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EFFICACY OF CENTELLAASIATICA LEAF POWDER -ENRICHED DIET TO AMELIORATE KHESARI DHAL -INDUCED OXIDATIVE IMPAIRMENTS IN BRAIN REGIONS OF YOUNG MICE.

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Abbreviations: 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, CA-*Centellaasiatica*, KD-Khesari dhal

ABSTRACT

Previously we demonstrated the prophylactic neuromodulatory effects of *Centellaasiatica* (CA), an extensivelyused medicinal plant in Ayurvedafor various ailments. The primary objective of this investigation was to examine its efficacy to ameliorate Khesari dhal (KD) -induced oxidative impairments in various brain regions of young mice. Neurolathyrism is a neurodegenerative disease caused by consumption of KD, containing a neurotoxin β-ODAP which causes inhibition of mitochondrial complex I and induces oxidative stress (OS)in brain. Mice were assigned to four groups (n=6). Group I: normal diet, group II: CA (1%) incorporated diet, group III:Khesari dhal (30%) incorporated diet and group IV:KD (30%) incorporated diet supplemented with CA (1%) for 30 days. Significant protection was observed against the KD –induced increase in ROS,hydroperoxide formation, MDA and protein carbonyls levels in cytosol of various brain regions of mice fed CA-enriched diet.Further, feeding KD in diet resulted in significantly enhanced antioxidant enzyme activities (GPx, GST and SOD) with concomitant decrease in GSH and TSH levelsincytosol of brain regions.Such alterations in activities were restored to normalcy among mice provided with CA-enriched diet.Taken together, this dietary study, clearly demonstrates the protective propensity of CA leaf powder against KD induced brain OSand related alterations in young adult mice and suggests its utility value as an adjuvant under OS mediated neuronal dysfunctions.

Key words: Centellaasiatica, Khesari dhal, oxidative stress, brain, neuroprotection

INTRODUCTION

Centellaasiatica (L) Urban (Umbelliferae) a plant native to countries such as India, Srilanka, Madagascar, South Africa and Malasia (1) is used in the Ayurvedic system of medicine to treat various ailments like headache, body ache, insanity, asthma, leprosy, ulcers, eczemas and wound healing (2-3). Recent studies have shown various neuropharmacological effects with *Centellaasiatica* (CA) and the major effects described in experimental animals comprise of memory enhancement and improvement in cognitive function (4-5), increased neurite elongation *in vitro* andacceleration of nerve regeneration *in vivo* (6). Besides,improving learning and memory (7), oral administration of an aqueous extract of CA was demonstrated to diminish malondialdehyde levels and enhance glutathione levels in the brain of adult rats (4). Further, CAis also shown to possess immunostimulatory activity *in vitro* (8) as well as adjuvant properties to antiepileptic drugs along with an added advantage of prevention of cognitive impairment.

Notable bioactive compounds of CA are the triterpenesaponins, madecassocide and asiaticoside with their respective ursane type sapogenins*viz.,*madecassic and asiatic acid (9). CA

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contains numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempferol, catechin, rutin, and naringin which are well known antioxidants (10-11). Treatment with CA extract during post natal period influenced neuronal morphology and promotes higher brain functions of juvenile/ young adult mice resulting in enhanced learning and memory (12). Previously,whole plant was demonstrated to improve general mental ability of mentally retarded children (13).

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 (14). 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. It has been postulated that the mechanistic basis of the neuroprotective activity of antioxidants does not rely 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 (15).

Consumption of the Khesari dhal (KD) for prolonged periods leads to a neurodegenerative disorder known as Neurolathyrism(NL) in humans (16) which manifests as damage of upper motor neurons, degeneration of anterior horn cells and loss of axons in pyramidal tracts of lumbar spinal cord (17). KD is shown to contain the neurotoxin β-ODAP which exerts neurotoxicity through the AMPA subclass of glutamate receptors (18) and causes inhibition of mitochondrial complex I (NADH-ubiquinone oxidoreductase) in motor cortex and lumbosacral cord (19) of mice. It has been postulated that oxidative stress plays a key role in the development of NL (20).

Earlier, our findings suggest thatCAleaf powder *per se*possess the ability to significantly reduce the basal levels of various oxidative markers in cytosol as well as mitochondria of brain regions of normal prepubertal mice (21).Further, we also obtained evidence showing the prophylactic propensity of CAaqueous extract to abrogate 3-nitropropionic acid -induced oxidative dysfunctions in various brain regions of mice (21-22). In the present study, we surmised that CA-enriched diet possess the propensity to modulate Khesari dhal -induced oxidative impairments in brain regions of young adult mice. The hypothesis was tested employing a short –term dietary paradigm.

