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Effect of *Centella asiatica* leaf powder on oxidative markers in brain regions of prepubertal mice *in vivo* and its *in vitro* efficacy to ameliorate 3-NPA-induced oxidative stress in mitochondria ☆

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Abstract

Centella asiatica (CA) is a common medicinal plant used in the ayurvedic system of medicine to treat various ailments and as a memory enhancer. Despite its extensive usage in children, data on its ability to modulate neuronal oxidative stress in prepubertal rodents are limited. Hence in the present study we have addressed primarily two questions (i) whether dietary intake of CA leaf powder possess the propensity to modulate endogenous oxidative markers in mouse brain regions and (ii) the efficacy of CA aqueous extract to abrogate 3-nitropropionic acid (3-NPA)-induced oxidative stress in brain mitochondria *in vitro*. Prepubertal male mice were fed CA-incorporated diet (0.5 and 1%) for 4 weeks, and biochemical markers of oxidative stress in brain regions were determined. Mice fed CA showed significant diminution in the levels of malondialdehyde (30–50%), reactive oxygen species (32–42%) and hydroperoxide levels (30–35%), which was accompanied by enhanced activities of antioxidant enzymes in all brain regions. While the levels of reduced glutathione and total thiols were elevated, the protein carbonyl content was decreased in brain among CA-fed mice. Interestingly, the oxidative markers among brain mitochondria of CA-fed mice were also significantly diminished (malondialdehyde, 25%; ROS, 30%; hydroperoxides, 35% and protein carbonyls, 28%). Further, the aqueous extract of CA showed significant free radical scavenging activity determined in established chemical test systems (viz., DPPH, superoxide and hydroxyl radical scavenging activity). Furthermore, the aqueous extract of CA markedly ameliorated the 3-NPA induced oxidative stress response in brain mitochondria under *in vitro* exposure,. Taken together, these data suggest that CA has the propensity to modulate both endogenous and neurotoxicant induced oxidative impairments in the brain and may be effectively employed as a neuroprotective adjuvant to abrogate oxidative stress *in vivo*.

Keywords: *Centella asiatica*; Brain regions; Oxidative markers; Prepubertal mice mitochondria; 3-NPA; *In vitro*

Abbreviations: CA, *Centella asiatica*; CAAE, *Centella asiatica* leaf aqueous extract; LPO, lipid peroxidation; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; DCF, 2′,7′-dichloro-fluorescein; DCF-DA, 2′,7′-dichloro-fluorescein diacetate; 3-NPA, 3-nitropropiponic acid.

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Introduction

Centella asiatica (L) Urban (Apiaceae) is a slender creeping plant native to countries like India, Srilanka, Madagascar, South Africa and Malasia (Kartnig, 1988). It is used in the ayurvedic system of medicine to treat various ailments like headache, body ache, insanity, asthma, leprosy, ulcers, eczemas and wound healing (Chopra et al., 1956; Shukla et al., 1999; Suguna et al., 1996). Earlier few clinical reports demonstrate the antidepressive–sedative effects (Chatterjee et al., 1992) of *Centella asiatica* (CA) powder and extracts as well as their ability to improve venous insufficiency (Kartnig, 1988). Further, studies have also shown the efficacy of CA extracts in improving memory and cognitive function in rats (Vaidyaratnam, 1994; Veerendra Kumar and Gupta, 2002, 2003).

Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanism is an important factor that has been implicated in various neurodegenerative diseases (Beal, 1995). The brain is highly susceptible to free radical damage because of its high utilization of oxygen and the presence of relatively low concentration of antioxidant enzymes and free radical scavengers. There have been efforts to find various therapeutic agents (both natural and synthetic) that could reduce oxidative stress and improve memory (Anekonda and Reddy, 2005). It has been postulated that the mechanistic basis of the neuroprotective activity of antioxidants rely not only on the general free radical trapping or antioxidant activity *per se* in neurons, but also on the suppression of genes induced by pro-inflammatory cytokines and other mediators released by glial cells (Wang et al., 2006). Recently, herbal treatments have been used in animal and cellular models of Alzheimer's disease (AD) and in clinical trials with AD subjects (Anekonda and Reddy, 2005). These extracts have multifunctional properties (such as procholinergic, antioxidant, antiamyloid, anti-inflammatory) and their increased human usage globally necessitates a better understanding of their potential for alleviating or reducing various neurological pathologies. Recent clinical data suggest the efficacy of a combination of antioxidants in preventing AD (Behl, 2005).

Despite the extensive human usage of CA, knowledge with regard to its ability to modulate endogenous oxidative markers in brain regions in experimental animals is lacking. Earlier studies showed that oral administration of an aqueous extract of CA reduces brain malondialdehyde levels and increases the glutathione levels in whole brain (Veerendra Kumar and Gupta, 2002) of adult rats. Recently, CA was demonstrated to accelerate nerve regeneration *in vivo* and increased neurite elongation *in vitro* (Soumyanath et al., 2005). Notable bioactive compounds of CA are the triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins viz., madecassic and asiatic acid (Mangas et al., 2006; Wijeweera et al., 2006). Further, CA is reported to contain numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempferol, catechin, rutin and naringin (Zainol et al., 2003), some of which have been shown to be potent antioxidants (Hussin et al., 2007). However, studies describing the potential of dietary CA to mitigate oxidative markers in various brain regions *in vivo* have not been attempted either in adult or prepubertal rodents.

3-Nitropropionic acid (3-NPA), a mitochondrial toxin, causes preferential neuronal degeneration in the striatum and produces anatomical changes similar to Huntington's disease in experimental animals (Beal et al., 1993). Enhanced ROS generation and MDA levels have been demonstrated in brain regions of rats challenged with 3-NPA, indicating the vital role of oxidative stress in the manifestation of neurotoxicity (Fu et al., 1995). Earlier, 3-NPA-induced neurotoxicity has been shown to be attenuated by taurine, and *S*-allylcysteine (Tadros et al., 2005; Herrera-Mundo et al., 2006). However, attempts to modulate 3-NPA-induced oxidative stress either *in vivo* or *in vitro* by specific phytochemicals have not been attempted. In view of this, we are investigating the propensity of CA extracts to modulate 3-NPA-induced oxidative response both *in vitro* and *in vivo* in various brain regions of prepubertal mice.

In the present study, we have addressed two issues: (a) whether dietary *Centella asiatica* leaf powder could significantly reduce the levels of endogenous oxidative markers in different brain regions of prepubertal mice and (2) the *in vitro* efficacy of an aqueous extract of *Centella asiatica* against 3-nitropropionic acid (3-NPA)-induced oxidative stress response in brain mitochondria.

