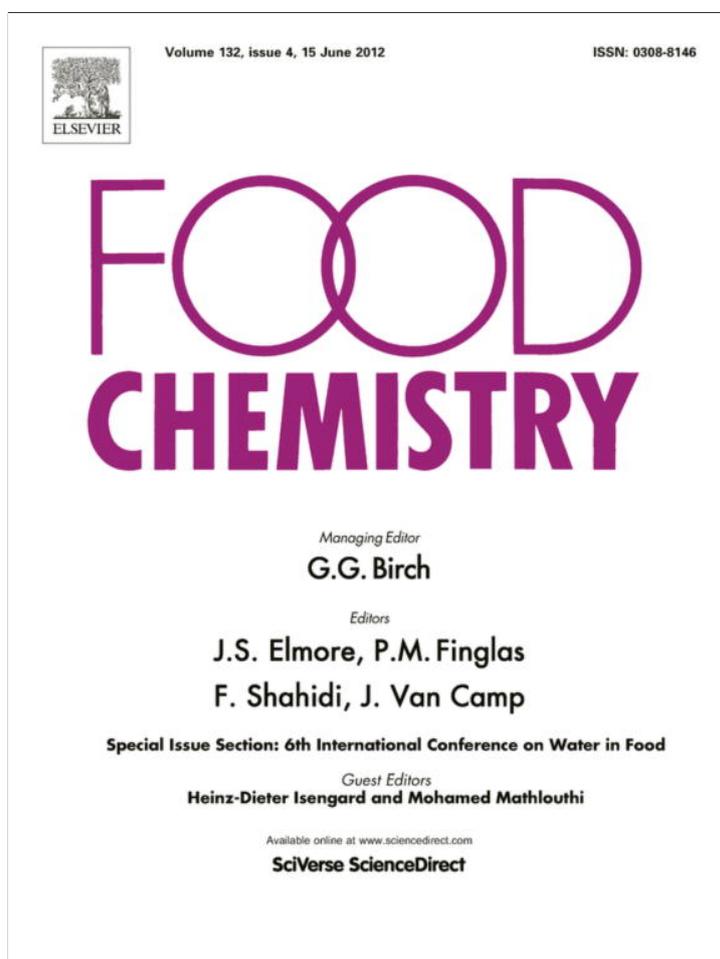


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## Food Chemistry

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## A novel cytoprotective antioxidant: 4-Hydroxyisophthalic acid

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## ARTICLE INFO

## Article history:

Received 21 September 2011

Received in revised form 18 November 2011

Accepted 8 December 2011

Available online 17 December 2011

## Keywords:

4-Hydroxyisophthalic acid

*Decalepis hamiltonii*

Antioxidant

ROS

Lipid peroxidation

Glutathione

## ABSTRACT

The antioxidant and cytoprotective properties of 4-hydroxyisophthalic acid (4-HIPA) isolated from the *Decalepis hamiltonii* roots was examined. 4-HIPA is a potent scavenger of superoxide ( $O_2^{\cdot-}$ ), hydroxyl ( $\cdot OH$ ), nitric oxide ( $\cdot NO$ ), and lipid peroxide ( $LOO\cdot$ ) physiologically relevant free radicals with  $IC_{50}$  values in the nanomolar (2–187) range. 4-HIPA also exhibits a concentration dependent secondary antioxidant activities like reducing power, metal chelating activity, and inhibition of protein carbonylation. Further, 4-HIPA at nanomolar concentration prevented  $CuSO_4$ -induced human LDL oxidation. 4-HIPA demonstrated cytoprotective activity in primary hepatocytes and Ehrlich Ascites tumour (EAT) cells against oxidative stress inducing xenobiotics apart from the *in vitro* free radical scavenging activity. The mechanism of cytoprotective action involved maintaining the intracellular glutathione (GSH), scavenging of reactive oxygen species (ROS), and inhibition of lipid peroxidation (LPO). Thus, 4-HIPA is a novel bioactive molecule with potential health implications.

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## 1. Introduction

There are over a thousand phytochemicals found in foods and one serving of a fruit or vegetable may have as many as 100 different phytochemicals (Carratu & Sanzini, 2005; Shahidi, McDonald, Chandrasekara, & Zhong, 2008). Antioxidant phytochemicals promote human health by exhibiting diversified physiological and pharmacological effects such as inactivating cancer-causing substances, stimulating the immune system, protecting the heart from free radical attack, and help preventing cataracts in the eye lens (Liu, 2004; Vincent, Bourguignon, & Taylor, 2010). These antioxidant phytochemicals influence multiple signaling pathways, including survival pathways such as those regulated by NF- $\kappa$ B, Akt, and growth factors; cytoprotective pathways dependent on Nrf2; and metabolic and angiogenic pathway (Nishino et al., 2005; Walker, 2006). Some of the common types of phytochemicals include flavonoids, indoles, isoflavones, alkaloids, nonprotein amino acids, isothiocyanate, phytosterols, carotenoids, and chlorophyll derivatives among others (Espin, Garcia-Conesa, & Tomas-Barberan, 2007). Currently, there is a great deal of interest in the study of natural compounds with free radical scavenging capacity and their role in human health.