MATERIALS AND METHODS

2.1 Preparation of Centellaasiatica(CA) supplemented diet

Centellaasiatica leaves along with the petiole were shade dried and powdered in a mill and sieved through a mesh (400 micron). Commercially available pellet diet for mice was powdered in a mill to coarse powder and CA leaf powder was mixed at two dietary levels (0.5 and 1.0 %) (21).

2.2 Preparation of KD –incorporated diet

Khesari Dhal was powdered and mixed with the commercial diet at 30% level(20).

2.3 Experimental protocol

Adult male mice (CFT-Swiss strain, 8 wks old) were randomly assigned to four groups (n=6). The treatment groups were as follows: Group I mice fed with normal diet served as the control group; Group II mice provided with CA (1%) enriched diet served as CA control; Group III mice were provided with Khesari dhal (30%) -incorporated dietand Group IV mice were fed KD (30%) incorporated diet supplemented with CA (1%). Dailyfood intake and weekly body weight gain were monitored throughout the experimental period of 30 days. Terminally, mice of all groups were sacrificed and biochemical determinations werecarried out in cytosol of selected brain regions.

Preparation of Cytosol Fractions

Cytosol was prepared by the removal of nuclear and mitochondrial content by differential centrifugation following published protocols with minor modifications(23-24). Briefly, 10% homogenates of the brain regions were prepared in ice-cold Tris-sucrose buffer (Tris-2mM; Sucrose-0.25 M, pH 7.4) using a glass–teflon grinder at 4^0 C. The homogenates were centrifuged at 1000xg for 10 min at 4^0 C to obtain the nuclear pellet. This pellet was discarded and the post-nuclear supernatant was centrifuged at 10,000xg for 20 min at 4^0 C. The pellet obtained which contains mainly mitochondria was removed and the supernatant was taken as crude cytosol and stored at -80^0 C until use.