Materials and methods

Chemicals

Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, 2′, 7′-dichloro-fluorescein (DCF), 2′, 7′-dichloro-fluorescein diacetate (DCF-DA) and other fine chemicals were procured from M/s Sigma Chemical Co., St Louis, USA. BHT (butylated hydroxyl toluene), DPPH (1,1-diphenyl-2-picrylhydrazyl), PMS (phenazine methosulphate), NADH (nicotinamide adenine dinucleotide reduced), NBT (nitroblue tetrazolium), trichloro acetic acid (TCA) were procured from M/s Sisco research Laboratories, Mumbai, India. All other chemicals used were of analytical grade

Animals and care

Prepubertal male mice (CFT-Swiss, 4-week old) were drawn from the stock colony of the 'institute animal house facility'. They were housed in rectangular polypropylene cages (three per cage) kept on racks built of slotted angles, and the cages were provided with dust-free paddy husk as a bedding material. The animals were housed in a controlled atmosphere with a 12 h light/dark cycle. They were acclimatized for 1 week prior to the start of the experiment and were maintained on a powdered diet and tap water *ad libitum*. The experiments were conducted strictly in accordance with the approved guidelines by the "Institute Animal Ethical Committee" regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Government of India, India.

Centella asiatica (CA) powder and aqueous extract

Centella asiatica plant was collected during early summer from the state of Kerala and authenticated by Prof. C.M Joy, Department of Botany, Sacred Heart College Thevara, Mahatma Gandhi University, Kerala, India. Fresh leaves of CA were shade dried and powdered in a mill without producing much heat and used for the dietary study.

For the preparation of aqueous extract, the leaf powder (5 g) was extracted with 8 parts of double-distilled water under boiling for 5 h, and cooled. The supernatant was then filtered through a 400-mesh cloth to collect the extract and rotary evaporated at 40 °C for 30 min. Later the product after flash evaporation was lyophilized to yield a greenish brown powder (total yield – 1.5 g). The triterpene content analysed by HPLC ranged between 8% and 8.6% on dry basis and the concentration of the known active component, asiaticoside, was found to be 0.8% w/w. The HPLC analysis was performed by following the method of Inamdar et al. (1996) with minor modifications. The chromatographic separation was performed with μBondapak, C_{18} 10 µm, 30×0.39 cm, SS column with a water – acetonitrile mobile phase with UV detection at 220 nm.

Experimental design

Centella asiatica leaf powder (shade dried, powdered) was mixed with diet (at 0.5% and 1.0%). Male mice ($n = 6$) were fed with either commercial diet or CA-incorporated diet for a period of 4 weeks. Mice fed with only commercial diet served as the normal controls. Diet was prepared twice every week. During the experimental period, food intake was monitored daily and individual body weights were recorded once a week. Mice from control and CA groups were sacrificed at 4 weeks and the following biochemical investigations were carried out.

Preparation of homogenates and mitochondria

Brain was excised and frozen immediately. The brain regions, cerebral cortex, cerebellum hippocampus and striatum were subsequently dissected over ice. The tissues were immediately homogenized in phosphate buffer (0.1 M, pH 7.4) using a glass-Teflon grinder, and the homogenate was centrifuged at 1000*g* for 10 min and the supernatant was used for the quantification of malondialdehyde (MDA), reactive oxygen species (ROS), protein carbonyls, total thiols and non-protein thiols levels. For GSH, GSSG and enzyme assays, the homogenate was centrifuged at 10,000*g* to obtain a cytosol fraction.

Mitochondria were prepared by differential centrifugation according to the method of Moreadith and Fiskum (1984) with minor modifications. Briefly, 10% homogenates of the brain regions were prepared in ice-cold Tris-sucrose buffer (0.25 M, pH 7.4) using a glass-teflon grinder at 4 °C. The homogenates were centrifuged at 1000*g* for 10 min at 4 °C to obtain the nuclear pellet. Mitochondria were obtained by centrifuging the post-nuclear supernatant at 10,000*g* for 20 min at 4 °C. The pellet was washed 3 times in mannitol–sucrose–HEPES buffer and resuspended in the same buffer and stored in ice.

Induction of oxidative damage: lipid peroxidation (LPO)

Induction of oxidative damage was ascertained by measuring the extent of LPO in brain (cortex, cerebellum, hippocampus and striatum) homogenates. LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Briefly, the reaction mixture contained 0.2 ml of brain region homogenate or mitochondria (1 mg protein), 1.5 ml of acetic acid (pH 3.5, 20%), 1.5 ml of 0.8% thiobarbituric acid (0.8% w/v) and 0.2 ml SDS (8% w/v). The mixture was heated to boiling for 45 min and TBARS adducts were extracted into 3 ml of 1-butanol and TBARS was measured in a UV–Visible spectrophotometer at 532 nm and quantified as malondialdehyde (MDA) equivalents using 1, 1, 3, 3-tetramethoxypropane as the standard (Ohkawa et al., 1979).

Measurement of ROS generation

ROS generation in brain regions was assayed using dihydro dichlorofluorescein diacetate (DCF-DA), a non-polar compound that, after conversion to a polar derivative by intracellular esterases, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein (Driver et al., 2000; Shinomol and Muralidhara, 2007). Briefly, the homogenate was diluted 1:20 times with ice-cold Locke's buffer to obtain a concentration of 5 mg tissue/ml. The reaction mixture (1 ml) containing Locke's buffer (pH 7.4), 0.2 ml homogenate or mitochondria (0.5 mg protein) and 10 µl of DCF-DA (5 µM) was incubated for 15 min at room temperature to allow the DCF-DA to be incorporated into any membrane-bound vesicles and the diacetate group cleaved by esterases. After 30 min of further incubation, the conversion of DCF-DA to the fluorescent product DCF was measured in a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of DCF-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS formation was quantified from a DCF-standard curve and data are expressed as p mol DCF formed/min/mg protein.

Measurement of hydroperoxide levels

An aliquot of tissue homogenate or mitochondria (100 μg protein) was added to 1 ml FOX reagent (100 μM xylenol orange; 250 μM ammonium ferrous sulphate; 100 μM sorbitol; 25 mM H_2SO_4) and incubated for 30 min at room temperature. The mixture was centrifuged at 600*g* and the supernatant was read at 560 nm in a spectrophotometer. Results were expressed as μmoles hydroperoxide/mg protein (Wolff, 1994).

Activity of antioxidant enzymes

Brain regions (cortex, cerebellum, hippocampus and striatum) were homogenized in phosphate buffer (50 mM, pH 7.4) and sonicated at 4 °C. The activities of enzymes viz., catalase, glutathione-*S*-transferase, glutathione peroxidase and superoxide dismutase were measured in cytosolic fractions which were obtained after centrifugation of the tissue homogenate at 10,000*g*. Catalase activity was assayed by the method of Aebi (1984). The enzyme activity was expressed as μmol H_2O_2 consumed/min/mg protein ($e = 43.6\,mM^{-1}\,cm^{-1}$). The activity of glutathione peroxidase was determined using *t*-butyl hydroperoxide as the substrate according to the method of Flohe and Gunzler, (1984) and the activity was expressed as η moles of NADPH oxidized/min/mg protein ($e_{340} = 6.22\,mM^{-1}\,cm^{-1}$). Glutathione-*S*-transferase was assayed by measuring the rate of enzyme-catalyzed conjugation of GSH with 1-chloro 2-4-dinitro benzene (CDNB) according to the method of Guthenberg et al. (1985) and the enzyme activity was expressed as η moles of *S*-2, 4, dinitrophenyl glutathione formed / min/mg protein (MEC- $9.6\,mM^{-1}\,cm^{-1}$). Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of ferricytochrome–c reduction using xanthine-xanthine oxidase as the source of O_2. One unit of SOD is calculated as the amount of protein required to inhibit 50% of the SOD-independent cytochrome ‘c’ reduction (McCord and Fridovich, 1969).

