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Original Article

Evaluate neuroprotective effect of silibinin using chronic unpredictable stress (cus) model

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Abstract: Chronic unpredictable stressors can produce a situation similar to clinical depression, and such animal models can be used for the preclinical evaluation of antidepressants. Many findings have shown that the levels of proinflammatory cytokines (e.g., TNF-α) and oxidative stress (increased lipid peroxidation, decreased glutathione levels, and endogenous antioxidant enzyme activities) are increased in patients with depression. Silibinin is the major active constituent of silymarin, a standardized extract of the milk thistle seeds, containing a mixture of flavonolignans consisting of silibinin, isosilibinin, silicristin, silidianin and exhibit antioxidant activity. Objectives The present study was designed to investigate the effect of silibinin on unpredictable chronic stressinduce behavioral and biochemical alterations in mice. Methods: Mice were subjected to different stress paradigms daily for a period of 45 days to induce depressive like behavior such as memory acquisition, and retention. Results: Chronic treatment with silibinin significantly reversed the unpredictable chronic stress-induced behavioral (improve memory function), biochemical changes (decreased glutathione levels, superoxide dismutas), and inflammation surge (serum TNF-α IL 1β) in stressed mice. Conclusion: The study revealed that silibinin exerted effects in behavioral despair paradigm in chronically stressed mice, specifically by modulating central oxidative stress and inflammation.

Keywords: Stress, silibinin, TNF-α, unpredictable chronic stress, antioxidants

Introduction

Memory impairment is a common and usual co morbidity associated with prolonged stress [1]. Chronic stress is known to influence cognitive performance in various psychiatric patients [2]. Chronic stress increases corticosterone secretion, which causes dysregulation of hypothalamic-pituitary-adrenocortical (HPA) axis and impairment of hippocampus-dependent learning and memory processes [3]. Secretion of corticosterone also triggers an increase in oxidative stress that ultimately leads to memory deficits [4]. These physiological consequences of stress depend on the intensity and duration of the stressor and on how an organism perceives and reacts to the noxious stimulus. Therefore, chronic unpredictable stress (CUS) model has been standardized to study the development and progress of stress and related problems [5]. Degeneration of cholinergic neurons is one of the major hallmarks in the brain of cognitive deficit patient [6]. Along with this, study report also suggest that neuronal functions are altered by generation of reactive oxygen species which leads to oxidative stress; a prominent feature in the pathogenesis of cognitive dysfunction [7]. Various antioxidants have been tried for their effectiveness in reducing deleterious effects on neurons due to oxidative stress [8]. Dietary and medicinal phyto-antioxidants are being used as an adjuvant therapy to limit their side effects and increase their effectiveness. Silibinin (INN), also known as silybin (both from *Silybum*, the generic name of the plant from which it is extracted), is the major active constituent of silymarin, a standardized extract of the milk thistle seeds, and has been extensively studied for its antioxidant [9, 10], anti-inflammatory and neuroprotective activities. Few studies showed inhibition of cognitive impairment against Aβ amyloid induced toxicity by treatment of Silybins [11]. These reported pharmacological properties of silibinin clearly suggest its beneficial role against stress induced cognitive impairment. In light of these

reports, the present study aims to investigate the protective effect of silibinin against chronic unpredictable stress induced cognitive deficits and oxidative damage in mice.

Materials and methods

Animals

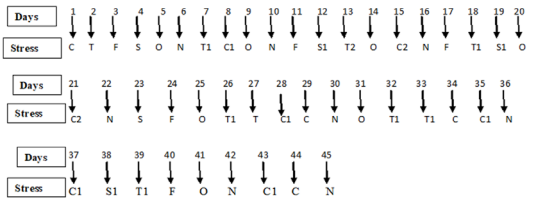
Three-month-old male Swiss albino mice (20-30 g) bred at Central Animal House (CAH), Sicra, Hyderabad, were used. They were housed (six mice per cage) under standard (25± 2°C, 60-70% humidity) laboratory conditions, maintained on a 12-h natural day-night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the test. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) and conducted according to the CPCSEA guidelines on the use and care of experimental animals (1821/PO/Re/S/15/CPCSEA).