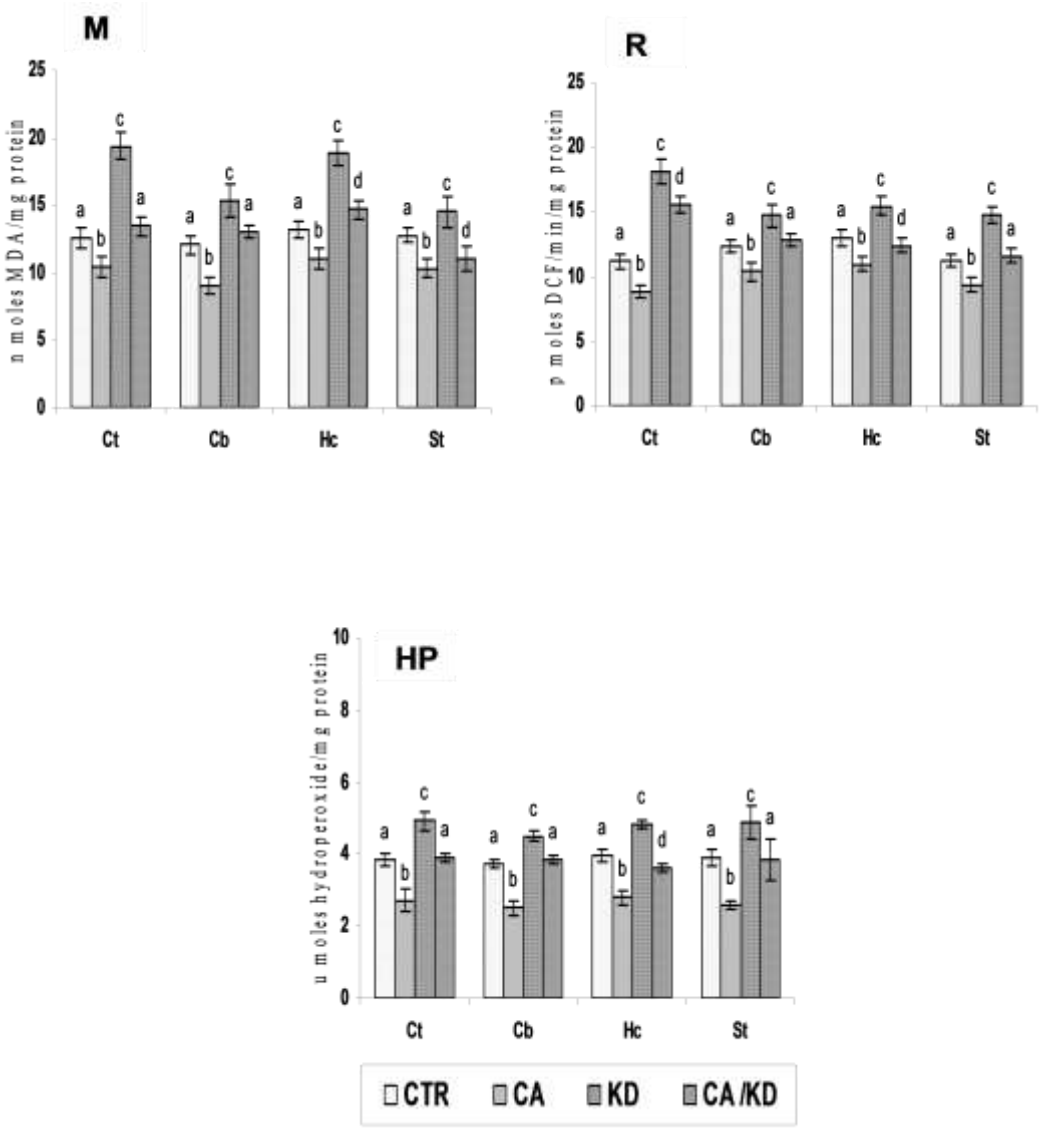

Fig: I Ameliorative effect of CA supplementationon KD-induced oxidative alterations in brain region cytosol of male mice fed Khesari dhal and CA incorporated diet for 30 days;
Values are mean± S.D of six determinations each; Data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.
CA-*Centellaasiatica* leaf powder (1%); KD- Khesari dhal (30%); MDA-Malondialdehyde ROS-Reactive oxygen species; HP-Hydroperoxides.

2.4 Assay methods

2.4.1 Lipid peroxidation (LPO)

Induction of oxidative damage was ascertained by measuring the extent of LPO in brain (cortex, cerebellum, hippocampus and striatum) cytosolic fractions. The extent of LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Briefly, the reaction mixture contained 0.2 ml of brain regions cytosol (1mg protein), 1.5 ml of acetic acid (pH 3.5, 20%), 1.5 ml of 0.8 % thiobarbituricacid (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 absorbance was measured in a UV-Visible spectrophotometer at 532 nm and expressed as malondialdehyde (MDA) equivalents using 1, 1, 3, 3- tetramethoxy-propane as the standard (25).

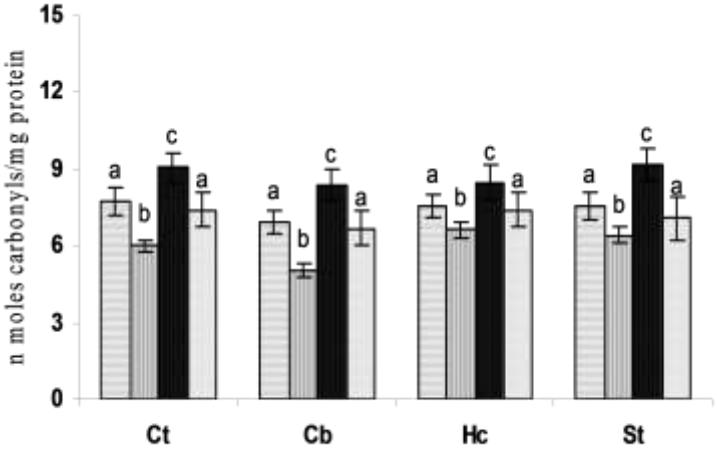

Fig II

Ameliorative effect of CA supplementation on KD-induced protein carbonyls in brain region cytosol of male mice;

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

CA *Centellaasiatica* leaf powder (1%); KD- Khesari dhal (30%); MDA-Malondialdehyde ROS-Reactive oxygen species; HP-Hydroperoxides

2.4.2 Reactive oxygen species generation

ROS generation in brain regions was assayed using dihydrodichlorofluoresceindiacetate (H_2 DCFH-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 (26,20). Briefly, the cytosol was diluted 1:20 times with ice-cold Locke's buffer to obtain a concentration of 5 mg /ml. The reaction mixture (1ml) containing Locke's buffer (pH 7.4), 0.2 ml cytosol (0.5mg protein) and 10 μl of DCFH-DA (5 μM) was incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After 30 min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured in a spectrofluorimeter with excitation wave length of 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-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.

