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ORIGINAL ARTICLE

2,4,8-trihydroxybicyclo [3.2.1]octan-3-one scavenges free radicals and protects against xenobiotic-induced cytotoxicity

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Abstract
Currently, there is a great deal of interest in the study of natural compounds with free-radical-scavenging activity because of their potential role in maintaining human health and preventing diseases. In this paper, we report the antioxidant and cytoprotective properties of 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one (TBO) isolated from the aqueous extract of *Decalepis hamiltonii* roots. Our results show that TBO is a potent scavenger of superoxide ($O_2^{\bullet-}$), hydroxyl (·OH), nitric oxide (·NO) and lipid peroxide (LOO·) – physiologically relevant free radicals with IC_{50} values in nmolar (42–281) range. TBO also exhibited concentration-dependent secondary antioxidant activities such as reducing power, metal-chelating activity and inhibition of protein carbonylation. Further, TBO at nmolar concentration prevented $CuSO_4$-induced human LDL oxidation. Apart from the *in vitro* free-radical-scavenging activity, TBO demonstrated cytoprotective activity in primary hepatocytes and Ehrlich ascites tumour (EAT) cells against oxidative-stress-inducing xenobiotics. The mechanism of cytoprotective action involved maintaining the intracellular glutathione (GSH), scavenging of reactive oxygen species (ROS) and inhibiting lipid peroxidation (LPO). Based on the results, it is suggested that TBO is a novel bioactive molecule with implications in both prevention and amelioration of diseases involving oxidative stress as well as in the general well-being.

Keywords: *2,4,8-trihydroxybicyclo [3.2.1]octan-3-one, Decalepis hamiltonii, antioxidant, ROS, lipid peroxidation, glutathione*

Introduction

Phytochemicals are non-nutritive plant-derived chemicals with biological effects. 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 [1,2]. It is well known that plants produce these chemicals to protect themselves against oxidative insult, but recent research has demonstrated that many phytochemicals could protect humans against diseases [3–5]. Antioxidant phytochemicals promote human health by exhibiting diversified physiological and pharmacological effects, namely, inactivate cancer-causing substances, stimulate the immune system, protect the heart from free radical attack and help prevent cataracts in the eye lens [6–8]. These antioxidant phytochemicals influence multiple signalling pathways, including survival pathways such as those regulated by NF-kB, Akt and growth factors; cytoprotective pathways dependent on Nrf2 and metastatic and angiogenic pathways. Some of the common types of phytochemicals include flavonoids, indoles, isoflavones, alkaloids, non-protein amino acids, isothiocyanate, phytosterols, carotenoids, chlorophyll derivatives, etc. [9]. 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 its alleged health-promoting properties. The roots are also used in folk medicine and ayurvedic preparations as general vitalizer and blood purifier [10]. We have earlier reported that the roots of *D. hamiltonii* possess potent antioxidant properties and have shown

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their hepatoprotective and neuroprotective potential [11–14]. We have isolated/characterized the antioxidant constituents of *D. hamiltonii* roots which could be associated with their alleged health benefits [15–17]. In this paper, we report the antioxidant properties of 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one (TBO) (Figure 1) isolated from the aqueous extract of *D. hamiltonii* roots using a battery of free-radical-scavenging assays and other additional antioxidant activity assays. Further, we demonstrate the cytoprotective activity of TBO in primary hepatocytes and Ehrlich ascites tumour (EAT) cells against oxidative-stress-inducing xenobiotics.

Methods

Chemicals

Thiobarbituric acid (TBA), glutathione (GSH), bovine serum albumin (BSA), tetraethoxypropane (TEP), HEPES buffer, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), phenazine methosulfate (PMS), cumene hydroperoxide (CHP), sodium nitroprusside (SNP), ethylenediamine tetraacetic acid (EDTA), Griess reagent and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), 5,5′dithiobis(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), deoxyribose, ascorbic acid, dimethyl sulphoxide (DMSO) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

Isolation of 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one

The roots of *Decalepis hamiltonii* were collected from B.R. Hills, Karnataka, India, and identity confirmed by a botany Professor 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. This root powder was used for extraction. We have earlier reported that an aqueous extract of *D. hamiltonii* roots shows high antioxidant activity [11]. The aqueous extract was prepared by homogenizing the root powder (200 g) in 1 L of warm water (50°C) and allowed to stand for 24 h, and filtering through Whatman No. 1 paper; the filtrate was lyophilized and weighed (34.75 g). The lyophilized aqueous extract (34.75 g) was re-extracted with methanol in a total volume of 500 ml and concentrated under reduced pressure; it was subjected to conventional purification techniques. The compound isolated was pure by RP-HPLC and TLC. The active compound was characterized as 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one (TBO) by classical (UV, IR, LC-MS and NMR) spectroscopic techniques that we have reported earlier [16].

