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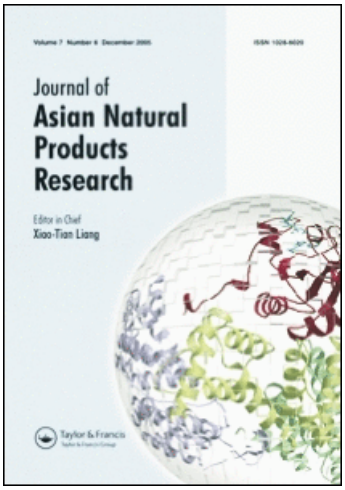

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Protective effect of curcumin (*Curcuma longa*) against d-galactose-induced senescence in mice

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Brain senescence plays an important role in cognitive dysfunction and neurodegenerative disorders. Curcumin was reported to have beneficial effect against several neurodegenerative disorders including Alzheimer's disease. Therefore, the present study was conducted in order to explore the possible role of curcumin against D-galactose-induced cognitive dysfunction, oxidative damage, and mitochondrial dysfunction in mice. Chronic administration of D-galactose for 6 weeks significantly impaired cognitive function (both in Morris water maze and elevated plus maze), locomotor activity, oxidative defense (raised lipid peroxidation, nitrite concentration, depletion of reduced glutathione and catalase activity), and mitochondrial enzyme complex activities (I, II, and III) as compared to vehicle treated group. Curcumin (15 and 30 mg/kg) and galantamine (5 mg/kg) treatment for 6 weeks significantly improved cognitive tasks, locomotor activity, oxidative defense, and restored mitochondrial enzyme complex activity as compared to control (D-galactose). Chronic D-galactose treatment also significantly increased acetylcholine esterase activity that was attenuated by curcumin (15 and 30 mg/kg) and galantamine (5 mg/kg) treatment. In conclusion, the present study highlights the therapeutic potential of curcumin against D-galactose induced senescence in mice.

Keywords: aging; curcumin; D-galactose; senescence; mitochondrial dysfunction; oxidative stress

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1. Introduction

Aging is a well-known complex physiological process, involved in several unknown cellular and molecular mechanisms leading to pathological states like Alzheimer's disease [1]. Many theories were put forward to explain aging, but biological mechanisms that underlie aging process are still unknown and poorly understood. Mitochondrial dysfunction and oxidative damage were well suggested in the aging process and related effects [1]. Aging process caused alteration in mitochondrial functions, characterized by opening of membrane permeability transition pore, reduction of mitochondrial membrane potential ($\Delta\psi_m$), and release of cytochrome *c* from mitochondria into the cytosol. Indeed, an important correlate of the oxidative stress theory is the mitochondrial theory of aging [2], which suggests that oxidative stress within mitochondria can lead to a vicious cycle in which damaged mitochondria increase reactive oxygen species (ROS) [3]. The result of these changes in physiological process accelerates the rate of accumulated damage. Several studies have indicated that Aβ might directly lead to mitochon-

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drial toxicity and diminish enzymatic activity of respiratory chain complexes [3].

Brain senescence plays an important role in cognitive dysfunction that is commonly associated with neurodegenerative disorders. Literatures suggest that administration of D-galactose causes senescent syndrome in experimental animals similar to humans aging (e.g. memory impairment), retrograde changes in neural cells, degeneration in immunological activity, and controlling abnormal genes expression [4]. It was reported that abnormal accumulation of galactitol from excess D-galactose by aldose reductase in cell, lead to osmotic stress and generation of ROS [5]. D-Galactose is a reducing sugar that reacts readily with free amines of amino acids in proteins and peptides both *in vivo* and *in vitro* to form advanced glycation end-products (AGE) through nonenzymatic glycation [6]. The AGE activates its receptors, coupled to biochemical pathways that stimulate free radical production. These animals also exhibit impairment of cholinergic neurons in the basal forebrain and neurogenesis in the hippocampus. Moreover, D-galactose-induced neurotoxicity was implicated also in the impairment of calcium homeostasis and mitochondrial dysfunction [7].

Curcuma longa (L) is one of the medicinal plants extensively used in the Indian system of medicine for management of various diseases.

Curcumin (‘**1**’) is an active polyphenol, isolated from *C. longa* rhizomes (Figure 1). ‘**1**’ was known for its antioxidant, anti-inflammatory, antimicrobial, antimutagenic, and anticarcinogenic activities [8].

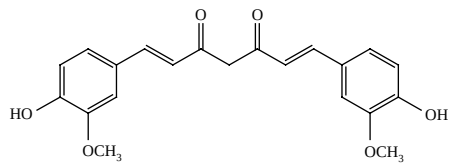

Figure 1. Structure of curcumin (**1**).

Study showed that ‘**1**’ inhibits apoptosis, platelet aggregation, cytokine production, cyclooxygenase lipoxygenase isoenzymes activities, and ROS production. In fact, ‘**1**’ can suppress oxidative damage, inflammation, cognitive deficits, and amyloid accumulation [9] that are the characteristic features of AD. Apart from AD, therapeutic benefits of ‘**1**’ were also demonstrated in ethanol induced oxidative injury in brain, CCl_4-induced hepatic injury, and cadmium-induced oxidative damage [10].

Therefore, the present study was designed to explore the possible role of ‘**1**’ against D-galactose-induced cognitive dysfunction, oxidative damage, and mitochondrial dysfunction in mice.

