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Ganoderma lucidum Ameliorates Spatial Memory and Memory-Related Protein Markers in Hypercholesterolemic and Alzheimer's Disease Model Rats

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Abstract

***Ganoderma lucidum* has been hailed as medicinal mushroom. Its effect on memory and learning related behavioral performance along with related protein markers has been evaluated using Alzheimer's disease (AD) and hypercholesterolemic model rats in the present study. Alzheimer's disease (AD) model rats were prepared infusing amyloid beta peptide into the right ventricles of the rats. Hypercholesterolemia was evoked feeding 1% cholesterol and 1% cholic acid with basal diet of the rats for 8 weeks. Hot water extract of *G. lucidum* was ingested orally (200 mg/kg bw) to the AD model rats. Memory and learning related behavioral tests were performed using Barnes maze while protein markers (BDNF, SNAP2, PSD-95, VAchT) were detected using ELISA. Observed findings suggest improved cognitive performance of the *Ganoderma lucidum* fed rats. Memory and learning related protein markers also substantiate this fruition. Thus, therapeutic potentiality of *Ganoderma lucidum* in AD amelioration seems promising.**

Keywords: Alzheimer's disease; Ganoderma lucidum; Hypercholesterolemia; Memory and learning; Protein markers of memory and learning

1. Introduction

Alzheimer's disease (AD) is neuro-degenerative and behavioral disorder affecting mainly the elderly people. People afflicted with AD suffer from declined memory and learning abilities due to the neuronal degeneration in the brain region associated with memory and learning [1]. Neurons of the AD patients become damaged due to the formation of amyloid beta (Aβ) plaques and/or neurofibrillary tangles (NFTs) [1]. As a consequence, levels of neurotransmitters become altered [2]. AD patients become confused about time and space, faces problem in planning and executing even errands [3]. At advanced stage, the AD patients suffer from difficulties in speaking and writing, sleeping and awakening and even cannot recall their own names [4].

They also face problem in remembering their personal history, recognizing relatives and family members [3]. They become solely dependent on the care-givers and ultimately bed-bound [4].

Currently, more than 40 million people are suffering with AD round the globe and the alarmingly increased rate of this fatal disorder adds extra burden to the ever increasing economical and societal sectors and demands immediate medico-healthcare oriented management [4]. Despite tremendous efforts in combating this global epidemic, there is hardly any AD medication available up to present date. The available therapeutic strategies are aimed at symptom - modifying targets rather than preventing the neurons from damages [5,6].

For example, the six drugs approved by the United States Food and Drug Administration (USFDA) can improve AD symptoms only through modulating brain neurotransmitter release [5,6]. The failure to achieve the ultimate goal in slowing AD progression might remain hidden into multiple causative factors encompassing oxidative stress (OS), hypercholesterolemia, hypertension, genetics, epigenetics as well as adaptive response to some stressors. Thus, effective management of the co-existing factors of AD has been highly regarded in the most recent recommendation from the Alzheimer's association [4].

Most of the AD modifying drugs have been suggested to be applied in the early stages rather than at the lower stages of AD development [5,6]. Currently available imaging and neurochemical AD biomarkers require invasive procedures and search for less invasive, reliable and easy-to-handle marker is badly needed [7]. In this context, early detection of protein bio-markers would be of immense prognostic importance in AD therapeutics.

Medicinal mushroomy *Ganoderma lucidum* beacons excellent in this context as it abounds with bioactive myconutrients [8-10]. Content of more than 400 gano-components had accredited *Ganoderma* as the "fungal biofactory", "panacea" and the "elixir of life" [11-13]. Medicinal importance of this mushroom include their role as, but not limited to, antioxidant, anti-inflammatory, antitumor, anticancer, antimicrobial, immunomodulatory and hepatoprotective agent [12-14]. Gano-components conferring medicinal values range from polysaccharides to triterpenes, sterols, proteins, peptides, fatty acids and vitamins [14]. Our previous studies have also found anti-oxidative, hypocholesterolemic and memory enhancing effect of the hot water extract (HWE) of *G. lucidum* [15-17].

Recently, its physostigmine like effect on brain acetylcholinesterase (AchE) activity led memory enhancing behavioral effect has been reported [18]. However, there is hardly any study featuring combined behavioral and neurotransmitter ameliorating effect of *G. lucidum* upon AD subjects. Thus, the present study has been designed to determine the AD ameliorating effect of this mushroom through memory related learning and behavioral tests and protein marker assessments using AD model rats.

2. Materials and Methods

2.1 Sample preparation

Purchased from Bangladesh National Mushroom Development Institute, the fruiting bodies of *G. lucidum* were powdered and boiled in distilled water at the ratio of 1: 20 (w/v) for 45 min. Cooling was followed by removal of the boiled mushrooms using Whatman No. 1 filter paper. Then, the hot water extracts (HWE) of *G. lucidum* were obtained using freeze-dryer (Labconco).

2.2 Animals

Ninety wistar male rats were divided into six groups, each group containing 15 rats, based on their weight range (120 ± 5 g). The groups were control (C), *G. lucidum* HWE fed control (CE), hypercholesterolemic (H), *G. lucidum* HWE fed hypercholesterolemic (HE), Alzheimer's diseased (A) and *G. lucidum* HWE fed Alzheimer's diseased (AE). The AE group received 200 mg/kg body weight *G. lucidum* HWE. All the experimental protocols had been approved by the ethical permission committee, Jahangirnagar University Animal Care and Use Committee (JUACUC) [Ethics reference no. JU/25/04/2018/MAR (R)].

