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RADICAL SCAVENGING ACTIVITY OF DECALPOLINE, A NOVEL COMPOUND CHARACTERIZED FROM *Decalepis hamiltonii*

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The ethanol extract of Decalepis hamiltonii *roots was subjected to antioxidant activity-guided fractionation by repeated silica gel column chromatography to get a pure antioxidant compound which was subjected to extensive analysis by UV, IR, LC-MS, and 2D HMQC NMR. The spectral data of the isolated compound was analyzed, and its structure was elucidated as decalpoline, a novel antioxidant compound not reported so far. Decalpoline exhibited multiple antioxidant properties like DPPH scavenging activity, superoxide radical scavenging activity, lipid peroxidation inhibitory activity, metal chelating and total reducing activity.*

Keywords: *Decalepis hamiltonii*, roots, decalpoline, antioxidant activity, DPPH scavenging activity, lipid peroxidation, metal chelating.

Roots of *Decalepis hamiltonii* Wight and Arn. (Asclepiadaceae) have a sweet, aromatic flavor and are used in India as food and beverage additives [1]. Recently the antimicrobial activity of different extracts and essential oil from *D. hamiltonii* roots against 15 different food-related microorganisms has been evaluated [2, 3]. The extracts have been shown to contain antioxidant properties [4, 5] and also insecticidal properties [6]. Several bioactive compounds were isolated from water and methanol extracts of *D. hamiltonii* roots and their health benefits were reported [7–10]. The dry powder and extracts of *D. hamiltonii* roots were extensively used for various therapeutic purpose such as to stimulate appetite and relieve flatulence, and as a vitalizer, analgetic, and stimulant in the form of a general tonic in *Ayurveda,* an ancient Indian system of medicine [11, 12, 4]. In the present investigation, the ethanol extract of *D. hamiltonii* root was used for isolation and characterization of its antioxidant compound.

Antioxidant Activity of the Extracts. Antioxidant activities of *D. hamiltonii* root extracts and their yields are presented in Table 1. The ethanol extract was selected for isolation and characterization of antioxidant compound because of its high DPPH radical scavenging activity and also high yield.

Purification of Antioxidant Compound. Purification of the ethanol extract by using silica gel column chromatography yielded about 24 fractions. Fractions having the same number of spots with similar R_f values on TLC plate were pooled into four fractions (Fr.1–Fr.4). All the fractions were tested for DPPH scavenging activity. Fraction 2 showed high DPPH scavenging activity (40.15%); hence it was selected for further purification. Further purification of the bioactive Fr. 2 yielded 17 fractions. Fractions having similar R_f values were pooled into two subfractions (Fr.2-1–Fr.2-2). Subfraction 2-2 obtained from the second step showed a single spot on TLC plate, and the purity of the compound was also confirmed by HPLC. This pure compound was subjected to various spectroscopic analyses for elucidation of the structure.

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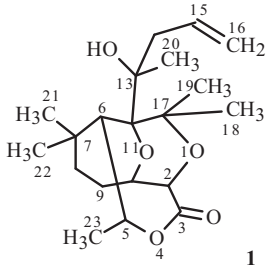
TABLE 1. Antioxidant Activity of Different Extracts of *Decalepis* Root

Extract	Yield, mg/g	DPPH scavenging activity, EC_{50} in mg/mL	Extract	Yield, mg/g	DPPH scavenging activity, EC_{50} in mg/mL
Toluene	48	1.2 ± 0.44	Ethanol	138	0.5 ± 0.18
Chloroform	54	2.4 ± 0.61	Water	128	0.6 ± 0.12
Ethyl acetate	90	1.9 ± 0.18	BHA	–	0.4 ± 0.13
Acetone	101	0.7 ± 0.21			