Drugs

Silibini was purchased from Yarrow chem. pvt. Ltd (Mumbai, Maharastra, India www.yarrow-pharma.com). Silibinin was administered daily for 40 days by oral gavage.

Experimental procedure for neuroprotective effect of silibinin on chronic unpredictable stress (CUS) model

Mice were exposed to a random pattern of mild stressors [12] daily for 45 days. The order of various stressors used in the present study is depicted below:



C - Cold swim (8 G, 5 min); T - Tail pinch (1 min); F - Food and water deprivation (24 h); S - Swimming at room temperature (24±2 G, 20 min); O - Overnight illumination; N - No stress; T1 - Tail pinch (1.5 min); C1 - Cold swim (10 G, 5 min); S1 - Swimming at room temperature (24±2 G, 15 min); T2 - Tail pinch (2 min); C2 - Cold swi m (6 G, 5 min).

Drug treatment

Randomly divided into eight experimental groups (n = 6-8). First and second group was named as normal and control (CUS) group respectively. Silibinin (0.01, 0.1 and 1 mg/kg, p.o.) were treated as group 3-5 respectively. Silibinin was prepared in 1% of Sodium CMC and administered orally on the basis of body weight (1 ml/100 g).

Solutions were made fresh at the beginning of each day of the drug treatment. Drugs were administered daily 30 minutes before CUS procedure (described in material and methods) for 40 days. The entire study was conducted in multiple phases.

Twenty four hour after the last treatment, all the animals were euthanized by cervical dislocation and the brain was dissected out from the cranial cavity. The brain was washed in 0.9% NaCl solution and kept in an ice cold PBS (pH 7.4) in a petriplate and was minced into small pieces. It was further homogenized immediately in Teflon homogenizer under the cold condition and cold centrifuged at 4°C to obtain 10% w/v brain tissue homogenate was subjected for estimation of total protein, reduced glutathione (GSH) [13], dismutase (SOD) [14], inflammatory mediators such as TNF α, IL 1β [15] and acetyl choline esterase (AchE) [16] and also performed behavioral pattern of mice.

Behavioral assessments

Elevated plus maze paradigm: The elevated plus maze (EPM) consists of two opposite black open arms, crossed with two closed walls of the same dimensions of 12 cm height. The arms were connected with a central square of dimensions 5 × 5 cm. The entire maze was elevated to a height of 25 cm from the floor. Acquisition and retention of memory processes were assessed as previously described [17] (Sharma and Kulkarni, 1992). Acquisition of memory was tested on day 20 of CUS procedure. Animal 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 initial transfer latency (ITL). Animal was allowed to explore the maze for 20 sec after recording the ITL and then returned to the home cage. If the animal could not enter

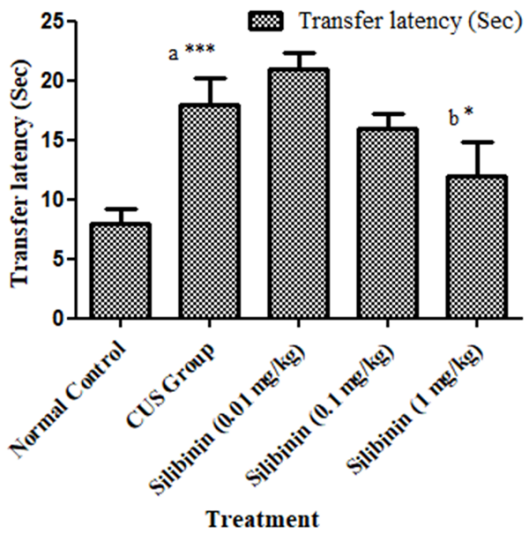


Figure 1. Effect of silibinin on transfer latency on Plus maze. All values are expressed as Mean ± SEM. a denotes significance (***P<0.001), difference between CUS group Vs normal control. b denotes significance (*P<0.05) difference between silibinin (1 mg/kg bd. wt.) Vs CUS group.