2.3 Preparation of AD model rats

Male wistar rats were prepared by injecting Aβ1-42 (ab120959, abcam, USA) in the right cerebral ventricles following the method of Abdullah et al. [19].

2.4 Memory and learning related behavioral tests

Evolutionarily, humans and rodents have very close root of origin and they share much common behavioral and survival strategies. Thus, behavioral studies on rodents exemplify human behavioral traits whose analyses aid greatly in identifying and managing pathophysiological alterations as well for establishing therapeutic approaches. Animal models of behavioral abnormalities, especially those for neurodegenerative diseases such as AD have been in vogue for the last few decades. Capacity of an animal to orient itself in its environment is essential for its existence as its feeding, mating and escaping from the enemies depend on this feature. Interestingly, orientation is an important criterion of spatial memory and AD patients gradually suffer from disorientation of time and place. At extreme cases, the AD patients cannot even locate their own houses though they had been living there since their birth. This manifests the spatial memory perturbation of the AD subjects. In memory and learning related behavior tests, spatial performance of experimental animals are studied using different types of mazes. In the present study, behavioral tests for spatial memory and learning were evaluated using the Barnes maze (BM) [20]. The principal of this test is based on the innate preference of the rodents to hide in the dark, enclosed space over illuminated open field. A stainless steel Barnes maze (BM) of 150 cm diameter was used [FIG. 1].

FIG 1. **Barnes Maze Test.**

It contained 18 holes, each having a diameter of 10.5 cm. One of the holes contained the dark escape box. The maze top has been placed on a metal stand and elevated 100 cm above the floor. Intra- and inter-maze cues of different types such as colored paper-shapes (round, square and triangle) had been placed as the landmarks for cognition and spatial memory. The surface of the maze had been brightly illuminated using flush (120 W) light based on the principle that the rodents tend to hide in the dark corner as compared to the illuminated open center of a circular surface. In Barnes maze, spatial learning and memory was measured based on the rodents' ability to learn and remember the location of the hidden escape box using the visual cues. After experimentation with each rat, the target hole and the whole maze were washed with 70% ethanol. All the sessions were recorded using video camera (HDR CX130E, Sony, Japan) and Arcsoft showbiz software and the video files were tracked with the tracking software kinovea.

2.5 Barnes maze experimentation

Every rat underwent three phases of experimentation: habituation, spatial acquisition training and probe (short- and long-term retention of reference memory) trial.

a. Habituation (1 day)

Each rat was allowed to become familiar with the BM environment and strategize the way to enter into the escape hole. Each rat kept in the 5 L transparent glass beaker was freed onto the center of the maze and let them explore the circular field. While exploration, they searched for and entered into the escape hole. Each rat was allowed exploration period of 5 min. When some rats were not finding the target hole, they were guided by gently nudging the tail. After completion of the 1-session habituation for all the rats, they were returned to their home cage.

b. Spatial acquisition phase (4 days)

Rats underwent 4 days of acquisition training with three trials per day. For every trial, each rat was placed through the beaker at the center of the maze. The direction of their placement (north, south, east and/or west) was random to allow them to avoid a fixed motor response to locate the escape hole. After entering into the escape hole, each rat was allowed to remain there for 30s and then returned to the holding cage. After each trial, the maze surface and escape box were cleaned with 70% ethanol. At the end of each trial, the maze was rotated 90º to the left or right to ensure the varied spatial position (hole no. 4, 8, 12 or 16) of the escape hole. For each trial, latency (time to find and the escape hole) and distance traveled were recorded by the tracking software kinovea. Errors (occurring from the situation when a rat dipped its head into a hole lacking the escape box) were also measured. Hole searching strategies (random, serial or spatial) of the rats were also evaluated. When a rat searched the holes in a haphazard fashion (moving here and there over the center, unorganized movement through the center, repeated entry into the center, repeated searching in the already searched region), its searching was termed as random [FIG. 2]. In addition to this unsystematic approach, failure to enter the escape hole within the assigned time (5 min) was also considered as random searching [FIG. 2]. In case of serial searching, a rat moved around the edge of the maze serially (clockwise or anti-clockwise) and/or made errors at adjacent holes before finding the escape hole [FIG. 2]. When some rats moved around the edge of the maze throughout the entire time frame but did not make any error before finding the escape hole, their search strategies were also classified as serial [FIG. 2]. Spatial search was identified when a rat directly entered into the escape hole

from the center of the maze and/or when a rat initially missed the escape hole but found it just after visiting any one hole [FIG. 1,2].

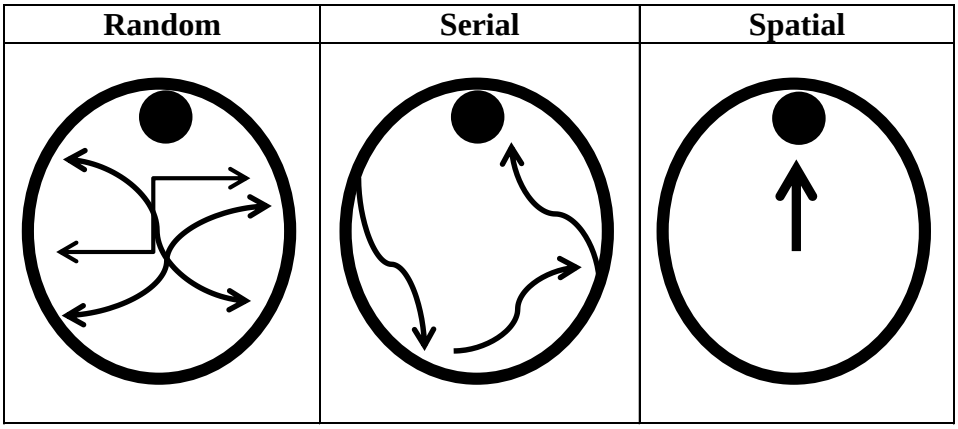

FIG 2. **Escape Strategies of the Rats.**

c. Probe (short- and long-term retention of reference memory) trial

The probe trial is usually performed to determine whether the animal remembers the location of its goal (here, the escape hole). One day following the last acquisition training day, the escape box was removed and the maze was divided into four equal quadrants (target, opposite, positive and negative). Each rat was to explore the maze for 2 min and time spent in each quadrant along with the number of holes searched was measured.

