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Isolation of ellagic acid from the aqueous extract of the roots of *Decalepis hamiltonii*: Antioxidant activity and cytoprotective effect

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Abstract

Roots of *Decalepis hamiltonii* are consumed as pickles and as a beverage and are believed to possess health-promoting properties. Roots of *D. hamiltonii* exhibit antioxidant properties. We have isolated an antioxidant compound from the aqueous extract of the roots of *D. hamiltonii* and identified it as ellagic acid (EA), based on NMR and MS. EA exhibited free radical-scavenging activity, inhibited LDL oxidation and showed cytoprotective effect against xenobiotic-induced oxidative stress in Ehrlich Ascites tumor cells. EA is a new addition to the list of antioxidant compounds in the roots of *D. hamiltonii*.
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Keywords: Antioxidant; Free radical scavenging; LDL oxidation; Cytoprotective

1. Introduction

Free radicals are implicated in the etiology of several diseases, such as atherosclerosis, neurodegenerative disorders, some forms of cancer and aging (Halliwell, 1999). 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 are believed to modulate oxidative stress and to prevent or delay degenerative disorders (Thatte, Bagadey, & Dahanukar, 2000). Naturally-occuring antioxidant phytochemicals are also of interest in the prevention of atherosclerosis which involves oxidation of LDL (Heinecke, 1997). Many plant-derived antioxidants are known to inhibit LDL oxidation in vitro (Vaya, Belinky, & Aviram, 1997). Protection of LDL from oxidation is considered to be an effective strategy to prevent or slow down the progression of atherosclerosis (Esterbauer, Gebicki, Puhl, & Jurgens, 1992). Examples of natural antioxidants include cinnamic acid derivatives, coumarins, flavonoids, polyfunctional organic acids and tocopherols (Pratt & Hudson, 1990).

Ellagic acid (EA), a dimeric derivative of gallic acid, is a polyphenolic antioxidant that occurs in its free form as a glycoside or is found as ellagitannins in fruits and nuts of several plants (Amakura, Okada, Tsuji, & Tonogai, 2000). Several studies have reported the antioxidant, antimutagenic, anti-inflammatory and cardioprotective activity of ellagic acid (Priyadarsini, Khopde, Kumar, & Mohan, 2002). The hepatoprotective property of ellagic acid has been reported both *in vitro* and *in vivo* (Singh, Khanna, & Chander, 1999a; Singh, Khanna, Visen, & Chander, 1999b). Protection against cellular injury, due to exposure to toxic chemicals, has been shown for some of the natural antioxidants (Malgorzata, Gilden, & Seibert, 2002; Tebbe et al., 1997). *In vitro* systems using cell culture are useful for studying cytotoxicity mediated by free radicals and to test the cytoprotective effect of antioxidants (Saraswat, Visen, & Agarwal, 2000; Srivastava & Shivanandappa,

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2006). Cytotoxic injury is believed to be integral to toxicological manifestation and cellular pathobiology. Compounds that ameliorate cytotoxic injury, therefore, are likely to exhibit health-promoting potential. In this study, we have demonstrated the cytoprotective action of EA isolated from *D. hamiltonii*.

D. hamiltonii (family: Asclepiadaceae), a climbing shrub, grows in the forests of peninsular India. Its tubers are consumed as pickles and juice for their alleged health-promoting properties. The roots are used in folk medicine and as a substitute for *Hemidesmus indicus* in ayurvedic preparations (Nayar, Shetty, Mary, & Yoganarshimhan, 1978). The roots of *D. hamiltonii* possess potent antioxidant properties which could be associated with their health benefits (Srivastava, Shereen, Harish, & Shivanandappa, 2006). Our recent work has shown that the extracts of the roots of *D. hamiltonii* are a cocktail of antioxidants (Harish, Divakar, Srivastava, & Shivanandappa, 2005; Srivastava, Harish, & Shivanandappa, 2006). In this paper, we report the isolation and characterization of EA from the aqueous extract of *D. hamiltonii* roots and evaluation of its free radical-scavenging properties, inhibition of LDL oxidation and, cytoprotective effect against oxidative stress-mediated cytotoxicity of xenobiotics.

2. Materials and methods

2.1. Chemicals

Human low density lipoprotein (LDL), butylated hydroxyanisole (BHA), nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), thiobarbituric acid (TBA), bovine serum albumin (BSA), glutathione (GSH), quercetin, tetraethoxy propane, trypan blue and ethylenediamine tetra-acetic 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 the reagents were of analytical grade.