Table 1. Effect of silibinin on swimming time in the target quadrant on Morris water maze

S.NO	Treatment	Swimming time in the target quadrant (Sec)
1	Normal Control	80±5.2
2	CUS Group	42±6.2^{**,a}
3	Silibinin (0.01 mg/kg)	52±4.3
4	Silibinin (0.1 mg/kg)	56±3.2
5	Silibinin (1 mg/kg)	67±1.8^{*,b}

All values are expressed in Mean ± SEM. CUS group significantly decrease in Swimming time in the target quadrant (**p<0.01), compared with normal control. Treatment with silibinin (1 mg/kg bd. wt.) significantly increase (*p<0.05), compared to CUS group.

closed arm within 90 sec, it was guided to the closed arm and ITL was given as 90 sec. Retention of memory was assessed on day 37 as first retention transfer latency (1st RTL) and on day 38 as the second retention transfer latency (2nd RTL) respectively, upto 40th day.

Morris water-maze test

Morris water-maze apparatus (MWM) is most commonly used model to test spatial memory [18]. The MWM procedure is based on the principle that animal dislikes swimming and hence when placed in a large pool of water its tendency is to escape it by searching for a platform. MWM consists of large circular pool (90 cm in diameter, 40 cm in height). The tank was divided into four equal quadrants. A submerged platform (10 cm in diameter and 26 cm high), painted white was placed in the middle of the target quadrant of this pool, 1 cm, below surface of water. The position of platform was kept unaltered throughout the training session. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the mice for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for four consecutive days from day 37-40. The mice received daily four consecutive training trials, with each trial having a ceiling time of 120 sec. For each trial, individual mouse was gently put into the water at one of four starting positions, the sequence of which being selected randomly and allowed 120 sec to locate submerged platform. Then, it was allowed to stay on the platform for 20 sec. If animal failed to find the platform within 120 sec, it was guided gently onto platform and allowed to remain there for 20 sec. Acquisition trial - Each mouse was subjected to four trials on each day (starting from day 37-40). A rest period of 1 hour was allowed in between each trial. Four trials per day were repeated for four consecutive days. Starting position on each day to conduct four acquisition trials was changed as described below and acquisition trials. Q4 was maintained as target quadrant in all.

Mean escape latency time (ELT) calculated for each day during acquisition trials was used as an index of acquisition.

Retrieval trial - On day 39, the platform was removed. Animal was placed in water maze and allowed to explore the maze for 120 sec. Mean time spent in the target quadrant, i.e. Q4 in search of missing platform provided an index of retrieval. Care was taken that relative location of water maze with respect to prominent visual clues was not disturbed during the total duration of study.

Y-maze task

Y-maze task is frequently used in monitoring spatial learning. Animals were allowed to learn

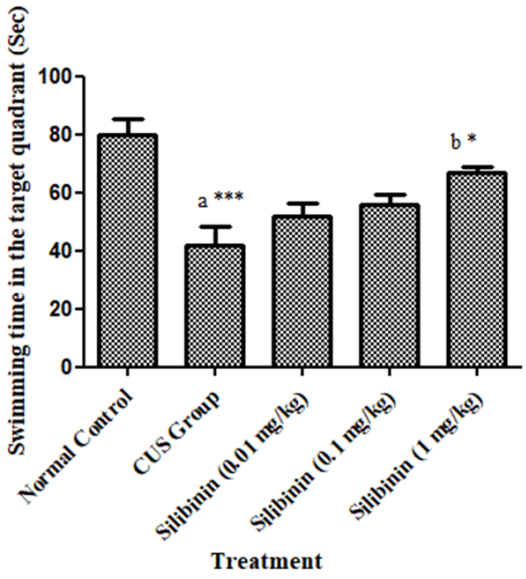


Figure 2. Effect of silibin on swimming time in the target quadrant of Morris water maze. All values are expressed as Mean ± SEM. a denotes significance (**P<0.001), difference between CUS group Vs normal control. b denotes significance (*P<0.05) difference between silibinin (1 mg/kg bd. wt.) Vs CUS group.