2.6 Memory and learning related marker assays

a. $A\beta_{(1-42)}$ oligomers

The levels of $A\beta_{(1-42)}$ oligomers in detergent soluble fraction (DSF) of hippocampus homogenates and brain derived neurotrophic factor (BDNF), synaptosomal associated protein 25 KD (SNAP 25), post-synaptic density protein 95 KD (PSD 95), vesicular acetylcholine transporter (VAchT) and tumor necrosis factor α (TNFα) in the cytosolic fractions of the rat hippocampus homogenates were detected utilizing sandwich ELISA. Briefly, ELISA microplates (Cellstar, Greiner-bio one, USA) were coated with 20 µl of the DSF and or cytoplasmic fraction of hippocampus using 0.1M carbonate buffer (pH 9.6). Blocking was done using 3% BSA in PBS followed by addition of primary antibody [rabbit polyclonal anti-Aβ, Abcam, ab 10148; mouse monoclonal anti-BDNF, Abcam, ab 10505; mouse anti-SNAP 25, Abcam, ab 31281; mouse anti-PSD 95, Abcam, ab 18258; rabbit polyclonal anti-VAchT, Abcam, ab 68984 and mouse monoclonal anti-TNFα, Abcam, ab 1793, USA, respectively] with dilution of 1:1000 and incubated at room temperature for 2 h. HRP-coupled anti-rabbit IgG (Abcam, ab 6721, USA) was used as the secondary antibody and incubated for 2 h at 37°C.

Then, tetramethyl benzidine substrate (TBS) (Sigma-Aldrich, USA) was added and incubated in dark for 30 min at 25°C followed by addition of stop solution (0.1 N HCl). Wells containing 0.1 M carbonate buffer (pH 9.6) only were used as the

negative controls. The wells were analyzed with a multiwell plate reader (Synergy H1, Gen 5 BioTek multimode plate reader, USA) at 450 nm.

b. Statistical analysis

Data have been presented as mean ± standard error of mean (SEM). Data have been analyzed by one-way ANOVA followed by Tukey's post hoc test using SPSS (version 20, USA). Probability level of 0.05 or less has been regarded as significant.

3. Results

3.1 Barnes maze study

a. Latency to locate the escape box

The *G. lucidum* HWE fed rats were capable of finding the escape (hidden) box placed under the target hole more quickly than their controlled counterparts as shown through timescale in the FIG. 3. Both the AD and the hypercholesterolemic rats took significantly ($P \leq 0.05$) higher time to find the escape cage compared to the controls and latency decreasing effect was significant ($P \leq 0.05$) in the extract fed controlled and the hypercholesterolemic rats (FIG. 3).

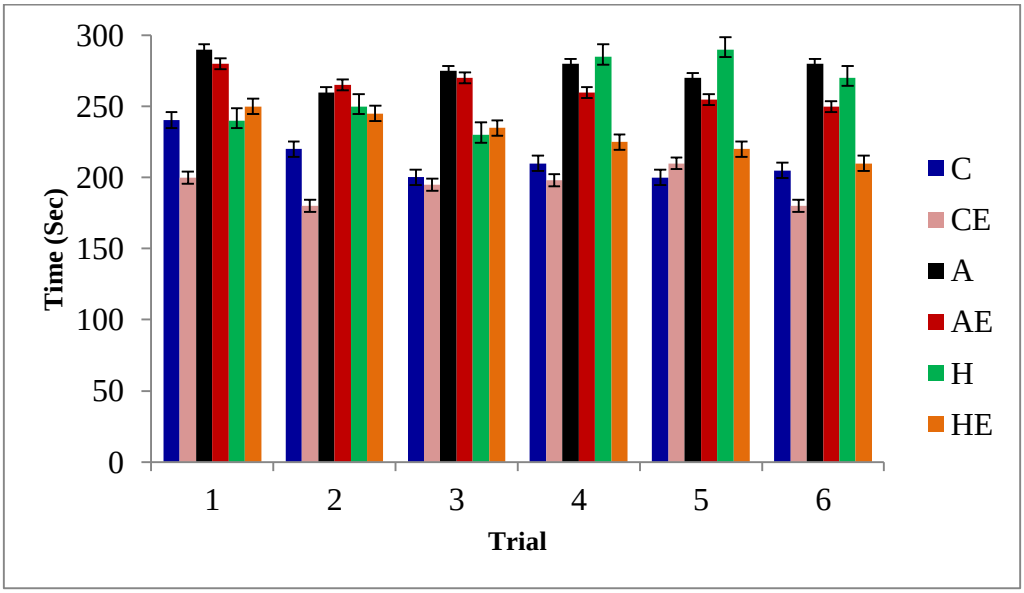

FIG 3. **Latency to Find the Escape Cage.**

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE=*G. lucidum* HWE fed AD rats, respectively.

b. Distance travelled to locate the escape box

Compared to the AD (16.69 m) and hypercholesterolemic (13.58 m) controls, the *G. lucidum* HWE fed rats travelled less distance (11.46 m and 12.44 m, respectively) to reach to the target hole (FIG. 4). Distance travelling effect was statistically significant ($P \leq 0.05$) in the extract fed AD rats (FIG. 4).