Determination of reduced glutathione (GSH)

GSH was measured according to the fluorimetric method of Mokrasch and Teschke (1984). Briefly, 100 μl of 10% homogenates (phosphate buffer, pH 7.4) was added to 2 ml formic acid (0.1 M) and centrifuged at 10,000*g* for 20 min. 100 μl of the supernatant was used for the assay. Concentration of GSH was calculated from the standard curve and the values were expressed as μg GSH/mg protein.

Oxidative damage: protein carbonyls

Protein carbonyl content was determined in supernatants obtained after centrifugation of tissue homogenates at 10,000*g* for 15 min by measuring the hydrazone derivatives between 360 and 390 nm according to the method of Levine et al. (1990).

Total thiols and non-protein thiols

Estimation of total thiols and non-protein thiols was done according to the methods of Ellman, (1959).

Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Ellmann et al. (1961). To the reaction mixture containing 2.85 ml phosphate buffer (0.1 M, pH 8.0), 50 μl of DTNB (10 mM), 50 μl sample and 20 μl acetylthiocholine iodide (78 mM) were added and the change in absorbance was monitored at 412 nm for 5 min in a spectrophotometer. The enzyme activity was expressed as η moles of substrate hydrolyzed/min/mg protein.

Determination of protein

Protein concentrations in the tissue homogenates and mitochondria were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Free radical scavenging activity of Centella asiatica aqueous extract

DPPH radical scavenging activity

The scavenging ability of CA extract on DPPH radical was studied employing the modified method described earlier (Yamaguchi et al., 1998). Briefly, 1.5 ml of DPPH solution (0.1 mM, in 95% ethanol) was incubated with varying concentrations of the CA extract. The reaction mixture was shaken well and incubated for 20 min at room temperature, and the absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

The synthetic antioxidant butylated hydroxytoluene (BHT) was included in experiments as a positive control.

Superoxide scavenging activity

The superoxide scavenging ability of the extract was assessed by the method of Nishikimi et al. (1972). The reaction mixture, containing CA extract, PMS (30 mM), NADH (338 mM) and NBT (72 mM) in phosphate buffer (0.1 M pH 7.4), was incubated at room temperature for 5 min, and the colour was read at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Inhibition of hydroxyl radical

Hydroxyl radical scavenging activity was determined according to the method of Chung et al. (1997). The Fenton reaction mixture consisted of 200 µl of $FeSO_4 \cdot 7H_2O$ (10 mM), EDTA (10 mM) and 2-deoxyribose (10 mM). Then, 200 µl of the extract and 1 ml of 0.1 M phosphate buffer (pH 7.4) were mixed together and made the total volume of 1.8 ml. Thereafter, 200 µl of 10 mM H_2O_2 was added and the reaction mixture was incubated at 37 °C for 4 h. After incubation, 1 ml of 2.8% TCA and 1 ml of 1% TBA were mixed and placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged (5 min, 395*g*) and the absorbance was measured at 532 nm with a UV–visible spectrophotometer. Percentage inhibition was calculated using the formula:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Determination of oxidative damage to deoxyribose

The deoxyribose assay was carried out essentially, as described by Halliwell et al. (1987). The reaction mixture (3.5 ml), which contained CA extract, deoxyribose (6 mmol/l), H_2O_2 (3 mmol/l), KH_2PO_4–K_2HPO_4 buffer (20 mmol/l, pH 7.4), $FeCl_3$ (400 µmol/l), EDTA (400 µmol/l) and ascorbic acid (400 µmol/l), was incubated at 37 °C for 1 h. To examine the extent of deoxyribose degradation, TBA (1 ml, 1%) and trichloroacetic acid (1 ml, 2.8%) were added to the mixture, which was then heated in a water bath at 100 °C for 20 min. The absorbance of the final mixture was read at 532 nm against a blank control, which contained all reagents except deoxyribose. The negative controls did not have the test compounds. The results are expressed as percent inhibition.

Effect of an aqueous extract of CA against 3-NPA-induced oxidative response in vitro

Modulation of 3-NPA-induced ROS generation in mitochondria

The modulatory effect of CAAE on 3-NPA-induced ROS generation in mitochondrial preparations of various brain regions *in vitro* were determined according to the procedure described earlier with modifications (Shinomol and Muralidhara, 2007). Briefly an aliquot of mitochondria (200 µg protein) was pre-incubated (30 min, 37 °C) with CAAE and later challenged with 3-NPA (2 mM) followed by incubation at 37 °C in a water bath for 1 h. After 30 min of further incubation, the conversions of DCF-DA to the fluorescent product DCF was measured in a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Appropriate blanks with and with out the extract and mitochondria were included to remove interferences with results. ROS formation was quantified from a DCF-standard curve, and results were expressed as ρmol DCF formed/min/mg protein.

Modulation of 3-NPA-induced hydroperoxide formation in mitochondria

An aliquot of mitochondria (100 µg protein) was pre-incubated with CAAE (30 min) and later challenged with 3-NPA (2mM). The mixture was incubated at 37 °C in a water bath for 1 h. 1 ml FOX reagent was added and incubated at room temperature, for 30 min. The mixture was centrifuged at 600*g* for 5 min and the supernatant was read at 560 nm in a spectrophotometer. Results were expressed as µmoles hydroperoxide/mg protein.

Modulation of 3-NPA-induced lipid peroxidation in mitochondria

The modulatory effect of CAAE on 3-NPA-induced lipid peroxidation in mitochondria of various brain regions *in vitro* were determined according to the procedure described earlier with modifications. An aliquot of mitochondria (200 µg protein) was added to Locke's buffer and pre-incubated with CAAE for 30 min at 37 °C and later challenged with 3-NPA and incubated further for 1 h. The reaction was stopped by addition of 200 µl of SDS (8%) followed by 1.5 ml acetic acid (20%, pH 3.5) and 1.5 ml TBA (0.8%), vortexed and kept in a boiling water bath for 45 min. The pink-colored complex was extracted into 1-butanol and read at 535 nm.

Statistical analysis of data

Data were analyzed by analysis of variance (one-way ANOVA) followed by Duncan's multiple range test (DMRT) with $p < 0.05$ taken as significant.

Results

Growth characteristics, food intake and liver weight

In mice fed CA-incorporated diet, there was no significant alteration in body weights except a slight decrease towards the fourth week (Table 1). The food

Table 1. Body weights of prepubertal male mice fed *Centella asiatica* leaf powder in the diet for 4 weeks

CA (%)/weeks	Body weights (g)				
	Initial	1	2	3	4
Control	22.5±0.55	23.8±0.75	27.5±1.12	33.8±1.02	37.6±1.11
0.5	21.8±0.75	22.6±1.0	27.6±1.07	32.7±1.00	36.5±0.50
1.0	21.8±1.01	22.4±0.50	25.5±1.11	32.5±1.21	35.8±0.50

Values are ±S.D ($n = 6$); data analyzed by one-way ANOVA.
No significant difference between control and treatment groups.

intake was comparable to untreated animals at both the concentrations (0.5% and 1%) of CA and there were no significant changes between control and different groups with respect to organ weights (data not shown).

