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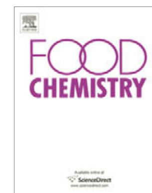

Neuroprotective effect of *Decalepis hamiltonii* roots against ethanol-induced oxidative stress

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ABSTRACT

The neuroprotective potential of the aqueous extract of the roots of *Decalepis hamiltonii* (*D. hamiltonii* root aqueous extract-DHRAE) was studied against ethanol-induced oxidative stress in the rat brain. Ethanol, single dose (5 g/kg body weight), induced oxidative stress in the rat brain which was evident from the increased lipid peroxidation and protein carbonylation, reduced glutathione, and suppressed activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase. Pretreatment of rats with multiple doses of DHRAE, 50 and 100 mg/kg b.w., for 7 consecutive days significantly prevented the ethanol-induced oxidative stress. DHRAE, as such, boosted the antioxidant status of the rat brain. The neuroprotective potential of DHRAE can be attributed to the known antioxidant constituents or its interaction with antioxidant response elements (AREs) which needs to be ascertained.

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1. Introduction

Brain is considered highly sensitive to oxidative damage as it consumes an inordinate fraction (20%) of the total oxygen consumed for its relatively small weight (2%), is relatively deficient in its antioxidant defenses, and is rich in oxidisable substrates like polyunsaturated fatty acids and catecholamines (Chong, Li, & Maiese, 2005; Somani et al., 1996). Abundance of redox active transition metal ions like iron in the brain makes it more susceptible to oxidative stress via the metal-catalysed formation of reactive oxygen species (ROS) (Ali, Barnham, Barrow, & Separovic, 2004). In addition, brain regions show differential sensitivity to oxidative stress because of the differential antioxidant profile, involving antioxidant enzymes and endogenous antioxidants like glutathione and vitamin E (Lieber, 2005; Srivastava & Shivanandappa, 2005). Oxidative stress has been shown to be linked to decline in the neurological functions and is implicated in neurological diseases like Parkinson's, Alzheimer's, and Amytropic lateral sclerosis amongst others (Lin & Beal, 2006).

Excessive and chronic ethanol consumption is known to result in damages to a number of organs including brain. A number of studies suggest that ethanol toxicity is associated with elevated generation of ROS (Das & Vasudevan, 2007) and concomitant reduction in endogenous antioxidant capacity (Bergamini, Gambetti, Dondi, & Cervellati, 2004; Lieber, 2005). Ethanol-induced neuronal oxidative stress has been shown to be ameliorated by antioxidant treatment (Shirpoora et al., 2009). Oxidative stress plays an important role in neuronal pathologies and there has been a spur in research focused towards developing novel antioxidant therapies. Amongst these therapies plant based (herbal) preparations have attracted much attention because of their safety profiles and powerful antioxidant constituents like polyphenols and flavonoids amongst others (Sun, Wang, Simonyi, & Sun, 2008).

Tuberous roots of *Decalepis hamiltonii* (Wight and Arn.) (family: Asclepiadaceae) are consumed as pickles and juice for its alleged health promoting properties in southern India. The roots are 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 isolated/characterised the antioxidant constituents (amongst them six are novel antioxidants, reported for the first time) which could be associated with their alleged health benefits (Harish, Divakar, Srivastava, & Shivanandappa, 2005; Srivastava, Harish, & Shivanandappa, 2006; Srivastava, Rao, & Shivanandappa, 2007; Srivastava, Shereen, Harish, & Shivanandappa, 2006). We have also shown the hepatoprotective potential of the roots of *D. hamiltonii* (Srivastava & Shivanandappa, 2006, 2009). As we have earlier proven that the aqueous extract of the roots of *D. hamiltonii* has high antioxidant properties, in this study we explored its neuroprotective activity against ethanol-induced neuronal oxidative stress in rat brain.