TABLE 2. NMR Data of Compound **1**

C atom	δ_H	δ_C	HMBC	C atom	δ_H	δ_C	HMBC
2	5.27 (d, J = 4.0)	77.53	C-3	14	2.37 (br.d, J = 16.5),	48.56	C-15, 13, 20
3		171.19			3.21 (br.d, J = 16.5)		
5	4.47 (m)	67.67	C-6, 23,	15	6.08 (dd, J = 11.0, 17.5)	146.46	C-13, 14, 20
6	2.13 (m)	42.54	C-5, 7, 12, 13	16	5.13 (dd, J = 1.5, 17.5)	108.72	C-13, 14, 15
7		33.57			4.87 (dd, J = 1.5, 11.0)		
8	2.18 (ddd, J = 3.0, 3.5, 14.5)	25.81	C-6, 7, 10, 21	17		81.07	
	1.37 (ddd, J = 3.0, 3.5, 14.5)			18	1.72 (s)	22.90	C-12, 17
9	1.78 (td, J = 3.5, 14.0)	35.77	C-10	19	2.11 (s)	19.63	C-18, 12
	1.04 (td, J = 3.0, 14.0)			20	1.34 (s)	29.24	C-13, 14, 15
10	4.46 (m)	73.33	C-2, 9	21	1.27 (s)	22.85	C-22, 6, 7
12		82.27		22	1.02 (s)	31.81	C-6, 7, 8, 9
13		74.99		23	1.44 (br.s)	18.56	C-6



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Elucidation of Structure of Decalpoline. Identification of specific functional groups was carried out using IR spectra. The O-H stretching observed at 3436 cm^{-1} is characteristic of a hydrogen-bonded OH group. The stretching at 1694 cm^{-1} was attributed to C=O stretching vibrations. The structure of the bioactive compound was elucidated after analyzing the data obtained by various spectroscopic techniques. The molecular weight was determined using LC-MS. The mass spectrum showed the parent molecular ion *m/z* at 353 $[M + 1]^+$. Elemental analysis (Vario EL III CHNS Elementar) revealed that the compound consists of 68.15% of carbon and 9.15% of hydrogen. Based on the molecular weight and elemental analysis, the empirical formula of the compound was determined as $C_{20}H_{32}O_5$.

The compound with the molecular formula $C_{20}H_{32}O_5$ seems to be a rearranged diterpene lactone **1** with three rings (a seven-membered ring containing an oxygen and gem-dimethyl group linked to a six-membered ring containing two oxygens in ether link along with a gem-dimethyl group and a bridge ring containing a methyl-substituted secondary lactone group connected to both the above rings). It was found to contain two gem-dimethyls attached at C-7 (δ 1.27 s, 3H; 1.02, s, 3H) and C-17 (1.72 s, 3H; 2.11, s, 3 H) from the ^1H NMR spectral data. Two more methyl groups were observed at 1.34 (3H, s) and 1.44 (3H, br.s). Spectral data [1.34 (3H, s); 5.13 (1H, dd, J = 1.5, 17.5 Hz); 4.87 (1H, dd, J = 1.5, 11.0 Hz); 6.08 (1H, dd, J = 11.0, 17.5 Hz); 2.37 (1H, br.d, J = 16.5 Hz); 3.21 (1H, br.d, J = 16.5 Hz)] also indicated the presence of an isopentenyl alcoholic group (4-methyl, 4-hydroxyl, 1-butenyl group) attached at the bridge carbon C-12. Protons of the methyl-substituted secondary lactone group are observed at δ 1.44 (3H, br.s); 4.47 (1H, m), and 2.13 (1H, m). Two methylene groups adjacent to two asymmetric carbons of the seven-membered ring are observed at 2.18 (1H, ddd, J = 3.0, 3.5, 14.5 Hz), 1.37 (1H, ddd, J = 3.0, 3.5, 14.5 Hz), 1.78 (1H, td, J = 3.5, 14.0 Hz), and 1.04 (1H, td, J = 3.0, 14.0 Hz). Two protons on the carbons adjacent to two ether oxygens are observed at 4.46 (1H, m) and 5.27 (1H, d, J = 4.0 Hz) (Table 2).

