

## **CALOCYBE INDICA POLYSACCHARIDES ALLEVIATES COGNITIVE IMPAIRMENT, MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS INDUCED BY D-GALACTOSE IN MICE**

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### **ABSTRACT**

The aim of this study was to investigate the protective effect of *Calocybe indica* crude polysaccharides (CICP) against D-galactose induced cognitive dysfunction, oxidative damage and mitochondrial dysfunction in mice. Mice were subcutaneously injected with D-galactose (150 mg/kg per day) for 6 weeks and were administered CICP simultaneously. Aged mice receiving vitamin E (100 mg/kg) served as positive control. Chronic administration of D-galactose significantly impaired cognitive performance oxidative defense and mitochondrial enzymes activities as compared to control group. The results showed that CICP (200 and 400 mg/kg) treatment significantly improved the learning and memory ability in Morris water maze test. Biochemical examination revealed that CICP significantly increased the decreased activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), mitochondrial enzymes-NADH dehydrogenase (ND), malate dehydrogenase (MDH), isocitrate dehydrogenase (ICDH), Na<sup>+</sup>K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>ATPase activities, elevated the lowered total anti-oxidation capability (TAOC); glutathione (GSH) and vitamin C decreased the raised acetylcholinesterase (AChE) activities; malondialdehyde (MDA), hydroperoxide (HPO), protein carbonyls (PCO), advanced oxidation protein products (AOPP) levels in brain of aging mice induced by D-gal in a dose-dependent manner. In conclusion, present study highlights the potential role of CICP against D-galactose induced cognitive impairment, biochemical and mitochondrial dysfunction in mice.

**Keywords:** *Calocybe indica*, oxidative stress, antioxidants, aging, neuro degenerative disease

### **INTRODUCTION**

Ageing is a complex natural phenomenon that is frequently accompanied by the occurrence of several diseases, such as schizophrenia, cognitive impairment, Alzheimer's and Parkinson's diseases and others. Anti-aging has already become a major public issue with the increasing elderly population in the world. Memory decline is characteristic of aging and age-related neurodegenerative disorders, which result in a progressive loss of cognitive function, specifically in spatial memory [1]. Abundant evidence has pointed to an important role of oxidative stress during the pathogenesis of brain aging, age-associated or neurodegenerative diseases [2–4]. Indeed, with increasing age, accumulation of oxidant damaged cellular macromolecules, such as DNA, proteins and lipids of cell membranes takes place [5,6]. Brain, with high oxygen demand, high level of unsaturated lipids, and relatively deficient in anti-oxidative defense mechanism, is the most susceptible organ to oxidative damage. Thus, antioxidant therapy may be an important avenue for managing neurodegenerative diseases.

Further, evidence suggests that mitochondria are both producers as well as targets of reactive oxygen species, which increases oxidative damage [7]. As a consequence, damaged mitochondria progressively become less efficient, losing their functional integrity and release more reactive oxygen molecules [8]. Increasing oxidative burden deteriorates functional mitochondria during aging. Mitochondria are the major source of energy or adenosine triphosphate (ATP) for the normal functioning of eukaryotic cells. Dysfunction of mitochondria is well known to generate reactive oxygen species (ROS), reduce mitochondrial ATP production, increased mitochondrial deoxyribonucleic acid (DNA) mutations, increase in abnormal mitochondrial criste structures and impairs intracellular calcium level [9]. Increased ROS generation with compromised mitochondrial function ultimately affects neurons and accelerates neurodegenerative process [10].

D-galactose (D-gal) is a physiological nutrient and can be metabolized at normal concentration. In animals, galactose is normally metabolized by D-galactokinase and galactose-1-phosphate uridylyltransferase but over-supply of D-galactose results its abnormal metabolism [11]. D-galactose converts into galactitol, which does not metabolize by above enzymes but accumulate in the cell, that leads to osmotic stress and ROS production [12]. D-galactose is a reducing sugar that reacts with free amines of amino acids in proteins and peptides to form advanced glycation end products (AGE), which in turn causes activation of receptor for advanced glycation end products. These sequences of events cause oxidative stress and cellular damage [13,14]. AGE increases with age and has been linked pathologically in many age related pathologies such as diabetes, arteriosclerosis, nephropathy and Alzheimer's disease. The D-gal-lesioned rodents have been used for brain aging studies, as D-gal induced behavioral and neurochemical changes can mimic many characters of the natural brain aging process [15-18].

Administration of antioxidants via oral gavage is thought to be an effective approach to prevent free radicals from oxidizing sensitive biological molecules, thus slowing aging process and preventing diseases. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. Nowadays more and more attention was cast on mushroom polysaccharide by biochemical and nutritional researchers due to their various biological activities used in health care food or medicine, especially antioxidant, immunostimulatory and antitumor effects (19-22). However, studies of the antiaging effects of *C. indica* crude polysaccharides (CICP) in animal models are scarce. Therefore, it is necessary to investigate the effect of CICP in an animal model to develop a neuroprotective drug. The aim of present study has been designed to explore the possible effect of CICP against D-galactose induced cognitive dysfunction oxidative damage and mitochondrial dysfunction in mice.