2.4.3 Measurement of hydroperoxide levels

Water soluble hydroperoxides were measured according to the original method using FOX 1 recipe. An aliquot of brain regions cytosol (100 μg protein) was added to 1ml FOX reagent (100μM xylenol orange; 250μM ammonium ferrous sulphate; 100 μM sorbitol; 25mM H_2SO_4/L) and incubated for 30

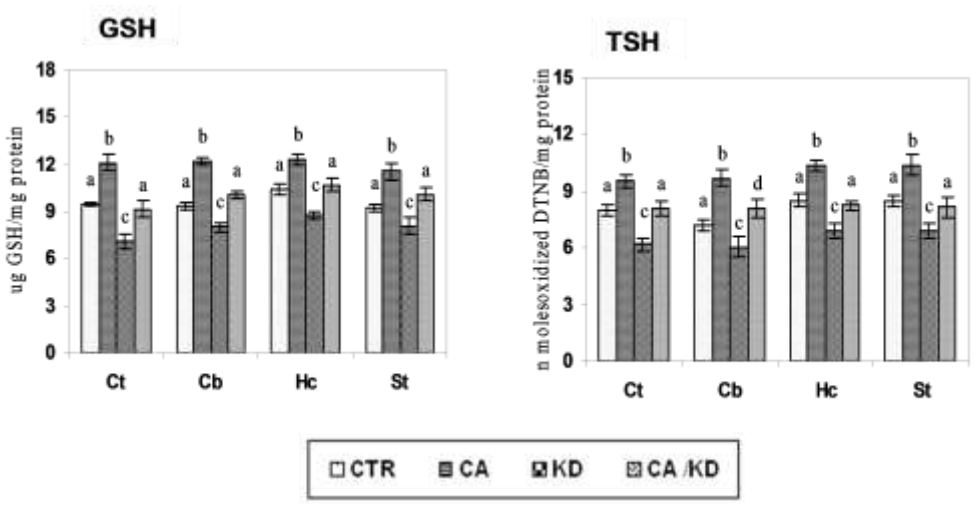

Fig. III

Mitigation of KD induced changes in reduced Glutathione levels and total thiols in cytosol of brain regions of male mice given CA supplementation; Values are mean± S.D of six determinations each; data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates.

Means followed by different letters differ significantly according to DMRT.CA-*Centellaasiatica leaf powder* (1%); KD- Khesari dhal (30%); GSH-Reduced glutathione TSH-Total thiols

min at room temperature. The mixture was centrifuged at 600xg and the supernatant was read at 560nm in a spectrophotometer. The concentration of hydroperoxides were calculated using the MEC of 1.5 X 10^{-4}mM and expressed as μmoles hydroperoxide /mg protein (27).

2.4.4 Determination of reduced glutathione (GSH)

GSH was measured according by a fluorimetric method. Briefly, 100 μl of 5mg/ml cytosol fraction (prepared in phosphate buffer pH 7.4) was added to 2 ml formic acid (0.1M) and centrifuged at 10,000xg for 20 minutes. The supernatant (100 μl) was mixed with 0.1ml buffered formaldehyde (1:4 (v/v), 37% formalin: 0.1M Na_2HPO_4).After 5 min, 1.0 ml of sodium phosphate buffer (0.1M, EDTA 5mM, pH 8.0) was added to each tube followed by 0.10 ml of *o*-phthalaldehyde. After 45 minutes at ambient temperature, the fluorescence was measured in a spectrofluorimeter with excitation wavelength at 345nm and emission at 425 nm. Concentration of GSH was calculated from the standard curve and the values were expressed as μg GSH /mg protein (28).

2.4.5 Total and non proteinthiols (TSH)

Estimation of total thiols was done according to the method of Ellman (29).To estimate total thiols, 125 μl (125μg Protein) of the cytosol was added to 375 μl of tris buffer (0.2M, pH.8.2), 25 μlof DTNB (10mM in absolute methanol) and 1.975ml of methanol and allowed to stand for 30 min at room temperature with occasional shaking. Following centrifugation at 3000xg for 15 min, the supernatant was read at 412 nm against distilled water blank and calculated using MEC 13.6 $mM^{-1}cm^{-1}$ and expressed as η mol oxidized DTNB formed/mg protein.