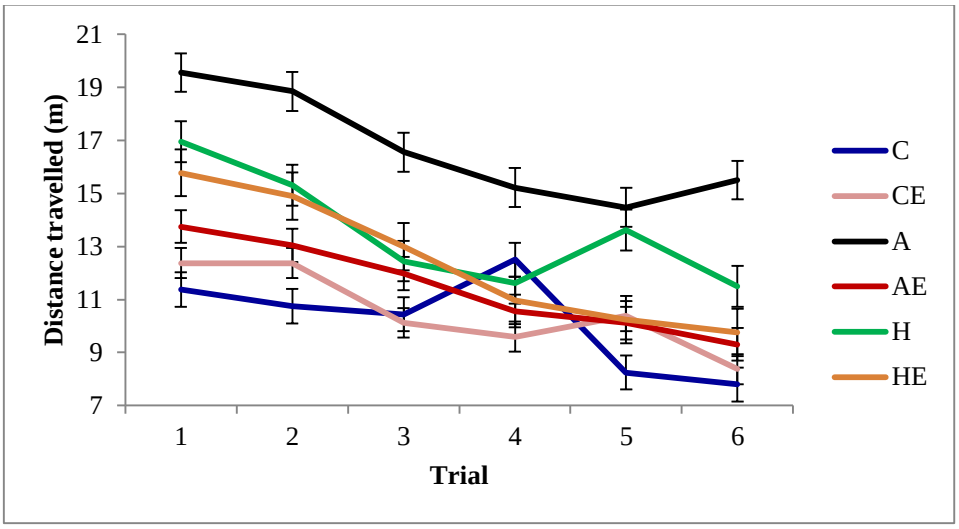

FIG 4. **Distance Travelled to Find the Escape Cage.**

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey' s HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE=*G. lucidum* HWE fed AD rats, respectively.

c. Strategies used to find the escape cage

All the rat groups adopted all the three escape strategies: random, serial and spatial. At the beginning of the experiments, random mode prevailed, followed closely by that of serial and spatial mode was of meager amount, as measured on percentage scale (TABLE 1). As time passed, rats' ability to strategize spatial memory increased differently in different groups (TABLE 1). The rate of spatial memory enhancement was higher in the extract fed rats than those of the non-fed. Rank of spatial strategy utilization was CE (39.17%)>AE (27.17%)>HE (31.83%) (TABLE 1).

TABLE 1. **Escape Strategies Used by the Rats.**

Strategy (%)	C	CE	A	AE	H	HE
Random	40.17 ± 2.34	41.0 ± 1.09	49.5 ± 1.99	43.33 ± 1.25	43.33 ± 2.05	39.17 ± 1.60
Serial	26.67 ± 1.6	29.33 ± 1.15	22.5 ± 1.10	20.67 ± 0.67	27.83 ± 1.56	27.5 ± 0.42
Spatial	35.5 ± 3.05	39.17 ± 4.10	22.83 ± 1.92	27.17 ± 2.03	28.33 ± 1.40	31.83 ± 1.51

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE=*G. lucidum* HWE fed AD rats, respectively.

d. Total errors

Measurement of the total errors made by the rats before entering into the hiding tunnel reinforces their escape strategy and also reflects their spatial memory and learning abilities. *G. lucidum* extract fed rats made less errors in finding the escape tunel and entering the hidden box than their respective control and AD littermates. During the initial period of the test, all the rat groups had almost equal number of errors (FIG. 5). As tests continued, the number of errors tended to decrease in the rat groups other than that of AD (FIG. 5). Up to the end of the test, the AD rats could not recover their mis-identification of the target hole paradigm whereas the extract fed AD rats' ability to locate the hole increased significantly ($P \leq 0.05$) (FIG. 5).

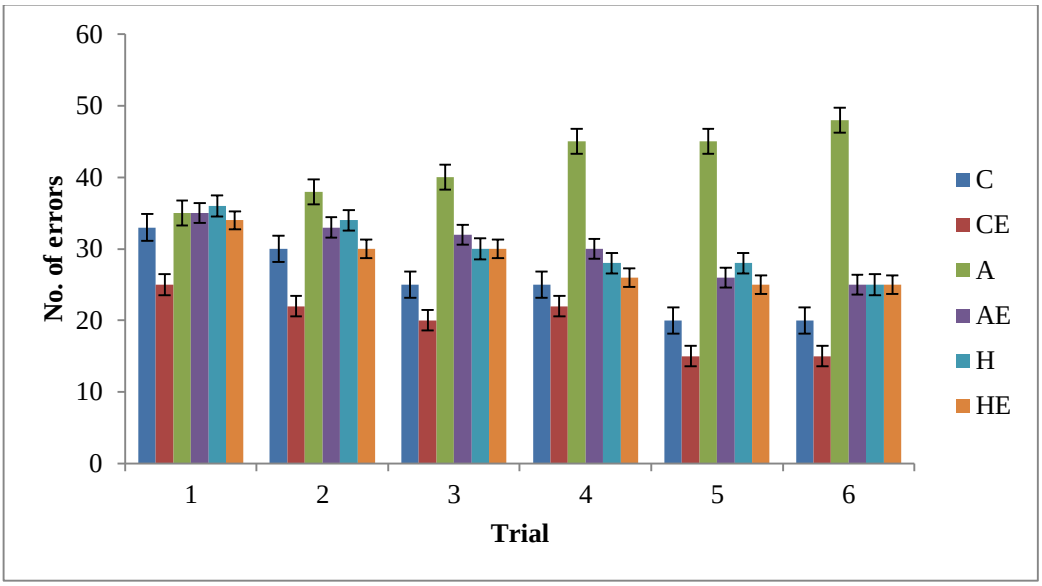

FIG 5. **Total errors of the rats in finding escape cage.**

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Turkey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE=*G. lucidum* HWE fed AD rats, respectively.