## **MATERIALS AND METHODS**

### **Animals**

Female Balb/C mice (20-25 g) were used. The mice were housed in the temperature and humidity controlled room (temperature  $23 \pm 2$  °C and humidity  $50 \pm 10\%$ ) with a 12 hr light-dark cycle with and water *ad libitum*. Experimental animals were handled according to the University and Institutional Legislation, 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.

### **Preparation of the extract**

Fruiting bodies of *Calocybe indica* were collected from a local mushroom farm. The fruiting bodies of the mushroom were cut into small pieces, dried at 40-50 °C for 48 h and powdered. The water soluble polysaccharides was prepared following the procedure described by Cheng *et al.* [23] with some modifications. Polysaccharides were extracted from dried mushrooms (~1.5 g) with boiling water (50 ml) for 4 h under agitation. The residue was then extracted with two further portions of boiling water over a total 6 h extraction. The resulting suspension was then centrifuged (4000 r/min for 10 min). It was then concentrated in a rotary evaporator under reduced pressure at 50 °C. The concentrated supernatants were then precipitated with three volumes of absolute ethanol (95%) and polysaccharides were precipitated overnight at 4 °C. The precipitated polysaccharides were collected after centrifugation at 3000 g for 40 min followed by filtration, before being lyophilised, resulting in a crude polysaccharide (CICP).

### **Experimental design**

The mice were randomly divided into five groups, each consisting of 10 animals. The five groups were designated as follows: group I (normal control group); group II (D-gal model group); group III (CICP + D-gal); group IV (CICP + D-gal); group V (Vit E + D-gal). Mice in group II to group V were subcutaneously intraperitoneally (i.p.) injected with D-gal (in normal saline) once daily at a dose of 150 mg/kg per day for 6 weeks, while mice in group I were treated with the same volume of physiological saline. From the first week, after the injection of D-gal, group III and group IV mice were orally administrated with crude mushroom polysaccharide fraction (CICP) at the doses of 200

and 400 mg/kg per day and group V mice were treated with Vit E by intragastric administration at the dose of 100 mg/kg per day, respectively. At the same time, the normal control group (group I) and the D-gal model group (group II) mice were given the same volume of physiological saline for 6 weeks by intragastric administration.

### Behavioral assessments

**Assessment of cognitive performance:** Learning and memory ability was detected by Morris water maze test [24]. In this test, mice were trained to find a platform (6 cm in diameter) hidden 1 cm below the water surface in a circular water tank (100 cm in diameter, 45 cm in height). Each mouse received four training sessions per day for 4 consecutive days. One day before the first trial, each mouse received 4 times of pre-training: mouse was put on the platform for 20s, then given a 30s free swim and placed on the platform, where it was allowed to rest for another 20s rest. For each trial, the mouse was placed in the water facing the pool wall at one of four starting quadrant point, and the time required for the mouse to find the hidden platform was recorded. A mouse that found the platform was allowed to stay on it for 20s and then returned to its cage for 40s inter-trial interval. If the mouse did not find the platform within 60s, it would be placed on it for 20s, and the escape latency (finding the submerged platform) was recorded as 60s. After the 4<sup>th</sup> training trial, the platform was removed and each mouse was allowed to swim freely for 60s as the probe test. The time that mice spent in the target quadrant (where the platform was once hidden) was measured.

**Preparation of brain tissue homogenate:** According the method of [24], all mice were deeply anesthetized and sacrificed by decapitation after behavioral testing. Brains were promptly dissected and the tissues were minced and homogenized (10% w/v) in Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 3000 g for 20 min at 4 °C.

**Preparation of mitochondrial fraction:** About 10% of the brain tissue homogenate of rats was prepared in 50 mM phosphate buffer (pH 7.0) containing 0.25 mol/l (w/v) sucrose. Homogenate was centrifuged initially at 3,000 g for 10 min and the supernatant was subjected to 11,000 g for 10 min at 4 °C in a cooling centrifuge. The mitochondrial pellets were washed twice with phosphate buffer to remove the sucrose and suspended in phosphate buffer.

**Biochemical assessments:** The brain homogenate were assayed for AChE, SOD, CAT, GPx, GR, GST, GSH, Vit C, LPO and HPO. The mitochondrial fraction were assayed for ND, MDH, ICDH, AOPP, PCO and TAOC.

**Determination of Acetylcholine esterase activity:** Acetylcholine esterase activity was estimated according to the method of Shinomol and Muralalidana [25, 26]. Acetylthiocholine is hydrolysed to thiocholine by acetylcholine esterase, which reacts with DTNB and give yellow colour chromophore 5-thio-2-nitrobenzoic acid (TNB), which is measured at 412 nm. The enzyme activity is expressed as  $\mu\text{M}$  of substrate hydrolyzed/min/mg protein.

**Determination of NADH dehydrogenase activity:** NADH dehydrogenase activity was determined by the method of King and Howard [27]. The method involves catalytic oxidation of NADH to  $\text{NAD}^+$  with subsequent reduction of cytochrome c and is assayed by measuring the increase in absorbance at 550 nm against reagent blank.