2.4.6 Determination of Protein carbonyls

Protein carbonyl content was determined according to the method of Levine *et al.,*(30). Briefly a 50ul of 5mg/ml cytosol in 20mM Tris-HCl- 0.14 M NaCl (pH 7.4) was made and centrifuged at 10,000X g for 10 min. at 4^0C.To 100 μl of the supernatant, 100 μl of 20% TCA was added and centrifuged at 10,000xg for 10 minutes at 4^0C .The supernatant was discarded and the pellet was re-suspended in 1ml of DNPH (10mM in 2N HCl) and kept at dark for 1 hour with occasional mixing. 500 μl of 20% TCA was added to precipitate protein and the pellet was washed in 1ml acetone and dissolved in 1ml of 2% SDS prepared in 20mM trisHCl. The absorbance was read at 360nm and the results were expressed as ηmoles carbonyls/mg protein using MEC-22.0$mM^{-1}cm^{-1}$.

2.4.7 Activity of antioxidant enzymes.

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 10,000x g. Catalase activity was assayed by the method of Aebi*,* (31).The enzyme activity was expressed as μmol H_2O_2 consumed/min/mg protein (MEC = 43.6 mM^{-1} cm^{-1}). Briefly, the 1ml of the reaction mixture containing 50 μl sample, 900 μl phosphate buffer (0.1M, pH 7.0) and 50 μL of H_2O_2 (8.8mM).The decrease in absorbance (at 240nm) wasfollowed for 5 min at room temperature using a UV-Visible spectrophotometer.The activity of glutathione peroxidase (GPx) was determined using t-butyl hydroperoxide as the substrate according to the method of Flohe and Gunzler, (32) 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 (GST) 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., (33) and the enzyme activity was expressed as η moles of S- 2, 4, dinitrophenyl glutathione formed /min/mg protein (M EC- 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 (34).

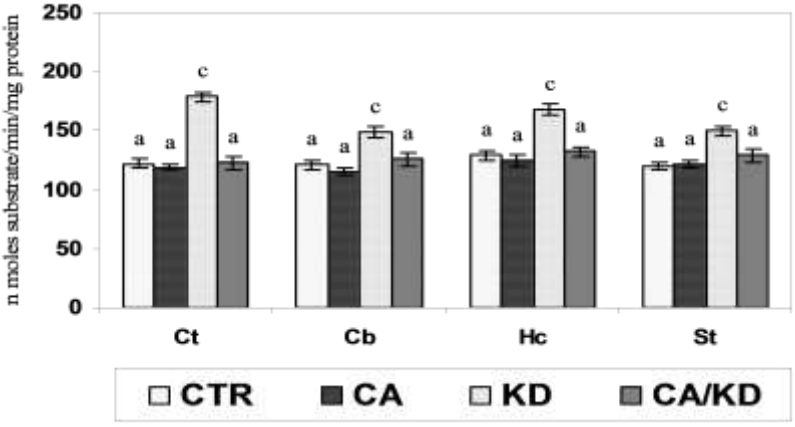

Fig.IV

Effect of CA supplementation on KD- induced alterations in Lactate dehydrogenase activity in the cytosol of brain regions of mice; .Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT. CA *Centellaasiatica* leaf powder (1%); KD- Khesari dhal (30%).MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride. LDH-Lactate dehydrogenase;

2.4.8Lactate dehydrogenase (LDH)

Lactate dehydrogenase was measured as per the method of Kornberg, (35). Tissue was homogenized in 10%w/v, Tris-HCl buffer (82.4 mM,pH 7.2 containing 210mM NaCl) and centrifuged at 10,000xg for 10 minutes at 4^0C. To 10ul of the supernatant 40 ul distilled water was added and 0.8 ml NADH (0.25mM, in Tris –HCl, pH 7.2)and 0.15 ml sodium pyruvate (10.66mM) was added. The absorbance was followed for 5 minutes at 340nm.Results are expressed as n mol NADH oxidized/min/mg protein.

2.4.9Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Ellmann*et al*., (36). To the reaction mixture containing 2.85 ml phosphate buffer (0.1 M, pH8.0), 50 μl of DTNB (10 mM), 50 μl sample and 20 μl acetylthiocholine iodide (150mM) were added

and the change in absorbance was monitored at 412 nm for 5 min in a spectrophotometer. The enzyme activity was expressed as ηmoles of substrate hydrolyzed /min/mg protein

2.5 Protein estimation

Protein concentrations incytosol wasdetermined according to the method of Lowry *et al* (37), using bovine serum albumin as the standard.