Tuberous roots of *Decalepis hamiltonii* (Wight and Arn.) (Family: Asclepiadaceae) are consumed in southern India as pickles and juice

for their alleged health promoting properties. The roots are also used in folk medicine and ayurvedic preparations as general vitalizer and blood purifier (Nayar, Shetty, Mary, & Yoganarshimhan, 1978). We have earlier reported that the roots of *D. hamiltonii* possess potent antioxidant properties and have hepatoprotective and neuroprotective potential (Srivastava, Shereen, Harish, & Shivanandappa, 2006; Srivastava & Shivanandappa, 2006b, 2010a, 2010b). We have isolated/characterised the antioxidant constituents of *D. hamiltonii* roots which could be associated with their alleged health benefits (Harish, Divakar, Srivastava, & Shivanandappa, 2005; Srivastava, Harish, & Shivanandappa, 2006; Srivastava, Rao, & Shivanandappa, 2007). In this paper, we report the antioxidant properties of 4-hydroxyisophthalic acid (4-HIPA) (Fig. 1) isolated from the aqueous extract of *D. hamiltonii* roots using a number of free radical scavenging and other antioxidant activity assays. Further, we demonstrated the cytoprotective activity of 4-HIPA in primary hepatocytes and Ehrlich Ascites tumour (EAT) cells against oxidative stress inducing xenobiotics.

## 2. Materials and methods

## 2.1. Chemicals

Thiobarbituric acid (TBA), glutathione (GSH), bovine serum albumin (BSA), tetraethoxypropane (TEP), HEPES buffer, 2,2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), cumene hydroperoxide (CHP), sodium nitroprusside (SNP), ethylenediaminetetraacetic acid (EDTA), Griess reagent, trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic

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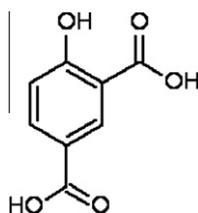


Fig. 1. Structure of 4-hydroxyisophthalic acid.

acid (TCA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), deoxyribose, ascorbic acid, sodium dodecyl sulphate (SDS), dimethyl sulphoxide (DMSO) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

## 2.2. Isolation of 4-hydroxyisophthalic acid

The roots of *D. hamiltonii* were collected from B.R. Hills, Karnataka, India and their identity confirmed by a botany Professor (Dr. G.R. Janardhana) at Mysore University. A voucher specimen was maintained in Botany Department of Mysore University, Mysore, Karnataka, India. 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 in a grinder (particle size 1003 µm). This root powder was used for extraction. We have earlier reported that an aqueous extract of *D. hamiltonii* roots shows high antioxidant activity (Srivastava et al., 2006). The aqueous extract was prepared by homogenising the root powder (200 g) in 1 L of warm water (50 °C) using a mixer and allowed to stand for 24 h, and filtering through Whatman No. 1 paper; the filtrate was lyophilised and weighed (34.75 g). The lyophilised aqueous extract was re-extracted with methanol in a total volume of 500 ml and concentrated under reduced pressure (−10 kPa, 37 °C); it was subjected to conventional purification techniques (silica gel, 60–120 mesh, column chromatography and preparative thin layer chromatography). The compound isolated was pure by RP-HPLC and TLC. The active compound was characterised as 4-hydroxyisophthalic acid (4-HIPA) by classical (UV, IR, LC-MS, and NMR) spectroscopic techniques which we have reported earlier (Srivastava et al., 2006).

## 2.3. Antioxidant activity

### 2.3.1. Superoxide radical-scavenging assay

Superoxide radical-scavenging activity was measured by NBT method. 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 different concentrations of 4-HIPA and the colour developed, due to NBT reduction, was read at 560 nm against a blank (Shimadzu UV-vis spectrophotometer UV-160, Kyoto, Japan). The radical-scavenging activity was measured as the decrease in the absorbance and was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - A_{\text{Sample}(560 \text{ nm})} / A_{\text{Control}(560 \text{ nm})}] \times 100$$

### 2.3.2. Hydroxyl radical-scavenging assay

Reaction mixtures containing different concentrations of 4-HIPA were incubated with deoxyribose (10 mM), H<sub>2</sub>O<sub>2</sub> (10 mM), FeCl<sub>3</sub> (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. 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 50 mM NaOH) in a boiling water bath for 15 min. The absorbance was measured at 532 nm against the reagent blank and inhibition of the oxidation of deoxyribose was calculated against the control.

### 2.3.3. Nitric oxide radical-scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which is measured by the Griess reaction. Sodium nitroprusside (5 mM) was mixed with different concentrations of 4-HIPA in phosphate-buffered saline and incubated at 25 °C for 150 min followed by the addition of Griess reagent (1% sulphanilamide, 2% H<sub>3</sub>PO<sub>4</sub> 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.

### 2.3.4. Inhibition of microsomal lipid peroxidation

Liver excised from adult male Wistar rats was homogenised (20% w/v) in 0.02 M Tris buffer (pH 7.4). Microsomes were isolated by the calcium aggregation method. Microsomes (0.5 mg protein) were mixed with FeSO<sub>4</sub> (1 mM) and ascorbic acid (1 mM) with or without 4-HIPA 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 (5000 g, 4 °C, 5 min). The absorbance of the supernatant was read at 532 nm and TBARS (thiobarbituric acid reactive substances) value of the supernatant was calculated using tetraethoxypropane as the standard. The TBARS value was taken as a measure of lipid peroxide generation.

### 2.3.5. Reducing power

The reducing power of 4-HIPA was quantified by the method described earlier with modifications. Briefly, 1 ml of reaction mixture, containing 4-HIPA in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50 °C for 20 min. The reaction was terminated by adding TCA solution (10% w/v) and centrifuged at 2000g for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1% w/v) solution and the resulting colour was read at 700 nm. The concentration required to get an OD<sub>700nm</sub> of 0.5 was used as measure of reducing power.