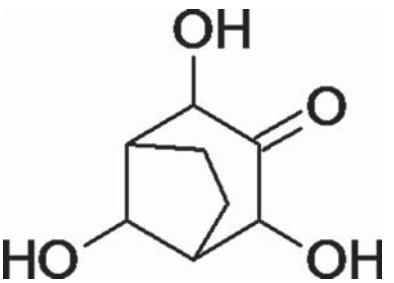

Figure 1. Structure of 2,4,8-trihydroxybicyclo [3.2.1]octan-3-one.

Antioxidant activity

Superoxide radical-scavenging assay. Superoxide radical-scavenging activity was measured by the method of Rao, Nishikimi and Yagi [18]. 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 TBO, and the colour developed, due to NBT reduction, was read at 560 nm against a blank. The radical-scavenging activity was measured as the decrease in the absorbance and was calculated using the following equation:

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

Hydroxyl radical-scavenging assay. Reaction mixtures containing different concentrations of TBO 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 [19]. 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.

Nitric oxide radical-scavenging assay. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which is measured by the Griess reaction [20]. Sodium nitroprusside (5 mM) was mixed with different concentrations of TBO 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.

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 [21]. Microsomes (0.5 mg protein) were mixed with $FeSO_4$ (1 mM) and ascorbic acid (1 mM) with or without TBO 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 [22]. The TBARS value was taken as a measure of lipid peroxide generation.

Reducing power. The reducing power of TBO was quantified by the method described earlier [23] with modifications. Briefly, 1 ml of reaction mixture, containing TBO 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 3000 rpm 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 $OD_{700\ nm}$ of 0.5 was used as a measure of reducing power.

Protein carbonyls. Rat liver homogenate (10% w/v) was prepared in 20 mM tris-HCl buffer (pH 7.4), centrifuged at 10,000 g for 10 min at 4°C. A sample of the supernatant (1 ml) was incubated with CCl_4 (1 mM) along with TBO for 1h and was precipitated with an equal volume of 20% TCA and centrifuged. 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. 0.5 ml of 20% TCA was added to the reaction mixture and centrifuged, 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 solubilize 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^{-1} cm^{-1} [24].

Metal ion chelation assay. The Fe^{2+}-chelating ability of TBO was assayed by measuring the formation of ferrous iron–ferrozine complex [25]. 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 mixture 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.

Inhibition of human low density lipoprotein oxidation. Human LDL (100 μg protein/ml) was oxidized with 10 μM $CuSO_4$ in the presence or absence of TBO in 10 mM PBS for 12 h, followed by the addition of 1 mM EDTA to stop the reaction. After incubation, aliquots of the reaction mixture were used for measuring TBARS formation and relative electrophoretic mobility (REM). TBARS was measured by 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 spectrofluorometer at 515 nm excitation and 553 nm emission wavelengths [26]. The electrophoretic mobility of the native or oxidized 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 oxidized LDL versus native LDL, was calculated [27].

Cell studies

Hepatocyte isolation. Hepatocytes were isolated from male Wistar rats (200–240 g) by collagenase perfusion of the liver as described earlier [28]. The preperfusion 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%.

Ehrlich ascites tumour (EAT) cells. EAT cells were cultured in the peritoneum of male Swiss albino mice [29]. 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.

Cell viability. Cells (10×10^6) suspended in 1 ml of HBSS were treated with the toxicant [for hepatocytes CCl_4 0.4 mM (dissolved in DMSO) or ethanol 1 mM; and for EAT cells CCl_4 0.4 mM or CHP 4 mM or HCH 1.6 mM (dissolved in DMSO)] at LC_{50} concentration with/without TBO and incubated 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 trypan blue exclusion method [30].