2.2. Preparation of the root powder and extraction

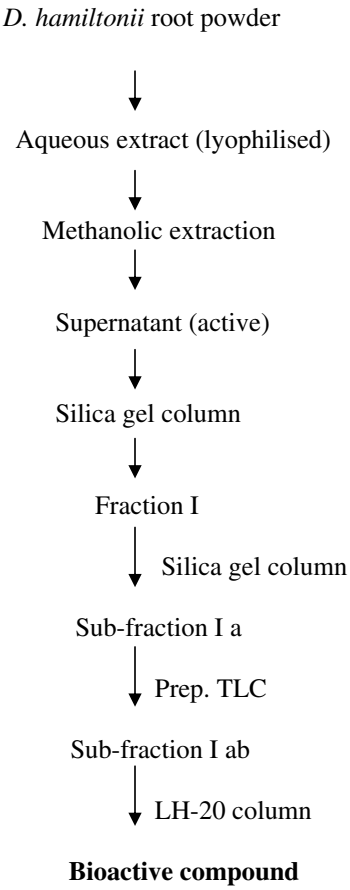
Tuberous roots of *D. hamiltonii* (10 kg) were procured from the local suppliers. The roots were washed with water, followed by crushing with a roller to separate the inner woody core from the outer fleshy layer. The fleshy portion was collected, dried at 40 °C in a hot air oven and finely powdered.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

We have earlier reported that an aqueous extract of *D. hamiltonii* shows high antioxidant activity (Srivastava, Shereen et al., 2006). The aqueous extract was prepared by homogenizing the root powder (200 g) in 1 l of warm water (50 °C) and allowing to stand for 24 h, and filtering through Whatman No. 1 paper; the filtrate was lyophilized and weighed (34.75 g).

2.3. Isolation

The lyophilized aqueous extract (34.75 g) was re-extracted with methanol, thrice, in a total volume of 500 ml and 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 ROS and DPPH radical-scavenging assays. Based on the antioxidant activity of the fractions and TLC profile, the active fractions were pooled (fraction I).

Fraction I was loaded on to a silica gel column (length: 32 cm, diameter: 2 cm) and eluted with a stepwise gradient of chloroform, ethyl acetate and methanol. The active sub-fractions collected were further separated by preparative TLC since the active isolate was not pure, it was again purified on a LH-20 column (length: 20 cm, diameter: 1.5 cm), using methanol as the eluant. The compound isolated was pure by RP-HPLC (Scheme 1).



Scheme 1. Activity-guided purification scheme for the isolation of ellagic acid from the aqueous extract of the roots of *D. hamiltonii*.

2.4. High performance liquid chromatography (RP-HPLC)

Reverse phase HPLC was performed 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. 21.2 mm) 5 μm particle size (Supelco, Sigma–Aldrich, St. Louis, USA), and eluting with an isocratic solvent comprising 0.1% TFA in water:methanol (70:30) at a flow rate of 1 ml/min and monitoring with a UV detector at 216 nm.

2.5. UV and infrared spectrometry

The purified compound (0.1 mg/ml in methanol) was used for recording the UV-spectrum at 200–800 nm in a Shimadzu UV/Vis spectrophotometer. IR spectra (10 mg/ml in DMSO) were recorded on a Perkin–Elmer FT-IR spectrometer at v 400–4000 cm^{-1}.

2.6. 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 μl loop, and a 4.6 i.d. 325 mm Devosil C30 UG-5 column (Nomura Chemical, Seto, Japan). LC was performed using an aqueous solution containing 1% 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 API-ES source with an ICIS II data system in the positive ion mode. API-ES 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 per Da.

2.7. ^{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 ppm relative to the internal tetramethylsilane standard. Two-dimensional heteronuclear multiple quantum coherence transfer spectra (2-D-HMQCT) were recorded in magnitude mode with sinusoidal shaped Z-gradients of strength 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 spectra were processed using unshifted and π/4-shifted since bell window function in F_1 and F_2 dimensions, respectively.

2.8. Quantification of the isolated compound in the crude extract

The isolated compound in the aqueous extract was quantified by RP-HPLC as described earlier. The calibration curve of the compound was used for calculating the quantity (mg)/g crude extract or per kg of root powder.

2.9. Antioxidant activity

2.9.1. General

Antioxidant activity of the compound was assayed by *in vitro* methods and compared with that of known antioxidants, quercetin and BHA.

2.9.2. DPPH radical-scavenging assay

DPPH radical-scavenging was assayed by the method of Shon, Kim, and Sung (2003). Briefly, 1 ml of DPPH solution (0.1 mM, in 95% ethanol) was mixed with different concentrations of the isolated compound, 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 and was calculated using the following equation:

$$\text{Scavenging effect } (\%) = [1 - A_{\text{Sample (517 nm)}}/A_{\text{Control (517 nm)}}] \times 100.$$

2.9.3. Superoxide radical-scavenging assay

Superoxide radical-scavenging was measured by the method of Nishikimi, Rao, and Yagi (1972). The reaction mixture, 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 with/without the isolated compound and the colour developed, due to NBT reduction, was read at 560 nm against a blank. The scavenging effect was calculated as in the case of DPPH radical-scavenging assay.