e. Visual cues

Both intra- and extra-maze cues were used to aid the rats find the escape box. Observed findings demonstrate inconsistent use of extra maze cues for the initial period of the experiment. This might be for the reason that the extra-maze cues were located slightly distant and outside of the maze compared to the intra-maze cues. Environmental configuration (curtain used to make aloof, its color, cue color) might have been perceived by the rats as a single component and provided no contrasting feature of memory and learning. More importantly, this might be because of the rats' initial preference towards random strategy of escape tunnel identification. As the animals' strategy shifted from random towards serial and spatial, their usage of extra-maze cues became much consistent. This indicates enhanced visuo-spatial memory of the experimental animals over time. And this enhancement was higher in the *G. lucidum* extract fed rats than the non-fed ones. Thus, though all the rats had remembered the escape hole relative to the visual cues, only the extract fed rats were capable of efficiently remembering and utilizing this information to locate the target hole later. Usage of extra-maze cues was consistent with the strategy to identify the target hole

i.e. the extract-fed rats used the spatial strategy and extra-maze cues much than other strategies and intra-maze cues, respectively.

f. Hole searched in the target quadrant

The maze was divided into four quadrants (positive, negative, target and opposite) and each group of rat had been expected to visit each quadrant by 25% by chance. The AD group fell behind the expected value (16.83%) reflecting their poor memory and learning capacities, whereas the control and extract-fed controls outnumbered the expected value (26% and 30%, respectively) in the target quadrant (FIG. 6). Improved memory and learning abilities in the *G. lucidum* extract fed AD rats had been observed as their hole search percentage improved up to 20.67% from 16.83% (FIG. 6).

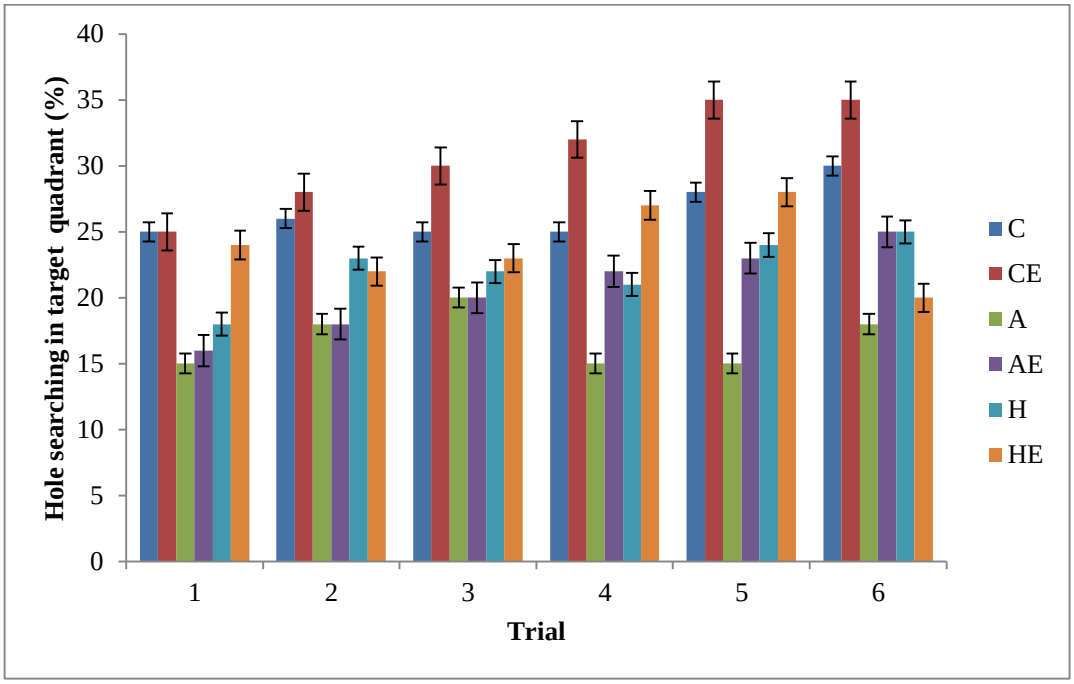

FIG 6. **Hole searched in the target quadrant.**

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

g. Time spent in target quadrant

The probe test was conducted for 120 sec and each group of rat (C, CE, A and AE) had 30% chance value of spending time in each of the quadrant. The AD rats showed impaired memory and learning abilities as they spent less time (22.67%) in the target zone than the control (60.33%) and extract fed control rats (70.83%) (FIG.7). Feeding of *G. lucidum* extract had ameliorating effect upon AD rats as reflected by the extract-fed rats' enhanced time spending (30%) in the target quadrant (FIG. 7).