**Determination of Malate dehydrogenase activity:** Malate dehydrogenase activity was estimated by the method of Mehler *et al.* [28]. It catalyses the reversible conversion of oxaloacetic acid to malic acid. The activity was expressed as  $\mu\text{moles}$  of NADH oxidized/ min/mg protein using the extinction coefficient of NADH  $6.22\text{mM}^{-1}\text{cm}^{-1}$ .

**Determination of Isocitrate dehydrogenase activity:** Isocitrate dehydrogenase activity was estimated according to the method of Varley [29]. ICDH catalyses the decarboxylative oxidation of trisodium-isocitrate by  $\text{NADP}^+$  yielding  $\alpha$ -ketoglutarate,  $\text{CO}_2$  and NADPH. The increase in absorbance associated with the reduction of NADP to NADPH is measured spectrophotometrically at 340 nm. The activity was expressed as micromoles of  $\text{NAD}^+$  reduced/min/mg protein using extinction coefficient  $6.22\text{mM}^{-1}\text{cm}^{-1}$ .

**Determination of total antioxidant capacity:** The total antioxidant capacity (TAOC) of the sample was assayed according to the method of Benzie and Strain [30] using ferric reducing ability as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe II-

tripirydyltriazine compound from colourless oxidized Fe III form by the action of electron donating antioxidants. The results are expressed as  $\mu\text{M}/\text{mg}$  protein.

**Determination of Superoxide dismutase activity:** Superoxide dismutase activity was assayed by the method of Das [31]. The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

**Determination of Catalase activity:** Catalase activity was assayed by the method of Sinha [32]. The enzyme assay was based on the utilization of  $\text{H}_2\text{O}_2$  by the enzyme. Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in presence of  $\text{H}_2\text{O}_2$ . The chromic acetate thus produced is measured spectrophotometrically at 610 nm. The activity of catalase was expressed as  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  consumed/min/mg protein.

**Determination of Glutathione peroxidase activity:** Glutathione peroxidase activity was measured by Ellman [33]. The assay measures the enzymatic reduction of  $\text{H}_2\text{O}_2$  by GPx through consumption of reduced glutathione (GSH). Glutathione was measured by its reaction with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to give a yellow derivative that absorbs at 412 nm. The activity was expressed in term of  $\mu\text{g}$  of glutathione oxidized/min/mg protein or ml of serum.

**Determination of Glutathione reductase activity:** Glutathione reductase activity assay was based on the method of Beutler [34]. The enzymatic activity was assayed photometrically by measuring NADPH consumption. In the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH, resulting in a decrease of absorbance at 340 nm. The activity was expressed as  $\mu\text{moles}$  of glutathione utilised/ min/mg protein.

**Determination of Glutathione-S-transferase activity:** Glutathione-S-transferase activity was measured by the method of Habig [35]. Glutathione-S-transferase catalyses the reaction of 1-chloro 2, 4 dinitrobenzene (CDNB) with the sulphhydryl group of glutathione. The CDNB- Glutathione conjugate absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance. The results were expressed as nmoles of CDNB conjugated/min/mg of protein.

**Determination of ATPase activity:**  $\text{Na}^+\text{K}^+$  ATPase was assayed by the method of Bonting [36],  $\text{Ca}^{2+}$ ATPase [37],  $\text{Mg}^{2+}$ ATPase [38] based on the inorganic phosphorus liberated, which is estimated by Fiske and Subbarow method.

**Determination of reduced glutathione:** Reduced glutathione level was measured according to the method of Moron [39] based on the formation of a yellow colored complex with Ellman's reagent measured at 412 nm. The amount of glutathione was expressed as  $\mu\text{g}/\text{mg}$  protein.

**Determination of vitamin C:** The level of vitamin C was determined by the method described by Omaye [40]. The dehydro-ascorbic acid formed from the oxidation of vitamin C by copper, formed a colored product on treatment with 2,4-dinitrophenylhydrazine, whose absorbance was measured at 520 nm. The results were expressed as  $\mu\text{g}/\text{mg}$  protein.

**Determination of protein carbonyl:** Protein carbonyl was estimated by the method of Levine *et al.* [41]. Protein carbonyl utilizes the DNPH reaction and the amount of protein hydrozone produced is quantified spectrophotometrically at an absorbance of 370 nm. The results were expressed as  $\mu\text{M}$  of carbonyl/ mg protein.

**Determination of advanced oxidation protein product:** Advanced oxidation protein products (AOPP) level was determined according to the method described of Kayali *et al.* [42]. The concentration of AOPP was calculated using the extinction coefficient  $26 \text{ mM}^{-1}\text{cm}^{-1}$  and expressed as  $\mu\text{M}/\text{mg}$  protein.

**Determination of Lipid peroxidation:** The extent of lipid peroxidation in the brain was determined quantitatively by performing the method as described by Niehius [43]. In this method malondialdehyde and other TBARS were estimated

by their reactivity with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore, which were read at 535 nm. MDA content was expressed as nmol per g tissue.

