxidant compounds; decalepin; lipid peroxidation**

INTRODUCTION

Free radicals are implicated in several degenerative diseases such as atherosclerosis, diabetes, arthritis, cancer, and aging (*1*)-. The harmful effects of free radicals on living systems could be attenuated by antioxidants that scavenge the free radials. Plants are rich sources of natural antioxidants, which play a vital role in the prevention or progression of the degenerative diseases (*2*). The consumption of fruits, vegetables, and herbs rich in antioxidants is associated with a decline in the incidence of degenerative diseases and cancer (*2*, *3*). Therefore, there is a great deal of interest in natural antioxidants in view of their health implications.

The Indian subcontinent is rich in biodiversity with more than 20000 species of flowering plants. *Decalepis hamiltonii* (family: Asclepiadaceae) grows as a climber in the hilly forests of eastern and western ghats of southern India, known by the vernacular name "Makali beru". The tuberous roots have a fleshy outer layer with an inner woody core and possess a strong aromatic odor. The roots are also used as a substitute for the scarce *Hemidesmus indicus* in the traditional Indian system of medicine because of the similar aromatic properties (*4*). The roots of *D. hamiltonii* are consumed as pickles and as a juice since they are believed to possess health-promoting properties. The dried roots could be stored for long periods without microbial or insect infestation (*5*). The antimicrobial properties of the roots of *D. hamiltonii* have been attributed to the presence of 2-hydroxy-4-methoxy benzaldehyde and vanillin (*6*). Earlier work has shown that the roots contain aldehyde, inositol, saponins, ketonic substances, sterols, amyrins, and lupeols (*7*−*9*). Thangaduarai et al. (*10*) and Nagarajan et al. (*11*) have reported several volatile flavor compounds including 4-methoxybenzaldehyde, vanillin, and salicylaldehyde in the essential oil extracts from the roots of *D. hamiltonii*. We have recently shown that the roots of *D. hamiltonii* possess antioxidant properties and hypothesized that antioxidants constituent present in the root extracts could contribute to the health-promoting potential (*12*, *13*). We now report the isolation and characterization of the antioxidant compounds from the methanolic extract of the roots of *D. hamiltonii*.

MATERIALS AND METHODS

Chemicals. Nicotinamide adenine dinucleotide reduced (NADH), nitroblue tetrazolium chloride (NBT), and phenazine methosulfate (PMS) were procured from Sisco Research Laboratories (India). 2-Deoxy-D-ribose, butylated hydroxy anisole (BHA), thiobarbituric acid (TBA), and 1,1-diphenyl-2 picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (United States). All other chemicals and solvents were purchased from Ranbaxy and Qualigens (India).

Isolation of the Antioxidant Compounds. Freshly harvested roots of *D. hamiltonii* were procured from the local suppliers in Mysore (India). The roots were washed with tap water, and the fleshy portions were separated from the inner woody core manually and cut into small pieces (0.2−0.3 cm thickness and 0.3 cm × 0.5 cm in size) and dried at 40 °C for 12 h. The dried pieces were ground to powder in a grinder.

* To whom correspondence should be addressed. E-mail: tshivanandappa@yahoo.com.
[†] Department of Food Protectants and Infestatin Control.
[‡] Department of Fermentation Technology & Bioengineering.

10.1021/jf051047c CCC: $30.25
Published on Web 09/08/2005

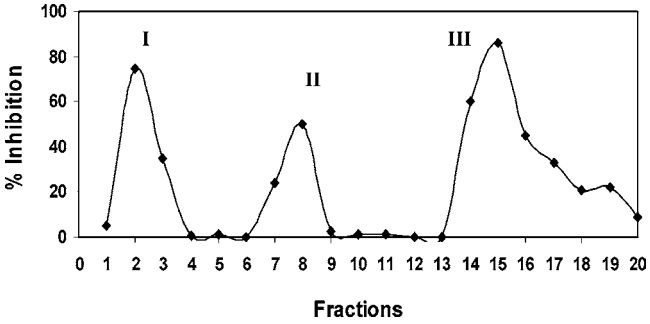
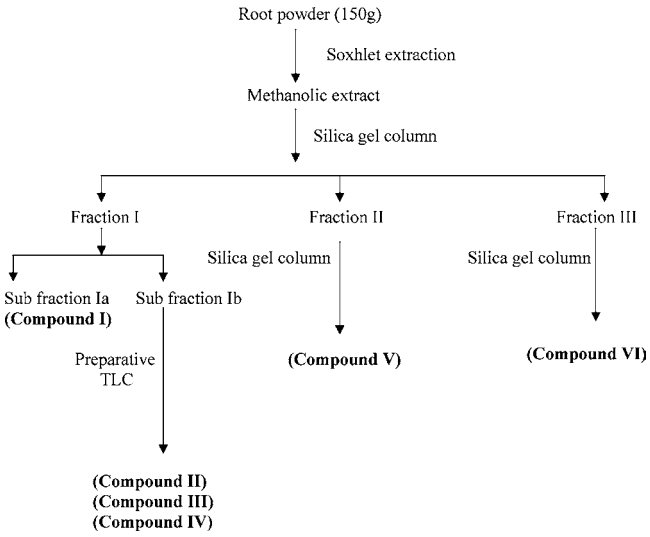