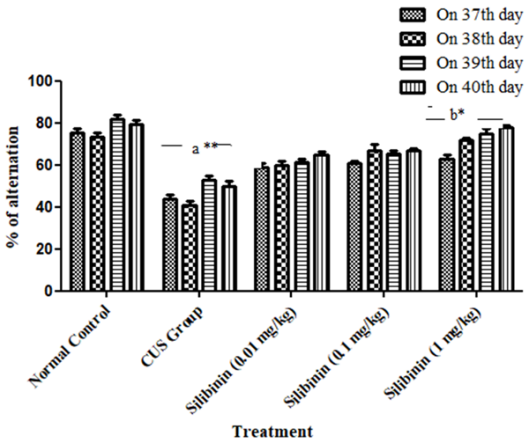


Figure 3. Effect of silibinin on memory function using Y-maze. All values are expressed as Mean ± SEM. a denotes significance (***P<0.01), difference between CUS group Vs normal control. b denotes significance (*P<0.05) difference between silibinin (1 mg/kg bd. wt.) Vs CUS group.

alternation between arms based on their memory of the previously visited arms. The experimental apparatus consisted of a white-painted Y-maze that is made from acryl. Each arm of the Y-maze was 30 cm long, 14 cm high, and 8 cm wide, and it is positioned at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 5 min session. Spontaneous alteration behavior was defined as the consecutive entry into all three arms in overlapping triplet sets. During each trial, spontaneous alternations were recorded. The percentage (%) of spontaneous alternation behavior was determined by dividing the total number of alternations by the total number of arm entries, subtracting 2, and then multiplying by 100 according to the following equation: % alternation = [(number of alternations)/(total number of arm entries-2)] × 100. One hour before the test, mice were orally administrated with vehicle. Acquisition trial - Each mouse was subjected to four trials on each day (starting from day 37-40). Silibinin (0.01, 0.1 and 1 mg/kg), and 30 min later, the mice were injected with vehicle. The Y-maze arms were thoroughly cleaned in between tests to remove residual odors.

Statistical analysis

All data are expressed as the means ± SEM. Statistical differences among the experimental groups were tested by using a one way analysis of variance (ANOVA) and Dunnet test was employed for multiple comparisons. *P*-values less than 0.05 were accepted as significant.

Results

Effect of silibinin on initial transfer latency in elevated plus maze test

Initial transfer latency was significantly increased in chronically stressed mice as compared to control mice (8±1.2 sec to 28±2.2 sec; ***P<0.001). Treatment with silibinin significantly and dose dependently decreased initial transfer latency in mice (silibinin (1 mg/kg 28± 2.2 sec to 12±2.8 sec; *P<0.05) when compared to CUS group (**Figure 1**).

Effect of silibin on swimming time in the target quadrant of Morris water maze

Time to reach target quadrant was significantly decreased in chronically stressed mice as compared to control mice (80±5.22 sec to 42±6.2 sec; **P<0.01) (**Table 1**; **Figure 1**). Treatment with silibinin significantly and dose dependently decreased time reach to target quadrant in mice silibinin (1 mg/kg) (42±6.2 sec to 67±1.8

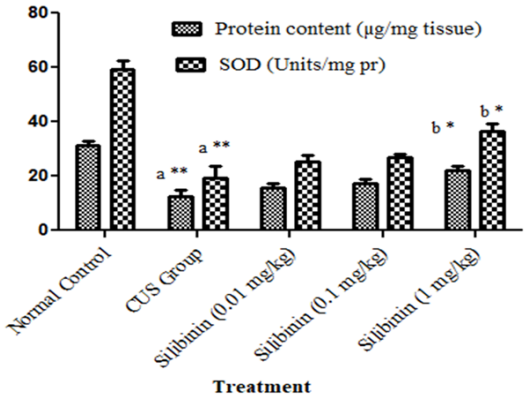


Figure 4. Effect of silibinin on brain protein content and SOD parameters against CUS rats. All values are expressed in Mean ± SEM. a denotes significant (a **P<0.01) difference between Normal Control vs CUS group. b denotes significant (b **P<0.05) difference between treatment vs CUS group.

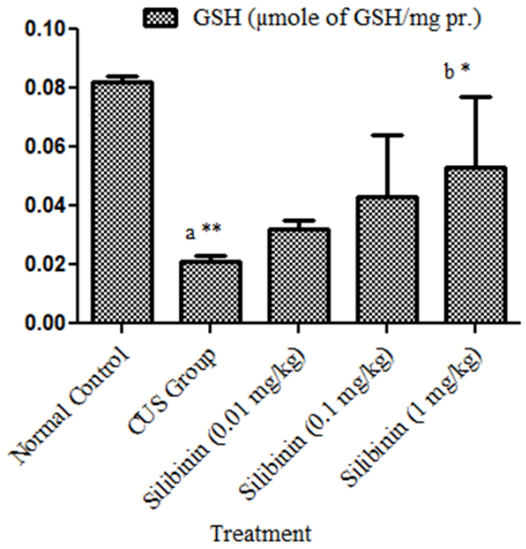


Figure 5. Effect of silibinin on brain GSH against CUS rats. All values are expressed in Mean ± SEM. a denotes significance (**P<0.01), difference between CUS group Vs normal control. b denotes significance (*P<0.05) difference between silibinin (1 mg/kg bd. wt.) Vs CUS group.