Lactate dehydrogenase leakage. After the incubation of cells (hepatocytes/EAT cells) in the presence of toxicant with/without TBO, cells were centrifuged and the supernatant was assayed for LDH with sodium lactate as the substrate [31]. The reaction mixture consisted of NADH (0.02 M), sodium pyruvate (0.01 M) and 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-sec interval for 3 min.

Lipid peroxidation. After incubation, as above, the cells (hepatocytes/EAT cells) were centrifuged 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. Fluorescence of the supernatant was measured in a fluorescence spectrophotometer at excitation and emission wavelengths of 532 nm and 553 nm, respectively [32]. Lipid peroxidation was quantified by the amount of malondialdehyde (MDA) formed, which was calculated using a standard curve prepared with tetraethoxypropane.

Reactive oxygen species (Superoxide anion). The cells (hepatocytes/EAT cells) (10×10^6) suspended in 1.0 ml HBSS were incubated with NBT (0.2 mM), toxicant (as mentioned earlier) and with/without TBO 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 the reduction of NBT [33].

Glutathione. Hepatocytes/EAT cells (10×10^6) suspended in 1 ml HBSS were treated with toxicant as mentioned above with/without TBO and incubated for 60 min in a shaking water bath at 37°C. At the end of incubation, cells were homogenized in 1 ml of 5% (w/v) trichloroacetic acid, centrifuged at 2,000 g for 5 min and glutathione (GSH) in the deproteinized supernatant was estimated by Ellman's reagent with a standard curve and represented as nmole/mg protein [34].

Protein estimation was done by the method of Lowry et al. [35] using BSA as the standard.

Statistical analysis

Data were expressed as mean ± S.E. of three separate experiments, and the significance was determined by the analysis of variance ($p < 0.05$) (Newman Keul's

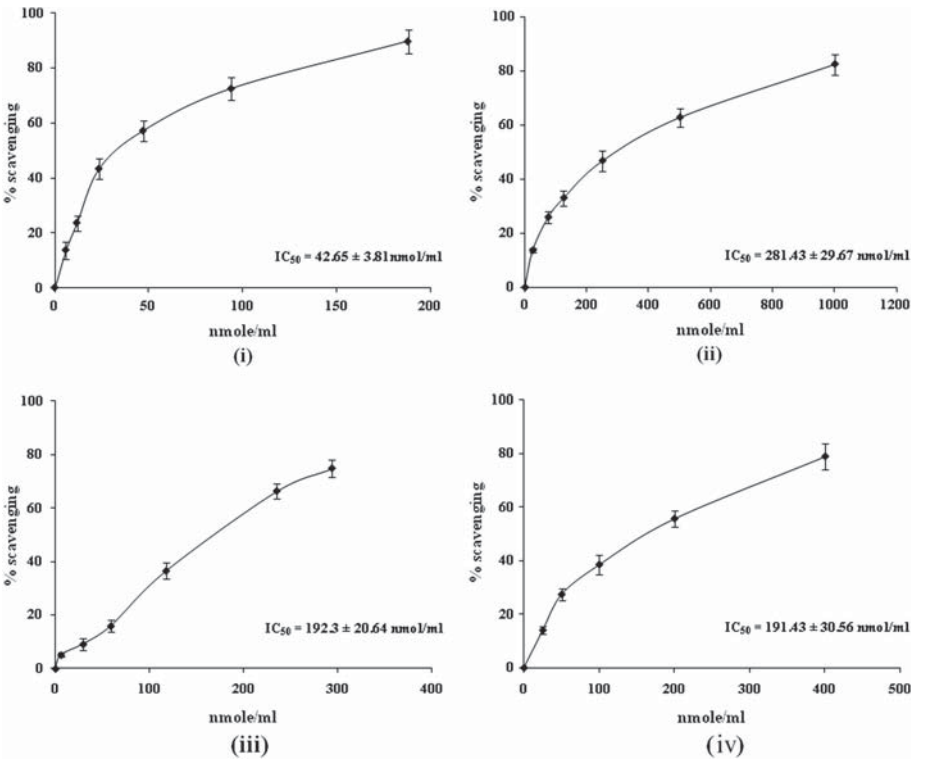

Figure 2. Free-radical-scavenging activity of TBO. (i) Lipid peroxide ($LOO^{\cdot}$) radical, (ii) Superoxide radical ($O_2^{\cdot-}$) radical, (iii) Hydroxyl ($^{\cdot}OH$) radical, (iv) Nitric oxide ($^{\cdot}NO$) radical.