Figure 1. Superoxide scavenging activity of the chromatographic fractions from the methanolic extract of the roots of *D. hamiltonii*.

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



Extraction. Root powder (150 g) was extracted with a series of solvents of increasing polarity, viz., hexane, chloroform, ethyl acetate, and methanol, in a Soxhlet apparatus. The solvent was evaporated in a flash evaporator, and the extracts were dissolved in a known volume and assayed for antioxidant activity by superoxide radical scavenging in vitro by the method of Nishikimi et al. (*14*). Methanolic extract, which showed maximum activity, was selected for the isolation of the antioxidant compounds.

Fractionation of the Methanolic Extract. The methanolic extract (20 g) was subjected to column chromatography using a glass column (450 mm × 40 mm) packed with silica gel (60−120 mesh) and eluted with chloroform followed by a stepwise gradient of ethyl acetate and methanol. Twenty fractions of 250 mL each were collected, concentrated under reduced pressure, and assayed for superoxide scavenging activity (*14*). Fractions showing radical scavenging activity were pooled into three major fractions (**Figure 1**).

Fraction I (**Figure 1**) comprised two subfractions, Ia and Ib. Subfraction Ia was eluted with chloroform and ethyl acetate (90:10), gradually precipitated, and was pure as confirmed by reverse phase high-performance liquid chromatography (RP-HPLC) and identified as 2-hydroxy-4-methoxy benzaldehyde (compound **I**). Subfraction Ib was loaded on to a preparative thin-layer chromatography (TLC) plate (200 mm × 200 mm), prepared with silica gel G for further purification, and eluted with hexane and chloroform (50:50). TLC showed nine spots of which two showed radical scavenging activity and were further purified by a second round of preparative TLC, and the purity was ascertained by RP-HPLC. The purified compounds were identified as *p*-anisaldehyde, vanillin, and borneol (compounds **II**, **III**, and **IV**, respectively).

Fraction II (**Scheme 1**) was concentrated and loaded on to a silica gel column (250 mm × 20 mm) and eluted with a stepwise gradient of chloroform and ethyl acetate (100:0, 50:50, 25:75, and 10:90). Sixteen fractions of 100 mL each were collected, concentrated, and assayed for antioxidant activity by the superoxide scavenging assay (*14*). Active fractions were pooled and checked for purity by RP-HPLC monitored at 220 nm, which showed a major peak and two minor peaks. The major peak was further subjected to silica gel column chromatography for further purification, which yielded one compound. The purified compound was identified as salicylaldehyde (compound **V**).

Fraction III was subjected to a further step of silica gel chromatography on a 450 mm × 40 mm column and eluted with chloroform and ethyl acetate (100:0) (0:100 v/v) and followed by a stepwise gradient of ethyl acetate and methanol (75:25−25:75 v/v). Seventeen fractions of 100 mL each were collected, concentrated, and assayed for the superoxide scavenging activity. Among the 17 fractions, eight active fractions were obtained of which one showing the highest activity was found to be pure by RP-HPLC. On the basis of the spectroscopic data, the compound was identified as bis-2,3,4,6-galloyl-α/β-D-glucopyranoside (compound **VI**).

HPLC. RP-HPLC was carried out in the Shimadzu-LC-8A instrument equipped with a rheodyne 7725i injection valve fitted with a 20 μL sample loop and a C_{18} column (5 μm particle size, 25 cm × 0.4 cm i.d; Suppelco, United States). 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 a UV detector at 220 nm.

UV and Infrared Spectrometry. UV−vis spectra were recorded on a Shimadzu UV−vis spectrophotometer. The sample was dissolved in methanol (0.1 mg/mL), and the spectrum at 200−800 nm was recorded. IR spectra were recorded with a Perkin-Elmer FT-IR spectrometer (Spectrum 2000) at 400−4000 cm^{-1}.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analysis of the purified molecules was carried out in Agilent 6890 GC equipped with a 5973N mass selective detector and Hp-5 Ms capillary column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 μm). Helium was the carrier gas at 1.0 mL/min and a constant flow rate; the oven temperature was maintained at 75 °C for 5 min and increased to 300 °C at the rate of 10 °C/min and held for 5 min at 300 °C; interface temperature, −150 °C; ion source temperature, −230 °C; and quadrupole temperature, −150 °C.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). MALDI-MS analysis was carried out on a Kompact MALDI mass spectrometer in the negative mode with α-cyano-4-hydroxycinnamic acid as the matrix prepared as a pinch in 0.1% ammonium acetate solution in water (50 μL) and shaken to get a saturated solution. The sample solution at 1 μg/mL was transferred to the MALDI plate by placing 0.5 μL of the sample solution and matrix solution on to the sample slide at the molar ratio of 1:100 to 1:50000.