### 2.3.6. Protein carbonyls

Wistar rat liver homogenate (10% w/v) was prepared in 20 mM Tris-HCl buffer (pH 7.4), centrifuged at 10,000g for 10 min at 4 °C. 1 ml of the supernatant was incubated with CCl<sub>4</sub> (1 mM) along with 4-HIPA (0.1–2.5 mmole/ml) for 1 h and was precipitated with an equal volume of 20% TCA and centrifuged (5000g, 4 °C, 5 min). The pellet was resuspended in 1 ml of DNPH (10 mM in 2 M HCl) and allowed to stand at room temperature for 60 min with occasional vortexing. TCA (0.5 ml, 20%) was added to the reaction mixture and centrifuged (5000g, 4 °C, 5 min), the pellet obtained was washed three times with acetone and 1 ml of 2% of SDS (in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.4) was added to solubilise the pellet. The absorbance of the sample was read at 360 nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M<sup>−1</sup> cm<sup>−1</sup>.

### 2.3.7. Metal ion chelation assay

The Fe<sup>2+</sup>-chelating ability of 4-HIPA was assayed by measuring the formation of ferrous iron-ferrozine complex. The reaction mixture containing FeCl<sub>2</sub> (2 mM) and ferrozine (5 mM) and the

isolated compound (0.1–50 mmole/ml) 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 earlier.

### 2.3.8. Inhibition of human low density lipoprotein oxidation

Human LDL (100 µg protein/ml) was oxidised with 10 µM CuSO<sub>4</sub> in the presence or absence of 4-HIPA 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 (12,000g, 4 °C, 10 min) and the fluorescence of the product formed was measured in a spectrofluorometer (Shimadzu RF-5000U Spectrofluorometer, Kyoto, Japan) at 515 nm excitation and 553 nm emission wavelengths. The electrophoretic mobility of the native or oxidised LDL was studied by agarose gel electrophoresis. Aliquots of the LDL reaction mixture were electrophoresed 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 oxidised LDL versus native LDL, was calculated.

## 2.4. Cell studies

### 2.4.1. Hepatocyte isolation

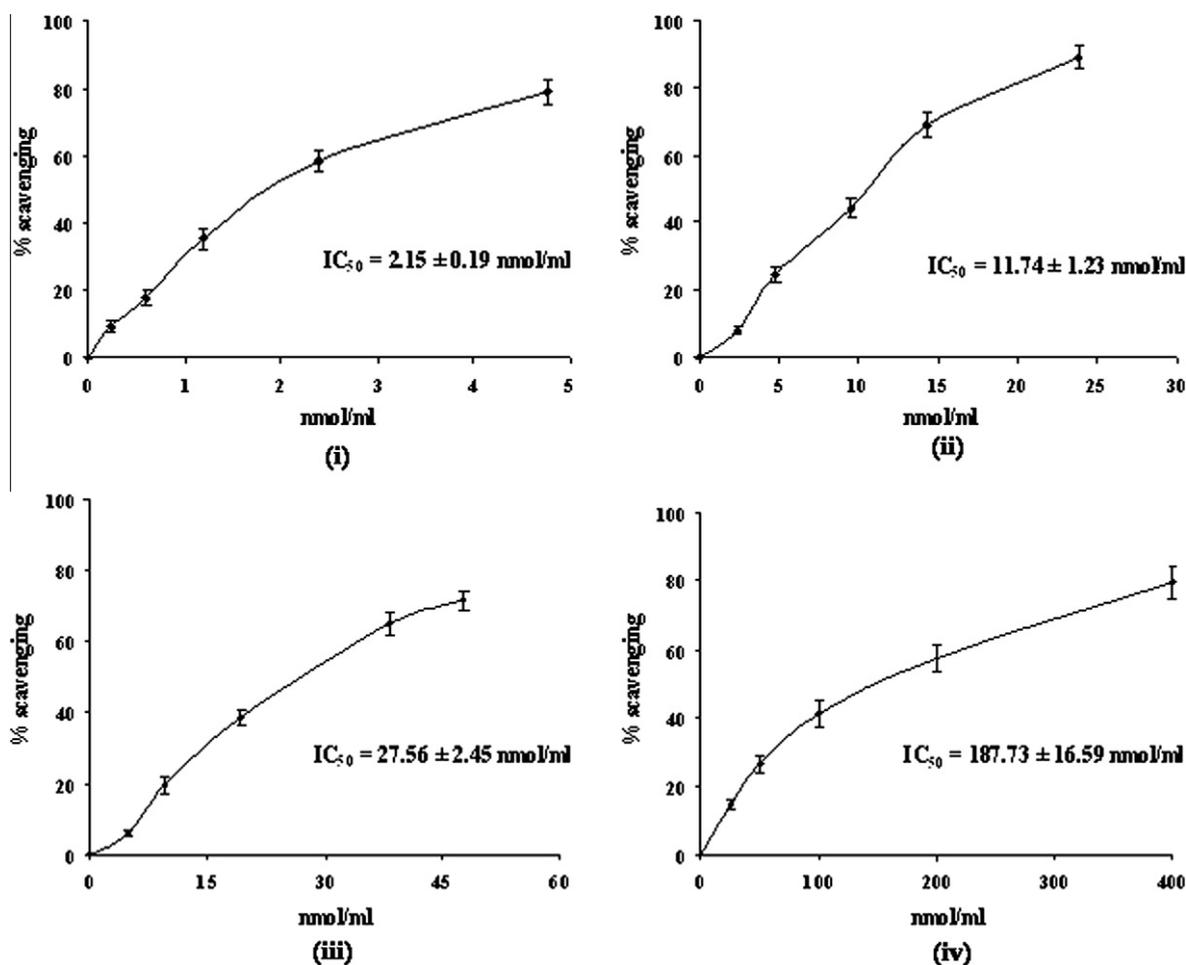
Hepatocytes were isolated from male Wistar rats (200–240 g) by collagenase perfusion of the liver. The pre-perfusion and perfusion solutions were buffered at pH 7.4 with 0.01 M HEPES. The total length of the perfusion was approximately 15–20 min. Aliquots of freshly isolated hepatocytes were immediately counted with a haemocytometer in 0.4% trypan blue solution containing 0.9% NaCl. The viability of the cells isolated by this method was always more than 90%.