2. Results

2.1 Effect of curcumin on memory performance in Morris water maze task in D-galactose-treated mice

In Morris water maze, D-galactose-treated mice showed an initial increase in escape latency, which progressively declined with continued training during acquisition phase on day 20 as compared to vehicle control group. However, galatamine (5 mg/kg) and curcumin (15 and 30 mg/kg) treatment for 6 weeks significantly decreased initial acquisition latency (IAL) to reach the platform in the pre-trained mice as compared to D-galactose treated group on day 20. Vehicle control and curcumin (30 mg/kg) *per se* group of animals quickly learned to swim directly to the platform in the Morris water maze on day 20 (Table 1).

Following training, the mean retention latencies (1st RL and 2nd RL) to escape onto the hidden platform were significantly decreased in vehicle control group on days 21 and 42, respectively, as compared to D-galactose treatment. On the contrary, the performance in the D-galactose-treated mice had changed after initial training in the water maze on days 21 and 42, with significant increase in mean retention latencies compared to IAL on day 20. The

Table 1. Effect of curcumin (15 and 30 mg/kg, p.o.) on memory performance in Morris water maze paradigm in D-galactose treated mice.

Treatment (mg/kg)	Day 20 (IAL)	Day 21 (1st RL)	Day 42 (2nd RL)
Vehicle control	48.1 ± 2.9	17.3 ± 3.2	12.3 ± 1.4
D-gal (100)	49.8 ± 3.0	58.0 ± 2.2[a]	55.1 ± 1.7[a]
Gal (5) + D-gal (100)	48 ± 2.5	32 ± 2.7[b]	16 ± 3.2[b,c]
CMN (30)	44.3 ± 1.5	13.0 ± 2.3	11.0 ± 2.1
CMN (15) + D-gal (100)	48.0 ± 2.6	37.5 ± 2.4[b]	35.6 ± 2.3[b]
CMN (30) + D-gal (100)	46.1 ± 2.1	27.5 ± 2.0[b,c]	24.3 ± 2.3[b,c,d]

Notes: The IAL on day 20 and retention latencies on days 21 (1st RL) and 42 (2nd RL) following D-galactose concurrent treatment were observed. Values are mean ± SEM ($n = 12$); [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group; [d]$P < 0.05$ as compared to Gal (15) + D-gal group (repeated measures two-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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results suggested that D-galactose caused significant cognitive impairment. However, there was a significant decrease in 1st RL and 2nd RL on days 21 and 42, respectively, when galatamine (5 mg/kg) and curcumin (15 and 30 mg/kg) were administered chronically to D-galactose-treated mice as compared to D-galactose (control)-treated mice (Table 1).

2.2 Effect of curcumin on memory performance in elevated plus maze paradigm in D-galactose-treated mice

In the elevated plus maze task, mean initial transfer latency (ITL) on day 20 for each mouse was relatively stable and showed no significant variation. All the mice entered the closed arm within 60 s. Following training, vehicle control and curcumin (30 mg/kg)-treated mice entered closed arm quickly, and mean retention transfer latencies (1st RTL and 2nd RTL) to enter closed arm on days 21 and 42 were shorter as compared to ITL on day 20 of each group, respectively. In contrast, D-galactose-treated (control) mice performed poorly throughout the experiment as compared to vehicle control group and did not show any significant change in the mean RTL on days 21 and 42 as compared to pre-training latency on day 20, demonstrating chronic D-galactose-induced marked memory impairment. Chronic administration of galatamine (5 mg/kg) and curcumin (15 and 30 mg/kg) significantly decreased the mean retention latencies on days 21 and 42 as compared to the control (D-galactose; $P < 0.05$; Table 2). The mean transfer latencies of

Table 2. Effect of curcumin (15 and 30 mg/kg, p.o.) on memory performance in elevated plus maze paradigm in D-galactose treated mice.

Treatment (mg/kg)	Day 20 (ITL)	Day 21 (1st RTL)	Day 42 (2nd RTL)
Vehicle control	77.5 ± 2.4	15.0 ± 2.7	10.0 ± 2.5
D-gal (100)	77.6 ± 2.3	87.8 ± 2.6[a]	84.3 ± 2.3[a]
Gal (5) + D-gal (100)	75.5 ± 2.5	34.75 ± 2.8[b]	24.5 ± 2.2[b,c]
CMN (30)	73.8 ± 1.6	13.1 ± 1.3	11.3 ± 1.4
CMN (15) + D-gal (100)	73.1 ± 1.7	51.5 ± 1.8[b]	49.5 ± 1.6[b]
CMN (30) + D-gal (100)	72.8 ± 1.4	31.8 ± 1.6[b,c]	29.8 ± 1.8[b,c]

Notes: The IAL on day 20 and retention latencies on days 21 (1st RL) and 42 (2nd RL) following D-galactose concurrent treatment were observed. Values are mean ± SEM ($n = 12$); [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures two-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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galatamine (5 mg/kg) and curcumin (15 and 30 mg/kg) in D-galactose-treated groups were significantly different from that of D-galactose (control)-treated group on days 21 and 42 ($P < 0.05$; Table 2).

2.3 Effect of curcumin on locomotor activity in D-galactose-treated mice

Chronic administration of curcumin (30 mg/kg) did not produce any significant effect on the locomotor activity as compared to the vehicle control group throughout the study period. Furthermore, both curcumin (15 and 30 mg/kg) and galatamine in D-galactose-treated mice did not produce any significant effect on the locomotor activity as compared to the control (D-galactose; Figure 2).