**Determination of Hydroperoxides:** The hydroperoxide was determined by the method of Jiang *et al* [44]. In this method, oxidation of ferrous ion (Fe<sup>2+</sup>) under acidic condition, in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm. Hydroperoxides were expressed as mM/g tissue.

**Determination of Protein:** The protein content was estimated by Lowry's method [45] using bovine serum albumin as a standard.

### Statistical analysis

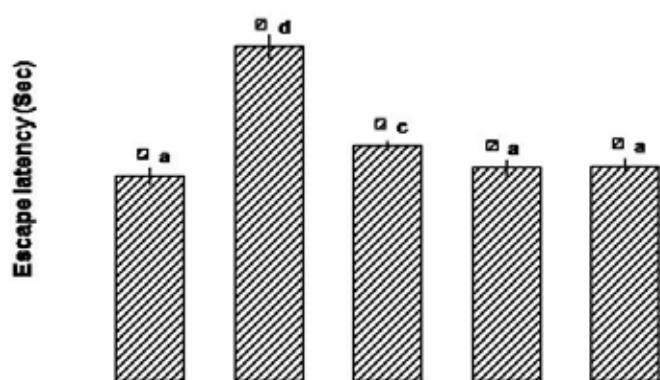
Values are expressed as mean ± SEM. The data are reported as the mean ± standard deviation and were analysed by SPSS (version 20.0 SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between the means were determined by Duncan's Multiple Range tests.  $p < 0.05$  was considered significant.

## RESULTS

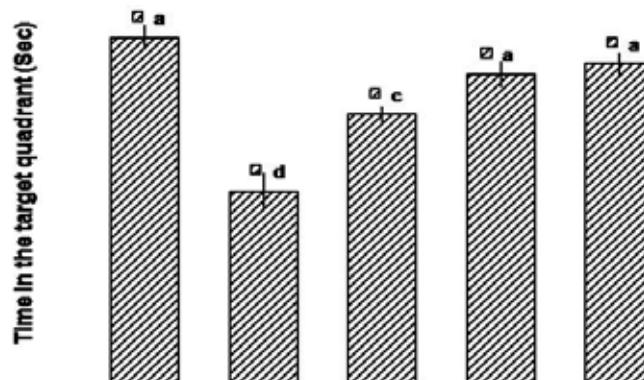
### Effect of CICP on memory performance in Morris water maze task in D-galactose treated mice

One of the age related declines of brain function is the cognitive behavioral deficit. It has been well established that water maze performance declines with aging of the animals and it is a sensitive method for revealing the impairment of spatial learning and memory [46]. As indicated in Fig. 1 the D-gal induced group took longer time to find the hidden platform as compared with the normal control group, suggesting that the D-gal treated mice had significant cognitive impairment. The prolonged escape latency in the D-gal model group was shortened by administration of CICP (200, 400 mg/kg per day) and Vitamin E (100 mg/kg day).

The time the mice spent swimming in the target quadrant on day 5<sup>th</sup> are shown in Fig. 2. The D-gal mice crossed the former location of the platform less frequently ( $p < 0.05$ ) than normal mice, but CICP (200, 400 mg/kg b.wt) and vitamin E (100 mg/kg) treatment could improve the impaired performance. In addition, mice in the CICP treated groups reached the target quadrant more rapidly and the time they spent swimming in the target quadrant were longer ( $p < 0.05$ ) than that of the D-gal mice. The above results indicated that CICP prevented D-gal induced spatial learning and memory dysfunction in mice.



**Figure 1.** Effect of CICP on the escape latency of mice in the water maze test on 5<sup>th</sup> day. Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p < 0.05$  by DMRT



**Figure 2.** Effect of CICP on the time that mice spent in swimming in the target quadrant on 5<sup>th</sup> day. Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p < 0.05$  by DMRT

Our present study demonstrated that long term administration of D-gal induced impairment of learning and memory in water maze tests. However, water maze tests suggested that CICIP significantly reversed the behavioral retrogression, such as learning and cognition indicating that CICIP had potential to prevent learning and memory deficits.

### Effect of CICIP on the activities of acetyl cholinesterase in D-gal induced mice

Cholinergic system is of predominant importance in learning and memory processes [47]. Acetyl cholinesterase, one of the specific cholinergic marker proteins, is responsible for degradation of acetyl cholinesterase to acetate and choline in the synaptic cleft. Therefore, we analyzed the acetyl cholinesterase activity in the brain of each group of mice. Figure 3 described the acetyl cholinesterase activity in the brain of all groups of mice. D-gal induced mice displayed a remarkable increase in AChE activity ( $p < 0.05$ ) compared with the normal control. The increase in AChE activity was restored to near normal in CICIP and vitamin E treated mice. CICIP at the dose of 400 mg/kg b.wt exhibited more significant effect than the lower dosage (200 mg/kg b.wt) and vitamin E at a dose of 100 mg/kg b.wt showed comparable effect to that of CICIP (400 mg/kg b.wt).