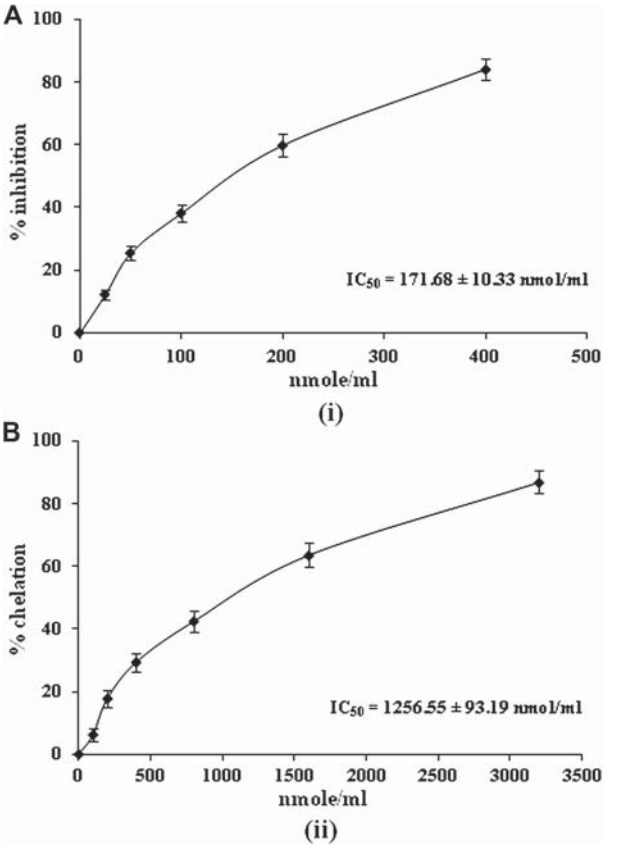

Figure 3. Additional antioxidant activity of TBO. (i) Inhibition of protein carbonylation, (ii) Metal-chelating activity.

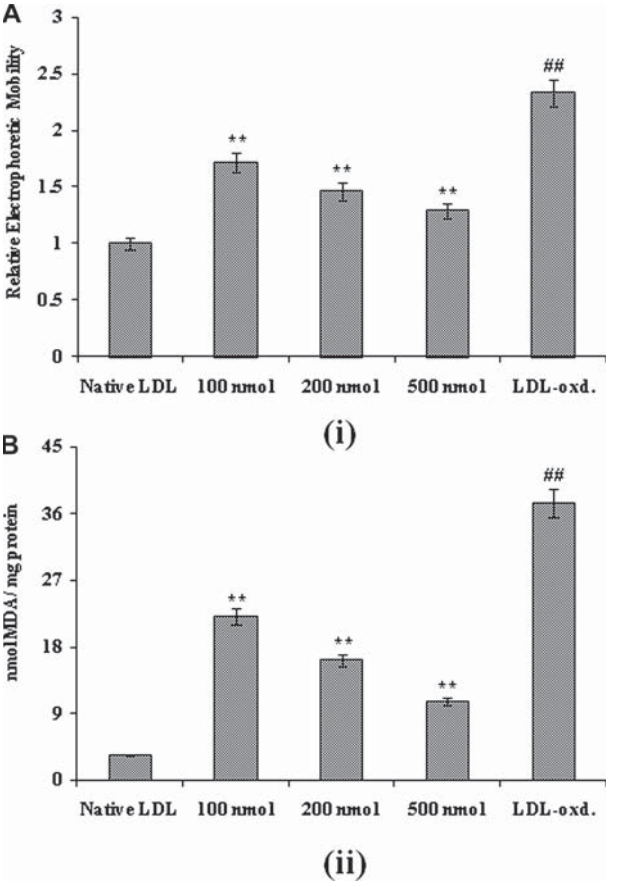

Figure 4. Inhibition of human LDL oxidation by TBO. (i) Relative Electrophoretic Mobility (REM), (ii) Thiobarbituric acid reactive substances (TBARS) content. Statistics was done by one way ANOVA. # represents comparison between control and $CuSO_4$ groups and *represents comparison between TBO + $CuSO_4$ treatment groups. ## or **represents $p < 0.05$.

posthoc test) using the computer programme Excel and Statistica software.