2.9.4. Hydroxyl radical-scavenging assay

Reaction mixtures containing different concentrations of the isolated compound were 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 (Halliwell, Gutteridge, & Cross, 1987). The reaction was terminated by adding TCA (5% w/v) and the reaction product was measured by the reaction with TBA (0.2% w/v) in a boiling water bath for 15 min. The absorbance was measured at 535 nm against the reagent blank and inhibition of the oxidation of deoxyribose was calculated against the control.

2.9.5. Nitric oxide radical-scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which is measured by the Griess reaction (Marcocci, Maguire,

& Droy-Lefaix, 1994). Sodium nitroprusside (5 mM) was mixed with different concentrations of the isolated compound in phosphate-buffered saline and incubated at 25 °C for 150 min followed by the addition of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the reaction was read at 546 nm and referred to the absorbance of standard solutions of potassium nitrite, treated in the same way with Griess reagent. The radical-scavenging activity was measured, using the equation described above for DPPH radical-scavenging assay.

2.9.6. Inhibition of microsomal lipid peroxidation

Liver excised from adult male Wistar rats was homogenized (20% w/v) in 0.02 M Tris buffer (pH 7.4). Microsomes were isolated by the calcium aggregation method (Kamath & Rubin, 1972). 100 μl (0.5 mg protein) of the liver microsomal suspension was mixed with $FeSO_4$ (1 mM) and ascorbic acid (1 mM) with or without the isolated compound 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 adding 1 ml each of TCA (10%) and TBA (0.67%), boiling in a water bath for 15 min and centrifuging. The absorbance of the supernatant was read at 535 nm and TBARS (thiobarbituric acid reactive substances) value of the supernatant was calculated using tetraethoxy propane as the standard (Buege & Aust, 1978). The TBARS value was taken as a measure of lipid peroxide generation.

2.9.7. Metal ion chelation assay

The Fe^{2+}-chelating ability of the isolated compound was assayed by measuring the formation of ferrous iron–ferrozine complex (Decker & Welch, 1990). The reaction mixture containing $FeCl_2$ (2 mM) and ferrozine (5 mM) and the isolated compound was adjusted to a total volume of 0.8 ml with methanol, shaken well, incubated for 10 min at room temperature and the absorbance of the resultant colour was read at 562 nm against a blank. EDTA was used as the positive control. The metal-chelating ability of the purified compound was calculated using the equation as described for DPPH radical-scavenging assay.

2.10. Inhibition of human low density lipoprotein oxidation

Human LDL was diluted in phosphate-buffered saline (PBS) to 200 μg of protein/ml and dialyzed overnight against PBS at 4 °C to remove the EDTA. LDL (100 μg protein/ml) was oxidized with 10 μM $CuSO_4$ in the presence or absence of the isolated *D. hamiltonii* compound in 10 mM PBS, 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 adding to 0.5 ml of the above aliquots, 1 ml each of 2.5% TCA and 1% TBA, vortexing and boiling for 30 min. After cooling to room temperature, the samples were centrifuged and the fluorescence of the product formed was measured in a spectrofluorimeter at 515 nm excitation and 553 nm emission wavelengths (Schuh, Faricough, & Haschemeyer, 1978). The electrophoretic mobility of the native or oxidized LDL was studied by agarose gel electrophoresis. Aliquots of the LDL reaction mixture were eletrophoresed in 0.7% agarose gel 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 (Jeong, Kim, Cho, Bae, & Lee, 2004).

2.11. EAT cells

2.11.1. General

Ehrlich Ascites Tumor cells were cultured in the peritoneum of male Swiss albino mice (Estrela et al., 1992). After harvesting, cells were suspended in Hank's balanced salt solution (HBSS) with 0.1% dextrose and 0.4% bovine serum albumin.

2.11.2. Cytoprotection

Cytotoxicities of the xenobiotics (hexachlorocyclohexane (HCH), carbon tetrachloride (CCl_4) and cumene hydroperoxide (CHP)) to EAT cells were assayed by measuring the cell viability after 60 min of exposure. LC_{50} values were determined by the dose–response curve. Cytoprotection experiments were done by incubating EAT cells (10×10^6) suspended in 1.0 ml of HBSS with xenobiotics (dissolved in DMSO) at LC_{50} concentration with/without the antioxidant compound for 60 min in a shaking water bath at 37 °C. At the end of incubation, an aliquot of cells was taken for viability assay by the trypan blue exclusion method (Frandsen & Schousboe, 1987).