TABLE 3. Antioxidant Activity of Decalpoline and Ethanol Extract

Antioxidant activity methods	Antioxidant activity EC_{50} (mg/mL)		
	ethanol extract	decalpoline	BHA
Inhibition of linoleic acid oxidation	1.0	0.71	0.48
Lipid peroxidation inhibitory activity	1.02	4.82	0.018
$O_2^{\bullet -}$ scavenging activity	40	20	10
DPPH radical scavenging activity	0.5	10.75	0.44
$^{\bullet}OH$ scavenging activity	60	80	30
Metal chelating activity	30	23.3	27.5

The carbon NMR spectra supported the proposed structure with the following signals. Eight carbon signals of the seven membered ring were observed at δ 42.54 (C-6), 33.57 (C-7), 25.81 (C-8), 35.77 (C-9), 73.33 (C-10), 82.27 (C-12), and 22.85, 31.81 (C-21 and 22 of the gem-dimethyl group). Four carbons of the 6-membered ring showed signals at δ 77.53 (C-2), 81.07 (C-17), and 22.90, 19.63 (C-18 and 19 of the gem-dimethyl group). The carbons of the *iso*-pentenyl alcoholic group were observed at 74.99 (C-13), 48.56 (C-14), 146.46 (C-15), 108.72 (C-16), and 29.24 (C-20 of the methyl group). The carbons of the methyl-substituted secondary lactone group are observed at 67.67 (C-5), 171.19 (C of the lactonic carbonyl), and 18.56 (C-23 of the methyl group) (Table 2).

Based on the above spectral data, the compound was identified as a diterpene lactone and named decalpoline (**1**).

Antioxidant Properties of Decalpoline (1). Various antioxidant properties such as DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation inhibitory activity, metal chelating activity, and linoleic acid oxidation inhibitory activity were tested for the decalpoline and ethanol extract. BHA was used as a standard for antioxidant assay.

DPPH Radical Scavenging Activity. The isolated compound showed moderate activity compared to BHA and the ethanol extract (Table 3). DPPH radical scavenging by antioxidants has been attributed to the hydrogen-donating ability of the OH and CH_3 groups [13, 14]. Compared to decalpoline, the source extract showed higher DPPH radical scavenging activity. This may be due to the synergistic effect of other phytochemicals present in the crude extract [15].

Superoxide Scavenging Activity. Interestingly, decalpoline showed a greater superoxide radical scavenging activity than its source extract (Table 3). The superoxide anion plays an important role in the formation of reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA [16–18]. The higher activity of decalpoline is a preferred property for development of pharmaceutical or neutraceutical products.

Lipid Peroxidation Inhibitory Activity. The lipid peroxidation inhibitory activity of decalpoline was higher than that of the ethanol extract and BHA (Table 3). The lipid peroxidation inhibitory activity is mainly attributed to the number of hydroxyl groups, solubility, and hydrophobicity of the compounds [19]. The presence of methyl groups in decalpoline may be responsible for its lipid peroxidation inhibitory activity, as in other compounds [19]. Lipid peroxidation causes destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer [20].

Metal Chelating Activity. Decalpoline is observed to have more potent metal chelating activity than its source extract and BHA (Table 3), which is due to -OH, -O-, and C=O functional groups. In addition, the presence of the 6 CH_3 functional groups may also be responsible for the synergistic effect of decalpoline [21–23]. It has been reported that structures containing two or more of the functional groups -OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S-, and -O- in a favorable structure–function configuration are responsible for their metal chelating activity [24, 25]. Ferrous ions can stimulate lipid peroxidation by the Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl groups [26]. Since they are the most effective proxidant in the food system [27], the high chelating abilities of decalpoline would be extremely beneficial.

Hydroxyl Scavenging Activity. The ability to scavenge hydroxyl radical is another measure of antioxidant activity [28]. Decalpoline showed moderate activity with an EC_{50} value of 80 mg/mL compared to BHA and the ethanol extract, with an EC_{50} value of 30 and 60 mg/mL, respectively (Table 3).

Linoleic Acid Oxidation Inhibitory Activity by the Thiocyanate Method. Decalpoline is a very good inhibitor of peroxide formation by linoleic acid, with an EC_{50} of 0.71 mg/mL. Its effects against auto-oxidation are studied using a linoleic acid model system (ferric thiocyanate method) [29]. It is better than the source extract (EC_{50} 1.0 mg/mL). The presence of

6 CH_3 groups may be one of the reasons for this activity. As reported before, in o/w (oil in water) emulsion auto-oxidation, the lipophilicity of the phenols is the determining factor because the least polar compounds bearing -CH_3 are found to be the most effective antioxidants [23].