^1H and ^{13}C NMR. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz for ^1H 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 and D_2O was used for recording the spectra at 25 °C. Chemical shift values were expressed in ppm relative to the internal standard, tetramethylsilane. Two-dimensional heteronulear multiple quantum coherence transfer spectra (2D HMQCT) were recorded in magnitude mode with sinusoidal-shaped *Z*-gradients of strengths 25.7, 15.42, and 20.56 G/cm with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The size of the computer memory used to accumulate the 2D data was 4K. The spectra were processed using unshifted and π/4 shifted since bell window function in F_1 and F_2 dimensions, respectively.

Antioxidant Activity Assay. Antioxidant activities of the methanolic extract and the purified compounds were assayed by the following methods.

Microsomal Membrane Lipid Peroxidation. Microsomes were prepared from the rat liver by the calcium aggregation method (*15*). The microsomal lipid peroxidation was assayed by the thiobarbituric acid method (*16*). To 100 μL of microsomes (suspended in 0.1 M phosphate buffer, pH 7.4) was added ferrous sulfate (100 μM) and ascorbate (100 μM) with or without the extract or purified compound in 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 1 h. This was followed by the addition of 2 mL each of 20% trichloroacetic acid and

Table 1. Antioxidant Activity of the Compounds Isolated from the Methanolic Extract of *D. hamiltonii*

	IC_{50} (μmol/mL)			
compounds	superoxide scavenging	microsomal lipid peroxidation	hydroxyl radical scavenging	DPPH radical scavenging
I. HMBA	0.57 ± 0.038	2.46 ± 0.16		
II. *p*-anisaldehyde	0.52 ± 0.047	1.38 ± 0.22	0.68 ± 0.07	0.20 ± 0.03
III. vanillin	2.33 ± 0.16	0.37 ± 0.04	0.16 ± 0.01	0.61 ± 0.02
IV. borneol	0.190 ± 0.010	1.91 ± 0.083	0.32 ± 0.016	0.18 ± 0.08
V. salicylaldehyde	0.66 ±. 0.04	12.2 ± 0.72	0.013±0.005	1.02 ± 0.09
VI. decalepin	1.64 ± 0.10	0.023 ± 0.002	2.9 ± 0.02	0.12 ± 0.01
BHA		0.018 ± 0.003		0.029 ± 0.004
crude methanolic extract (mg/mL)	1.8 ± 0.12	0.40 ± 0.05	2.7 ± 0.1	0.18 ± 0.01

[a] Values expressed are means ± SD of three replicates.

1% thiobarbituric acid. The mixture was heated in a boiling water bath for 10 min, cooled, centrifuged, and the color in the supernatant was read at 535 nm in a spectrophotometer. The percent inhibition was calculated against a control.

DPPH Radical Scavenging. Scavenging of the stable radical, DPPH, was assayed by the method of Hatano et al. (*17*). The extract or the compound was added to a 0.5 mL solution of DPPH (0.25 mM in 95% ethanol). The mixture was shaken well and allowed to stand at room temperature for 30 min, and the absorbance was read at 517 nm in a spectrophotometer.

Superoxide Anion Scavenging. Superoxide anion was generated by the reaction of NADH and PMS coupled to the reduction of NBT (*18*) with slight modification. The reaction mixture contained NBT (100 μM) and NADH (300 μM) with or without the extract or the compound to be assayed in a total volume of 1 mL of tris buffer (0.02 M, pH 8.3). The reaction was started by adding PMS (30 μM) to the mixture, and the absorbance change was recorded at 560 nm every 30 s for 2 min. The percent inhibition was calculated against a control without the extract/compound.

OH^- Radical Scavenging. OH^- radical scavenging activity was assayed by the method of Halliwell et al. (*18*). The reaction mixture containing deoxyribose (2.8 mM), $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM), and ascorbate (100 μM) in a final volume of 1.0 mL of phosphate buffer (20 mM, pH 7.4) was incubated at 37 °C for 1 h, followed by the addition of 2 mL each of 20% TCA and 1% TBA. The mixture was heated on a boiling water bath for 10 min and cooled. The color was read at 535 nm in a spectrophotometer.