### 2.4.2. Ehrlich Ascites tumour (EAT) cells

EAT cells were cultured in the peritoneum of male Swiss albino mice. After harvesting, cells were suspended in Hanks Balanced Salt Solution (HBSS) with 0.1% dextrose and 0.4% bovine serum albumin. Cells were used for the experiments within 6 h after harvesting. EAT cells were chosen because they offer a good model to study ROS induction and consequent oxidative stress by xenobiotics.

### 2.4.3. Cell viability

Cells ( $10 \times 10^6$ ) suspended in 1 ml of HBSS were treated with the toxicant [for hepatocytes CCl<sub>4</sub> 0.4 mM (dissolved in DMSO) or ethanol 1 mM; and for EAT cells CCl<sub>4</sub> 0.4 mM or CHP 4 mM or HCH 1.6 mM (dissolved in DMSO)] at LC<sub>50</sub> concentration with/without 4-HIPA and incubated for 60 min in a shaking water bath



**Fig. 2.** Free radical scavenging activity of 4-HIPA. (i) Lipid peroxide (LOO<sup>·</sup>) radical, (ii) Superoxide radical (O<sub>2</sub><sup>·-</sup>) radical, (iii) Hydroxyl (·OH) radical, (iv) Nitric oxide (·NO) radical. Values are expressed as means with standard error of mean.

at 37 °C. At the end of incubation, an aliquot of cells was taken for viability assay by trypan blue exclusion method.

#### 2.4.4. Lactate dehydrogenase leakage

After incubation of cells (hepatocytes/EAT cells) in the presence of toxicant with/without 4-HIPA cells were centrifuged (2000g, 25 °C, 2 min) and the supernatant was assayed for LDH with sodium lactate as the substrate. The reaction mixture consisted of NADH (0.02 M), sodium pyruvate (0.01 M), sodium phosphate buffer (0.1 M, pH 7.4) in a total volume of 3 ml. The changes in the absorbance were recorded at 340 nm at 30 s interval for 3 min.

#### 2.4.5. Lipid peroxidation

After incubation, as above, the cells (hepatocytes/EAT cells) were centrifuged (2000g, 25 °C, 2 min) and the cell pellet was washed in saline. The cell pellet was boiled in TCA (5.5%) and TBA (0.34%) for 15 min, cooled and centrifuged (5000g, 4 °C, 5 min). Fluorescence of the supernatant was measured in a fluorescence spectrophotometer at excitation and emission wavelengths of 532 and 553 nm, respectively. Lipid peroxidation was quantified by the amount of malondialdehyde (MDA) equivalents formed which was calculated using a standard curve prepared with tetraethoxypropane (0–100 nmole/ml).

#### 2.4.6. Reactive oxygen species (superoxide anion)

The cells (hepatocytes/EAT cells) ( $10 \times 10^6$ ) suspended in 1.0 ml HBSS were incubated with NBT (0.2 mM), toxicant (as mentioned

earlier), and with/without 4-HIPA 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.

#### 2.4.7. Glutathione

Hepatocytes/EAT cells ( $10 \times 10^6$ ) suspended in 1 ml HBSS were treated with toxicant as mentioned above with/without 4-HIPA and incubated for 60 min in a shaking water bath at 37 °C. At the end of incubation, cells were homogenised in 1 ml of 5% (w/v) trichloroacetic acid, centrifuged at 2000g for 5 min and glutathione (GSH) in the deproteinized supernatant was estimated by Ellman's reagent with a standard curve (0–500 nmole/ml) and represented as nmole/mg protein.

Protein estimation was done by Lowry's method using BSA as the standard.

### 2.5. Statistical analysis

Data were expressed as mean  $\pm$  standard error of three separate experiments and the significant difference was determined by the analysis of variance ( $p < 0.05$ ) (Newman Keul's post hoc test) using Statistica software (Statistica, Version 5.5, 99th ed., Stat-soft Inc., Tulsa, OK, USA).