### Effect of CICIP on the activities of brain mitochondrial NADH dehydrogenase, MDH and ICDH in D-gal induced mice

Administration of D-Gal for 6 weeks caused significant alterations in mitochondrial enzyme activities. D-galactose treatment significantly decreased NADH dehydrogenase, MDH, ICDH activities as compared to control mice ( $p < 0.05$ ). However, CICIP (200 and 400 mg/kg b.wt) and Vit E (100 mg/kg b.wt) treatment significantly restored the mitochondrial enzyme activities to near normal (Table 1).

**Table 1.** Effect of polysaccharides from *C.indica* on the activities of NADH dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase in mitochondrial brain of D-gal induced mice

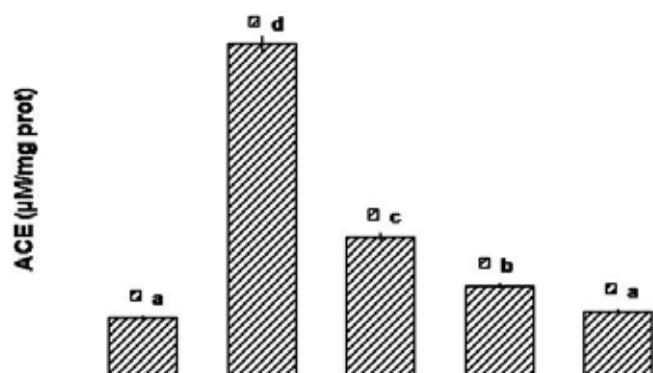
Parameters	Normal control	D-Gal (150 mg/kg)	CICIP (200 mg/kg) + D-Gal	CICIP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
NADH DH	168.55 ± 3.92 <sup>c</sup>	95.84 ± 2.66 <sup>a</sup>	120.97 ± 4.44 <sup>b</sup>	141.11 ± 4.67 <sup>c</sup>	154.83 ± 3.97 <sup>d</sup>
MDH	4.66 ± 0.12 <sup>d</sup>	3.34 ± 0.11 <sup>a</sup>	4.10 ± 0.13 <sup>bc</sup>	4.19 ± 0.09 <sup>c</sup>	4.01 ± 0.14 <sup>b</sup>
ICDH	3.31 ± 0.12 <sup>d</sup>	2.40 ± 0.10 <sup>a</sup>	2.88 ± 0.12 <sup>b</sup>	3.14 ± 0.13 <sup>c</sup>	3.46 ± 0.15 <sup>d</sup>

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p < 0.05$  by DMRT; Units: NADH DH -  $\mu\text{M}$  of NADH oxidized/min/mg protein; MDH-  $\mu\text{M}$  of succinate oxidized/min/mg protein; ICDH- $\mu\text{M}$ /min/mg protein.

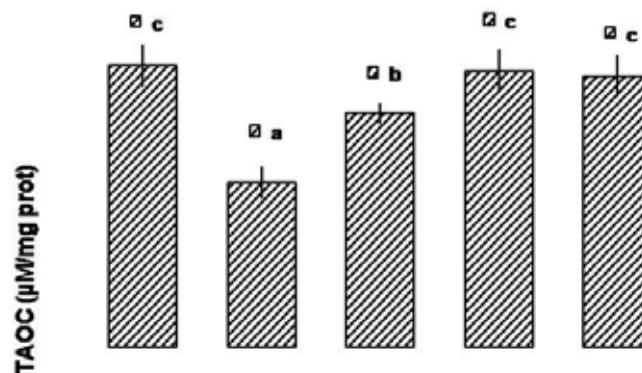
The results of the present study indicate that chronic administration of D-galactose caused an impairment in mitochondrial enzyme activities as indicated by decrease in the NADH dehydrogenase, MDH and ICDH activities. The observed alteration in mitochondrial enzymes activities could be involved in aging and its complications, which could be due to free radicals as well as reduction in both mitochondrial transcription and translation levels. Moreover, it has been reported that disruption of mitochondrial activity associated with inhibition of enzymes in the electron transport chain which, lead to increase in electron leakage from the mitochondria [48], production of ROS like the superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\text{OH}^-$ ). Mitochondrial dysfunction might be the key factor for the production of ROS, which leads to oxidative damage in D-galactose induced aging.

### Effect of CICIP on the levels of TAOC in D-gal induced mice

TAOC represents the free radical scavenging capacity of antioxidants. Decline of TAC level is an important part of the pathophysiological changes that occur during ageing, which is one of the reasons for the increased accumulation of ROS. D-galactose significantly decreased the TAOC level compared to control mice ( $p < 0.05$ ). CICIP treatment (200 and 400 mg/kg b.wt) significantly enhanced the TAOC levels in the brain in a dose dependent manner. Vitamin E also reversed these altered changes to near normal levels (Fig. 4).