2.4 Effect of curcumin on brain lipid peroxidation and nitrite in D-galactose-treated mice

Chronic administration of D-galactose significantly increased malondialdehyde (MDA) and nitrite concentration as compared to vehicle control group ($P < 0.05$). However, chronic curcumin (15 and 30 mg/kg) and galatamine (5 mg/kg) treatment for 6 weeks significantly attenuated the rise in MDA and nitrite concentration as compared to control group (D-gal-treated group; Figures 3 and 4). Furthermore, curcumin (30 mg/kg) *per se* treatment did not produce any significant effect as compared to vehicle control group.

2.5 Effects of curcumin on catalase levels, reduced glutathione, glutathione-S-transferase activity, and superoxide dismutase levels in D-galactose-treated mice

Chronic administration of D-galactose significantly depleted reduced glutathione, superoxide dismutase (SOD), glutathione-*S*-transferase activity, and catalase activity in brain as compared to vehicle control group ($P < 0.05$). Chronic curcumin (15 and 30 mg/kg) and Gal (5 mg/kg) treatment

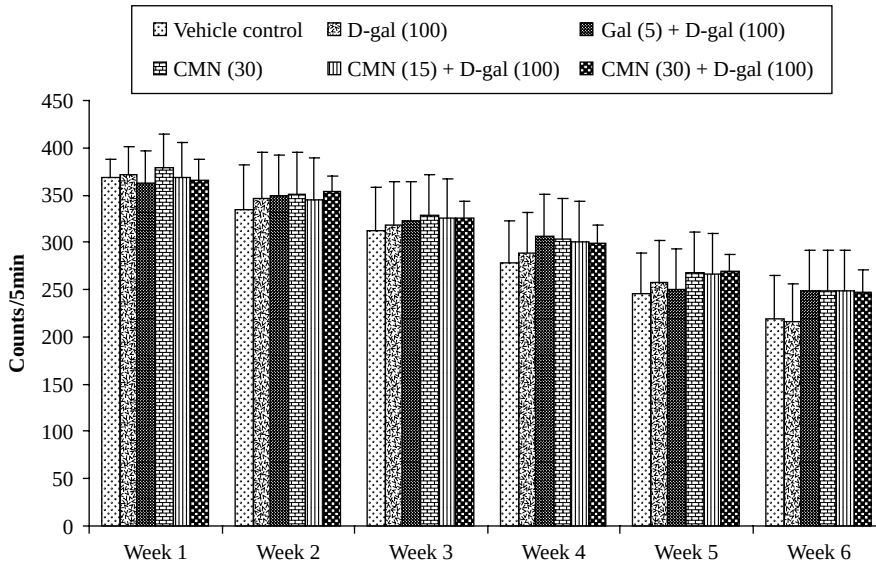

Figure 2. Effect of curcumin (15 and 30 mg/kg, p.o.) on locomotor activity in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). Data were analyzed by two-way ANOVA; D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

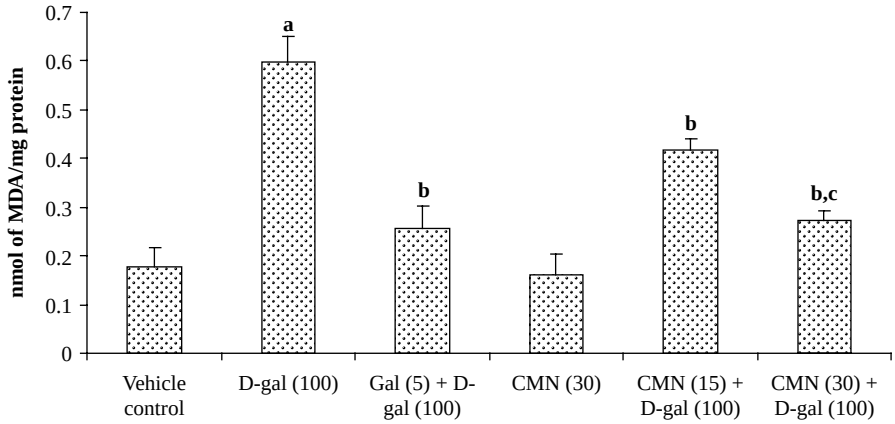

Figure 3. Effect of curcumin (15 and 30 mg/kg, p.o.) on lipid peroxidation in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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for 6 weeks significantly caused restoration of reduced glutathione, SOD, glutathione-*S*-transferase, and catalase activity as compared to control (D-gal treated mice; Figures 5–8). Furthermore, curcumin (30 mg/kg) *per se* treatment did not produce any significant effect on these stress parameters as compared to vehicle control mice.

2.6 Effect of curcumin on acetylcholinesterase activity in D-galactose-treated mice

D-galactose treatment for 6 weeks caused a significant increase in acetyl cholinesterase (AChE) activity as compared to vehicle control group ($P < 0.05$). However, chronic curcumin (15 and 30 mg/kg,