Statistical Analysis. IC_{50} values from the inhibition data were calculated by regression analysis.

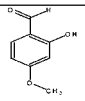
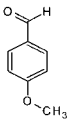
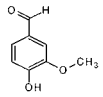
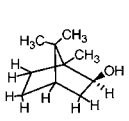
RESULTS AND DISCUSSION

Although some of the chemical constituents of *D. hamiltonii* roots have been isolated (*8*−*11*), compound(s) with antioxidant activity have not been reported. The antioxidant activity of the extracts of the roots of *D. hamiltonii* was shown by us earlier (*12*, *13*). In this study, we have isolated six bioactive compounds showing antioxidant properties from the methanolic extract of *D. hamiltonii* (**Table 1**).

Identification of the Compounds. The six compounds showing antioxidant activity were characterized by spectroscopic analysis (**Tables 2** and **3**).

Compound I (2-Hydroxy-4-methoxybenzaldehyde). Compound **I** showed UV absorption maxima (**Table 2**) at 256 nm ($\pi\rightarrow\pi^*$ transition of phenolic group OH) and 309 nm ($n\rightarrow\pi^*$ transition). IR spectra showed OH stretching at 3345 cm^{-1} and carbonyl stretching at 1730 cm^{-1}. GC-MS indicated the molecular ion at *m*/*z* 152. Proton NMR data showed aldehyde and OH groups at 9.72 and 11.49 ppm, respectively, along with the

Table 2. Spectral Characterization of the Isolated Compounds from the Methanolic Extract of the Roots of *D. hamiltonii*

compound	UV λ max	IR	NMR	structure
I. 2-hydroxy-4-methoxybenzaldehyde (M.W. 152)	256nm, 309nm	3345 cm^{-1} (OH stretching) 1730 cm^{-1} (C=O stretching 1207 cm^{-1} (C-O-CH_3 stretching)	^{1}H ppm (d_6- DMSO, 500.13 MHz): CHO - 9.72, 2-OH - 11.49, H-6 – 7.44 ppm (d, 1H, 8.7Hz), H-5 – 6.55 ppm (t, 1H, 8.7 Hz, 2.2 Hz), H-3 – 6.44 ppm (d, 1H, 2.2 Hz), 4-O-CH_3 – 3.84 ppm.	
II. *p*-anisaldehyde (M.W. 136)	308nm	3342 cm^{-1} (OH stretching) 1796 cm^{-1} (C=O stretching)	^{1}H ppm (d_6- DMSO, 500.13 MHz): OCH_3 – 3.71 ppm (s, 3H), 2,6-H – 6.99 ppm (d, 8.5 Hz), 3, 5-H – 7.23 ppm (d, 8.5 Hz), CHO – 9.75 ppm (s, 1H).	
III. vanillin (M.W. 152)	304, 278 & 229nm	3205 cm^{-1} (OH stretching) 1676 cm^{-1}(C=O stretching)	^{1}H ppm (d_6- DMSO, 500.13 MHz): OCH_3 – 3.8 ppm (s, 3H), H-5 – 7.21 ppm (d, 1H, 9.0 Hz), H-2 – 7.37 ppm (s, 1H), H-6 – 7.55 ppm (d, 1H, 9.0 Hz), CHO – 9.9 ppm (s, 1H).	
IV. borneol (M.W. 154)	213nm	3793 cm^{-1}(OH stretching)	^{1}H ppm (d_6- DMSO, 500.13 MHz): 7,8-CH_3 – 0.99 ppm (s, 3H) & 0.98 ppm (s, 3H), 1-CH_3 – 1.13 ppm (s, 3H), OH – 3.25 ppm (s, 1H), 2-H – 3.62 ppm (m,1H), 3,5,6 – $H_{a\&b}$ – 1.05 – 1.5 ppm (m, 6H), 4H - 2.05 ppm (s, 1H).	
V. salicylaldehyde (M.W. 122)	272nm	3452 cm^{-1} (OH stretching) 3070 cm^{-1} (CH stretching) 1696 cm (C=O stretching)	^{1}H ppm (d_6- DMSO, 500.13 MHz): H-3 – 6.85 ppm (d, 1H, 7.3 Hz), H-4 – 7.31 ppm (t, 1H, 7.0 Hz), H-5 – 7.02 ppm (t, 1H, 7.7 Hz), 6-H – 7.29 ppm (d, 1H, 7.7), CHO – 9.9 ppm (s, 1H). ^{13} C NMR C (d_6- DMSO, 125 MHz): C_1 – 119.9 ppm, C_2 – 158.7 ppm, C_3 – 118.0 ppm, C_4 – 130.7 ppm, C_5 – 118.5 ppm C_6 – 130.8 ppm, CHO – 196.1 ppm	

aromatic proton at 7.44, 6.55, and 6.44 ppm with characteristic coupling constants corresponding to a ABX pattern of trisubstituted aromatic compound. A single peak at 3.84 ppm, corresponding to the proton indicative of a $O-CH_3$ group, was also detected. The compound was identified as 2-hydroxy-4-methoxybenzaldehyde (HMBA).