sec; *P<0.05) when compared to CUS group (**Figure 2**).

Effect of silibinin on % of alteration on 40th day on Y-maze task

Percentage of alteration was significantly decreased in chronically stressed mice as compared to control mice (79.4±2.1% to 50±2.2%; **P<0.01) (**Table 1**; **Figure 1**). Treatment with silibinin significantly and dose dependently decreased percentage of alteration in mice. The efficacy of silibinin (1 mg/kg) was comparable to that of CUS group. Silibinin (1 mg/kg) administration to unstressed mice show significant effect on percentage of alteration latency (50±2.2% to 77.7±1%; *P<0.05) (**Figure 3**).

Effect of silibinin on brain biochemical parameters against CUS rats

Brain protein content was significantly decreased in CUS group (31.3±1.5 µg/mg to 12.6±2.1 µg/mg; **P<0.01) compare to normal control. Treatment with silibinin significantly and dose dependently increased protein content in mice (silibinin 1 mg/kg) (12.6±2.1 µg/mg to 22.1±1.5 µg/mg; *P<0.05) when compared to CUS group.

Brain SOD levels was significantly decreased in CUS group (59.43±3.2 U/mg to 19.32±4.2 U/mg; ***P<0.01) compare to normal control. Treatment with silibinin significantly and dose dependently increased SOD activity in mice CUS group (silibinin 1 mg/kg 19.32±4.2 U/mg to 36.41±2.9 U/mg; *P<0.05) when compared to CUS group (**Figure 4**).

Brain GSH activity was significantly decreased in CUS group (0.082±0.002 µ.mol to 0.021±0.002 µ.mol; **P<0.01) compare to normal control. Treatment with silibinin significantly and dose dependently increased GSH activity in mice (silibinin 1 mg/kg 12.6±2.1 µ.mol to 22.1±1.5 µ.mol; *P<0.05) when compared to CUS group (**Figure 5**).

Brain AchE activity was significantly increased in CUS group (0.02±0.02 µ.mol to 0.92±0.03 µ.mol; **P<0.01) compare to normal control. Treatment with silibinin significantly and dose dependently increased AchEactivity in mice (silibinin 1 mg/kg 0.92±0.03 µ.mol to 0.23±0.02 µ.mol; *P<0.05) when compared to CUS group (**Figure 6**).

Effect of silibinin on brain TNF alfa and IL 1 beta in CUS rats

Brain inflammatory mediator levels such as TNF α (1400±14.3 pg/gr tissue to 17545±32.4 pg/gr tissue; ***P<0.001) and IL 1β (3400±41.5 pg/gr tissue to 43292±23.6 pg/gr tissue; ***P<0.001) was significantly increased in CUS group compare to normal con-

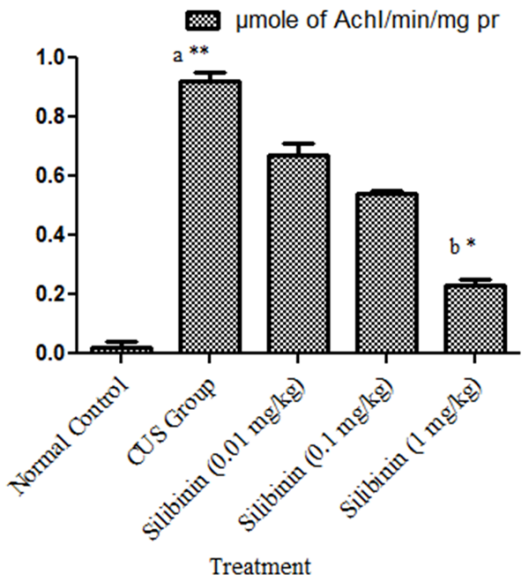


Figure 6. Effect of silibinin on brain Acetyl choline esterase activity against CUS rats. All values are expressed in Mean ± SEM. a denotes significance (**P<0.01), difference between CUS group Vs normal control. b denotes significance (*P<0.05) difference between silibinin (1 mg/kg bd. wt.) Vs CUS group.