**Figure 3.** Effect of polysaccharides from *C.indica* on the activity of acetyl choline esterase in brain of D-gal induced mice. Values are mean  $\pm$  SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p<0.05$  by DMRT



**Figure 4.** Effect of polysaccharides from *C.indica* on the levels of TAOC in brain of D-gal induced mice. Values are mean  $\pm$  SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p<0.05$  by DMRT

TAOC, an indicator of enzymatic and non enzymatic antioxidants, reflects the total antioxidant capabilities. When antioxidant defenses are weakened, body cells and tissues become more prone to develop dysfunction and/or disease. The maintenance of adequate antioxidant levels is essential in preventing and managing a great number of conditions, so TAOC could be a reliable diagnostic biomarker. C150 which possessed strong antioxidant activity noticeably increased TAOC levels in galactose induced mice. Thus the observed beneficial effect of C150 might be either due to its antioxidant or free radical scavenging activity [49].

### Effect of C150 on the activities of antioxidant enzymes in D-gal induced mice

As shown in Table 2 it is evident that SOD, CAT, GPx, GR, and GST activities in brain decreased significantly ( $p<0.05$ ) in D-galactose induced mice compared with normal mice. Administration of C150 extract (200 and 400 mg/kg b.wt) increased the activity of antioxidant enzymes in a dose dependent manner. C150 at 400 mg/kg b.wt exhibited stronger antioxidant effect which was comparable to that of vitamin E. A vast number of evidence implicates that aging is associated with a decrease in antioxidant status and age dependent increases in lipid peroxidation [50], being in agreement with our present study. The major antioxidant enzymes, including SOD, GPx and CAT are regarded as the first line of the antioxidant defense system against reactive oxygen species generated *in vivo* during oxidative stress. SOD dismutates superoxide radicals to form hydrogen peroxide, which in turn is decomposed to water and

**Table 2.** Effect of polysaccharides from *C. indica* on the activities of enzymic antioxidants in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	C150 (200 mg/kg) + D-Gal	C150 (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
SOD	5.94 $\pm$ 0.20 <sup>d</sup>	3.63 $\pm$ 0.14 <sup>a</sup>	5.12 $\pm$ 0.13 <sup>b</sup>	5.77 $\pm$ 0.21 <sup>d</sup>	5.42 $\pm$ 0.18 <sup>c</sup>
CAT	12.04 $\pm$ 0.35 <sup>c</sup>	6.34 $\pm$ 0.23 <sup>a</sup>	11.12 $\pm$ 0.33 <sup>b</sup>	11.89 $\pm$ 0.52 <sup>c</sup>	12.02 $\pm$ 0.56 <sup>c</sup>
GPx	3.36 $\pm$ 0.10 <sup>c</sup>	1.64 $\pm$ 0.05 <sup>a</sup>	2.75 $\pm$ 0.07 <sup>b</sup>	3.24 $\pm$ 0.12 <sup>c</sup>	3.27 $\pm$ 0.14 <sup>c</sup>
GR	5.87 $\pm$ 0.21 <sup>d</sup>	3.60 $\pm$ 0.16 <sup>a</sup>	4.74 $\pm$ 0.21 <sup>b</sup>	5.66 $\pm$ 0.19 <sup>cd</sup>	5.43 $\pm$ 0.24 <sup>c</sup>
GST	421.17 $\pm$ 18.84 <sup>d</sup>	233.36 $\pm$ 9.48 <sup>a</sup>	389.45 $\pm$ 13.93 <sup>b</sup>	411.01 $\pm$ 11.03 <sup>cd</sup>	402.06 $\pm$ 17.98 <sup>bc</sup>

Values are mean  $\pm$  SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p<0.05$  by DMRT.

Units: SOD - inhibition of 50% nitrite formation/min/ mg protein; CAT-  $\mu$ M of H<sub>2</sub>O<sub>2</sub> consumed/min/ mg protein; GPx -  $\mu$ M of glutathione oxidized/min/ mg protein; GR -  $\mu$ M of glutathione utilized/min/ mg protein; GST -  $\mu$ mole of CDNB-GSH conjugate formed/min/mg protein.

oxygen by GPx and CAT, thereby preventing the formation of hydroxyl radicals [51]. Therefore, these enzymes act cooperatively at different sites in the metabolic pathway of free radicals. Reduction in the activity of GPx in D-galactose induced mice indicates that the glutathione was consumed during the reaction with oxygen and peroxide radicals. Inhibition of this enzyme, which eliminates hydrogen peroxide and lipid peroxide leads to accumulation of these oxidants and thus the subsequent oxidation of lipids [52].

GR is the family of homologous proteins, whose members are dimeric, NADPH dependent and FAD containing enzymes. The increase in GR activity implies that CICP protects the brain from oxidative damage by GSH regenerated from its oxidized form (GSSG). The decrease in GST activity in D-galactose induced mice may favour the excretion of oxidised GSH, thereby maintaining the thiol redox status in tissues. The decreased activity of GST in the present study may be due to the decreased availability of GSH. The increased activity of GST in CICP treated aged mice hastens the detoxification of the lipid peroxides.