*Compound **II** (p-Anisaldehyde).* Compound **II** showed a UV absorption maxima at 308 nm indicative of an $n\rightarrow\pi^*$ transition. IR spectra showed an OH stretching band at 3342 cm^{-1} and carbonyl stretching at 1796 cm^{-1}. GC-MS indicated the molecular ion at *m*/*z* 136. Proton NMR data showed a OCH_3 peak at 3.71 ppm and a signal at 9.75 ppm indicating an aldehyde group. Aromatic peaks (doublets) corresponding to the two proton peaks at 6.99 and 7.23 ppm with a common coupling constant at 8.5 Hz indicated that these two protons are adjacent. NMR spectra indicated that the compound is para-substituted (**Table 2**). The compound was identified to be paramethoxybenzaldehyde (*p*-anisaldehyde).

*Compound **III** (Vanillin).* Compound **III** exhibited UV absorption maxima at 304, 278 ($n\rightarrow\pi^*$ transition), and 229 ($n\rightarrow\pi^*$ transition) nm. IR spectra showed a OH stretching band at 3205 cm^{-1} and a carbonyl stretching at 1676 cm^{-1}. GC-MS indicated the molecular ion at *m*/*z* 152. Proton NMR data showed a singlet at 9.9 ppm indicating an aldehyde group. Doublet with a coupling constant of 9 Hz at 7.21 ppm, another doublet at 7.55 ppm corresponding to a singlet with a coupling constant of 9 Hz, and a singlet at 7.37 ppm (**Table 2**) indicated that the compound is a trisubstituted benzene. The NMR spectral data matched with that of vanillin.

*Compound **IV** (Borneol).* Compound **IV** showed a UV absorption maxima at 213 nm indicative of an $\sigma\rightarrow\sigma^*$ transition. IR spectra showed a OH stretching band at 3793 cm^{-1}. GC-MS indicated the molecular ion at *m*/*z* 154. Proton NMR data showed three singlets consisting of three protons at 0.99, 0.98, and 1.13 ppm indicating that three methyl groups are present in the molecule. Multiple peaks correspond at 3.62 ppm indicated that a CH is attached to a OH group. A singlet at 3.25 ppm indicated a OH group. Six protons with a complex coupling pattern were observed between 1.05 and 1.5 ppm indicating that they are from CH_2 groups. A singlet proton at 2.05 ppm for a single proton was also observed (**Table 2**). From the NMR data, the compound was deduced to be borneol.

*Compound **V** (Salicylaldehyde).* Compound **V** showed a UV absorption maxima at 272 nm indicative of an $n\rightarrow\pi^*$ transition of a phenolic OH. IR spectra showed a OH stretching band at 3452 cm^{-1}, CH stretching at 3070 cm^{-1}, and a carbonyl stretching at 1696 cm^{-1}. GC-MS indicated the molecular ion at *m*/*z* 122. NMR data showed a doublet for a single proton at 6.85 ppm, triplet corresponding to another single proton at 7.02 ppm, and another doublet corresponding to another single proton at 7.29 indicating that the compound is a disubstituted benzenoid. A singlet at 9.9 ppm indicated an aldehyde group. ^{13}C NMR spectra showed seven carbon signals. A signal at 158.7 ppm and another at 196.1 ppm **(Table 2)** indicated that the molecule is salicylaldehyde.