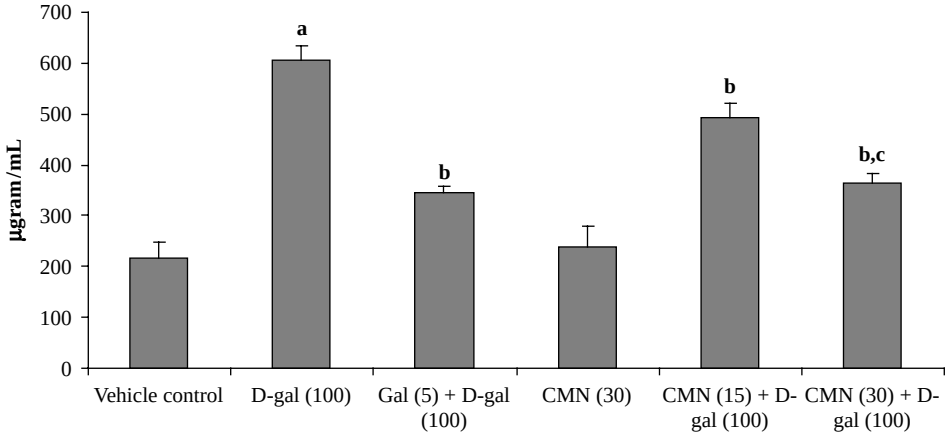

Figure 4. Effect of curcumin (15 and 30 mg/kg, p.o.) on nitrite levels in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

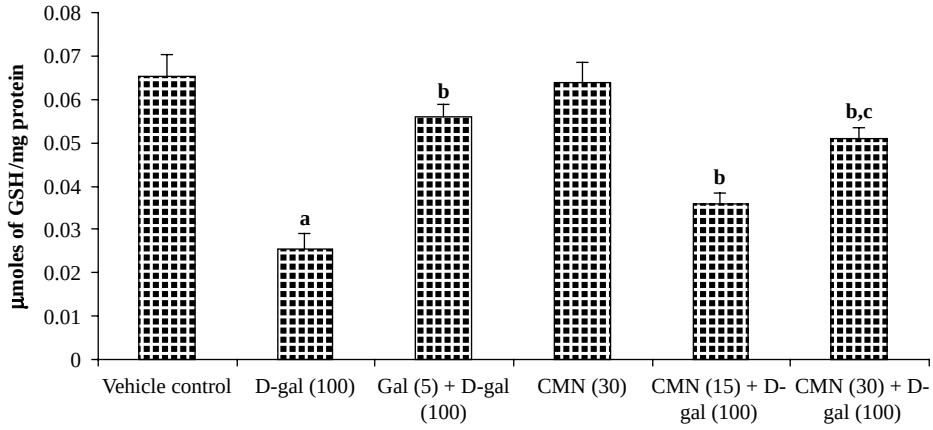

Figure 5. Effect of curcumin (15 and 30 mg/kg, p.o.) on reduced glutathione levels in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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p.o.) and galantamine (5 mg/kg) treatment significantly decreased acetylcholinesterase activity as compared to the control (D-gal-treated mice; Figure 9). Furthermore, curcumin (30 mg/kg) *per se* treatment did not produce any significant effect on acetylcholinesterase activity as compared to vehicle control group.

2.7 Effect of curcumin on NADH dehydrogenase activity, succinate dehydrogenase activity, and MTT ability in D-galactose-treated mice

Chronic administration of D-galactose significantly impaired mitochondrial enzyme complex activities (NADH dehydrogenase, succinate dehydrogenase, and MTT ability)

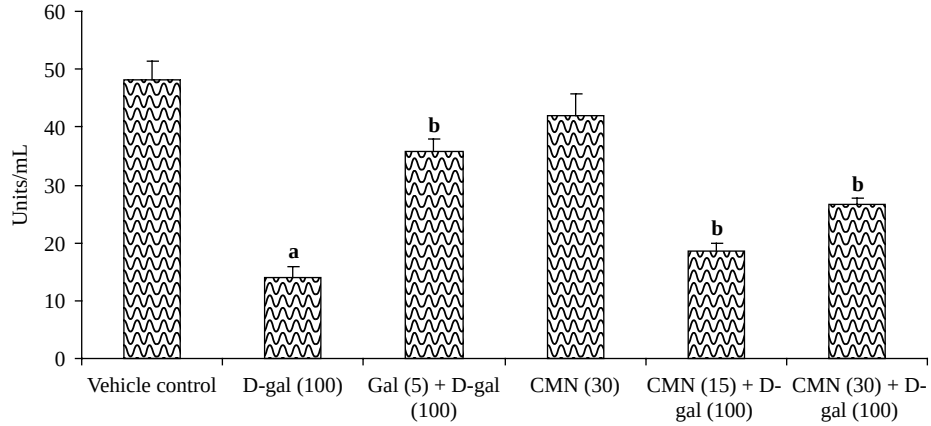

Figure 6. Effect of curcumin (15 and 30 mg/kg, p.o.) on SOD in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal-treated group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

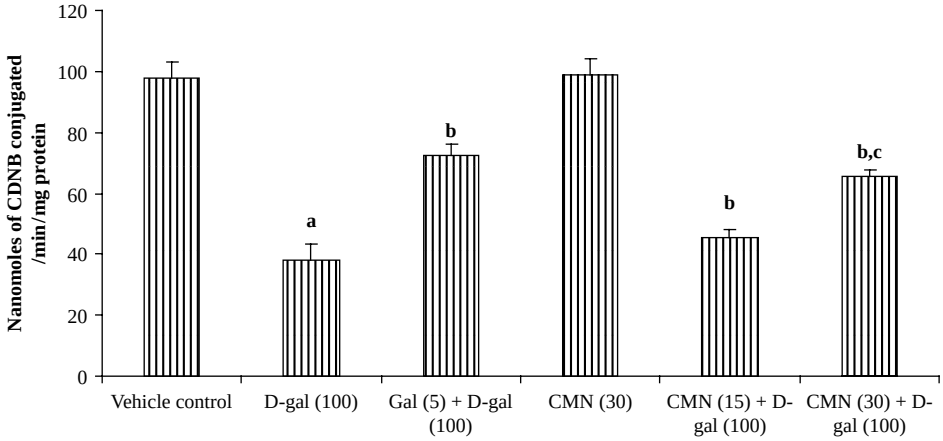

Figure 7. Effect of curcumin (15 and 30 mg/kg, p.o.) on glutathione-*S*-transferase levels in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal-treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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as compared to vehicle control group ($P < 0.05$). Chronic curcumin (15 and 30 mg/kg) treatment and galantamine (5 mg/kg) significantly restored the alterations in mitochondrial enzyme complex activities as compared to the control (D-gal-treated mice; Figure 10). However, chronic curcumin (15 and 30 mg/kg) *per se* treatment did not produce any significant effect on NADH dehydrogenase, succinate dehydrogenase, and MTT ability as compared to vehicle control group.