Effect of CA on oxidative markers in brain regions

In general, brain regions of mice fed CA diet showed markedly reduced MDA levels (Table 2). The reduction was dose related, and at higher dose the percent reduction was cortex, 50%; cerebellum, 42%; hippocampus, 43%; striatum, 31%. A similar trend of reduced MDA levels was evident in mitochondrial fractions of brain regions (Fig. 1A), although the degree was relatively lower compared to homogenates (cortex, 27%; cerebellum, 26%; hippocampus, 26%; striatum, 22%).

The ROS levels in brain regions of mice fed CA were significantly diminished in both homogenates and mitochondria (Table 2, Fig. 1B). At the higher dose, the decrease in ROS levels was more robust (cortex, 33%; cerebellum, 42%; hippocampus, 40%; striatum, 38%) in brain homogenates of CA-fed mice. In mitochondria, however, significant decrease in ROS levels was evident only at the higher dose (cortex, 30%; cerebellum, 31%; hippocampus, 35%; striatum, 28%).

There was a general reduction of basal HP levels in brain regions of mice fed CA in diet (Table 2). The reduction was dose dependent, and at higher doses the percent reduction was: cortex, 31%; cerebellum, 33%: hippocampus, 35%: striatum, 36%. A similar trend in decrease (cortex, 24%; cerebellum, 36%: hippocampus, 21%: striatum, 20%) was also evident in mitochondria, even though the degree of decrease was less robust (Fig. 1C).

Reduced glutathione (GSH) and thiol levels

In general, a moderate increase in GSH levels were observed in the brain regions of CA-fed animals (Table 3, Fig. 2A). Although the increase was marginal at the lowest dose, significant elevation was evident at the higher dose (cortex, 29%; cerebellum, 27%; hippocampus, 30%; striatum, 20%) in homogenates. In mitochondria also significant increase (22%) in GSH levels was observed at the higher dose (Table 3).

A marginal increase of total thiols as well as non-protein thiols was observed in brain regions at the higher dose of CA. While the increase in total thiols was marginal (10–15%) in all brain regions (Table 3), increase in non-protein thiols was more robust (cortex, 28%; cerebellum, 35%; hippocampus, 38%; striatum, 28%) at the higher dose (data not shown). A similar

Table 2. Status of endogenous oxidative markers in brain regions of prepubertal male mice fed *Centella asiatica* in the diet for 4 weeks

	Centella asiatica leaf powder		
	0%	0.5%	1%
MDA[1]			
Cortex	10.19±0.98^{a}	8.78±1.02^{b}	5.03±0.87^{c}
Cerebellum	8.18±0.65^{a}	7.58±0.92^{b}	5.04±0.91^{c}
Hippocampus	11.1±0.89^{a}	9.92±1.02^{ab}	7.12±0.78^{c}
Striatum	10.17±0.78^{a}	9.02±0.89^{b}	6..99±0.89^{c}
ROS[2]			
Cortex	4.84±0.15^{a}	3.49±0.22^{b}	3.04±0.16^{c}
Cerebellum	4.18±0.26^{a}	3.60±0.26^{b}	2.54±0.33^{c}
Hippocampus	4.85±0.45^{a}	4.35±0.45^{b}	3.05±0.26^{c}
Striatum	4.52±0.59^{a}	3.70±0.39^{b}	2.79±0.29^{c}
HP[3]			
Cortex	2.89±0.09^{a}	2.45±0.10^{b}	1.98±0.11^{c}
Cerebellum	2.78±0.16^{a}	2.23±0.23^{b}	1.87±0.16^{c}
Hippocampus	2.93±0.20^{a}	2.46±0.19^{b}	1.91±0.19^{c}
Striatum	2.99±0.25^{a}	2.43±0.19^{b}	1.93±0.20^{c}

The values are ±S.D of six determinations each; data analysed by one-way ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.
MDA, malondialdehyde; ROS, reactive oxygen species; HP, hydroperoxides.
[1] η moles MDA/mg protein.
[2] ρ moles DCF/min/mg protein.
[3] μ moles hydroperoxides/mg protein.

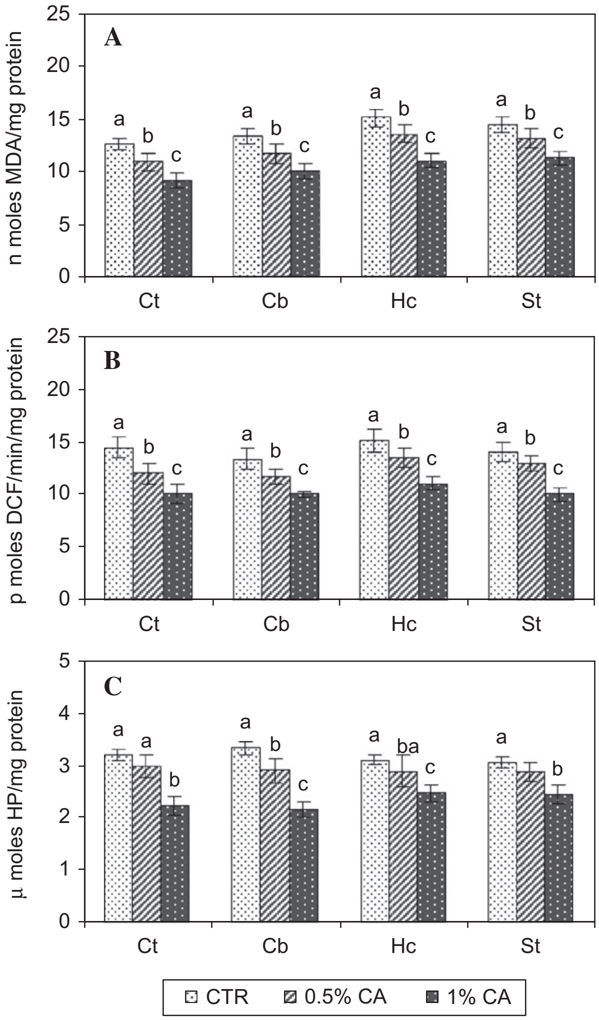

Fig. 1. Status of lipid peroxidation measured as malondialdehyde (MDA) levels (A), reactive oxygen species (ROS) generation(B) and hydroperoxide (HP) levels(C) in mitochondria of brain regions (Ct – cortex, Cb – cerebellum, Hc – hippocampus and St – striatum) of prepubertal male mice fed *Centella asiatica*-incorporated diet for 4 weeks. Values are ±S.D ($n = 6$); data analyzed by ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

trend of increase in total thiols was observed in mitochondria in all brain regions (Fig. 2B).