*Compound **VI** (Decalepin).* Compound **VI** showed UV absorption maxima at 231 and 276 ($n\rightarrow\pi^*$ transisition) nm, indicating phenolic OH groups. IR spectra showed a broad OH stretching band at 3766 cm^{-1}, CH stretching at 3182 cm^{-1}, and carbonyl stretching at 1809 cm^{-1}. The compound showed a specific rotation $[\alpha]_D^{25}$ of 1.42. MALDI-MS, in the negative mode, showed peaks at 1392.2, 826.5, and 657.9. The *m*/*z* of 826.5 fragment corresponds to digalloellagate attached to glucopyranose. The *m*/*z* 657.9 fragment could be due to the loss of one gallate from digalloellagate moiety attached to glucopyranose. ^1H and ^{13}C NMR data from 2D HMQCT were complex. ^1H data showed very few aromatic protons between 6.23 and 6.65 ppm. ^1H data showed signals between 3.09 and 4.9 ppm also indicating that the molecule is a carbohydrate. The chemical shift values of the individual protons indicated that the molecule is glucose. ^{13}C NMR spectra confirmed two anomeric carbons at 92.3 and 102.1 ppm (**Table 3**) indicating that the molecule is a mixture of α- and β-anomers of glucose. Besides, ^{13}C NMR data showed a huge number of peaks between 106 and 130 ppm indicating large number of aromatic carbons. Two-dimensional HMQCT spectra showed good correlation between ^1H and ^{13}C signals from glucose and aromatic protons. A large number of carbon singlets were detected from the one-dimensional ^{13}C spectra. Upon the basis of MALDI-MS and 2D NMR data, the structure was assigned to be bis-2,3,4,6-galloyl-α/β-D-glucopyranoside. Because this compound was a new molecule, it was named "decalepin". Among the six compounds isolated and identified in this study, five have been reported earlier as flavor or antimicrobial compounds (*6*, *10*, *11*). However, we report for the first time the compounds showing antioxidant activity from the roots of *D. hamiltonii*.

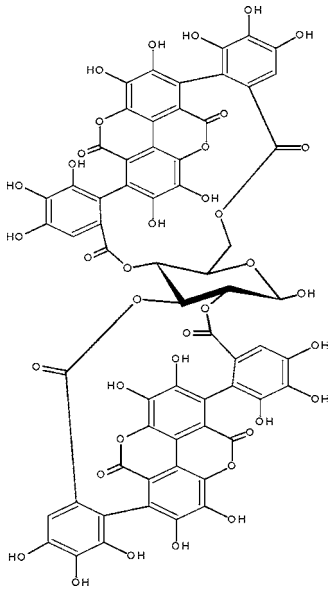
Antioxidant Activity. All of the compounds isolated from the methanolic extract of the roots of *D. hamiltonii* exhibited antioxidant activity in various in vitro assays.

Superoxide Radical Scavenging Activity. Although superoxide is a relatively weak oxidant, it gives rise to strong reactive oxygen species such as singlet oxygen, hydroxyl radical, and hydrogen peroxide via Fenton reaction, which initiates peroxidation of lipids (*19*). Reactive oxygen radicals produce oxidative damage to carbohydrates, proteins, lipids, and DNA (*20*). In the present study, superoxide anion generated by the NADH−PMS reaction was inhibited by the antioxidant molecule, which is a measure of their superoxide scavenging activity. **Table 1** shows inhibition of superoxide radical generation by the purified compounds from *D. hamiltonii*. IC_{50} values for the compounds ranged from 0.19 to 2.33 μmol/mL. Among the compounds, borneol showed the highest superoxide scavenging activity and vanillin, the lowest. *p*-Anisladehyde, HMBA, and salicylaldehyde were intermediate in their superoxide scavenging potential. Decalepin was somewhat lower in potency. *p*-Anisaldehyde has been reported to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (*21*). We report, for the first time, that it is a good scavenger of superoxide.

Inhibition of Lipid Peroxidation. In a biological system, lipid peroxidation involves oxidative degradation of polyunsaturated fatty acid in the cell membrane generates malondialdehyde (MDA), which is considered to be the principal cause of membrane damage and cellular injury (*22*). In the present study, the potential of the purified compounds to inhibit LPO in the rat liver microsomes induced by $FeCl_2$−ascorbate system measured as thiobarbiutric acid reactive substance (TBARS) is shown in (**Table 1**). IC_{50} values for the inhibition of LPO were in the following order: decalepin > vanillin > *p*-anisaldehyde > borneol > HMBA > salicylaldehyde. The crude methanolic extract of the roots showed an IC_{50} of 0.4 mg/mL. Our results show that decalepin, the new molecule from *D. hamiltonii*, is a potent inhibitor in LPO and was comparable to BHA (**Figure 2**). Decalepin, the galloyl ester of glucose, is therefore a novel antioxidant molecule of biological relevance.

Inhibition of Hydroxyl Radical Scavenging. The hydroxyl radical scavenging potential of the purified compounds is presented in (**Table 1**). The order of potency as judged from the IC_{50} values was salicylaldehyde > vanillin > borneol >