## 3. Results

### 3.1. Free radical scavenging activity

Free radicals like lipid peroxide ( $\text{LOO}^\cdot$ ), superoxide ( $\text{O}_2^{\cdot-}$ ), hydroxyl ( $\cdot\text{OH}$ ), and nitric oxide ( $\cdot\text{NO}$ ) are physiologically relevant

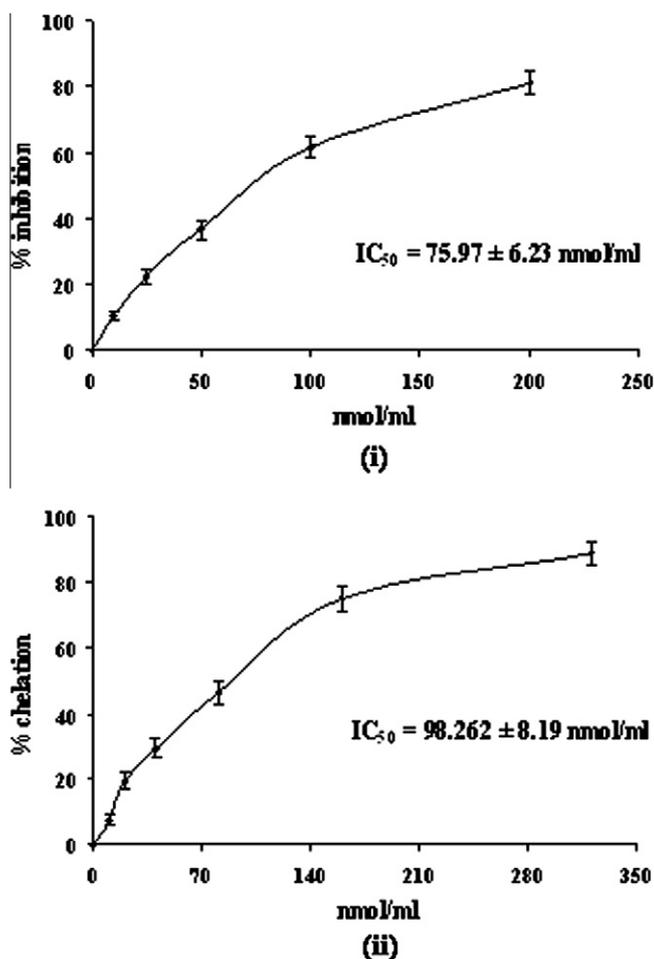


Fig. 3. Additional antioxidant activity of 4-HIPA. (i) Inhibition of protein carbonylation. (ii) metal chelating activity. Values are expressed as means with standard error of mean.

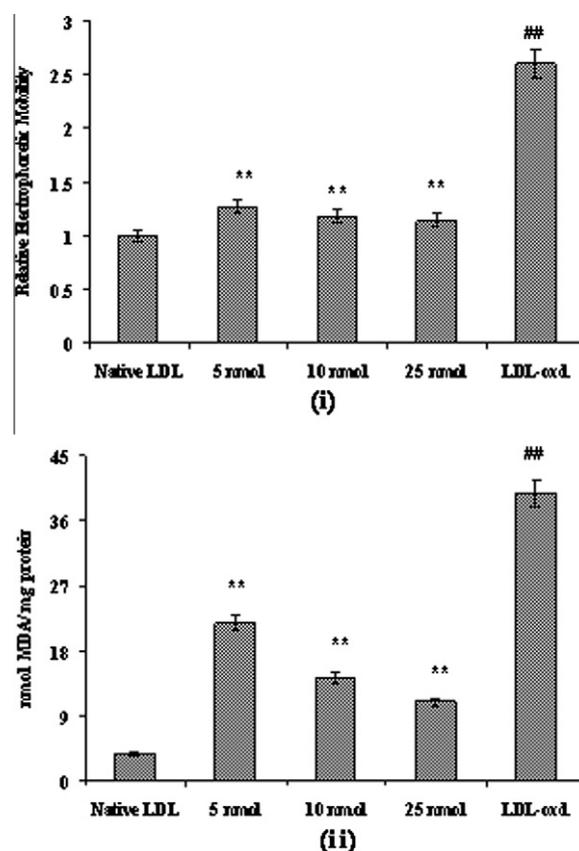


Fig. 4. Inhibition of human LDL oxidation by 4-HIPA. (i) Relative Electrophoretic Mobility (REM), (ii) thiobarbituric acid reactive substances (TBARS) content. Statistics was done by one way ANOVA. # represents comparison between control and  $\text{CuSO}_4$  groups and \* represents comparison between 4-HIPA +  $\text{CuSO}_4$  treatment groups. # or \* represents  $p < 0.05$ ; ## or \*\* represents  $p < 0.01$ .

and when in excess cause oxidative stress which can lead to pathological conditions. We tested the efficacy of 4-HIPA in scavenging these free radicals. 4-HIPA showed a concentration dependent scavenging of free radicals (Fig. 2). The free radical scavenging activity of 4-HIPA was as potent as known antioxidants, quercetin and butylated hydroxyanisole (BHA). The calculated  $IC_{50}$  values were in nanomole range indicating its high potency. We also found that 4-HIPA is a potent scavenger of DPPH radical, a synthetic free radical (data not shown).

### 3.2. Additional antioxidant activity

One of the major consequences of oxidative stress is irreversible protein modification such as generation of protein carbonyls which lead to their fragmentation, increased aggregation, and enzyme dysfunction. 4-HIPA in a dose dependent fashion prevented  $CCl_4$  induced protein carbonyl formation in rat liver tissue (Fig. 3). The metal ion chelating capacity plays a significant role in the antioxidant mechanism because it prevents oxyradical generation and the consequent oxidative damage. 4-HIPA showed significant ferrous ion-chelating activity, as a measure of its antioxidant capacity (Fig. 3). Ferric reducing antioxidant power assay is conventionally used to measure the reducing ability of antioxidants. 21.79 nmol/ml of 4-HIPA was sufficient to get an  $OD_{700nm}$  of 0.5 compared to 45.72 nmol/ml of quercetin, a well known natural antioxidant.