Protein carbonyls levels

There was a dose-related decrease in basal levels of protein carbonyls in all the brain regions of mice fed CA both in homogenates and mitochondria (Table 3 and Fig. 2C). At the higher dose, the basal levels of protein carbonyls were significantly diminished (cortex, 46%; cerebellum, 36%; hippocampus, 36%; striatum, 42%) in homogenates and a similar trend of decrease (22–28%) was observed in mitochondria in all brain regions.

Table 3. Status of reduced glutathione, total thiols and protein carbonyls in brain regions of prepubertal male mice fed *Centella asiatica* in the diet for 4 weeks

	Centella asiatica leaf powder		
	0%	0.5%	1%
GSH[1]			
Cortex	7.41 ± 0.89^{a}	8.41 ± 0.77^{b}	9.23 ± 0.45^{c}
Cerebellum	7.08 ± 0.78^{a}	8.34 ± 0.85^{b}	9.56 ± 0.95^{c}
Hippocampus	6.89 ± 0.66^{a}	7.99 ± 0.89^{b}	8.67 ± 0.89^{c}
Striatum	7.30 ± 0.29^{a}	8.03 ± 0.46^{b}	8.73 ± 0.76^{c}
Total thiols[2]			
Cortex	6.53 ± 0.51^{a}	6.69 ± 0.67^{a}	7.01 ± 0.56^{b}
Cerebellum	6.15 ± 0.45^{a}	6.36 ± 0.46^{b}	6.99 ± 0.78^{c}
Hippocampus	6.01 ± 0.32^{a}	6.21 ± 0.45^{b}	6.76 ± 0.55^{b}
Striatum	6.46 ± 0.66^{a}	6.85 ± 0.56^{b}	7.25 ± 0.45^{c}
Protein carbonyls[3]			
Cortex	7.54 ± 0.45^{a}	6.52 ± 0.44^{b}	4.72 ± 0.82^{c}
Cerebellum	5.51 ± 0.58^{a}	4.62 ± 0.98^{b}	3.45 ± 0.67^{c}
Hippocampus	6.02 ± 0.67^{a}	5.23 ± 0.78^{b}	4.01 ± 0.49^{c}
Striatum	6.28 ± 0.26^{a}	5.30 ± 0.36^{b}	3.64 ± 0.25^{c}

The values are ±S.D of six determinations each; data analysed by one way ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

[1]µg GSH/mg protein.

[2]ηmoles oxidized DTNB/mg protein.

[3]ηmoles carbonyls/mg protein.

Antioxidant enzyme activities

Varying degree of increase was evident in the activities of antioxidant enzymes such as CAT, GSH-Px and SOD in all the brain regions (Table 4). At the higher dose the activity of CAT showed significant increase (cortex, 36%; cerebellum, 36%; hippocampus, 30%; striatum, 29%), while GSH-Px activity increased significantly only at the higher dose (cortex, 37%; cerebellum, 25%; hippocampus, 24%; striatum, 37%). While SOD activity at the high dose was uniformly elevated (34%) in all brain regions, the activity of GST showed no significant changes. The activities of both GSH-PX (Fig. 3A) and SOD (Fig. 3B) were uniformly elevated (by 25%) in mitochondria in all brain regions, while the activity of GST was unaffected (data not shown).

Acetylcholinesterase (AChE) activity

In general, the AChE activity was significantly elevated in all brain regions of CA-fed mice at both doses. While the low dose caused only marginal elevation in cerebellum and hippocampus (12%), the

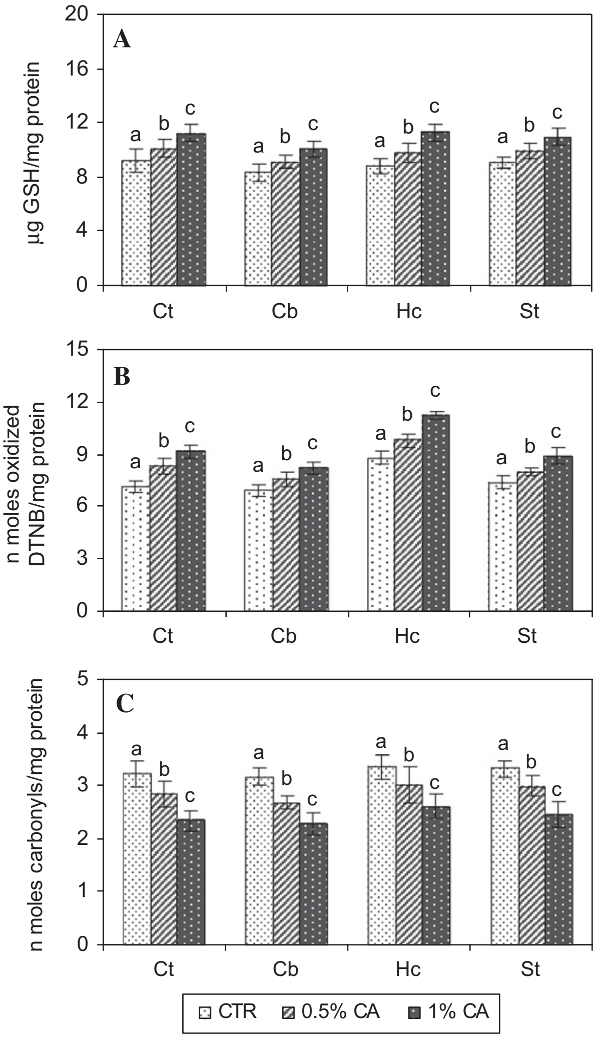

Fig. 2. Reduced glutathione (GSH) levels (A), total thiols (B) and protein carbonyls (C) in mitochondria of brain regions (Ct – cortex, Cb – cerebellum, Hc – hippocampus and St – striatum) of prepubertal mice fed *Centella asiatica*-incorporated diet for 4 weeks. Values are ±S.D ($n = 6$); data analyzed by ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Table 4. Activities of antioxidant enzymes in cytosol of brain regions of prepubertal male mice fed *Centella asiatica* in the diet for 4 weeks

	Centella asiatica		
	0%	0.5%	1%
CAT[1]			
Cortex	1.84 ± 0.11^a	2.05 ± 0.15^b	2.37 ± 0.17^c
Cerebellum	2.22 ± 0.12^a	2.47 ± 0.13^b	2.75 ± 0.18^c
Hippocampus	2.01 ± 0.11^a	2.12 ± 0.11^b	2.56 ± 0.20^c
Striatum	2.03 ± 0.13^a	2.13 ± 0.11^b	2.61 ± 0.10^c
GST[2]			
Cortex	17.06 ± 0.98^a	18.73 ± 1.22^b	20.45 ± 1.45^c
Cerebellum	19.72 ± 1.22^a	20.15 ± 1.78^b	20.45 ± 1.11^b
Hippocampus	16.12 ± 1.45^a	18.42 ± 1.03^b	20.59 ± 1.99^c
Striatum	17.82 ± 0.31^a	18.37 ± 0.31^b	18.81 ± 0.41^b
GSH-Px[3]			
Cortex	8.09 ± 1.02^a	20.22 ± 1.34^b	26.20 ± 1.22
Cerebellum	20.68 ± 1.66^a	22.68 ± 1.77^b	27.12 ± 1.45
Hippocampus	25.11 ± 1.01^a	28.12 ± 1.33^b	30.65 ± 1.45
Striatum	21.85 ± 0.91^a	23.68 ± 1.01^b	29.85 ± 0.81
SOD[4]			
Cortex	34.20 ± 1.03^a	43.76 ± 2.04^b	45.75 ± 2.22^c
Cerebellum	38.88 ± 2.01^a	45.25 ± 2.67^b	52.34 ± 3.07^c
Hippocampus	40.45 ± 2.10^a	43.44 ± 2.67^b	52.45 ± 3.01^c
Striatum	40.71 ± 1.11^a	44.10 ± 0.98^b	52.21 ± 2.11^c

The values are ±S.D of six determinations each; data analysed by one way ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione *S*-transferase; SOD, superoxide dismutase.