EXPERIMENTAL

Sample Collection. Fresh and healthy *D. hamaltonii* roots were procured from the local market, Mysore, India. The roots were washed, sliced, and dried in a hot air oven at 50°C for 72 hours and powdered to 60 meshes in an Apex grinder (Apex Constructions, London).

Extraction Procedure. *D. hamaltonii* root powder was extracted using solvents of increasing polarity with toluene, chloroform, ethyl acetate, acetone, ethanol, and water so as to extract both non-polar and polar antioxidants. The root powder (100 g) was extracted in 1 L of the solvent in a conical flask on a shaker for 10–12 h at room temperature. The extract was filtered and dried using a rotary evaporator.

Isolation of Antioxidant Compound. Fractionation of the Ethanol Extract. Activated silica gel (60–120 mesh) was packed onto a glass column (450 × 40 mm) using toluene solvent. For large-scale isolation of the antioxidant compound, about 10 g of crude ethanol extract was loaded and eluted stepwise with 400 mL of toluene, 1000 mL of toluene–chloroform (50:50 to 0:100, v/v), and 1000 mL of chloroform–methanol (50:50 to 0:100, v/v). About 24 fractions measuring 100 mL each were collected and concentrated by using the rotary evaporator.

Thin Layer Chromatography (TLC). An aliquot of all the concentrated fractions was loaded on activated silica gel TLC plates (20 × 20 cm). The plates were developed using hexane–chloroform (10:30), ethyl acetate–hexane (30:70), and chloroform–methanol (90:10) solvents. The spots were located by exposing the plate to iodine fumes. Fractions having the same number of spots with similar R_f values on the TLC plate were pooled into four fractions (Fr.1–Fr.4). All four fractions were tested for DPPH scavenging activity.

Purification of the Antioxidant Compound (Fr. 2). Since Fraction 2 obtained from the first step of column chromatography showed high antioxidant activity, it was selected for further purification. About 2.5 g of the bioactive fraction (Fr. 2) was further purified using a silica gel (60–120 mesh) column (450 × 20 mm). The column was eluted stepwise with 200 mL of toluene, 400 mL of toluene–chloroform (90:10 to 0:100, v/v), 700 mL of ethyl acetate–acetone (90:10 to 0:100, v/v), and 400 mL of acetone–methanol (90:10 to 0:100, v/v). About 17 fractions measuring 100 mL each were collected and concentrated on a rotary evaporator. Aliquots of all the fractions were loaded on the TLC plate, and fractions having similar R_f values were pooled into two subfractions (Fr.2.1–Fr.2.2). These two subfractions were tested for antioxidant activity. Among these, subfraction 2.2 obtained from the second chromatographic step showed a single spot on the TLC plate. This pure compound was subjected to various spectroscopic analyses for elucidation of the structure.

High-Performance Liquid Chromatography (HPLC). The purified antioxidant compound was tested for its purity by HPLC using an LC-10AT liquid chromatograph (M/s Shimadzu, Singapore) equipped with a C-18 column (Prevail C-18 5 m, 150 mm from M/s Alltech, Germany; fitted with a refillable guard column from M/s Waters India Ltd., India) and acetonitrile–water (50:50) as a mobile phase with a flow rate of 1.5 mL/min. UV detection was carried out with a diode array detector (M/s Shimadzu, Singapore). The detector was operated at an ultraviolet wavelength detection of 210 nm.

Characterization of the Purified Compound. IR Spectrometry. The IR spectrum of isolated compound was recorded on a Thermo Nicolet FTIR spectrometer (Model 5700, USA). It consists of a computer-assisted data acquisition and the OMNIC analysis program. The IR spectrum from 400–4000 cm^{-1} was recorded in the absorbance mode. A single bounce horizontal attenuated total reflectance (HATR) accessory with Zn–Se crystal was used to obtain the spectrum of the sample.

Mass Spectrometry. The mass spectrum of the isolated compound was recorded on an HP 1100 MSD series mass spectrometer (Palo Alto, CA) by the electron spray ionization (ESI) technique with a flow rate of 0.2 mL/min on a C-18 column at a total run time of 40 min. A diode array was used as the detector. About 1 mg of isolated compound dissolved in 5 mL of methanol was used for recording the spectrum.