2.11.3. Lactate dehydrogenase leakage

After incubation of cells in the presence of xenobiotics with/without the antioxidant compound, cells were centrifuged and the supernatant was assayed for LDH with sodium lactate as the substrate (Bergmeyer & Bernt, 1974).

2.11.4. Lipid peroxidation

After incubation, as above, the cells were centrifuged and the cell pellet was washed in saline and the pellet was boiled in TCA (5.5%) and TBA (0.34%) for 15 min, cooled and centrifuged. Fluorescence of the supernatant was measured in a fluorescence spectrophotometer at excitation and emission wavelengths of 532 and 553 nm, respectively (Cereser, Boget, Parvaz, & Revol, 2001). LPO, i.e. thiobarbituric acid reactive substances (TBARS) value, was calculated using a tetraethoxypropane standard curve.

2.11.5. Reactive oxygen species (superoxide anion)

The cells (10×10^6) suspended in 1.0 ml HBSS were incubated with NBT (0.2 mM) with or without xenobiotics (in DMSO) and antioxidant compound in a shaking water bath at 37 °C. The generation of ROS by cells (respiratory burst) was measured by the formation of coloured formazan due to reduction of NBT (Pompeia, Cury-Boaventura, & Curi, 2003).

2.11.6. Glutathione content

EAT cells (10×10^6) suspended in 1.0 ml HBSS were treated with xenobiotics (dissolved in DMSO) at LC_{50} concentration with/without the antioxidant compound and incubated for 60 min in a shaking water bath at 37 °C. At the end of incubation, cells were homogenized in 1.0 ml of 5% (w/v) trichloroacetic acid, centrifuged at 2000*g* for 20 min and glutathione (GSH) in the deproteinized supernatant was estimated by Ellman's reagent with a standard curve (Ellman, 1959).

Protein estimation of the microsomes was done by the method of Lowry, Rosenburg, Farr, and Randall (1951) using BSA as the standard.

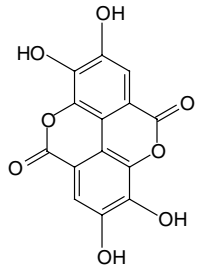
2.12. Statistical analysis

Data were expressed as means ± SE of three separate experiments and the significance was determined by the analysis of variance ($p < 0.05$) using the computer programme Excel and Statistica software.

3. Results

3.1. Identification of the compound

The purified compound was obtained as a colourless amorphous powder. The UV spectrum of the compound showed absorption λ_{max} 214 and 273 nm and IR spectrum indicated carbonyl absorption at ν 1700 cm^{-1} and hydroxyl absorptions at ν 3426, 3762 cm^{-1}. The ^1H NMR spectrum of the purified compound showed two aromatic protons at δ 6.91 (s) and three hydroxyl signals at δ 8.80 (s), 9.15 (s) and 12.20 (s), which disappeared on deuterium exchange. The ^{13}C NMR spectrum of the compound exhibited five signals at δ 110.0 (C-5,6,5′,6′), 120.8(C-1,1′), 138.0 (C-3,3′), 144.4(C-2,2′,4,4′), 170.1(C-7,7′). The HMQC spectrum indicated correlation between the signals at δ 6.91 (s, H-5,5′) and 110.0 (C-5,5′). API positive-ion mass spectroscopy (API-ES-MS) of the purified compound in methanol gave a quasi-molecular ion peak at *m*/*z* 364 $[M-2H+2MeOH]^+$, which corresponded to the molecular formula of $C_{14}H_6O_8$. The above spectral data indicated that the compound was ellagic acid. The identity of the compound was further confirmed by co-TLC with an authentic sample (Sigma, USA). The concentration of EA in the root extract was 2.27 mg/g extract or 0.39 g/kg of root powder.



Ellagic acid

3.2. Antioxidant activity

The compound isolated from *D. hamiltonii* showed concentration dependent scavenging of DPPH radical. Based on the IC_{50} value, the radical-scavenging capacity of the isolated compound was comparable to that of quercetin and it was much more potent than BHA. The IC_{50}values for superoxide radical-scavenging showed that EA exhibited very high activity compared to quercetin whereas BHA did not scavenge superoxide. EA is also a scavenger of hydroxyl radical and nitric oxide. Inhibition of microsomal lipid peroxidation (LPO) by EA was much higher than those of the standard antioxidants. The metal-chelating activity of EA was concentration-dependent. Overall, EA isolated from *D. hamiltonii* showed antioxidant activity in all the assays and its potency was equal to or higher than those of quercetin and BHA (Table 1).