### 3.3. Inhibition of LDL oxidation

Oxidation of human LDL is considered to be an essential step in the pathogenesis of atherosclerosis. LDL oxidation is characterised by alterations in structure and biological properties of lipids and

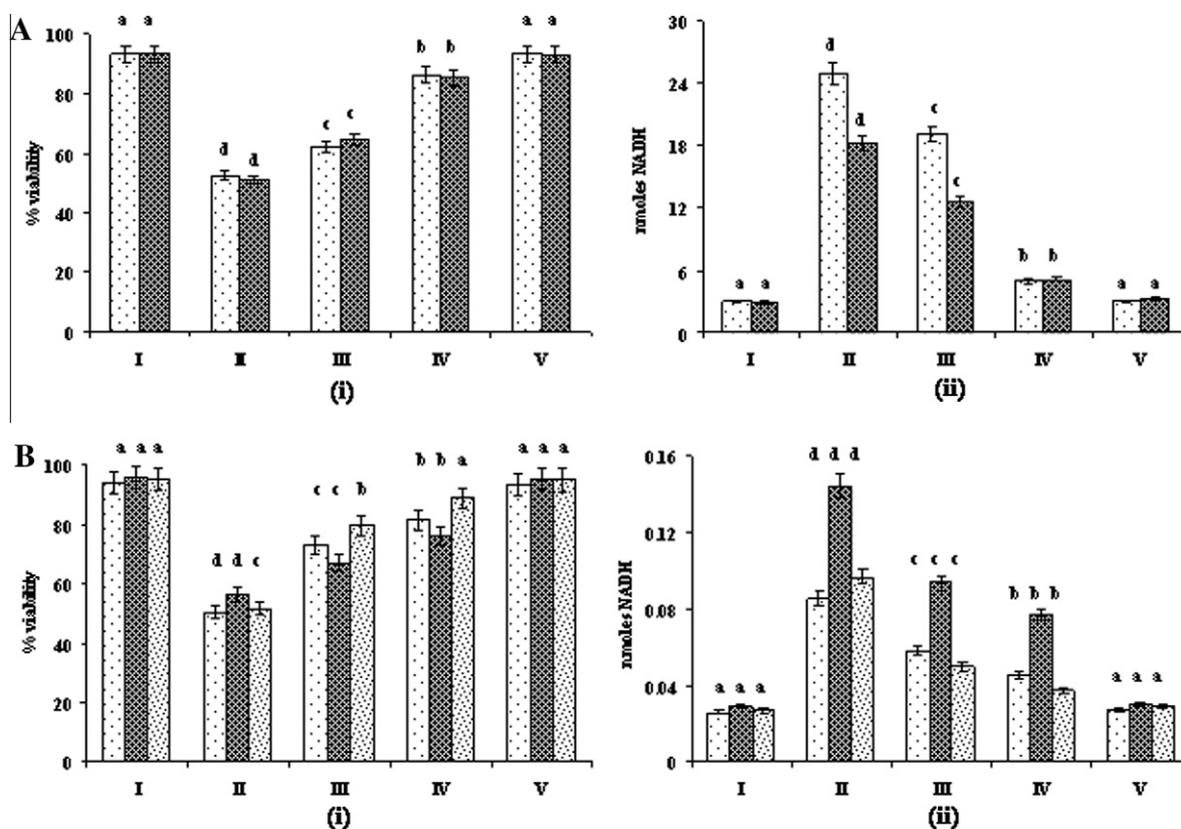
apolipoprotein B (apo B). 4-HIPA showed a dose dependent protection against copper-induced human LDL oxidation as measured by relative electrophoretic mobility (REM) and TBARS formation (Fig. 4). At equimolar concentration the protective effect of 4-HIPA was comparable to quercetin and BHA.

### 3.4. Cell viability

*In vitro* studies using cell cultures offer a good model system to understand the mechanism of xenobiotic-induced cell injury/death and its amelioration by phytochemicals. Ehrlich Ascites tumor (EAT) cells offer a good model to study oxidative stress mediated cytotoxicity involving LPO and ROS induction by xenobiotics. Primary hepatocytes are also a useful *in vitro* cell model for pharmacological and toxicological studies of xenobiotics. Xenobiotics such as hexachlorocyclohexane (HCH), carbon tetrachloride ( $CCl_4$ ), ethanol, and cumene hydroperoxide (CHP) are well known inducers of oxidative stress in cell systems. To assess the cytoprotective effect of 4-HIPA (hepatocytes and EAT cells) were exposed to different xenobiotics at  $LC_{50}$  concentration (hepatocytes –  $CCl_4$  and ethanol) (EAT cells – HCH, CHP, and  $CCl_4$ ). 4-HIPA dose dependently ameliorated toxicant-induced cytotoxicity as measured by trypan blue method and lactate dehydrogenase leakage. 4-HIPA, by itself, was not toxic to the cells at the highest concentration used (Fig. 5).