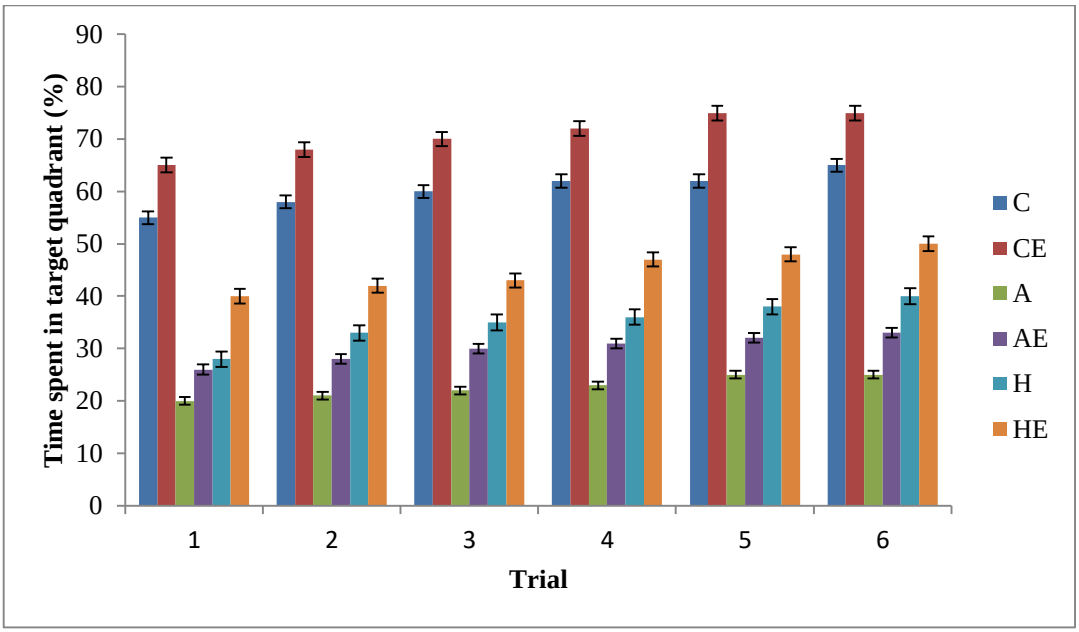

FIG 7. **Time Spent in Target Quadrant.**

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE=*G. lucidum* HWE fed AD rats, respectively.

3.2 Neuro - biochemical tests of memory and learning related protein markers

As depicted in the TABLE 2, the HWE of *G. lucidum* had ameliorating effect upon the memory and learning related neuro-biochemical and neuro-immunological markers of the rats such as BDNF, SNAP 25, PSD 95 and VAchT. On the other hand, decreased levels of anti-$A\beta_{(1-42)}$ oligomers and TNFα levels were observed in all the rat groups treated with *G. lucidum* HWE (TABLE 2).

TABLE 2. **Memory and Learning Related Marker Assays.**

% of control	CE	A	AE	H	HE
Anti-$A\beta_{(1-42)}$ Oligomer	85.30 ± 0.49^{a}	141.85 ± 0.60^{b}	119.80 ± 0.44^{c}	$107.03 \pm 0.35^{d,e}$	$102.56 \pm 0.50^{d,e}$
BDNF	$109.57 \pm 0.91^{a,e}$	$84.00 \pm 1.78^{b,c}$	$90.43 \pm 1.74^{b,c,d}$	$95.72 \pm 1.03^{b,c,d}$	$103.60 \pm 0.29^{a,e}$
SNAP 25	104.80 ± 0.71^{a}	85.33 ± 0.69^{b}	89.16 ± 0.57^{c}	$93.25 \pm 0.63^{d,e}$	$94.09 \pm 0.78^{d,e}$
PSD 95	102.44 ± 1.25^{a}	$91.56 \pm 0.94^{b,c,d,e}$	$94.23 \pm 0.42^{b,c,d,e}$	$94.83 \pm 0.83^{b,c,d,e}$	$95.57 \pm 0.98^{b,c,d,e}$
TNFα	90.02 ± 0.80^{a}	120.50 ± 1.34^{b}	111.05 ± 0.83^{c}	103.62 ± 1.00^{d}	97.56 ± 0.90^{e}
VAchT	$109.12 \pm 1.27^{a,c}$	$91.98 \pm 0.68^{b,d}$	$106.04 \pm 0.57^{a,c,e}$	$96.48 \pm 0.87^{b,d}$	$111.89 \pm 1.13^{a,c,e}$

Data are expressed as mean ± SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Turkey's HSD test ($P \leq 0.05$). Here, C=Control, CE=*G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively. BDNF stands for brain derived neurotrophic factor, SNAP 25 for synaptosomal associated protein 25 KD, PSD 95 for post-synaptic density protein 95 KD, TNFα for tumor necrosis factor α and VachT for vesicular acetylcholine transporter, respectively.

4. Discussion

4.1 Barnes maze study

Compared to MWM, Barnes maze (BM) introduces less stress towards the experimental animals and etho-logically is much relevant with the rats. As, the *G. lucidum* HWE fed rats took less time (s) and travelled less distance (m) to find the escape box as well as utilized spatial searching strategy much, made less error and passed much time in the target quadrant than their non-fed littermates, the findings of the current Barnes maze test are indicative of the spatial memory improving potentiality of *G. lucidum*. However, not enough reports incorporating BM and mushrooms are available and perhaps, this is the first demonstration of memory and learning related behavioral studies utilizing *G. lucidum* HWE and BM. Available information points towards improved Barnes maze performance of both the wild and APPswe/PS1dE9 transgenic mice had followe by feeding of vitamin D2 (ergosterol derivative) enriched *A. bisporus* [21]. Interestingly, much improved memory had been noticed in the mushroom-fed transgenic mice than the mushroom-fed wild mice [21]. Biochemical and immunohistochemical studies had linked vitamin D2 with reducing the level of brain Aβ and glial fibrillary acidic protein (GFAP) and increasing IL-10 in the *A. bisporus* fed mice [21]. Content of ergosterol derivative in the *G. lucidum* might act similarly upon the current experimental animals and thus improved cognitive and memory related learning abilities.