GST offers protection against LPO by promoting the conjugation of toxic electrophiles with GSH [53]. GST plays a physiological role in inactivating the detoxification of potential alkylating agents. GST activities were significantly reduced in D-galactose induced mice and were reverted to near normal after treatment with the CICP. The major antioxidant enzymes, including SOD, CAT and GPx are regarded as the first line of the antioxidant defense system against ROS *in vivo* during oxidative damage [54]. Non-enzymatic antioxidants, such as the total antioxidant capacity (T-AOC) and GSH level, also play a significant role in intracellular antioxidant defense in the body [55]. The enhanced activities of SOD, and CAT as well as the increases in T-AOC and GSH levels in the aging mice could be very effective in clearing various types of oxygen free radicals and their products, thus reversing D-gal induced cognitive and motor performances deficits resulted from oxidative damage.

### Effect of PFCI on the activities of ATPases in D-gal induced mice

Na<sup>+</sup>K<sup>+</sup>-ATPase, the main biomolecule for maintaining cation homeostasis, is responsible for generating and maintaining membrane potential necessary for neuronal excitability. Thus disturbance in its activity could have grave consequences for central nervous system (CNS) functioning [56]. Table 3 depicts the changes in the activities of Na<sup>+</sup>K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase in brain induced by D-gal. Treatment with D-gal showed a decline in Na<sup>+</sup>K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase activities ( $p < 0.05$ ), whereas the effect was significantly restored by CICP and vitamin E. CICP at a dose of 400 mg/kg b.wt exhibited more significant effect ( $p < 0.05$ ) than the dose of 200 mg/kg b.wt and vitamin E.

It has been reported that Na<sup>+</sup>K<sup>+</sup>-ATPase might play a relevant role in mechanism for learning [57], as well as in activity dependent synaptic plasticity, such as long term potentiation (LTP) [58]. As a result of oxidative damage, Na<sup>+</sup>K<sup>+</sup>-ATPase activity decreased age dependently in mice brain [59]. Our results showed that oxidative damage led to the decline of Na<sup>+</sup>K<sup>+</sup>-ATPase activity, whereas CICP could protect Na<sup>+</sup>K<sup>+</sup>-ATPase.

**Table 3.** Effect of polysaccharides from *C.indica* on the activities of ATPase in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
Na <sup>+</sup> K <sup>+</sup> ATPase	609.52 ± 19.62 <sup>c</sup>	374.55 ± 12.99 <sup>a</sup>	555.21 ± 16.88 <sup>b</sup>	593.02 ± 18.03 <sup>c</sup>	598.38 ± 27.93 <sup>c</sup>
Ca <sup>2+</sup> ATPase	610.08 ± 16.37 <sup>c</sup>	323.49 ± 7.66 <sup>a</sup>	450.64 ± 20.15 <sup>b</sup>	588.10 ± 26.20 <sup>c</sup>	595.77 ± 15.98 <sup>c</sup>
Mg <sup>2+</sup> ATPase	457.07 ± 16.35 <sup>d</sup>	291.86 ± 11.02 <sup>a</sup>	356.96 ± 15.96 <sup>b</sup>	414.84 ± 18.55 <sup>c</sup>	451.48 ± 13.00 <sup>d</sup>

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p < 0.05$  by DMRT.

Units: ATPases - nM Pi liberated/min/mg protein.

### Effect of CICIP on the levels of non-enzymic antioxidants in D-gal induced mice

The level of non enzymatic antioxidants (GSH and vitamin C) in D-gal induced mice decreased significantly ( $p<0.05$ ) in brain as compared with the normal control group (Table 4). Treatment with CICIP(200 and 400 mg/kg b.wt) significantly and dose dependently increased the non-enzymic antioxidant levels in brain. Glutathione is a major, non protein thiol in living organisms which performs a key role in coordinating the innate antioxidant defense mechanisms. It is involved in the maintenance of the normal structure and function of cells, probably by its redox and detoxification reactions [60].

**Table 4.** Effect of polysaccharides from *C.indica* on the levels of non enzymic antioxidants in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICIP (200 mg/kg) + D-Gal	CICIP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
GSH	5.99 ± 0.16 <sup>d</sup>	2.23 ± 0.10 <sup>a</sup>	4.98 ± 0.20 <sup>b</sup>	5.88 ± 0.18 <sup>cd</sup>	5.68 ± 0.21 <sup>c</sup>
Vitamin C	5.83 ± 0.15 <sup>d</sup>	2.68 ± 0.10 <sup>a</sup>	4.17 ± 0.17 <sup>b</sup>	5.30 ± 0.27 <sup>c</sup>	5.73 ± 0.21 <sup>d</sup>

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p<0.05$  by DMRT; Units: GSH, Vitamin C - µg/mg protein.