2.6 Statistical analysis

Experimental data obtained were expressed as mean ± standard deviation (SD) and analysed by one way analysis of variance (ANOVA) using SPSS software for windows (version 10.0.1., SPSS Inc, New york, 1999). The *in vitro* data in all chapters were analyzed only by one way ANOVA. Post hoc multiple comparisons were performed between groups using Duncan's Multiple Range Test (DMRT) in all the studies. P value less than 0.05 was taken as significant through out the studies.

RESULTS

3.1 Food intake, growth and organ weights

No significant alteration was evident in food intake among thevarious treatment groups throughout the experiment period (Data not shown). However, a marginal decrease in body weight, reduction in liver (23%) and kidney weights (29%) was observed among mice fed KD. However, no significant change in organ weights was evident among mice fed CA *per se*(Data not shown).

3.2 Amelioration of oxidative stress markers in brain regions

In general, CA *per se*reduced the basal levels of oxidative stress markers in cytosol (Fig.1) of brain regions. Interestingly, the levels of MDA, ROS and HP in brain region cytosol were elevated among KD fed mice. There was a significant (13-53%) increase in MDA levels in cytosoland CA supplementation completely restored the levels in all brain regions of KD fed mice. While theROS levels were differentially enhanced inbrain regions of KD fed mice (Ct-63%; Cb-19%; Hc-19%; St-32%),varying degree of protection ensuedwith CA supplementation.Likewise, elevated HP levels in brain region of KD fed mice werealsonormalizedamong mice provided with CA supplements.

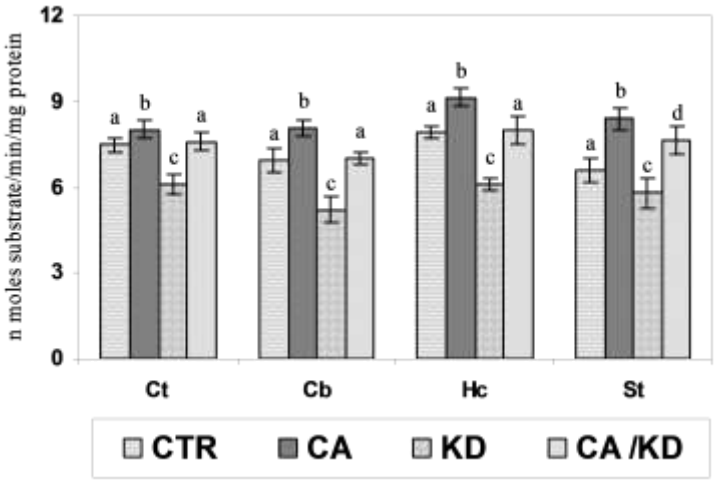

Fig.V

Effect of CA supplementation on KD- induced alterations in Acetylcholinesterase activity in brain regions of mice; Values are mean± S.D of six determinations each; data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT. CA-*Centellaasiatica* leaf powder (1%); KD- Khesari dhal (30%)

3.3 Protein carbonyl levels

The levels of protein carbonyl (PC) in cytosolic fractions(12-28%) were significantly reduced in the brain region of mice provided with CA*per se*. Interestingly, among KD fed mice, thelevels were enhanced levels (13-21%), while CA supplementation

resulted in restoration of KD-induced oxidative damage to proteins(Fig.2).

of mice fed CA *per se*. However, among KD fed mice there was a decrease in GSH (Ct-25%; Cb-14%; Hc-15%; St-12%) and TSH (Ct-23%; Cb-16%; Hc-20%; St-

Table 1 : Effect of CA supplementation on KD- induced alterations in antioxidant enzymes in cytosol of brain regions of young mice.