Table 3. Spectral Characterizations of Decalepin Isolated from the Methanolic Extract of the Roots of *D. hamiltonii*



compound	UV λ max	IR	specific rotation	MALDI-MS	NMR
VI. bis-2,3,4,6-galloyl α/β D-glucopyranoside (decalepin) (MW 1384)	231 nm 276 nm	3766 cm^{-1} (OH stretching) 3182 cm^{-1} (CH stretching) 1809 cm^{-1} (C═O stretching)	$[\alpha]_D^{25}$ = +1.42	1392.2 (negative mode) 657.9	^{1}H ppm (d_6-DMSO, 500.13 MHz) (ppm): H-$6_{a,b}$, 3.66 and 3.62; H-2, 3.09; H-5, 3.11; H-4, 3.39; H-1, 4.9 and 5.1; C-aromatic, 6.23–6.65 ^{13}C NMR (d_6-DMSO, 125 MHz) (ppm): C-6, 61.0; C-2, 77.0; C-5, 71.6; C-4, 72.5; C-1, 92.3 and 102.1

p-anisaldehyde > decalepin. IC_{50} values ranged from 0.013 to 2.9 μmol/mL, salicylaldehyde being the most potent and decalepin, the least. The methanolic extract of *D. hamiltonii* showed good OH^- radical scavenging activity with an IC_{50} value of 2.7 mg/mL. In the dose−response curve, it was observed that OH^- radical scavenging gets saturated at a point and does not increase with concentration. The low potency of decalepin in hydroxyl radical scavenging could be attributed to the competition in the oxidation of ascorbate and the phenolic hydroxyl group of decalepin by the Fenton reaction, which will attack the phenolic hydroxyl group of decalepin in more fascile manner than that of ascorbate, thereby reducing the effective concentration of dehydroascorbic acid for reaction with deoxyribose. This could also be the reason for BHA showing no hydroxyl scavenging activity.

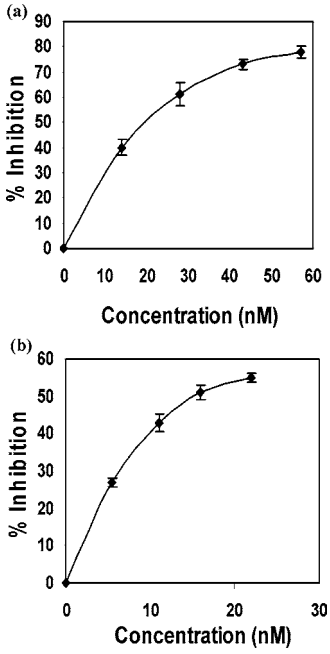


Figure 2. Inhibition of microsomal membrane lipid peroxidation by (**a**) decalepin and (**b**) BHA.

DPPH Radical Scavenging. The DPPH radical scavenging property of the compounds from *D. hamiltonii* is shown in (**Table 1**). All of the compounds except HMBA showed potent scavenging effect. The potency of a molecule for scavenging the radical is due to the number of hydrogens available for donation by the hydroxyl groups (*23*). However, the radical scavenging potential of decalepin, borneol, and *p*-anisaldehyde was rather high when compared to vanillin and salicylaldehyde. It has been reported earlier that vanillin is a good scavenger of DPPH radical and also inhibits lipid peroxidation in microsomes (*24*), which is inconsistent with our results. The root extract of *D. hamiltonii* shows an IC_{50} value of 0.18 mg/mL for DPPH radical scavenging.

Several polyphenolic compounds exhibit antioxidant property by virtue of their phenolic−hydroxyl groups, which contribute protons, thereby aiding in oxidation of the antioxidant molecule (*25*). Decalepin, the new molecule from *D. hamiltonii*, showed strong antioxidant activity as compared to the other compounds, which could be attributed to the presence of 10 phenolic OH groups. Although all of the isolated compounds from *D. hamiltonii* exhibited free radical scavenging activity to varying degrees depending on the assay, it is difficult to assign ranking on the overall antioxidant activity. It is also true for the well-known antioxidant, BHA. However, some of the antioxidant

assays such as inhibition of microsomal membrane lipid peroxidation and superoxide scavenging have physiological relevance as detailed above. Our study shows that the roots of *D. hamiltonii* are a source of natural antioxidants, which could contribute to the health benefits. However, in vivo studies are needed to confirm the health-promoting potential of the roots of *D. hamiltonii*.

ACKNOWLEDGMENT

We acknowledge the encouragement by Dr. V. Prakash, Director of the Institute, for supporting this study. MALDI-MS and NMR analyses were done at the Sophisticated Instrument Facility, Indian Institute of Science, Bangalore.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. C.; Cross, C. E. Free radicals, antioxidants and human disease; where are we now? *J. Lab. Clin. Med.* **1992**, *119*, 598−620.

(2) Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915−7922.

(3) Weisburger, J. H. Mechanisms of action of antioxidants as exemplified in vegetables, tomatoes and tea. *Food Chem. Toxicol.* **1999**, *37*, 943−948.

(4) Nayar, R. C.; Pattan Shetty, J. K.; Mary, Z.; Yoganarasimhan, S. N. Pharmacognostical studies on the root of *Decalepis hamiltonii* Wight & Arn. and comparison with *Hemidesmus indicus* (L.). *Proc. Natl. Acad. Sci. U.S.A.* **1978b**, *87*, 37−48.