[1] μmoles H_2O_2 degraded/min/mg protein.

[2] ηmoles NADPH/min/mg protein.

[3] η moles conjugate/min/mg protein.

[4] Units/mg protein.

elevation was more robust in cortex (20%). However at the higher dose, a consistent increase was evident irrespective of the brain region (cortex, 40%; cerebellum, 36%; hippocampus, 38%; striatum, 22%) (data not shown).

Free radical scavenging potential of CA aqueous extract *in vitro*

Data on the total DPPH scavenging potential of the CA extract at varying concentrations measured are depicted in Fig. 4A. Significant DPPH radical scavenging activity was evident at all concentrations tested. The preparation was able to reduce the stable free radical DPPH to the yellow-colored 1, 1-diphenyl-2-picrylhydrazyl with an $IC_{50} = 12.18$ μg/ml. The synthetic antioxidant, BHT at similar conditions showed an IC_{50} of 4.15 μg/ml.

The inhibitory effect of CA extract on superoxide radicals was also significant and concentration-related. The IC_{50} concentration of CA extract for superoxide scavenging was 21.87 μg/ml. The IC_{50} value for caffeic acid at similar conditions was 4.5 μg/ml (Fig. 4B). On the other hand, the IC_{50} concentration of CA extract for hydroxyl radical scavenging was 1.22 mg/ml, while for BHT the IC 50 value was 6.85 μg/ml (Fig. 4C). Further, CA extract also exhibited significant protection against deoxyribose oxidation and the IC_{50} value was 352.9 μg/ml (Fig. 4D).

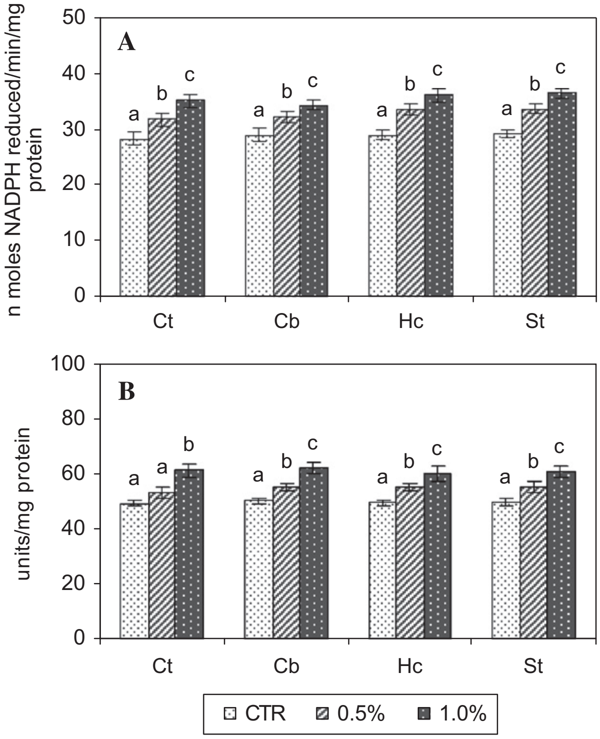

Fig. 3. Glutathione peroxidase (A) and superoxide dismutase (B) in mitochondria of brain regions (Ct – cortex, Cb – cerebellum, Hc – hippocampus and St – striatum) of mice fed *Centella asiatica*-incorporated diet for 4 weeks. Values are ±S.D ($n = 6$); data analyzed by ANOVA ($p<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Attenuation of 3-NPA-induced oxidative stress in brain region mitochondria in vitro

Oxidative response induction by 3-NPA

On exposure to 3-NPA, mitochondria of all brain regions showed a concentration-dependent increase in all the markers of oxidative damage. ROS levels were markedly elevated in all brain regions (cortex, 56–151%; cerebellum, 56–121%; hippocampus, 42–113%; striatum, 60–143%). Similarly MDA levels were also significantly increased (cortex, 10–64%; cerebellum, 25–120%; hippocampus, 17–67%; striatum, 60–124%). Concomitantly, the hydroperoxide levels were also significantly enhanced in all brain regions (cortex, 70–172%; cerebellum, 65–135%; hippocampus, 71–173%; striatum, 71–185%) (Fig. 5).

Modulation of 3-NPA-induced ROS, lipid peroxidation and hydroperoxide levels

Aqueous extract of CA (0.5 μg/ml) provided complete protection to mitochondria of all brain regions against 3-NPA-induced ROS generation (Fig. 6B). CAAE *per se* reduced the basal levels of MDA in mitochondria (cortex, 28%; cerebellum, 20%; hippocampus, 17%; striatum, 20%). Further, the extract provided marked protection (cortex, 100%; cerebellum, 80%, hippocampus, 85%; striatum, 80%) against 3-NPA-induced lipid peroxidation (Fig. 6A). A similar trend of protection was also evident in all brain regions against 3-NPA-induced hydroperoxide formation (cortex, 60%; cerebellum, 85%; hippocampus, 100%; striatum, 100%) (Fig. 6C).

Discussion

The extensive use of CA in ayurvedic system of medicine necessitates a basic understanding of its possible mechanism of action (Dash et al., 1996). *In vitro* evidences (Hamid et al., 2002) suggest that the CA extract has numerous antioxidant compounds which may be responsible for some of the neuropharamcological effects observed with CA powder and extracts *in vivo* (Veechai et al., 1984; Veerendra Kumar and Gupta, 2002, 2003; Rao et al., 2005; Wijeweera et al., 2006). However, evidences demonstrating the direct involvement of antioxidative action of CA *in vivo* are limited (Gnanapragasam et al., 2004). More importantly, data on its potency to modulate endogenous markers of oxidative stress in various brain regions are lacking. Hence, we determined the implications of feeding CA powder in the diet for 4 weeks to prepubertal mice. The criterion for selection of prepubertal mice was based on the following: (i) the brain in a 4-week-old mice is still in the process of development of new inter neuronal connections and will continue during the postnatal development till the adult architecture is established by about 6 weeks (Rao et al., 2005), (ii) the prepubertal brain may be more responsive, (iii) the enhancement of memory and increased antioxidant levels are necessary in the growing stage. Hence, in the present study we examined the efficacy of CA in diminishing the basal oxidative stress markers and antioxidant enzyme levels in different regions of pubertal mice. Further, the efficacy of aqueous extract of CA to abrogate 3-NPA-induced oxidative dysfunctions in brain mitochondria *in vitro* was also examined.