4.2 $A\beta_{(1-42)}$ oligomer lowering effect of *G. lucidum* HWE

As $A\beta_{(1-42)}$ is the culprit for memory disruption, its measurement in the brain homogenate is apparent. Thus, the level of $A\beta_{(1-42)}$ oligomer was measured using antibody against it in the DSF of the rat hippocampus as $A\beta_{(1-42)}$ oligomer is a soluble protein. Significantly increased level of $A\beta_{(1-42)}$ oligomer in the AD rats (41% higher than those of the controls) were found in the present study (TABLE 2). This may be due to the combined effect of increased generation per se and deposition of those through cerebro-ventricular infusion. On the contrary, significantly decreased ($P \leq 0.05$) level of $A\beta_{(1-42)}$ oligomer was observed in the *G. lucidum* HWE fed rats (lowered up to 19%) (TABLE 2). Although, hypercholesterolemia led 7% increased generation of $A\beta_{(1-42)}$ oligomer, feeding of the *G. lucidum* HWE could lower this level up to 2% (TABLE 2). This lowering of $A\beta_{(1-42)}$ oligomer burden in the AD and hypercholesterolemic rats' brain are indication of AD ameliorating effect of *G. lucidum*. Among multiple strategy, *G. lucidum* mediated decreased biosynthesis of cholestersol might be responsible for generating comparatively lowered level of $A\beta_{(1-42)}$ [15-17]. Content of phenolics in the HWE of *G. lucidum* might also contribute to this effect [15-17]. *G. lucidum* mediated stimulation of the P13K and ERK signaling cascades resulting in stimulation of the non-amyloidogenic pathway and enhanced secretion of sAPPα might also have been involved [22]. Current findings are also compatible with those of Wang et al., who found that *G. lucidum* powder at 0.3%, 0.6% and 1.8% of diet can significantly lower Aβ burden in the mouse brain, improve memory and learning abilites along with enhanced anti - oxidative enzymatic levels [23]. Thus, lowering of brain Aβ burden along with increasing anti - oxidative defense might confer the AD ameliorating effect of the *G. lucidum* HWE.

4.3 Effect of *G. lucidum* HWE on memory and learning related markers

4.3.1 Neurotransmission maintenance effect of *G. lucidum* HWE

Brain derived neurotrophic factor (BDNF) is a neuroprotectin group of growth factor involved in neuronal survival and functioning. Significantly lowered level of BDNF in the AD rats compared to the controls (84% compared with 100% of control value) was found in the present study (TABLE 2). Feeding of *G. lucidum* HWE resulted in increased BDNF level both in the AD (from 84%, raised up to 90.43%) and the hypercholesterolemic (from 95.72%, increased up to 103.60%) rats and the increasing effect was significantly high in the latter ($P \leq 0.05$) (TABLE 2).

BDNF participates in both pre- and post-synaptic neurotransmissions by binding with the tyrosine kinase receptor B (TrkB) and thus plays important role in memory and learning activities [24]. Its impairment has been found to affect memory related learning and behavioral performances in different organisms and in different conditions [24]. AD patients possess considerably lower amount of BDNF [25]. Deteriorated memory emanating from intra-cerebroventricular infusion of Aβ had been reported to be due to the lowering level of BDNF [26]. Learning activities increases BDNF level in the rodent hippocampus and cortex [25-26]. As described in the Barnes maze test section, the *G. lucidum* HWE fed rats performed better than those of the non-fed ones in the learning tests and the mushroom fed rats were supposed to contain higher level of BDNF. Observed neuro - biochemical findings are compatible with those of the behavioral test findings of the present study. This claim is substantiated by the finding that BDNF not only forms spatial memory but also regulates retention and recalling of it [27]. Previous studies had shown that the tri-terpenoids present in *G. lucidum* provide BDNF enhancing effect [28]. In our another study, several tri-terpenoids have been detected in the HWE of *G. lucidum* that might have been involved in increasing the BDNF level of the mushroom fed rats' hippocampus and thus improved their memory and learning abilities [15-17]. In addition, phenolics present in the HWE of *G. lucidum* might also confer effects towards increasing the hippocampal BDNF level [15-17].

4.3.2 Maintenance of Pre-Synaptic Membrane and Long-Term Potentiation by *G. lucidum* HWE

Synaptosomal - associated protein 25 KD (SNAP 25) is a pre-synaptic membrane protein necessary for long - term potentiation (LTP) and working memory [29]. Its decreased level had been noticed in AD subjects [30]. Compared with the controls, significantly lowered ($P \leq 0.05$) level of SNAP in the hippocampus of the AD rats was observed (TABLE 2). In case of the *G. lucidum* HWE fed rats, SNAP 25 level increased significantly ($P \leq 0.05$). Previous studies indicate that spatial recognition is mediated by SNAP 25 and rats' performance in MWM had been found to be negatively affected with the anti-SNAP 25 antisense oligonucleotide [31]. Aβ leads towards loss of synaptophysin, another pre - synaptic protein, in the caspase - dependent way [32]. Thus, improved spatial recognition of the *G. lucidum* HWE fed rats in different spatial memory and learning related behavior tests in the present study might be attributed by the enhanced SNAP 25 level in the respective rats.