3.3. Inhibition of human LDL oxidation

EA showed protection against copper-induced oxidation of LDL as measured by relative electrophoretic mobility (REM) and thiobarbituric acid-reactive substances (TBARS) formation. At equimolar concentration, the relative protective effect was in the following order: EA > Quercetin > BHA (Table 2).

Table 1
Comparison of free radical-scavenging and metal-chelating activities of ellagic acid isolated from the roots of *D. hamiltonii* with quercetin and BHA

Compounds	Free radical-scavenging activity[a] IC_{50} (nmol/ml)					Metal-chelating activity[a] IC_{50} (nmol/ml)
	LOO·	DPPH	·OH	O_2^{-}	·NO	
EA	1.01 ± 0.89	9.34 ± 0.12	857 ± 74.23	1.5 ± 0.14	660 ± 54.3	1038 ± 98.4
BHA	1.45 ± 1.29	34.4 ± 4.12	–	0	0	2116 ± 164
Quercetin	1.86 ± 0.92	8.61 ± 0.79	–	336 ± 31.2	415 ± 39.7	557 ± 43.3

[a] IC_{50} values were calculated from a dose–response curve.

Table 2
Inhibition of LDL oxidation by ellagic acid isolated from the roots of *D. hamiltonii*

Treatment*	TBARS (nmol MDA/mg protein)	REM
LDL	3.56 ± 0.29^{a}	1.0^{a}
LDL + $CuSO_4$	38.2 ± 2.98^{e}	2.48 ± 0.13^{e}
LDL + $CuSO_4$ + DMSO	37.7 ± 3.02^{e}	2.46 ± 0.12^{e}
LDL + $CuSO_4$ + EA	9.54 ± 0.10^{b}	1.14 ± 0.04^{b}
LDL + $CuSO_4$ + BHA	26.9 ± 2.73^{d}	1.61 ± 0.06^{d}
LDL + $CuSO_4$ + Quercetin	16.3 ± 1.63^{c}	1.4 ± 0.05^{c}

Values with non-identical superscripts are significantly different ($p < 0.05$) by DMRT.
* EA, quercetin and BHA were used at equimolar concentrations (5 μM); $CuSO_4$ concentration = 10 μM.

3.4. Cytoprotective action

EA was tested for its cytoprotective effect against xenobiotic-induced cytotoxicity employing EAT cells. The cytotoxicants used were hexachlorocyclohexane (HCH), by cumene hydroperoxide (CHP) and carbon tetrachloride (CCl_4) at LC_{50} concentration. EA prevented xenobiotic-induced cell injury/death measured by the trypan blue method and lactate dehydrogenase (LDH) leakage. EA, by itself, was not toxic at the highest concentration used (Figs. 1 and 2). Xenobiotic-induced reactive oxygen species (ROS) production (i.e. respiratory burst) in EAT cells was inhibited by EA (Fig. 3). Similarly, induction of LPO was also inhibited by EA in EAT cells (Fig. 4). Depletion of

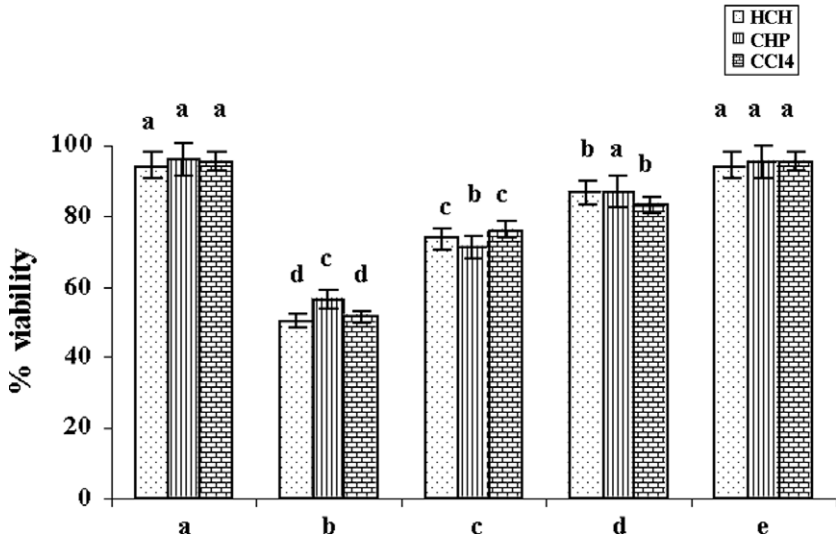


Fig. 1. Protective effect of EA isolated from *D. hamiltonii* on xenobiotic-induced cytotoxicity to EAT cells. (a) Control, (b) X (HCH 1.6 mM, CHP 4 mM, CCl_4 3 mM), (c) X + EA (0.13 mM), (d) X + EA (0.27 mM), (e) EA (0.27 mM). Each bar represents the mean ± SE; bars with different superscripts differ significantly at $p < 0.05$ level (DMRT).