Groups	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
CAT(μ moles H_2O_2degraded/min/mg protein)		2.23±0.09 [a]	2.30±0.05 [a]	2.11±0.08 [a]
CTR2.19±0.10[a]		3.02±0.12 [b]	3.12±0.08 [b]	3.05±0.11 [b]
CA2.70±0.11[b]				
KD	1.28±0.08 [c]	1.88±0.07 [c]	1.79±0.06 [c]	1.99±0.07 [c]
CA /KD	2.30±0.11 [a]	2.19±0.08 [a]	2.35±0.10 [a]	2.24±0.08 [a]
GST(η moles conjugate/min/mg protein)		26.21±1.02 [a]	26.45±1.11 [a]	25.12±1.59 [a]
CTR26.12±1.56 [a]				
CA	26.02±0.98 [a]	25.23±0.83 [a]	25.67±0.78 [a]	24.92±0.58 [a]
KD	31.02±0.33 [b]	30.01±0.45 [b]	29.90 ±0.78 [b]	30.32±0.35 [b]
CA /KD	26.72±0.67 [a]	27.67±0.54 [a]	26.78±0.98 [a]	25.16±0.60 [a]
GPx (η moles NADPH/min/mg protein)		22.18 ±0.60 [a]	20.19±0.52 [a]	20.20±0.49 [a]
CTR21.22±0.72 [a]				
CA	25.14±0.83 [b]	25.56 ±0.70 [b]	25.12±0.81 [b]	25.24±0.70 [b]
KD	26.77±0.94 [b]	28.45 ±0.74 [c]	27.12±0.80 [c]	27.77±1.02 [c]
CA /KD	21.91±0.78 [a]	21.56 ±0.90 [a]	21.99±0.75 [a]	20.21±0.76 [a]
SOD				
Units/mg protein CTR	35.67±1.23 [a]	36.77±0.89 [a]	35.30±0.90 [a]	35.67±0.97 [a]
CA	42.56±1.40 [b]	42.16±1.01 [b]	40.21±1.02 [b]	41.21±0.98 [b]
KD	48.90±1.22 [c]	48.81±1.03 [c]	39.65±1.02 [c]	38.23±0.90 [c]
CA /KD	38.99±0.95 [c]	39.77±1.07 [c]	35.70±1.10 [a]	35.78±1.12 [a]

3.4 Effect on Antioxidant enzymes

Significant enhancement was observed in the activity levels of antioxidant enzyme among CA fed mice in the brain cytosol. In the KD group, there was significant decrease in CAT activity (Ct-42%; Cb-16%; Hc-22%) and marginal increase in activity of GST (Ct-19%; Cb-14%; Hc-13%; St-21%). Likewise significant increase was also evident in the activity of GPx (Ct-26%; Cb-28%; Hc-34%; St-37%) and SOD (Ct-37%; Cb-33%; Hc-12%) in KD fed mice. Interestingly,theseperturbations in antioxidant enzyme activities were normalized among CA supplemented KD group (Table 1).

3.5 Modulatory effect on glutathione and thiol status

Enhanced levels of GSH (19-30%) and total thiol (20-34%) were evident in the cytosol of brain regions

19%) levels in cytosol. Both these perturbations were normalized among micesupplemented with CA along with KD (Fig.3). Likewise, GSSG levels were elevated (Ct-32%; Cb-19%; Hc-26%; St-13%) and a decrease in npSH (16- 21%) levels were also observed in the KD fed group(Data not shown). In CA supplemented KD group, all these alterations were attenuated and the levels were comparable to that of controls.

3.6Amelioration of LDH activity

There was no significant alteration in LDH activity in cytosol of brain region of CA fed mice. Interestingly, in KD fed animals significant increase (Ct-46%; Cb-23%; Hc-30%; St-24%) in LDH activity was observed in all brain regions while in CA supplemented KD group no significant changes wasobserved (Fig.4).

3.7 Acetylcholinesterase activity

The activity levels of AChEwere consistently elevated (14-27%) in all brain regions of mice fed CA. However, KD caused significant reduction in the activity of AChEin all brain regions (Ct-18%; Cb-25%; Hc-23%; St-12%), while CA supplementation resulted in total restoration of activity to normal levels (Fig.5).

DISCUSSION

Our earlier findings clearly demonstrated the efficacy of CA leaf powder supplementation to prepubertal mice in elevating the antioxidant status in both cytosol and mitochondria of various brain regions (21). We have earlier demonstrated the efficacy of CA leaf aqueous extract to ameliorate 3-NPA induced oxidative stress and mitochondrial dysfunctions in cytosol /mitochondria of brain regions in prepubertal mice *in vivo* and *in vitro* (21-22).In this study, we examined the hypothesis that CA leaf powder is likely to ameliorate Khesari dhal (KD) induced oxidative damage in brain regions of male mice.Previously, CA supplementation was shown tooffersignificant protective value as it exhibited potent antioxidant activity against arsenic sensitive biochemical variables in blood, and moderate ability to chelate arsenic (38). Further experimental evidence suggests its ability to improve cognition and memory (39).