Results

Free-radical-scavenging activity

Free radicals such as lipid peroxide (LOO·), superoxide ($O_2^{\cdot -}$), hydroxyl (·OH) and nitric oxide (·NO) are physiologically relevant and when excess cause oxidative stress, which can lead to pathological conditions. We tested the efficacy of TBO in scavenging these free radicals. TBO showed concentration-dependent scavenging of free radicals (Figure 2). The free-radical-scavenging activity of TBO was as potent as compared with known antioxidants, quercetin and butylated hydroxyl anisole (BHA) (data not shown). The calculated IC_{50} values were in nmole range, indicating its high potency. We also found that TBO is a potent scavenger of DPPH radical, a synthetic free radical (data not shown).

Additional antioxidant activity

Apart from scavenging physiologically relevant free radicals, TBO also demonstrated additional antioxidant activity [16]. One of the major consequences of oxidative stress is irreversible protein modifications such as generation of protein carbonyls, which lead to their fragmentation, increased aggregation and enzyme dysfunction. TBO in a dose-dependent fashion prevented CCl_4-induced protein carbonyl formation in rat liver tissue (Figure 3(i)). The metal ion-chelating capacity plays a significant role in the antioxidant mechanism because it prevents oxyradical generation and the consequent oxidative damage. TBO showed significant ferrous ion-chelating activity, as a measure of its antioxidant capacity (Figure 3(ii)). Ferric reducing antioxidant power assay is conventionally used to measure the reducing ability of antioxidants. 18.43 ± 1.27 nmol/ml of TBO was sufficient to get an $OD_{700\ nm}$ of 0.5.

Inhibition of LDL oxidation

Oxidation of human LDL is considered to be an essential step in the pathogenesis of atherosclerosis. LDL oxidation is characterized by alterations in structure and biological properties of lipids and apolipoprotein B (apo B). TBO showed dose-dependent