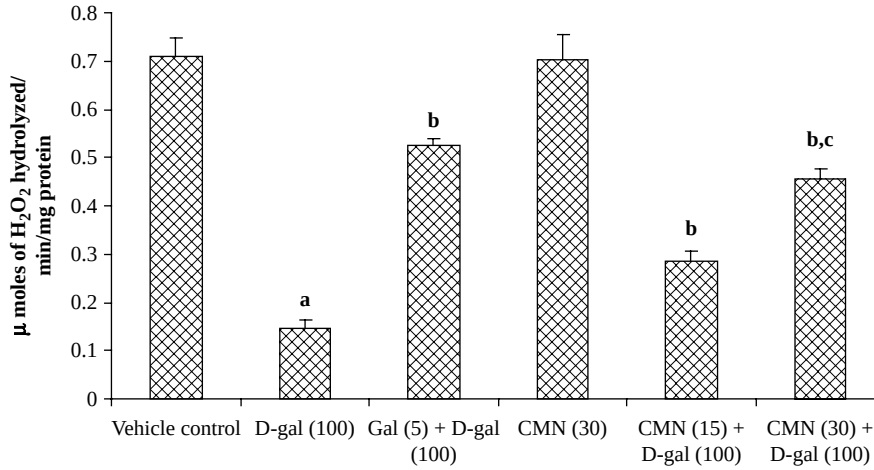

Figure 8. Effect of curcumin (15 and 30 mg/kg, p.o.) on catalase levels in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal-treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group; (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

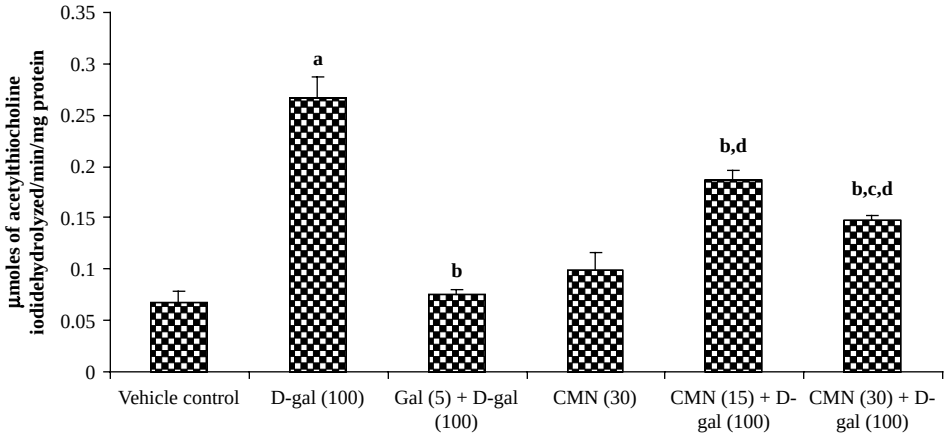

Figure 9. Effect of curcumin (15 and 30 mg/kg, p.o.) on acetylcholinesterase activity in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal-treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group; [d]$P < 0.05$ as compared to Gal (15) + D-gal group (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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3. Discussion

The present study suggested that chronic '**1**' treatment significantly attenuated cognitive dysfunction in D-galactose-induced senescence in mice. '**1**' treatment significantly attenuated oxidative stress and restored mitochondrial enzyme complex activity in D-galactose-induced senescence mice.