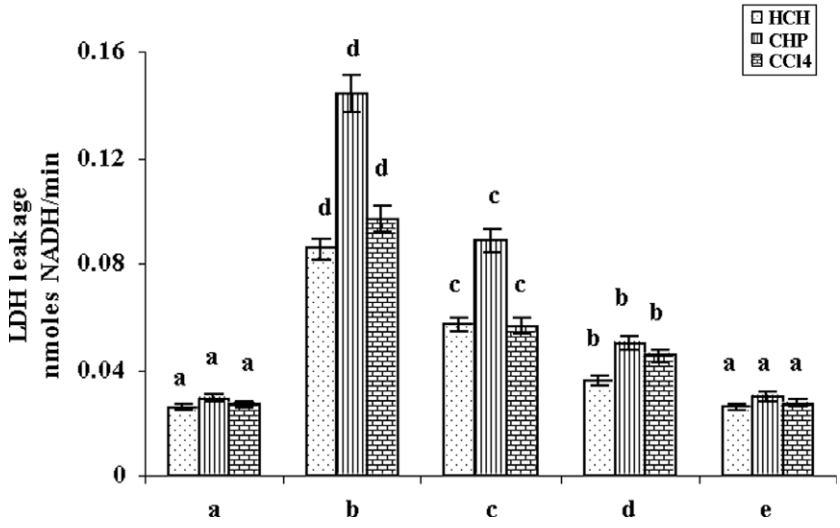


Fig. 2. Protective effect of EA from *D. hamiltonii* on EAT cells exposed to cytotoxic xenobiotics: LDH leakage. (a) Control, (b) X (HCH 1.6 mM, CHP 4 mM, CCl_4 3 mM), (c) X + EA (0.13 mM), (d) X + EA (0.27 mM), (e) EA (0.27 mM). Each bar represents the mean ± SE; bars with different superscripts differ significantly at $p < 0.05$ level (DMRT).

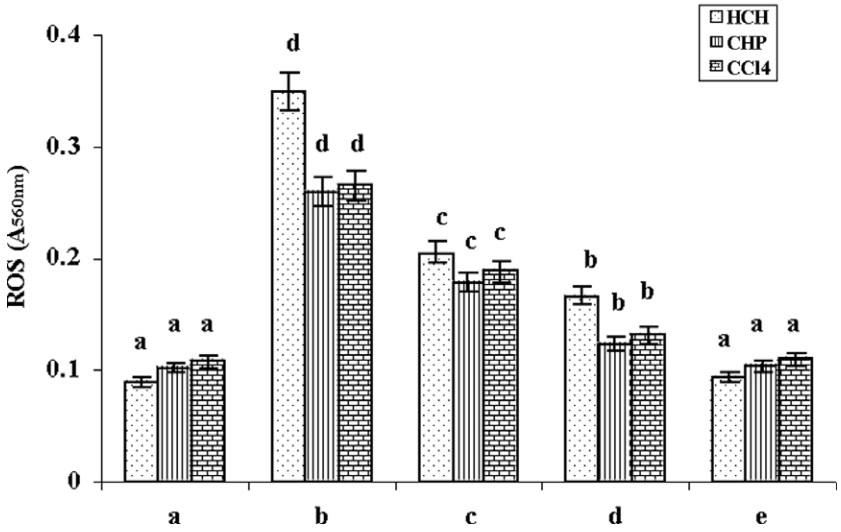


Fig. 3. Suppression of ROS production by EA from *D. hamiltonii* in EAT cells exposed to xenobiotics. (a) Control, (b) X (HCH 1.6 mM, CHP 4 mM, CCl_4 3 mM), (c) X + EA (0.13 mM), (d) X + EA (0.27 mM), (e) EA (0.27 mM). Each bar represents the mean ± SE; bars with different superscripts differ significantly at $p < 0.05$ level (DMRT).