In the present study, dietary KD induced significant elevations in oxidative stress markers incytosol of brain regions as evidenced by increased LPO and ROS, while CA supplemented mice showed normal levels of oxidative markers suggesting the protective efficacy of CA. Earlier studies have shown that β-ODAP- induced neurotoxicity, was prevented in a dose-dependent manner by focal co-injection of potential free radical scavengers; dimethyl sulphoxide (1750–7000 ηmol), dimethylthiourea (8000 ηmol), dimethylformamide (7000 ηmol) and mannitol (1000 ηmol). These findings suggest that hippocampal damage induced by β-ODAP- involves an interaction between AMPA receptors and free radicals (40). However, there exist no reports on the use of phytochemicals in modulating KD–induced oxidative stress.We observedsignificant increase in the levels of hydroperoxideswhich is indicative of the potency of KD to induce hydrogen peroxide formation and this may partially account for the oxidative damages observed. Interestingly, we evidenced complete normalization of hydroperoxide levels among CA supplemented mice which is indicative of the ability of CA to scavenge hydrogen peroxide.

GSH depletion is a robust and significant alteration in the antioxidant defense which is demonstratedin several neurodegenerative diseases such as Parkinson's disease andNeurolathyrism is certainly not an exception. We have reported earlier a marginal decrease in the GSH content of brain regions following consumption of KD diet (20). Apart from functions such as in GSH-peroxidase dependent metabolism of hydoperoxides and direct scavenging of reactive oxygen species, GSH may contribute to antioxidant defense by networking with other major antioxidants such as vitamins E and C (41). Hence, significant GSH depletion in cytosol of brain regions of KD fed mice clearly suggestsa state of compromised antioxidant status. Since GSH is associated with many critical redox regulatory genes (42), GSH depletion (~ 20-30%) can impair the cell's defense against the toxic actions of ROS and may lead to cell injury/ death (43). Further in the present study, among CA supplemented KD mice, there were no significant reduction in GSH levels which is indicative of the ability of CA to modulate redox status of cells. Additionally, there was significant depletion in total thiol levels in cytosol of brain regions of mice fed KD, while in CA supplemented group, no such alterations were discernible.

GSH depletion was associated with significant alterations in the activities of antioxidant enzyme activities in both mitochondria and cytosol of KD fed mice. Significant increase was observed in the activities of GSH-Px, GST and SOD in cytosol followed by a decrease in catalase activity. The decrease in catalase and increase in GSH-Px and SOD may be due to the increase in ROS and hydroperoxides. GST is directly involved in detoxification mainly of xenobiotics (44) and the significant increase in its activity in cytosol of brain regions of KD fed mice can be explained as a response to β-ODAP toxin. Further, increase in GST may also be due to the increase in the levels of malondialdehyde formed in all brain regions following consumption of KD

Carbonyl content of proteins are indicative of oxidative stress mediated protein-oxidation (30).The accumulation of oxidized proteins is a complex function of the rates and kinds of ROS formed, levels of numerous antioxidants systems ,and the rates of degradation of oxidized proteins by a multiplicity of proteases (45). Further protein oxidation *in vivo*

affect variety of cellular functions involving proteins, receptors, signal transduction mechanisms, transport systems and enzyme (46). The significant increase in protein carbonyls in cytosol of brain regions of KD fed mice suggests a state of oxidative stress. CA supplementation resulted in normalization of those levels indicating its ability to protect against severe protein oxidation probably by scavenging the free radicals and thus preventing the chain reactions leading to protein oxidative damage.

Earlier reports have shown that LDH leakage from brain slices on exposure to β-ODAP *in vitro* (47). In the present study significant elevation in LDH activity was observed in brain regions of mice fed KD. LDH elevation may be due to increased ROS and subsequent oxidative stress leading to membrane damages.CA supplementation resulted in amelioration of these perturbations indicative of the protective property of CA against mitochondrial dysfunctions. Further, there was a decrease in AChE activity on feeding KD which may be due to increased ROS generation (20). CA supplementation resulted in normalized AChE activity in all brain regions of mice fed KD indicative of the protective efficacy of CA against cholinergic dysfunctions.

In conclusion, we have for the first time provided evidence which clearly indicates that supplementation of CA leaf powder provides significant protection against KD induced oxidative stress in young adult mice brain regions.In view of this, we speculate that CA supplements are likely to prevent the major side effects which may arise due to consumption of KD and thus provide a therapeutic alternative under similar conditions.

CONFLICT OF INTEREST

None

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