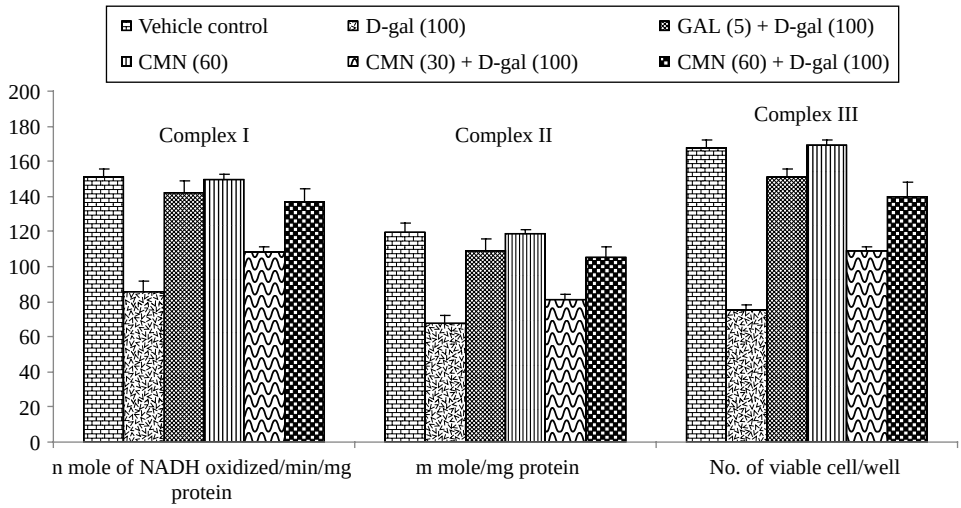

Figure 10. Effect of curcumin (15 and 30 mg/kg, p.o.) on NADH dehydrogenase activity, succinate dehydrogenase activity, and MTT ability in D-galactose-treated mice. Values are mean ± SEM ($n = 12$). [a]$P < 0.05$ as compared to vehicle control group; [b]$P < 0.05$ as compared to D-gal-treated group; [c]$P < 0.05$ as compared to CMN (15) + D-gal group (repeated measures two-way ANOVA followed by Tukey's test for multiple comparisons); D-gal, D-galactose; CMN, curcumin; Gal, galatamine.

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Evidence suggests that free radical generation is involved in the physiological process of aging [11]. The free radical theory of aging suggests that oxygen-free radicals interact with cellular macromolecules that result in cellular senescence and aging. D-Galactose-induced aging was used as an experimental model for studying aging and to design suitable drug strategies against aging. The exact cellular mechanism underlying D-galactose-induced aging has not been well understood so far. Existing data indicate that the oxidative stress that increases oxidants might be one of the main possible reasons [7].

The D-galactose-treated mice have a decreased number of new neurons in the subgranular zone in the dentate gyrus, a reduction of migration of neural progenitor cells, and an increase in the death of newly formed neurons in the granular cell layer [6]. The effect of D-galactose on synaptic morphology has demonstrated a reduction in the density of synapses on the catecholaminergic region and a forebrain cholinergic neuronal loss. So, there is a strong correlation between oxidative stress and reduced density of synapses and neurodegenerative disorders. D-Galactose induces several behavioral and biochemical alterations that are very much similar to the natural aging.

The results of the present study indicated that chronic administration of D-galactose significantly deteriorated spatial memory in both Morris water maze and elevated plus maze task paradigms, suggesting impairment of memory. Poor cognitive performance is a well-known fact in aged population.

In the present study, chronic administration of D-galactose caused significant oxidative stress as indicated by increase in lipid peroxidation, nitrite concentration, and depletion of reduced glutathione level, catalase, SOD, and glutathione-*S*-transferase activity in D-galactose-induced senescence mice suggesting oxidative stress. In our previous report, we have demonstrated that D-galactose produced oxidative damage in aged animals [12,13]. The oxygen metabolism of D-galactose produces many ROS, which could be responsible for impaired learning and memory tasks [14].

Mitochondria contribute significantly to both generation of ROS and their policing within cells due to the failure of mitochondrial electron transport via increase in permeability of mitochondrial transition pore. Therefore, mitochondria might be expected to perturb oxidative damage in cells. Furthermore, potential involvement of the mitochondrial permeability transition was one of possible mechanisms that could also be involved by which mitochondria may be drawn into these death cascades. Mitochondrial decay was shown as a major contributor to aging and age-associated degenerative changes. Antioxidants and mitochondrial nutrients were shown to delay age and ameliorate age-associated diseases in different aging models [15]. The results of the present study indicate that chronic administration of D-galactose results in mitochondrial complex enzyme activities as indicated by decreasing in the NADH dehydrogenase, succinate dehydrogenase activity, and MTT ability. It seems that free radical generation could be responsible for the mitochondrial damage. Our laboratory earlier reported that D-galactose produced mitochondrial dysfunction in the aged brain of mice [12,13]. Present study characterized the protective effect of '**1**' for the first time against D-galactose-induced aging mice.

Since oxidative stress and aging are strongly correlated, agents that modulate ROS may be potentially useful to retard aging process. Chronic administration of '**1**' was found to retard not only the memory dysfunction, but also attenuated oxidative damage induced by chronic D-galactose administration. '**1**' as such did not produce any significant effect on oxidative stress and memory performance, but significantly

attenuated D-galactose-induced oxidative damage and improved memory dysfunction suggesting its potential role in aging process. The effect was comparable to galantamine treatment. '**1**' is well-known antioxidant acting by free radical scavenging action. Not only the parent compound, but also its major metabolite tetrahydrocurcumin is a strong antioxidant and was demonstrated to scavenge free radicals and inhibit lipid peroxidation and formation of hydroperoxides. In the present study, '**1**' treatment significantly attenuated the increased lipid peroxidation, nitrite, restored the reduced glutathione, catalase, glutathione-*S*-transferase, and SOD level on D-galactose-treated animals, suggesting its antioxidant-like property. In earlier studies, we have shown that '**1**' reverses all the oxidative parameters in aluminum-induced neurotoxicity in rats [12]. It was reported that '**1**' restored the decreased level of reduced glutathione in ethanol-intoxicated rats [16]. This lends support to our findings that '**1**' restores the reduced level of glutathione. Besides, reports indicate that '**1**' improves the levels of SOD and catalase in irradiated mice [17]. It seemed that '**1**' produced its potential beneficial effect with virtue of its anti-oxidant effect. Furthermore, our experimental findings revealed that '**1**' markedly restored the mitochondrial respiratory enzymes like NADH dehydrogenase, succinate dehydrogenase activity, and MTT ability in D-galactose-induced senescence mice. '**1**' inhibits iNOS expression and causes free radical scavenging action that might contribute to the restoration of altered mitochondrial complex activity. These results further indicated that neuroprotective effect of '**1**' might involve its potential effect through mitochondrial oxidative pathway. In the present study, D-galactose caused a significant increase in the AChE activities that were probably responsible for cognitive deficit which was significantly attenuated by chronic '**1**' treatment in D-galactose-treated animals. Interestingly, we also found that chronic treatment of galatamine (5 mg/kg) which was a positive control, produced comparable effect as that of chronic '**1**' treatment in D-galactose mice.