### 3.5. Markers of oxidative stress

4-HIPA dose dependently ameliorated xenobiotic-induced oxidative stress in both hepatocytes as well as EAT cells. Oxidative stress was measured by lipid peroxidation, reactive oxygen species (ROS) production i.e. “respiratory burst”, and depletion of



**Fig. 5.** Cytoprotective effect of 4-HIPA. (i) Trypan blue exclusion method, (ii) LDH leakage. (A) Rat primary hepatocytes: Groups-I: control, II: toxicant [ $CCl_4$  (0.4 mM) or Ethanol (1 mM)], III: toxicant + 4-HIPA (0.054 mM), IV: toxicant + 4-HIPA (0.22 mM), V: 4-HIPA (0.22 mM) (B) EAT cells: Groups-I: Control, II: toxicant [HCH (1.6 mM) or CHP (4 mM) or  $CCl_4$  (3 mM)], III: toxicant + 4-HIPA (0.27 mM), IV: toxicant + 4-HIPA (0.54 mM), V: 4-HIPA (0.54 mM). Different letters indicate statistical significance at  $p < 0.05$ .

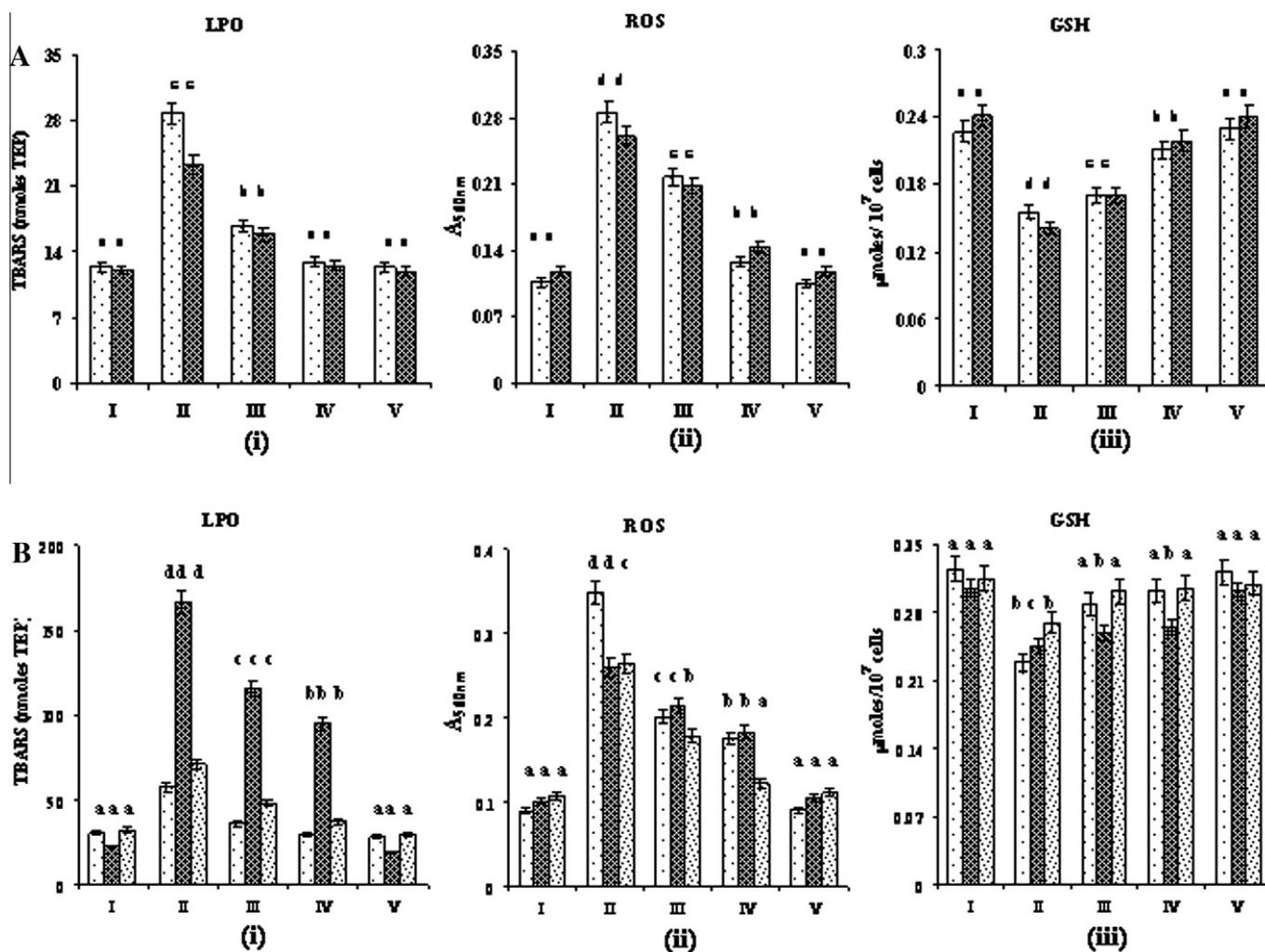


Fig. 6. Amelioration of oxidative stress by 4-HIPA. (i) Inhibition of lipid peroxidation (LPO), (ii) reactive oxygen species (ROS) scavenging, (iii) restoration of glutathione (GSH) levels. (A) Rat primary hepatocytes: Groups-I: Control, II: toxicant [ $\text{CCl}_4$  (0.4 mM) or ethanol (1 mM)], III: toxicant + 4-HIPA (0.054 mM), IV: toxicant + 4-HIPA (0.22 mM), V: 4-HIPA (0.22 mM). B. EAT cells: Groups-I: Control, II: toxicant [HCH (1.6 mM) or CHP (4 mM) or  $\text{CCl}_4$  (3 mM)], III: toxicant + 4-HIPA (0.27 mM), IV: toxicant + 4-HIPA (0.54 mM), V: 4-HIPA (0.54 mM). Different letters indicate statistical significance at  $p < 0.05$ .