As an endogenous antioxidant, GSH plays a critical role in intracellular antioxidant defense in the brain. GSH scavenges ROS by directly reacting with it. On the other hand, GSH can prevent H<sub>2</sub>O<sub>2</sub> induced hydroxyl radical formation. So, GSH level parallels the antioxidant defense capacity in the brain. Vitamin C is capable of maintaining sulfhydryl groups in reduced state in several redox reactions [61]. Remarkable decrease in the level of vitamin C in D-galactose induced mice is due to the increased utilization of vitamin C in antioxidant defense against increased reactive oxygen species or due to the decrease in GSH levels, since GSH is required for recycling of vitamin C. Vitamin C, a water soluble antioxidant and also pro oxidant is a potential and one of the most effective scavengers of oxygen free radicals and other derived species. It imparts its protection by undergoing oxidation to dehydroascorbate. For the back conversion to ascorbate, GSH is required. Consequently when GSH is reduced, there is fall in the level of vitamin C [62]. The increased level of vitamin C in aged mice treated with the CICIP is due to its antioxidant property.

### Effect of CICIP on the levels of PCO and AOPP in D-gal induced mice

D-galactose treatment for 6 weeks significantly ( $p<0.05$ ) raised the levels of protein carbonyl (PCO) and advanced protein glycation products (AOPP) in the brain of D-gal model group as compared to control mice (Table 5). CICIP (200 and 400 mg/kg b.wt) treatment significantly attenuated the increase in PCO and AOPP levels as compared to control (D-galactose treated) group in a dose dependent manner.

**Table 5.** Effect of polysaccharides from *C.indica* on the levels of protein carbonyl and AOPP in mitochondrial brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICIP (200 mg/kg) + D-Gal	CICIP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
PCO	1.54 ± 0.03 <sup>b</sup>	2.28 ± 0.09 <sup>e</sup>	1.73 ± 0.04 <sup>d</sup>	1.64 ± 0.07 <sup>c</sup>	1.47 ± 0.05 <sup>a</sup>
AOPP	6.93 ± 0.15 <sup>a</sup>	10.69 ± 0.28 <sup>d</sup>	8.25 ± 0.21 <sup>c</sup>	7.07 ± 0.20 <sup>a</sup>	7.87 ± 0.23 <sup>b</sup>

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p<0.05$  by DMRT.

Units: PCO - µM of carbonyl/min/mg protein; AOPP- µM/mg protein.

## Effect of CICP on the levels of LPO and HPO in D-gal induced mice

MDA and TBARS levels are indexes of membrane damage and the elevated levels of MDA and TBARS result in reducing membrane fluidity inactivating membrane-bound proteins and changing the structure and function of the membranes [54].

The MDA production, a main index of lipid peroxidation and HPO was significantly increased ( $p < 0.05$ ) in brain of D-galactose induced mice when compared with the normal group (Table 6). Mice Treated with CICP(200 and 400 mg/kg b.wt) and vitamin E decreased ( $p < 0.05$ ) the MDA level in a dose dependent manner. The result suggested that administration of CICP extract (200 and 400 mg/kg b.wt) and vitamin E resulted in an effective inhibition of lipid peroxidation in D-galactose induced mice.

**Table 6.** Effect of polysaccharides from *C. indica* on the levels of lipid peroxidation and hydroperoxides in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
LPO	1.50 ± 0.04 <sup>a</sup>	3.11 ± 0.12 <sup>c</sup>	1.98 ± 0.08 <sup>d</sup>	1.78 ± 0.04 <sup>c</sup>	1.62 ± 0.07 <sup>b</sup>
HPO	3.24 ± 0.08 <sup>a</sup>	6.49 ± 0.29 <sup>d</sup>	4.99 ± 0.69 <sup>c</sup>	4.15 ± 0.15 <sup>b</sup>	3.20 ± 0.14 <sup>a</sup>

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at  $p < 0.05$  by DMRT; Units: LPO - nM of MDA formed/min/mg protein; HPO - mM/g tissue.

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of poly unsaturated lipids. Under normal physiological conditions, only low levels of lipid peroxides occur in body tissues. The excessive generation of free radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation [63]. MDA, a secondary product of lipid peroxidation, is used as an indicator of tissue damage [64]. Aging has been reported to be associated with increased disruption of membrane lipids leading to subsequent formation of peroxide radicals. In the present investigation, such a disruption of membrane lipids possibly accounted for the observed increase in MDA levels in the organs of D-galactose induced mice when compared to normal mice. In addition, insufficient levels of antioxidants to scavenge peroxy radicals during aging [65] could also have contributed to the elevated level of MDA in the D-galactose induced mice. Antioxidants are necessary for preventing the formation of free radicals and they inhibit some of the deleterious actions of reactive oxygen species on lipids, DNA and proteins.

## CONCLUSION

The present results reveal that the administration of CICP in D-galactose induced rats helps to significantly elevate the activity of antioxidant enzymes, reduce the levels of lipid peroxidation products, and keep the balance of oxidative and antioxidative systems during severe oxidative stress in rats. Therefore, CICP should be further explored as a functional antioxidant food for the prevention of ageing-related diseases. The possible mechanism of the protective effect of CICP against oxidative stress in D-gal-induced ageing rats may be through its strong antioxidant activity. Nevertheless, our results need to be confirmed by more comprehensive and detailed human trials to determine whether these effects might be extrapolated to humans. In conclusion, present study highlights the potential role of CICP against D-galactose induced cognitive impairment, biochemical and mitochondrial dysfunction in mice.

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