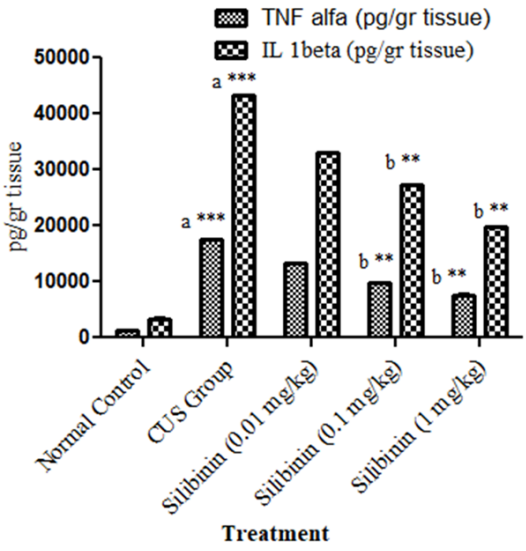


Figure 7. Effect of silibinin on TNF alfa and IL 1 beta against CUS rats. All values are expressed in Mean ± SEM. a denotes significant (***P<0.001) difference between Normal Control vs CUS group. b denotes significant (**P<0.05) difference between treatment vs CUS group.

trol. Treatment with silibinin significantly and dose dependently decreased TNF α (17545± 32.4 pg/gr tissue to 7654±19.8 pg/gr tissue; ***P<0.001) and IL 1β (43292±23.6 pg/gr tissue to 19843±21.7 pg/gr tissue; ***P<0.05) in mice (silibinin 1 mg/kg 0.92±0.03 pg/gr tissue to 0.23±0.02 pg/gr tissue; **P<0.05) when compared to CUS group (**Figure 7**).

Discussion

There seem to be a complex relationship between stressful situations, mind and body's reaction to stress, and the onset of cognitive disturbances [19]. Chronic administration of various uncontrollable stresses, a procedure known as chronic unpredictable stress, is generally thought to be the most reliable and valuable experimental model to study stress pathology [20]. Chronic unpredictable stress (CUS) has been shown to influence brain regions which play a critical role in spatial navigation and memory [21]. Thus in the present study, silibinin has been tried as a drug strategy against chronic unpredictable stress induced oxidative damage and cognitive deficits in mice.

In the present study, memory performance was evaluated by Morris water maze (MWM), Y Maze (YM) as well as elevated plus maze (EPM). Though elevated plus maze test is primarily used for anxiety, it can also be employed as an experimental model for evaluation of long term memory in rodents [17]. In the present study, chronic unpredictable stress resulted in a significant impairment of cognitive performance in MWM, YM and EPM tests as compared to normal mice. These results are consistent with the previous finding [22].

Silibinin treatment for 40 days significantly improved cognitive performance in MWM, YM and EPM indicating its therapeutic potential against chronic stress induced memory impairment. The results are in accordance with previous studies by Rinwa P *et al.* [23], which showed a significant decrease in cognitive function CUS mice. Further, Piperine in a dose dependent manner significantly restored the cognitive function in chronic unpredictable strss mice. Hippocampus has been well known to play a key role in spatial learning and memory [24]. Since hippocampus has abundant inputs from the basal forebrain cholinergic system and thus acetylcholine (ACh) plays a crucial role in learning and memory [25]. Acetylcholine is degraded by the enzyme acetylcholinesterase, terminating the physiological action of the

neurotransmitter. Cognitive dysfunction affects cholinergic system resulting in increased activity of acetylcholinesterase [26]. Stress has been well documented to induce alterations in acetylcholinesterase enzyme activity [27]. In the present study, CUS caused a significant increase in acetylcholinesterase activity leading to memory deficits, but later was significantly attenuated by chronic silibinin treatment, implicating its role in cholinergic transmission processes.

Disclosure of conflict of interest

None.

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