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2. Materials and methods

2.1. Chemicals

Nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (GR), cumene hydroperoxide (CHP), pryogallol, bovine serum albumin (BSA), tetraethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), 5,5′dithiobis(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

2.2. Preparation of the root powder and extraction

The roots of *D. 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. Tuberous roots of *D. hamiltonii* 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 fine powdered. The powder was used for extraction. We have earlier reported that aqueous extract of *D. hamiltonii* shows high antioxidant activity amongst the different solvent extracts. The aqueous extract was prepared by homogenising the root powder in warm water (50 °C) and allowed to stand for 24 h, filtered with Whatman paper No. 1 and the filtrate was lyophilised and weighed (17% yield). To quantify/characterise the extract composition total polyphenolic content was measured (13.8 mg/g extract). Aqueous extract of *D. hamiltonii* was chosen for this study as it shows highest antioxidant activity amongst the different solvent extracts.

2.3. Animals and treatment

Wistar male rats were procured from the animal colony maintained at the institute. Sixty days old adult male rats (180–200 g) were divided into different groups, of eight each. The Institute Animal Ethics Committee guidelines were followed for the animal experiments. Neuroprotective activity of the *D. hamiltonii* root aqueous extract (DHRAE) was tested with multiple dose (7 consecutive days) oral pretreatment at the doses of 50 and 100 mg/kg b.w. The toxicant was administered orally on the 7th day 1 h after the DHRAE administration. The dose of the toxicant was ½LD_{50} of ethanol. Animals were sacrificed by ether anaesthesia after 16 h of toxicant administration; the brain perfused with saline was processed immediately for biochemical assays.

2.4. Experimental design and groupings

2.4.1. Groups

Group I-Control; Group II-DHRAE (100 mg/kg b.w.) (7 days); Group III-DHRAE (50 mg/kg b.w.) (7 days) + ethanol (5 g/kg b.w.); Group IV-DHRAE (100 mg/kg b.w.) (7 days) + ethanol (5 g/kg b.w.); Group V-ethanol (5 g/kg b.w.) (diluted in saline).

2.5. Lipid peroxidation

Lipid peroxidation (LPO) in the tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS). Brain tissue was homogenised (10% w/v) in ice-cold 50 mM phosphate buffer (pH 7.4), centrifuged at 10,000*g* for 20 min. at 4 °C and the supernatant was used to assay the TBARS.

Tissue homogenate was mixed with TCA (10%) and TBA (0.34%) and boiled in a water bath for 15 min, cooled and centrifuged. Absorbance of the supernatant was read at 535 nm. TBARS was calculated using tetraethoxypropane as the standard.

2.6. Glutathione

A 10% (w/v) tissue homogenate prepared in 5% (w/v) trichloroacetic acid, centrifuged at 5000*g* for 5 min and glutathione (GSH) in the deproteinised supernatant was estimated by Ellman's reagent with a standard curve.

2.7. Protein carbonyls

Tissue homogenates (10% w/v) were prepared in 20 mM Tris–HCl buffer, pH 7.4 with 0.14 NaCl, centrifuged at 10,000*g* for 10 min at 4 °C and 1 ml of the supernatant 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 3 times with acetone and solubilised in 1 ml of 2% of SDS (in 20 mM Tris–HCl, 0.1 M NaCl, pH 7.4). The absorbance was read at 360 nm in a UV–Visible Spectrophotometer and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

2.8. Antioxidant enzymes

Brain tissue was homogenised (10% w/v) in ice-cold 50 mM phosphate buffer (pH 7.4), centrifuged at 10,000*g* for 20 min. at 4 °C and the supernatant was used to assay the enzyme activities.

Superoxide dismutase (SOD) activity was measured using pyrogallol (2 mM) autoxidation in Tris buffer. Catalase (CAT) activity was measured using H_2O_2 (3%) as the substrate in phosphate buffer. Glutathione peroxidase (GPx) activity was measured by the indirect assay method using glutathione reductase. Cumene hydroperoxide (1 mM) and glutathione (0.25 mM) were used as substrates and coupled oxidation of NADPH by glutathione reductase (0.25 U) in Tris buffer (50 mM, pH 7.6) was monitored at 340 nm. Glutathione reductase (GR) activity was assayed in a reaction mixture containing oxidised glutathione (20 mM) and NADPH (2 mM) in potassium phosphate buffer. Glutathione transferase (GST) activity was assayed by using glutathione (20 mM) and CDNB (30 mM) as the substrates in phosphate buffer, change in absorbance at 344 nm was monitored in a UV–Visible Spectrophotometer.