NMR Spectroscopy. Different types of NMR spectra were recorded on a Bruker AQC 500 Avance NMR instrument (Rheinstetten, Germany). About 5 mg of isolated compound dissolved in 0.5 mL of CD_3OD was used for recording the spectra. Operation at 500 MHz for ^1H and 125 MHz for ^{13}C at room temperature and the region from 0–12 ppm for ^1H and 0–200 ppm for ^{13}C was employed to record proton and carbon spectra of the compound. Signals were referred to internal standard tetramethylsilane. Proton NMR spectra was recorded with the Zg30 pulse program, and the ^{13}C NMR spectra was recorded with the Zgdc pulse program.

Antioxidant Properties of Isolated Bioactive Compound. About 0.5 mL/0.5 mg of the plant extracts was mixed with linoleic acid emulsion (2.5 mL, 0.02 M, pH 7.0) and phosphate buffer (2 mL, 0.2 M, pH 7.0) and incubated at 37°C. The antioxidant activity was evaluated from the peroxide value determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate at various intervals during incubation [30].

Iron (Fe^{++}) Chelating Activity. One milliliter (0–1 mg) of the plant extract was mixed with 3.7 mL of deionized water and the mixture reacted with $FeCl_2$ (2 mM, 0.1 mL) and ferrozine (5 mM, 0.2 mL) for 10 min. The absorbance at 562 nm was determined using a spectrophotometer [31].

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity. The DPPH assay was carried out according to the method of Yamaguchi et al. [27]. Plant extracts were mixed with 800 μL of 100 mM Tris HCI buffer (pH 7.4) and then added to 1.0 mL of 500 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 20 min in the dark. The absorbance by DPPH was measured at 517 nm against a blank of ethanol in place of the extract.

Lipid Peroxidation in Erythrocyte Ghost (Lipid Peroxidation Inhibitory Activity). Fifty microliters of ghost membrane (50 μg of protein) was mixed with 100 μM $FeSO_4$, 1 mM ascorbic acid, and 4 mM BHA in 500 μL Tris buffered saline (10 mM, pH 7.8, 50 mM NaCl). The mixture was incubated at 37°C for 60 min with and without the extract as well as standards at the same concentration (1.0 mg); then 1.0 mL of 1% TBA was added, the whole boiled for 15 min, and the absorbance read at 535 nm [32].

Superoxide Radical Scavenging Activity. The superoxide radical scavenging assay was carried out according to the method of Liu et al. [33]. Superoxide radicals were generated in 1 mL of Tris–HCl buffer (0.02 M, pH 8.3) containing 0.1 mM NADH, 0.1 mM NBT, 10 LMPMs and the crude extract, and the purified antioxidant compound. The color reaction of superoxide radicals and NBT was detected at 560 nm using a UV-Vis spectrophotometer (M/s Shimadzu, Japan; model UV-1601).

Hydroxyl Radical Scavenging Activity. The hydroxyl radical was generated from a Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction), which attacks the deoxyribose and set off a series of reactions that eventually result in formation of malondialdehyde – TBA chromogen that has a λ_{max} at 532 nm. The reaction mixture (1 mL) contained deoxyribose (2.8 mM), KH_2PO_4–KOH (20 mM, pH 7.4), $FeCl_3$ (100 mM), EDTA (104 μM), H_2O_2 (1 mM), and ascorbate (100 μM). The reaction mixture was incubated at 37°C for 1 h; color developed as described above, and the spectra was measured at 532 nm [34].

Conclusions. Antioxidant-activity-guided fractionation of ethanol extract of *Decalepis hamiltonii* root by repeated silica gel chromatography yielded a pure compound. The structure of the compound was elucidated by analyzing the spectral data. The compound was identified as a diterpene lactone with molecular formula $C_{20}H_{32}O_5$. The compound has been named as decalpoline. Decalpoline exhibited multiple antioxidant activity, such as metal chelating activity, $O_2^{\bullet -}$ scavenging activity, and inhibition of linoleic acid oxidation activity. These biological properties of decalpoline and other extracts are of pharmaceutical interest.

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