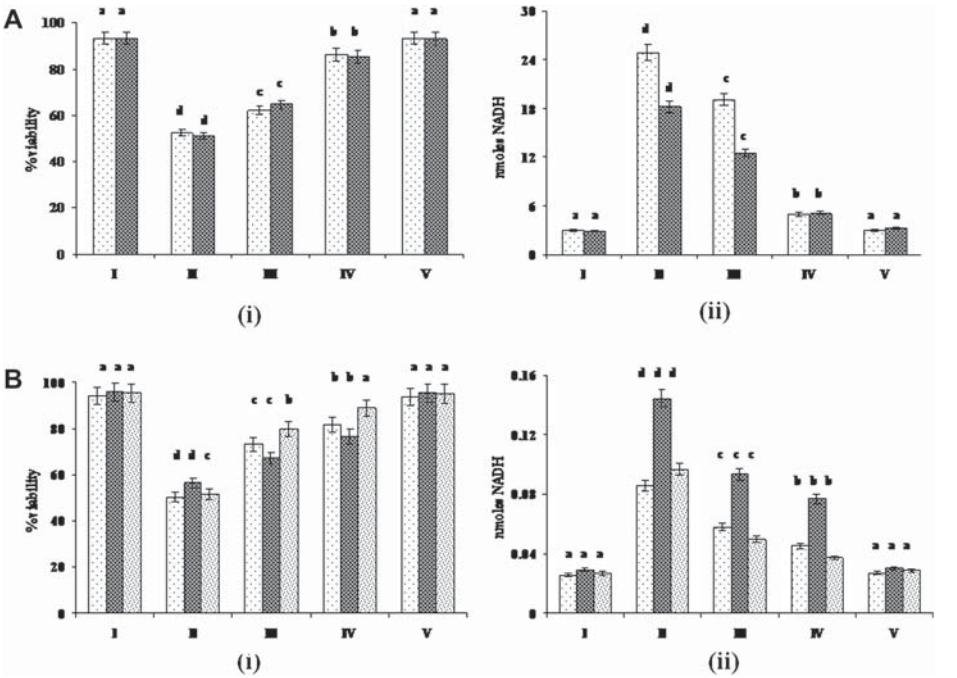

Figure 5. Cytoprotective effect of TBO. (i) Trypan blue exclusion method, (ii) LDH leakage. A. Rat primary hepatocytes: Groups-I: Control, II: Toxicant [CCl_4 (0.4 mM) or Ethanol (1 mM)], III: Toxicant + TBO (0.058 mM), IV: Toxicant + TBO (0.232 mM), V: TBO (0.232 mM). B. EAT cells: Groups-I: Control, II: Toxicant [HCH (1.6 mM) or CHP (4 mM) or CCl_4 (3 mM)], III: Toxicant + TBO (0.29 mM), IV: Toxicant + TBO (0.58 mM), V: TBO (0.58 mM). Different letters indicate statistical significance at $p < 0.05$.

protection against copper-induced human LDL oxidation as measured by relative electrophoretic mobility (REM) and TBARS formation (Figure 4). The comparison of TBO with butylated hydroxyanisole (BHA) and quercetin in inhibiting LDL oxidation was reported earlier [16].

Cell viability

To assess the cytoprotective effect of TBO, 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). TBO dose dependently ameliorated toxicant-induced cytotoxicity as measured by trypan blue method and lactate dehydrogenase leakage. TBO, by itself, was not toxic to the cells at the highest concentration used (Figure 5).

Markers of oxidative stress

TBO 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 glutathione level (Figure 6). The toxicants induced oxidative stress to different degree; in hepatocytes, CCl_4 induced more oxidative stress compared to ethanol at LC_{50} concentration. In EAT cells, HCH induced the highest production of ROS, and CHP caused most LPO at LC_{50} concentration. TBO decreased ROS by either inhibiting their production or scavenging them, leading to decreased LPO, and maintained the GSH level. TBO alone did not have any adverse effect on oxidative stress parameters of hepatocytes and EAT cells.

Discussion

Free radicals such as O_2·-, ·OH, ·NO and LOO· when produced in excess react with lipids, proteins and DNA to cause oxidative stress. Unabated oxidative stress leads to cardiovascular diseases, degenerative diseases, cancer and aging. The beneficial health effects of antioxidants have been attributed to their ability to scavenge free radicals [36]. There is overwhelming evidence that phytochemicals could be used as effective antioxidants for improving human health by preventing or delaying degenerative diseases [37]. Our results show that TBO, isolated from *D. hamiltonii*, is a potent scavenger of O_2·-, ·OH, ·NO and LOO· – physiologically relevant free radicals. Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction [38]. Chelating agents, which form bonds with a metal, are effective as secondary antioxidants as they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. It is believed that antioxidant activity and reducing power are related [39]. Reductones donate