(5) George, J.; Ravishankar, G. A.; Pereira, J.; Divakar, S. Bioinsecticide from swallow root (*Decalepis hamiltonii* Wight & Arn) protects food grains against insect infestation. *Curr. Sci.* **1999**, *77*, 501−502.

(6) Phadke, N. Y.; Gholap, K.; Ramakrishnan; Subbulakshmi, G. Essential oil of *Decalepis hamiltonii* as an antimicrobial agent. *J. Food Sci. Technol.* **1994**, *31*, 472−475.

(7) Murti, P. B. R.; Seshadri, T. R. A study of the chemical components of the roots of *Decalepis hamiltonii* (Makali veru). Part II. Chemical composition of the roots. *Proc. Indian Acad. Sci.* **1941a**, *A13*, 221−232.

(8) Murti, P. B. R.; Seshadri, T. R. A study of the chemical components of the roots of *Decalepis hamiltonii* (Makali veru). Part III. Comparison with Hemidesmus indicus (Indian sarsaparilla). *Proc. Indian Acad. Sci.* **1941b**, *A13*, 399−403.

(9) Murti, P. B. R.; Seshadri, T. R. A study of the chemical components of the roots of *Decalepis hamiltonii* (Makali veru). Part IV. Resinols of *D.hamiltonii* and *Hemidesmus indicus*. *Proc. Indian Acad. Sci.* **1941c**, *A14*, 93−99.

(10) Thangadurai, D.; Anitha, S.; Pullaiah, T.; Reddy, P. N.; Ramachandraiah, O. Essential oil constituents and *in vitro* antimicrobial activity of *Decalepis hamiltonii* roots against food borne pathogens. *J. Agric. Food Chem.* **2002**, *50*, 3147−3149.

(11) Nagarajan, S.; Jagan Mohan Rao, L.; Gurudutt, K. N. Chemical constituents of the volatiles of *Decalepis hamiltonii* (Wight & Arn). *Flavour Fragrance J.* **2001**, *16*, 27−29.

(12) Shereen; Srivastava, A.; Shivanandappa, T. Antioxidant properties of the swallow root, *Decalepis hamiltonii*. 71st Annual Meeting of the Society of Biological Chemists, Hyderabad, India, 2001; Abstract P2B28, p 176.

(13) Srivastava, A.; Shereen; Harish, R.; Shivanandappa, T. Antioxidant activity of the roots of *Decalepis hamiltonii* (Wight & Arn.). *Food Sci. Technol.* In press.

(14) Nishikimi, M.; Rao, A.; Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849−854.

(15) Vijayalakshmi, R.; Anandatheerthavarada, H. K. Preparation of brain microsomes with cytochrome P450 activity using calcium aggregation method. *Anal. Biochem.* **1990**, *187*, 310−313.

(16) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302−310.

(17) Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root; their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* **1988**, *36*, 2090−2097.

(18) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: A simple method test tube assay for determination of rate constant for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215−219.

(19) Dhal, M. K.; Richardson, T. Photogenesis of superoxide anion in serum of bovine milk and in model system containing riboflavin and amino acids. *J. Dairy Sci.* **1978**, *61*, 400−407.

(20) Nabasree, D.; Bratati, D. Antioxidant activity of piper betle L. leaf extract in vitro. *Food Chem.* **2004**, *88*, 219−224.

(21) Kubo, I.; Kinst Hori, I. Tyrosinase inhibitors from anisine oil. *J. Agric. Food Chem.* **1998**, *46*, 1268−1271.

(22) Yoshikawa, T.; Naito, Y.; Kondo, M. Food and diseases. In *Free Radicals and Diseases*; Hiramatsu, M., Yoshikawa, T., Inoue, M., Eds.; Plenum Press: New York, 1997; pp 11−19.

(23) Chen, C. W.; Ho, C. T. Antioxidant properties of polyphenols extracted from green and black tea. *J. Food Lipids* **1995**, *2*, 35−46.

(24) Liu, J.; Mori, A. Antioxidant and pro-oxidant activities of *p*-hydroxybenzyl alcohol and vanillin: Effects on free radicals, brain peroxidation and degradation of benzoate, deoxyribose, amino acids and DNA. *Neuropharmacology* **1993**, *32*, 659−669.

(25) Gow-Chin, Y.; Pin-Der, D.; Hui-Ling, T. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chem.* **2002**, *79*, 307−313.

Received for review May 6, 2005. Revised manuscript received August 4, 2005. Accepted August 5, 2005. The first and third authors are recipients of Research Fellowship from CSIR, New Delhi.

JF051047C