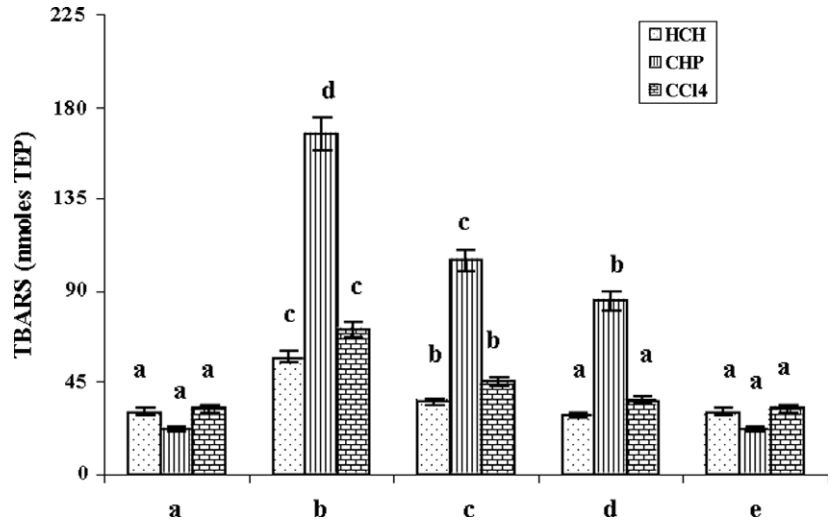


Fig. 4. Inhibition of xenobiotic-induced LPO by EA isolated from *D. hamiltonii* in EAT cells. (a) Control, (b) X (HCH 1.6 mM, CHP 4 mM, CCl_4 3 mM), (c) X + EA (0.13 mM), (d) X + EA (0.27 mM), (e) EA (0.27 mM). Each bar represents the mean ± SE; bars with different superscripts differ significantly at $p < 0.05$ level (DMRT).

glutathione (GSH) content in the cells by xenobiotics was prevented by EA (Fig. 5).

4. Discussion

Ellagic acid is one of the widely distributed antioxidant compounds in fruits and nuts (Seeram, Lee, & Heber, 2004). Plant extracts rich in EA, such as pomegranate juice, are promising health foods (Amakura et al., 2000). The beneficial health effects of antioxidants have been attributed to their ability to scavenge free radicals (Halliwell, 1999). Several studies have shown that plant-derived polyphenolic antioxidants exhibit anti-inflammatory, anticancer and antiatherosclerotic activities. Herbal extracts, including traditional medicines, possess antioxidant properties (Zheng & Wang, 2001). There is overwhelming evidence that phytochemicals could be used as effective antioxidants for improving human health and preventing or delaying degenerative diseases, including cardiovascular diseases (Cui, Dai, Li, Zhang, & Fang, 2000).

The plant-derived antioxidants are characterised by their ability to scavenge free radicals. Proton radical-scavenging action is an important attribute of antioxidants which is measured by the DPPH radical-scavenging assay *in vitro* (Yamaguchi, Takamura, Matoba, & Terao, 1998). The superoxide anion is produced in cells during the course of normal metabolism which is removed by enzymatic detoxication. Although superoxide anion is, by

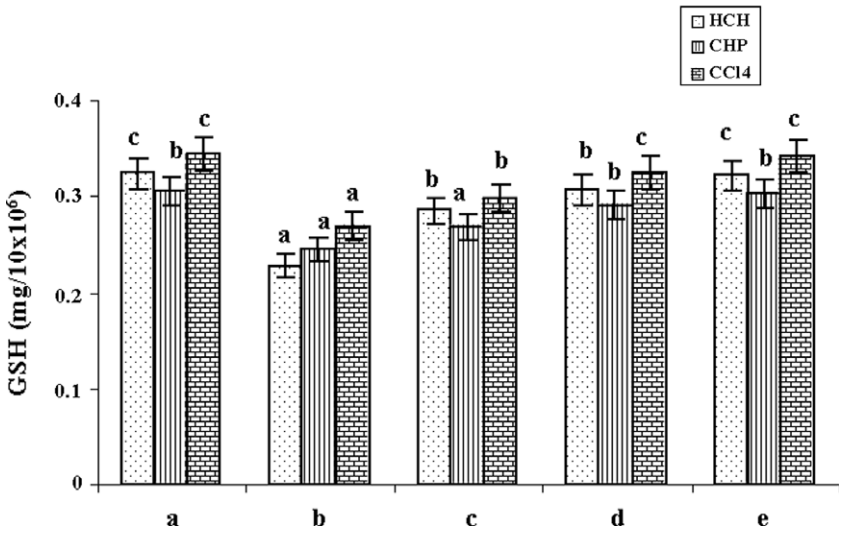


Fig. 5. Effect of EA isolated from *D. hamiltonii* on xenobiotic-induced glutathione depletion in EAT cells. (a) Control, (b) X (HCH 1.6 mM, CHP 4 mM, CCl_4 3 mM), (c) X + EA (0.13 mM), (d) X + EA (0.27 mM), (e) EA (0.27 mM). Each bar represents the mean ± SE; bars with different superscripts differ significantly at $p < 0.05$ level (DMRT).

itself, a weak oxidant, when it is produced in excess it gives rise to the highly reactive hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Aruoma, 1998). 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 (Aruoma, 1998). The hydroxyl radical is also a quick initiator of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. Nitric oxide, a physiologically active molecule, acts as a reactive radical, damages tissues and when it reacts with superoxide anion radical, forms the even stronger oxidant peroxynitrite (Moncada, Palmer, & Higgs, 1991).