Results of our *in vivo* study clearly demonstrated that CA-fed mice exhibited a substantial reduction in the basal levels of ROS, MDA and hydroperoxides both in cytosol and mitochondria of all brain regions. ROS have been implicated to be the most likely candidate responsible for producing the neuronal changes in neurodegenerative disorders (Cantuti et al., 2000), causing the progressive modification or degradation of cellular biochemicals including DNA, protein, lipids and carbohydrates when produced at elevated non-physiological concentrations (Halliwell, 2006). Although H_2O_2

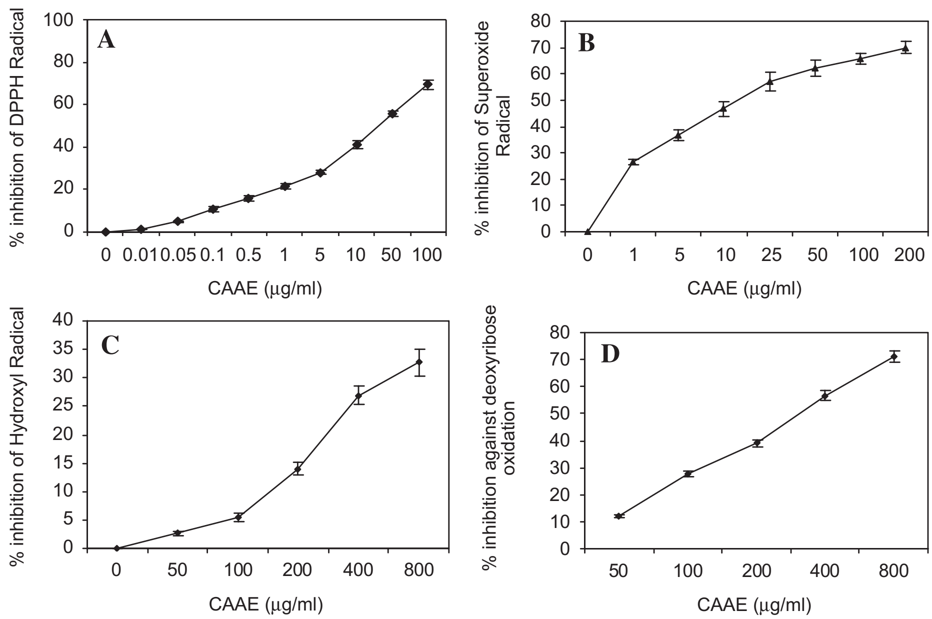

Fig. 4. Free radical scavenging activity of *Centella asiatica* aqueous extract (CAAE) as measured by DPPH radical scavenging assay (A), superoxide radical scavenging assay (B), hydroxyl radical scavenging assay (C) and inhibition of deoxyribose oxidation (D). Values are represented as means ± S.D of four determinations.

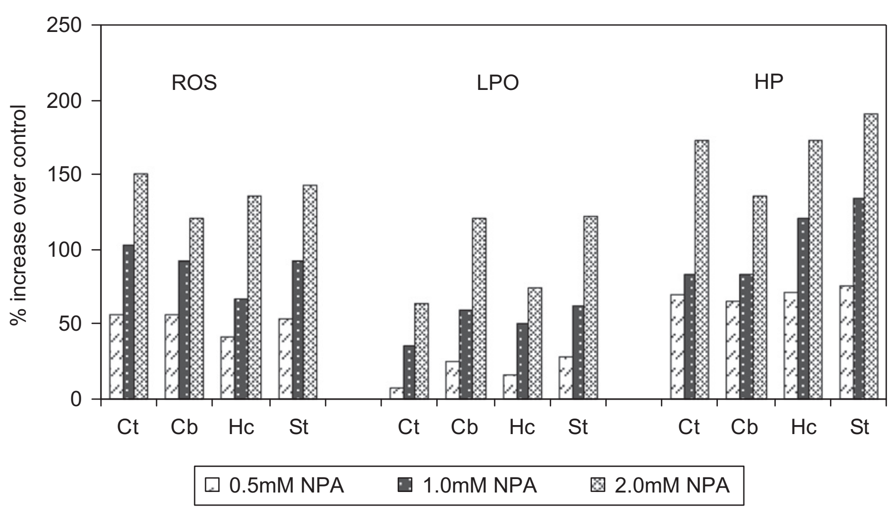

Fig. 5. 3-NPA-induced elevations in malondialdehyde (MDA), reactive oxygen species (ROS) and hydroperoxide (HP) levels in mitochondria of brain regions (Ct – cortex, Cb – cerebellum, Hc – hippocampus and St – striatum) of prepubertal mice *in vitro*. Values are ±S.D ($n = 6$); data analyzed by ANOVA ($p < 0.05$) appropriate to completely randomized design with replicates.

may freely diffuse out of mitochondria (Cadenas and Davies, 2000), it is having a relatively long half life and a unique property of being soluble both in lipid and aqueous media (Droge, 2002). Moreover, cytochrome C present in the mitochondrial inner membrane is an iron-containing protein and mitochondrial oxidative damage can result in its release into the cytoplasmic compartment (Kroemer et al., 1998), promoting secondary ROS generation from non-specific iron catalyzed reaction (Hazel, 2006). CA may provide protection against this secondary reaction by chelating iron released from the inner mitochondrial membrane and thus prevent the ROS generation. This is consistent with recent reports which have demonstrated CA to chelate metals such as arsenic *in vivo* (Gupta and Flora, 2005) and iron *in vitro* (Shinomol and Muralidhara, unpublished data).

Further, in the present study, CA significantly reduced the basal levels of MDA in all brain regions of mice, suggesting its protective ability against lipid peroxidation events. Peroxidation of lipids represents a