Protein content was estimated by the method of Lowry, with bovine serum albumin as the standard. All the described methods were followed from published literature and are well established.

2.9. Statistical analysis

The data were expressed as means ± S.E.M. of eight observations ($n = 8$) and significant difference between each of the groups was statistically analysed by Duncan's multiple range test (Statistica Software, 1999), represented by alphabets for each level of significance. A difference was considered significant at $p < 0.05$.

3. Results

The food consumption and body weights of the DHRAE treated rats were comparable to the control group. Ethanol increased lipid

peroxidation (LPO) in the rat brain. DHRAE pretreatment ameliorated the ethanol-induced LPO, interestingly the higher dose showed complete prevention. In addition, ethanol increased protein carbonyls (PC) content in the rat brain. Ethanol-induced carbonylation of proteins was significantly reduced by DHRAE pretreatment. Furthermore, a significant depletion in glutathione (GSH) content was observed in the brain of ethanol treated rats. DHRAE pretreatment not only significantly prevented the decrease in GSH due to ethanol treatment but also substantially enhanced GSH levels in the rat brain. Ethanol decreased superoxide dismutase (SOD) activity in brain, which was prevented by pretreatment of both the doses of DHRAE. DHRAE (100 mg/kg b.w.) could restore the SOD activity back to normal. DHRAE, *per se*, raised the SOD activity which was significantly higher compared to control groups. Treatment with ethanol reduced catalase (CAT) activity in the rat brain. DHRAE pretreatment, both doses, could restore the CAT activity to normalcy. Further, DHRAE significantly elevated CAT activity in rat brain. Brain glutathione peroxidase (GPx) activity was decreased in ethanol treated rats. DHRAE pretreatment prevented the ethanol-induced decrease in GPx activity. DHRAE treatment boosted the GPx activity in the rat brain. Furthermore, the activity of glutathione reductase (GR) was suppressed in the brain of ethanol treated rats, which was completely reversed back by both the doses of DHRAE pretreatment. DHRAE, by itself, raised the GR activity in rat brain. In addition, glutathione-S-transferase (GST) activity in the brain of rats administered ethanol was decreased. DHRAE prevented this decrease and at high dose it could normalise to control levels. DHRAE alone increased GST activity in rat brain (Table 1).

4. Discussion

Brain is considered highly vulnerable to oxidative stress than other organs of the body as it consumes high amounts of oxygen; contains high amounts of PUFA and low levels of antioxidant enzymes (Chong et al., 2005; Somani et al., 1996). Excessive ethanol consumption has been shown to result in damages to a number of organs including the brain due to the induction of oxidative stress (Das & Vasudevan, 2007). Increased oxidative stress is directly caused by ethanol and its oxidation products. It has been demonstrated that ethanol induces synthesis of cytochrome P450 2E1 in the brain which is an important source of ethanol-induced oxidative stress (Somani et al., 1996).

Lipid peroxidation (LPO), a consequence of oxidative stress, is associated with progressive loss in membrane potential, increase in membrane permeability, and finally cell death. Acute ethanol intake induces an increment in TBARS levels in brain, an effect that is abolished by antioxidants (Dey & Cederbaum, 2006). The mechanism of ethanol-induced depletion of GSH involves conjugation of GSH with acetaldehyde, the reactive intermediate of ethanol oxidation, or enhanced utilisation of GSH for the detoxification of free radicals and oxidants produced as a result of ethanol exposure (Calabrese et al., 2000). Increased protein carbonyl formation, one of the commonly used indicators of oxidative protein damage, has been observed following alcohol exposure (Altomare, Grattagliano, Vendemiale, Palmieri, & Palasciano, 1996). Our study is in agreement with earlier observations on ethanol-induced oxidative stress in the rat brain (Shirpoora et al., 2009). In this study, the aqueous extract of the roots of *D. hamiltonii* prevented LPO and protein carbonylation induced by ethanol in the rat brain. Further, *D. hamiltonii* aqueous extract pretreatment restored the GSH level and as such boosted the basal GSH level.