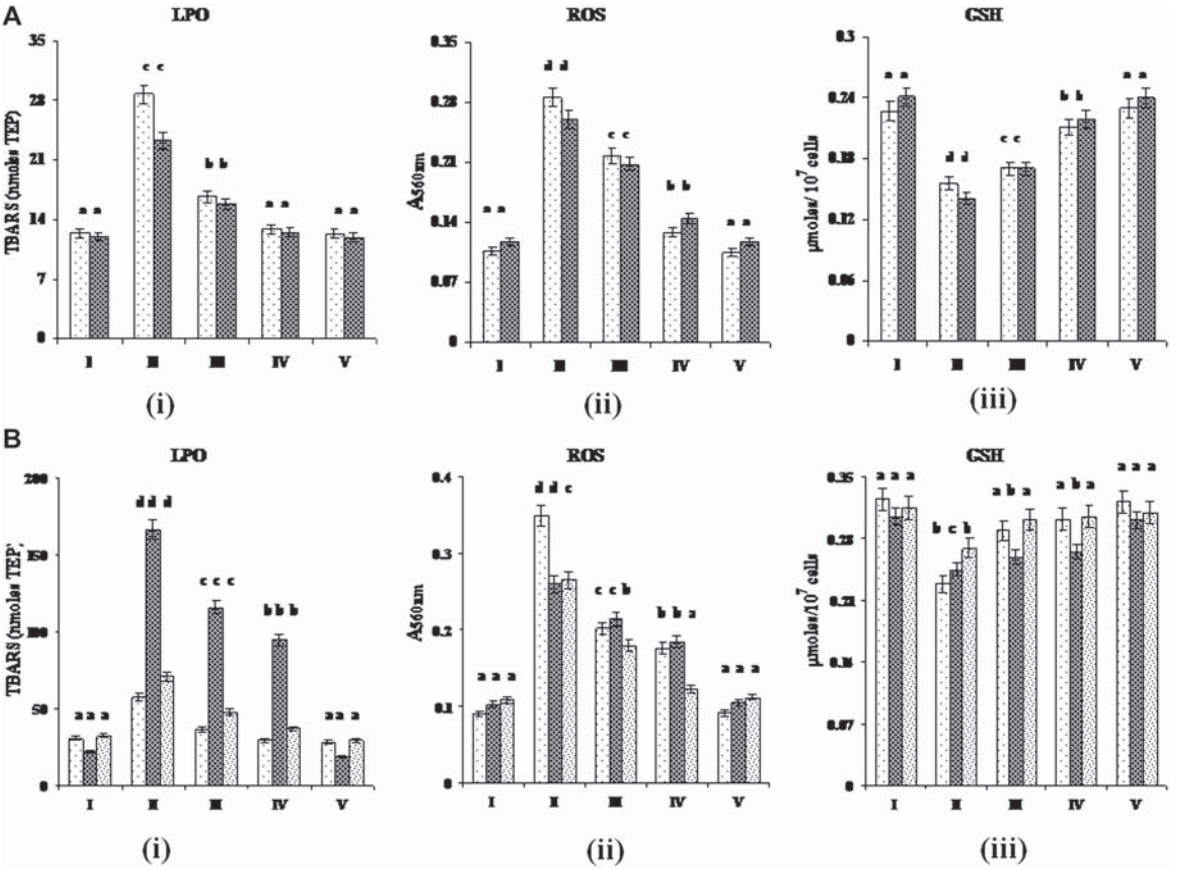

Figure 6. Amelioration of oxidative stress by TBO. (i) Inhibition of lipid peroxidation (LPO), (ii) Reactive Oxygen Species (ROS) scavenging, (iii) Restoration of glutathione (GSH) levels. A. Rat primary hepatocytes: Groups – I: Control, II: Toxicant [CCl_4 (0.4 mM) or Ethanol (1 mM)], III: Toxicant + TBO (0.058 mM), IV: Toxicant + TBO (0.232 mM), V: TBO (0.232 mM). B. EAT cells: Groups – I: Control, II: Toxicant [HCH (1.6 mM) or CHP (4 mM) or CCl_4 (3 mM)], III: Toxicant + TBO (0.29 mM), IV: Toxicant + TBO (0.58 mM), V: TBO (0.58 mM). Different letters indicate statistical significance at $p < 0.05$.

a hydrogen atom and inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction [40]. Oxidative stress leads to irreversible protein modification, such as generation of carbonyls or loss of thiol residues [41]. These oxidative modifications alter the biological properties of proteins, leading to their fragmentation, increased aggregation and enzyme dysfunction. By virtue of its free-radical-scavenging, metal-chelating, high-reducing and protein carbonylation inhibiting properties, TBO 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 [42,43]. Transition-metal-induced oxidation of LDL is one of the classical models of oxidation employed in research [44,45]. TBO showed dose-dependent protection against copper-induced human LDL oxidation. The protection of LDL oxidation by TBO could be due to: (a) scavenging of various radical species in the aqueous phase, (b) interaction with peroxyl 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 α-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 [46,47]. Several phytochemicals have been evaluated for their protective activity against xenobiotic-induced toxicity in experimental models *in vitro* and *in vivo* conditions [48,49]. 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 [47,50]. TBO inhibited xenobiotic-induced LPO in both EAT cells and hepatocytes. Further, the results show that TBO 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 TBO. Our results show that TBO 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 the 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, TBO is a potent antioxidant and inhibits human LDL oxidation. Further, TBO prevents 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 inhibiting LPO.

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Declaration of interest

Authors declare no conflicts of interest.

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