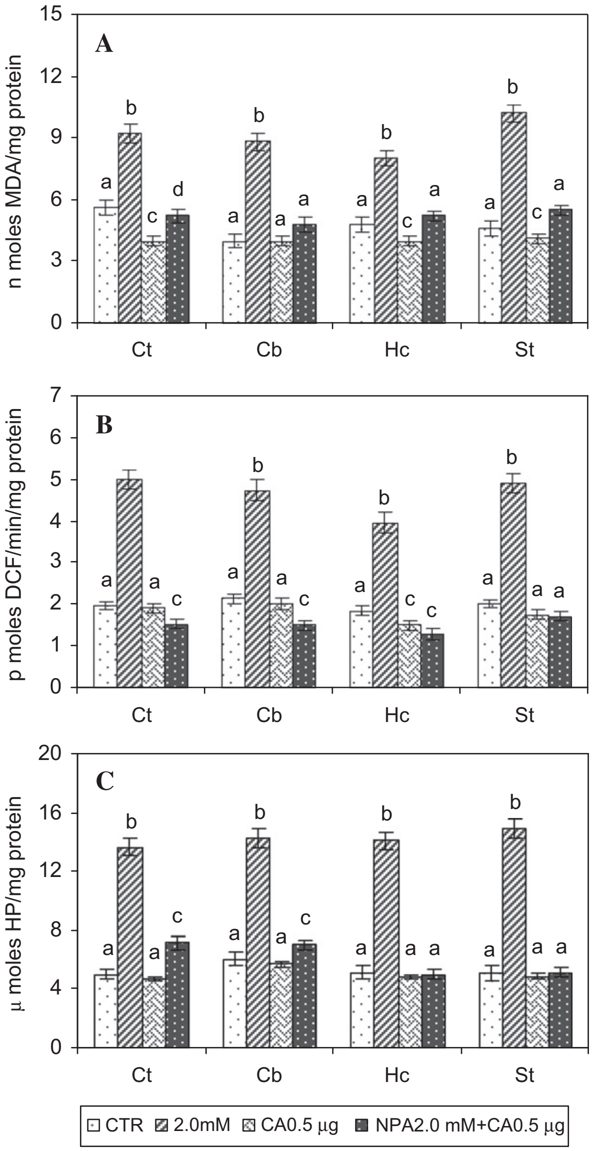

Fig. 6. Modulation of 3-NPA-induced MDA (A), ROS (B) and HP (C) by CA aqueous extract in mitochondria of brain regions (Ct – cortex, Cb – cerebellum, Hc – hippocampus and St – striatum) of prepubertal mice *in vitro* . Values are ±S.D ($n = 6$); data analyzed by ANOVA ($p < 0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

primary consequence of cellular oxidative stress (Halliwell, 2006) and the oxidation of membrane phospholipids in the plasma membrane, as well as within internal organelle membranes such as the mitochondria, leading to biophysical changes that disrupt membrane and organelle function which may promote cell death. In addition, lipid peroxidation may lead to the production of additional reactive species like 4-HNE (Awasthi et al., 2004). Our findings are consistent with earlier reports of reduced LPO in erythrocytes of CA-fed adult rats (Hussin et al., 2007), reduction in MDA in whole brain of adult rats (Veerendra Kumar and Gupta, 2002). However, our results show for the first time that CA has a uniform modulatory effect on oxidative markers in various brain regions of prepubertal mice.

In the present model, a significant increase in both GSH and thiols (total and non protein thiols) was discernible in all brain regions of mice fed CA both in cytosol and mitochondria, clearly suggesting that the active components of CA possess the propensity to enhance the thiol-containing antioxidant molecules. The thiol compounds have critical importance in the 3 tier antioxidant defense of our body (Russel, 1998), and increasing the thiol-containing antioxidants in brain provides protection against a wide range of oxidative and toxic insults to the prepubertal brain. Moreover, these elevations may explain at least in part for the reduction in endogenous levels of ROS, MDA, hydroperoxides and protein carbonyls in brain regions of CA-fed mice. Further, CA also caused a significant increase in the activities of antioxidant enzymes such as CAT, GPx and SOD. This finding is consistent with the previous reports of elevated activities of SOD and catalase in whole brain homogenate of adult rats fed CA (Veerendra Kumar and Gupta, 2002). These results allow us to speculate that there is not only suppression of certain genes but also activation of genes, which results in the increased antioxidant status of the animal.

Among the various oxidative modifications of amino acids in proteins, protein carbonyl formation may be an early marker for protein oxidation (Levine et al., 1990). Further, it also reflects a very low rate of oxidized protein degradation and or low repair activity since oxidized forms of some proteins and proteins modified by lipid peroxidation products are not only resistant to proteolysis, but also can inhibit the ability of proteases to degrade the oxidized forms of other proteins and form aggregates (Dalle-Donne et al., 2003). Following CA consumption, protection against protein oxidative damage was also evident from the reduction in the endogenous protein carbonyl levels in all brain regions of growing mice. Since CA-fed mice exhibited relatively lower basal ROS levels, we speculate that the lowered protein carbonyl level may also be due to the radical scavenging property of CA. Alternatively it may also be due to an over expression of enzymes like carbonyl reductase apart from the direct free radical scavenging effect.

In the present model, terminally, significant enhancement in AChE activity in different brain regions of CA-fed animals was evident, suggesting altered cholinergic function. Earlier workers have shown that oral administration of *Clitoria terneata* causes increased AChE activity and elevated acetylcholine content in rat brain (Taranalli and Cheeramkuzhy, 2000). Interestingly, up regulation of AChE activity is reported to be sufficient to reverse memory deficits (Parent and Baxer, 2004).

Our finding of increased AChE levels might reflect the enhancement of acetylcholine release which would facilitate in synaptic transmission of CA3 pyramidal neurons which are still branching during the growth spurt period (Rao et al., 2005). This thinking is consistent with a recent study in rats employing intracerebroventricular streptozotocin model of Alzheimer's disease in which CA was demonstrated to significantly mitigate oxidative stress and enhance cognitive behavior (Veerendra Kumar and Gupta, 2003). The enhancement of cognitive functions by CA may be attributed to its ability to elevate brain AChE activity.

Further, we determined the free radical scavenging activity of CA extract in four well-established in vitro test systems. The antioxidant activity of extract was discernible in the DPPH radical assay, which primarily evaluates proton radical-scavenging ability. DPPH is one of the compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). Further, it is well established that the DPPH-free radical scavenging by antioxidants is due to their hydrogen-donating ability, and the concentration-dependent scavenging of DPPH radical by CA extract is attributable to its hydrogen-donating ability. Interestingly, CA extract also exhibited significant activity as reflected in the scavenging of both superoxides and hydroxyl radicals, clearly suggesting its multiple antioxidant potential.

In a separate study, we investigated the efficacy of CA extract to abrogate 3-NPA-induced oxidative stress in mitochondria isolated from various brain regions of prepubertal mice. Mitochondria exposed to 3-NPA showed significant concentration-dependent elevation in all the markers viz., ROS, MDA and hydroperoxide levels, suggesting induction of oxidative stress. These data are consistent with earlier findings of 3-NPA-induced oxidative stress *in vivo* (Fu et al., 1995). In the presence of aqueous extract of CA, 3-NPA-induced generation of ROS and hydroperoxides was brought to normalcy in mitochondrial fraction, clearly suggesting its propensity in modulating neuronal dysfunctions even at the subcellular level. CA extract also provided protection against 3-NPA-induced lipid peroxidation *in vitro* in mitochondria in all brain regions. We have also obtained evidences which show that pretreatment of prepubertal mice with aqueous extract of CA dramatically protects against 3-NPA-induced oxidative stress response *in vivo* (Shinomol and Muralidhara, unpublished).

In conclusion, dietary CA markedly diminished the levels of endogenous oxidative markers, increased the antioxidant molecules and enzymes *in vivo* in both homogenates/mitochondria and markedly mitigates 3-NPA-induced mitochondrial oxidative stress response *in vitro*. These results necessitate further understanding of the biochemical mechanisms underlying the neuroprotective effects of CA against select neurotoxicants *in vivo* in order to optimize its usage in humans. Our results provide direct evidence to show that antioxidant properties may in part be responsible for the modulatory effects of *Centella asiatica* leaf powder *in vivo* and can be better exploited to protect against neuronal dysfunctions among children.

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