Ethanol is known to suppress the activities of antioxidant enzymes like SOD, CAT, etc. (Shirpoora et al., 2009). In this study also a significant reduction in the activities of antioxidant enzymes was observed. DHRAE pretreatment abolished the adverse effect of ethanol on the antioxidant enzyme activities in the rat brain. Moreover, DHRAE treatment alone boosted the antioxidant capacity of the rat brain. The exact mechanism by which the plant extracts enhance the antioxidant enzyme levels is not clearly understood. It is suggested that the enhancement of phase II enzymes by antioxidants present in aqueous plant extracts, is achieved by upregulating the corresponding genes by interaction with antioxidant response elements (AREs) that transcriptionally regulate these genes (Mandel et al., 2008; Moskaug, Carlsen, Myhrstad, & Blomhoff, 2005). There is a possibility that the antioxidant compounds present in DHRAE are interacting with AREs *in vivo*, which needs further investigation.

Antioxidant intervention in therapeutic strategy for treatment of neurological disorders is gaining significance (Joshi et al., 2005). It is known that dietary antioxidants and herbal extracts can significantly contribute to the modulation of complex mechanisms of neurodegenerative diseases (Aruoma, 2002). This study demonstrates the neuroprotective potential of the roots of *D. hamiltonii* which could provide an explanation for the health promoting properties attributed to its traditional consumption. Antioxidant constituents from DHRAE have been identified and are shown to possess potent antioxidant properties. Further studies are underway which will provide the exact mechanism of DHRAE's health promoting effect.

Table 1
Neuroprotective effect of *D. hamiltonii* aqueous extract pretreatment (multiple dose) against ethanol toxicity in rats.

Group	TBARS[A]	SOD[B]	CAT[C]	GPx[D]	GR[D]	GST[E]	GSH[F]	PC[G]
I	$1.73^a \pm 0.14$	$0.57^c \pm 0.04$	$1.17^b \pm 0.09$	$17.64^b \pm 1.44$	$91.23^b \pm 8.23$	$172.48^c \pm 14.38$	$14.56^c \pm 1.23$	$30.23^a \pm 2.94$
II	$1.69^a \pm 0.13$	$0.71^d \pm 0.03$	$1.4^c \pm 0.13$	$20.04^c \pm 1.83$	$120.36^c \pm 10.28$	$193.25^d \pm 16.74$	$16.95^d \pm 1.49$	$30.09^a \pm 2.71$
III	$2.13^b \pm 0.19$	$0.48^b \pm 0.04$	$1.15^b \pm 0.11$	$16.32^b \pm 1.47$	$87.48^b \pm 7.15$	$148.22^b \pm 12.27$	$11.23^b \pm 0.09$	$35.36^b \pm 3.21$
IV	$1.87^a \pm 0.16$	$0.59^c \pm 0.03$	$1.21^b \pm 0.11$	$17.54^b \pm 1.51$	$93.61^b \pm 8.27$	$170.83^c \pm 14.35$	$13.87^c \pm 0.11$	$33.29^b \pm 2.93$
V	$3.38^c \pm 0.28$	$0.22^a \pm 0.02$	$0.91^a \pm 0.08$	$14.03^a \pm 1.23$	$64.24^a \pm 5.83$	$121.54^a \pm 11.06$	$9.56^a \pm 0.08$	$39.18^c \pm 3.76$

Group I-control; Group II-DHRAE (100 mg/kg b.w.) (7 days); Group III-DHRAE (50 mg/kg b.w.) (7 days) + ethanol (5 g/kg b.w.); Group IV-DHRAE (100 mg/kg b.w.) (7 days) + ethanol (5 g/kg b.w.); Group V-ethanol (5 g/kg b.w.).
TBARS-thiobarbituric acid reactive substances; SOD-superoxide dismutase; CAT-catalase; GPx-glutathione peroxidase; GR-glutathione reductase; GST-glutathione-S-transferase; GSH-glutathione; PC-protein carbonyls.
Means with different superscript letters differ significantly ($p < 0.05$).
[A] nmoles MDA/mg protein.
[B] Units/mg proteins.
[C] µmole H_2O_2/min/mg protein.
[D] nmoles NADPH/min/mg protein.
[E] µmole CDNB conjugate/min/mg protein.
[F] µg/mg protein.
[G] µmole/mg protein.

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