The present study highlights that the therapeutic potential of '**1**' against D-galactose induced aging. The present study further provided a hope that '**1**' could be used as an effective adjunct in the treatment of aging and related problems.

4. Materials and methods

4.1 Animals

Male Laca mice (25–30 g), 2–3 months old (Central Animal House, Panjab University, Chandigarh, India) were used. Animals were acclimatized to the laboratory conditions at room temperature prior to the experiment. Animals were kept under standard condition of 12-h light/dark cycle with food and water *ad libitum* in plastic cages with soft bedding. Experiment was performed between 9.00 and 17.00 h. The protocol was approved by the Institutional Animal Ethics Committee and was carried out in accordance with the Indian National Science Academy Guidelines for the use and care of animals.

4.2 Drugs and treatment

Curcumin (Sigma chemicals Co., St Louis, MO, USA) and galatamine (Jannsen Pharmaceutica, NJ, USA), were suspended in 0.25% w/v sodium carboxy-methyl cellulose and administered orally in a dose of 1 ml/100 g body weight. D-Galactose (CDH, India) solution was made freshly at the beginning of each experiment. D-Galactose was dissolved in distilled water for subcutaneous (s.c.) administration. Animals were randomized into 6 groups, 12 animals in each.

Group I. Vehicle control group received 0.25% w/v sodium carboxy-methyl-cellulose.

Group II. Received D-galactose (100 mg/kg) subcutaneously.

Group III. Galatamine (5 mg/kg) + D-

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gal (100)-treated mice.
Group IV. CMN (15 mg/kg) + D-galactose (100 mg/kg)-treated mice.
Group V. CMN (30 mg/kg) + D-galactose (100 mg/kg)-treated mice.
Group VI. CMN (30 mg/kg) *per se*.

The doses of CMN and D-galactose were selected based on our report in the literature [12,13,18]. The study was carried out for a period of 42 days (6 weeks).

4.3 Behavioral assessment

4.3.1 Assessment of cognitive performance

(a) Morris water maze task. The acquisition and retention of memory was evaluated by using Morris water maze [12]. Morris water maze consisted of large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water at 23 ± 1°C). Pool was divided into four equal quadrants with the help of two threads, fixed at right angle to each other. The pool was placed in an illuminated light room among the several colored clues. These external clues were unchanged throughout the experimental period and were used as reference memory. A circular platform (4.5 cm diameter) was placed in one quadrant of the pool, 1 cm above the water level during the acquisition phase. A similar platform was placed 1 cm below the water level for the retention phase. The position of the platform was not changed in any quadrant during assessment of both the phases. Each animal was subjected to four consecutive trials with the gap of 5 min. The mouse was gently placed in the water of the pool between the quadrants facing the wall of pool and allowed 120 s to locate the platform. Then, it was allowed to stay on the platform for 20 s.

Maze acquisition phase (training). Animals received a training session consisting of four trials on day 20. Starting position was different in all the four trials. The time taken by the mouse to reach the visual platform was taken as the IAL. At the end of each trial, mice were returned to their respective home cages.
Maze retention phase (testing for the retention of the learned task). Following 24 h (day 21) and 21 days (day 42) after IAL, mouse was released randomly at one of the edges facing the wall of the pool to assess for memory retention. Time taken by mice to find the hidden platform on days 21 and 42 following the start of D-galactose administration was recorded, termed as first retention latency (1st RL) and second retention latency (2nd RL), respectively.

(b) Elevated plus maze paradigm. The elevated plus maze consists of two opposite white open arms (16 × 5 cm), crossed with two closed walls (16 × 5 cm) with 12 cm high walls. The arms were connected with a central square of dimensions 5 × 5 cm. The entire maze was placed 25 cm high above the ground. Acquisition of memory was tested on day 20. A mouse was placed individually at one end of the open arm facing away from the central square. The time taken by the animal to move from the open arm to the closed arm was recorded as the ITL. Animals were allowed to explore the maze for 10 s after recording ITL. If the animal did not enter the enclosed arm within 90 s, then it was guided to the enclosed arm manually and the ITL was recorded as 90 s. The retention of memory was assessed by placing the mouse in an open arm on day 21 and day 42 of the ITL, termed as the first retention transfer latency (1st RTL) and second retention transfer latency (2nd RTL), respectively [12].

4.3.2 Assessment of gross behavioral activity

Gross behavioral activity was observed at weekly interval. Each animal was placed in a square (30 cm) closed arena equipped

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with infrared light sensitive photocells using digital actophotometer. The animal was observed for a period of 5 min and values were expressed as counts/5 min. The apparatus was placed in a darkened, light, and sound attenuated and ventilated test room [12].

4.4 Mitochondrial complex estimation

4.4.1 Isolation of mice brain mitochondria

The whole brain (excluding cerebellum) was used for mitochondrial isolation. Mice brain mitochondria were isolated by differential centrifugation [13]. The mice brain is homogenized in 10 ml of homogenizing buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, and pH 7.4. The homogenate is brought to 30 ml with the same buffer and centrifuged at 2000*g* for 3 min at 40°C. The pellet is discarded and the supernatant is divided into 2 tubes and centrifuged at 12,000*g* for 10 min. The pellet containing the mixture of synaptosomes and mitochondria is suspended in 10 ml of homogenization buffer containing 0.02% digitonin to lyse the synaptosomes, followed by centrifugation at 12,000*g* for 10 min to pellet down both extra-synaptosomal and intrasynaptosomal mitochondria. The mitochondrial pellet is washed twice in the same buffer without EGTA, BSA, and digitonin.