glutathione level (Fig. 6). The toxicants induced oxidative stress to different degree, in hepatocytes  $\text{CCl}_4$  induced more oxidative stress compared to ethanol at  $\text{LC}_{50}$  concentration. In EAT cells HCH induced highest production of ROS and CHP caused most LPO at  $\text{LC}_{50}$  concentration. 4-HIPA decreased ROS either by inhibiting their production or scavenged them leading to decreased LPO and maintained the GSH level. 4-HIPA, *per se*, did not have any adverse effect on oxidative stress parameters of hepatocytes and EAT cells.

#### 4. Discussion

The beneficial health effects of antioxidants have been attributed to their ability to scavenge free radicals (Chandrasekara & Shahidi, 2011a, 2011b; Halliwell, 1999). There is overwhelming evidence that phytochemicals could be used as effective antioxidants for improving human health by preventing or delaying degenerative diseases (de Kok, de Waard, Wilms, & van Breda, 2010). Our results show that 4-HIPA, isolated from *D. hamiltonii*, is a potent scavenger of  $\text{O}_2^{\cdot-}$ ,  $\cdot\text{OH}$ ,  $\cdot\text{NO}$ , and  $\text{LOO}^{\cdot}$  physiologically relevant free radicals. Iron is known to generate free radicals through the Fenton and Haber–Weiss reaction (Halliwell & Gutteridge, 1990). Chelating agents which form r-bonds with a metal are effective as secondary antioxidants as they reduce the redox potential,

thereby stabilizing the oxidised form of the metal ion. It is believed that antioxidant activity and reducing power are related (Moon & Shibamoto, 2009). Reductones donate a hydrogen atom and inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction (Huang, Ou, & Prior, 2005). Oxidative stress leads to irreversible protein modification, such as generation of carbonyls or loss of thiol residues (Berlett, Levine, Chock, Chevion, & Stadtman, 2001). These oxidative modifications alter the biological properties of proteins, leading to their fragmentation, increased aggregation and enzyme dysfunction. By virtue its free radical scavenging, metal-chelating, high reducing, and protein carbonylation inhibiting properties 4-HIPA can act as a powerful antioxidant *in vivo* as well.

Oxidation of low density lipoprotein (LDL) is considered to be an essential step in the pathogenesis of atherosclerosis (Chisolm & Steinberg, 2000). Transition-metal induced oxidation of LDL is one of the classical models of oxidation employed in research (Corti, Barbato, & Baggio, 1997). 4-HIPA showed a dose dependent protection against copper-induced human LDL oxidation. The protection of LDL oxidation by 4-HIPA could be due to: (a) scavenging of various radical species in the aqueous phase, (b) interaction with peroxy radicals at the LDL surface, (c) partitioning into the LDL

particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals, (d) regenerating endogenous  $\alpha$ -tocopherol back to its active antioxidant form, and (e) metal ion chelation.

*In vitro* studies on cell cultures are often used as a model system to study the mechanism of xenobiotic-induced cell injury/death and its amelioration by phytochemicals (Robertson & Orrenius, 2000). Several phytochemicals have been evaluated for their protective activity against xenobiotic induced toxicity in experimental models *in vitro* and *in vivo* conditions (Fraga, 2007). A number of prooxidant drugs and other chemicals (including Cumene hydroperoxide, hexachlorocyclohexane, carbon tetrachloride, and ethanol) have been implicated in the oxidative stress and cell injury resulting from the intracellular production of injurious ROS (Djordjevic, 2004; Srivastava & Shivanandappa, 2006a). 4-HIPA inhibited xenobiotic-induced LPO in both EAT cells and hepatocytes. Further, the results show that 4-HIPA scavenged the ROS produced, which could be responsible for the amelioration of cytotoxic cell death. Furthermore, xenobiotic-induced GSH depletion in EAT cells and hepatocytes was restored by 4-HIPA. Our results show that 4-HIPA protects the cells from xenobiotic-induced cell injury/death by relieving oxidative stress and, at least in part, by restoring the cellular redox status. Our results support the view that induction of ROS in cells by xenobiotics is correlated with oxidative stress-mediated cell death and antioxidants can be successfully used to ameliorate such oxidative stress induced cytotoxicity.

In conclusion, our results show that 4-HIPA has potent free radical quenching activity, exhibits secondary antioxidant properties, and inhibits human LDL oxidation. We have further demonstrated the ability of 4-HIPA to prevent xenobiotic-induced cellular damage in hepatocytes and EAT cells. The mechanism of cytoprotective action appears to involve maintaining the intracellular GSH, scavenging of ROS, and inhibition of LPO. Based on the results it is suggested that 4-HIPA is a novel bioactive molecule. This study opens up avenues for exploiting food sources such as *D. hamiltonii* for applications of novel bioactive molecules in both prevention and amelioration of degenerative diseases as well as general well being.

## Acknowledgement

This work was done at Central Food Technological Research Institute, Mysore, India. The authors wish to thank the Director of the institute for his keen interest in this study. The first author acknowledges Council for Scientific and Industrial Research, New Delhi for awarding the research fellowship.

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