The root extracts of *D. hamiltonii* are cocktails of diverse antioxidant compounds (Harish et al., 2005; Srivastava et al., 2006). This study reports that EA is one of the antioxidants present in high amounts in the roots of *D. hamiltonii* and a new addition to its antioxidant profile. Our results show that EA, isolated from *D. hamiltonii*, was a potent free radical-scavenger in all the assays performed. Our results are consistent with earlier reports of antioxidant activity of EA wherein lipid peroxidation inhibition and DPPH radical scavenging activity have been demonstrated (Mertens-Talcott, Talcott, & Percival, 2003; Seeram et al., 2005). The health-promoting potential of pomegranate juice is attributed to a high content of polyphenols, including ellagic acid (Clifford & Scalbert, 2000).

Metal ion-chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage (Duh, Tu, & Yen, 1999). Chelating agents which form σ-bonds with a metal are effective as secondary antioxidants as they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. By virtue of both metal-chelating properties and radical-scavenging ability, antioxidant phytochemicals from *D. hamiltonii*, therefore contribute toward the prevention of free radical formation and the consequent damage. Our results show that EA is equal to, or, better than quercetin in its overall antioxidant activity.

Oxidation of LDL is considered as an essential step in the etiology of atherosclerosis (Chisolm & Steinberg, 2000; Steinberg, 2005). Transition metal-induced oxidation of LDL is a widely used in vitro experimental model of LDL oxidation. We have earlier reported that the aqueous extract contains at least five novel antioxidant compounds which also inhibit LDL oxidation *in vitro* (Srivastava, Harish et al., 2006). In this study, inhibition of LDL oxidation by EA isolated from *D. hamiltonii* was demonstrated by both electrophoretic mobility and lipoprotein lipid peroxidation. Inhibition of LDL oxidation could be attributed to the metal-chelating activity of EA in addition to its free radical-scavenging activity. EA, being a new addition to the antioxidant compounds of *D. hamiltonii*, adds significantly to the health effects by its anti-atherogenic potential.

In vitro studies, using cell cultures, offer a good model system to study the mechanism of xenobiotic-induced cell injury/death and its amelioration by phytochemicals (Robertson & Orrenius, 2000; Srivastava & Shivanandappa, 2006). Several phytochemicals have been evaluated for their protective activity against xenobiotic-induced toxicity in experimental models under *in vitro* and *in vivo* conditions. EA has been reported to exhibit antimutagenic, anti-inflammatory, anticlastogenic and anticarcinogenic activities (Castonguay, Boukharta, & Teel, 1998; Kaur, Grover, & Kumar, 1997; Priyadarsini et al., 2002). Hepatoprotective effect of EA against CCl_4-induced damage to hepatocytes has been reported (Singh et al., 1999b). In this study, we have demonstrated its ability to prevent xenobiotic-induced cellular damage in EAT cells. Hexachlorocyclohexane, carbon tetrachloride and cumene hydroperoxide are well

known inducers of oxidative stress in cells (Ahmed et al., 2000; Srivastava & Shivanandappa, 2006; Tseng, Wang, Kao, & Chu, 1996). Our results show that EA isolated from *D. hamiltonii*, inhibited xenobiotic-induced LPO, suppressed ROS production and ameliorated cytotoxic cell death in EAT cells. Induction of ROS in cells by xenobiotics is associated with oxidative stress-mediated cytotoxicity (Sreekumar et al., 2005). Our results are consistent with earlier reports wherein EA has been shown to suppress xenobiotic-induced ROS production in cells (Festa et al., 2001; Fedeli, Berrettini, Gabryelak, & Falcioni, 2004). Further, we have demonstrated, for the first time, that xenobiotic-induced GSH depletion in EAT cells was modulated by exposure to EA. Depletion of GSH leads to severe oxidative stress and alters the redox status of cells (Cao & Li, 2002). Our results show that exposure of cells to EA protects against xenobiotic-induced cell injury by relieving oxidative stress and, at least in part, by restoring the redox potential.

Overall, the present study adds EA as one of the antioxidant constituents and adds significantly to our earlier work, in that the root extract of *D. hamiltonii* is a rich cocktail of diverse antioxidant molecules and, therefore, contributes to its health-promoting potential.

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