4.4.2 Complex-I (NADH dehydrogenase activity)

Complex-I was measured spectrophotometrically by the method of King and Howard [19]. The method involves catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome *c*. The reaction mixture contained 0.2 M glycyl glycine buffer of pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer, and 10.5 mM cytochrome *c*. The reaction was initiated by the addition of requisite amount of solubilized mitochondrial sample and followed by absorbance change at 550 nm for 2 min.

4.4.3 Complex-II (succinate dehydrogenase activity)

SDH was measured spectrophotometrically according to King [20]. The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer of pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and the absorbance change was followed at 420 nm for 2 min.

4.4.4 Complex-III (MTT ability)

The MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria by the method of Liu *et al.* [21]. Briefly, 100 µl of mitochondrial samples were incubated with 10 µl of MTT for 3 h at 37°C. The blue formazan crystals were solubilized with dimethylsulfoxide and measured by an ELISA reader at 580 nm filter.

4.5 Biochemical assessment

Biochemical tests were conducted 24 h after last behavioral test. The animals were sacrificed by decapitation. Brains were removed and rinsed with ice-cold isotonic saline. Brains were then homogenized with ice-cold 0.1 mmol/l phosphate buffer (pH 7.4). The homogenates (10% w/v) were then centrifuged at 10,000*g* for 15 min, and the supernatant was used for the biochemical estimations.

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4.5.1 Measurement of lipid peroxidation

The extent of lipid peroxidation in the brain was determined quantitatively by performing the method as described by Wills [22]. The amount of MDA was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer Lambda 20 spectrophotometer. The values were calculated using the molar extinction co-efficient of chromophore (1.56×10^5 - $(\text{mol/l})^{-1}\,\text{cm}^{-1}$).

4.5.2 Estimation of nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide, was determined by a colorimetric assay with Greiss reagent (0.1% *N*-(1-napththyl) ethylene diamine dihydrochloride, 1% sulphanilamide, and 5% phosphoric acid) [23]. Equal volumes of the supernatant and the Greiss reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using Perkin Elmer Lambda 20 spectrophotometer. The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve.

4.5.3 Estimation of reduced glutathione

Reduced glutathione was estimated according to the method described by Ellman *et al.* [24]. One milliliter of supernatant was precipitated with 1 ml of 4% sulphosalicylic acid and cold digested for 1 h at 4°C. The samples were then centrifuged at 1200*g* for 15 min at 4°C. To 1 ml of the supernatant obtained, 2.7 ml of phosphate buffer (0.1 mmol/l, pH 8) and 0.2 ml of 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB) were added. The yellow color developed was measured at 412 nm using Perkin Elmer Lambda 20 spectrophotometer. Results were calculated using molar extinction coefficient of the chromophore ($1.36 \times 10^4\,(\text{mol/l})^{-1}\,\text{cm}^{-1}$).

4.5.4 SOD activity

SOD activity was assayed by the method of Kono [25]. The assay system consists of EDTA 0.1 mM, sodium carbonate 50 mM, and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml of hydroxylamine, and 0.05 ml of the supernatant were added and auto-oxidation of hydroxylamine was measured for 2 min at 30 s interval by measuring absorbance at 560 nm using Perkin Elmer Lambda 20 spectrophotometer.

4.5.5 Catalase activity

Catalase activity was assessed by the method of Luck [26], wherein the break-down of H_2O_2 is measured. Briefly, the assay mixture consists of 3 ml of H_2O_2 phosphate buffer and 0.05 ml of the supernatant of the tissue homogenate. The change in absorbance was recorded for 2 min at 30 s interval at 240 nm using Perkin Elmer Lambda 20 spectrophotometer. The results were expressed as micromoles of hydrogen peroxide decomposed per minute per milligram of protein.

4.5.6 Glutathione-S-transferase activity

The activity of glutathione transferase was assayed by the method of Habig and Jakoby [27]. Briefly, the assay mixture consisted of 2.7 ml of phosphate buffer, 0.1 ml of reduced glutathione, 0.1 ml of 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and 0.1 ml of supernatant. The increase in the absorbance was recorded at 340 nm for 5 min at 1 min interval using Perkin Elmer Lambda 20 spectrophotometer. The results were expressed as nanomoles of CDNB conjugated per minute per milligram of protein.

4.5.7 Estimation of AChE activity

AChE activity is a marker of loss of cholinergic neurons in the forebrain. The AChE activity was assessed by Ellman

method [28]. The assay mixture contained 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide, and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 s interval at 412 nm using Perkin Elmer Lambda 20 spectrophotometer. Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed per minute per milligram of protein.

4.5.8 Protein estimation

The protein content was estimated by Biuret method [29] using bovine serum albumin as a standard.

4.6 Statistical analysis

Values are expressed as mean ± SEM. The behavioral assessment data were analyzed by a repeated measures two-way analysis of variance (ANOVA) with drug-treated groups as between-subjects factors and sessions as the within-subjects factors. The biochemical estimations were separately analyzed by one-way ANOVA. *Post hoc* comparisons between groups were made using Tukey's test. $P < 0.05$ was considered significant.

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