4.3.3 Maintenance of Post - Synaptic Density by *G. lucidum* HWE

Post-synaptic density protein 95 KD (PSD - 95) is involved in maturation of the excitatory synapses and in maintenance of post - synaptic density [33]. Increased Aβ accumulation and impaired memory have been reported to be associated with its disruption and AD patients exhibit lowered level of PSD 95 in the hippocampus [34]. Decreased level of PSD 95 in the AD and hypercholesterolemic rats (94.43% and 95.57%, respectively) compared with the control (100%) were noted in the present study (TABLE 2). This might be due to Aβ guided loss of PSD 95 in the caspase - dependent way [32]. However, feeding of

G. lucidum HWE to the rats resulted in increased level of PSD-95 in the hippocampus. Thus, the improved memory and learning abilities of the *G. lucidum* HWE fed rats in the present study might be accrued from inter alia increased PSD 95 level in the hippocampi of the mushroom fed rats.

4.3.4 Neuro-Inflammation Lowering Effect of *G. lucidum* HWE

TNFα acts as a neurotoxin and thus AD amelioration strategy points towards its reduced level [35]. As depicted in TABLE 2, the AD rats had significantly ($P \leq 0.05$) higher level of TNFα (120.5% compared to 100% of the controls). Feeding of *G. lucidum* HWE significantly decreased (up to 1116.05%) TNFα level. Similar effect was observed in case of the mushroom fed hypercholesterolemic rats (lowered from 103.62% to 97.60%). At 400 µg/mL, the methanolic extract of *G. lucidum* had been found to significantly lower the production of microglial TNFα and prevents the dopaminergic neurons [36]. At the same dosage, it could lower the expression of TNFα mRNA up to 90% [36]. Presence of phenolic anti-inflammatory and anti-oxidant substances in the *G. lucidum* HWE might also cause TNFα lowering effect [37]. Content of β-D glucan, triterpenoids and polysaccharides had been supposed to confer this protecting effect of *G. lucidum* [36]. *Ganoderma lucidum* polysaccharide (GLPS) administered to the traumatic spinal cord injured rats also showed neuroprotective effect through lowering TNFα level along with reduced production of malondialdehyde (MDA) [38]. In addition to the TNFα lowering effect, MDA lowering effect of the HWE of *G. lucidum* was also observed in the present investigation (data not shown for the sake of brevity). Thus, the current findings are compatible with those published ones as similar lowering effect of *G. lucidum* HWE upon TNFα level (TABLE 2) and presence of similar bio-components have been observed in our another study [17].

4.3.5 Enhancement of Cholinergic Neurotransmission by *G. lucidum* HWE

Vesicular acetylcholine transporter (VAchT) transports acetylcholine (ACh) from the presynaptic membrane of the cholinergic neurons into the secretory vesicles (SV), from where ACh is released into the synaptic cleft [39]. Activity of VAChT highly regulates cholinergic neurotransmission and VAChT deficiency leads towards neuromuscular abnormalities [39]. Impaired cognitive function in AD subjects had been linked with reduced VAChT level [39]. In the present study, significantly lower ($P \leq 0.05$) level of VAChT was noted in the AD model rats (91.98% in AD, compared to 100% for the controls) while the *G. lucidum* HWE fed rats had been observed with significantly higher level of VAChT (106.04%) (TABLE 2). Reduced level of AChT in the hypercholesterolemic rats (96.48%) brings testimony towards hypercholesterolemia induced impairment of cholinergic neurons and VAChT activity [40].

Thus, memory and learning related poor performances of the AD and the hypercholesterolemic rats may be due to inter alia disrupted VAChT activity and diminished cholinergic neurotransmission [41]. In line with this, the ameliorated memory and learning related performances of the *G. lucidum* HWE fed rats might be the offshoot of ameliorated VAChT activity of the cholinergic neurons in the respective rats.

5. Conclusion

Infusion of soluble Aβ1-42 to the rat cerebral ventricles affected AD model rats' memory and learning related behavioral tasks indicating the effectiveness of the current model of AD studies. Hypercholesterolemic model rats also showed poor performance in behavioral tests. Feeding of *G. lucidum* HWE to the AD and hypercholesterolemic rats improved their memory

and learning abilities. Memory-related protein marker tests also indicate hypercholesterolemia and AD ameliorating effect of *G. lucidum*. Thus, *G. lucidum* could be regarded as an AD and hypercholesterolemia ameliorating agent. However, further study is needed for formulating therapeutic dosage.

6. Abbreviations

Aβ: Amyloid beta; A / AD: Alzheimer's disease; AE: *G. lucidum* hot water extract fed AD model rats; BDNF: Brain derived neurotrophic factor; BM: Barnes maze; C:Control; CE: *G. lucidum* HWE fed control; GL: *Ganoderma lucidum;* HWE: Hot water extract; H / HC: Hypercholesterolemic; HE: *G. lucidum* hot water extract fed hypercholesterolemic model rats; PSD 95: Post-synaptic density protein 95 KD; SNAP 25: Synaptosomal associated protein 25 KD; TNFα: Tumor necrosis factor α; VachT: Vesicular acetylcholine transporter.

7. Ethics Approval and Consent to Participate

All the experimental protocols had been approved by the ethical permission committee, Jahangirnagar University Animal Care and Use Committee (JUACUC) [Ethics reference no. JU/25/04/2018/MAR (R)]. Animals had been handled following the guidelines mentioned in "Guidelines for the care and use of laboratory animals, 8th edition, the national academies council, Washington DC, USA".

8. Consent for Publication

Not applicable.

9. Availability of Data and Material

Not applicable.

10. Competing Interests

The authors declare that they have no competing interests.

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12. Authors' Contribution

Study design, sample collection and preparation, Laboratory experiments, data collection and analysis, interpretation of results and drafting of manuscript were done by Mohammad Azizur Rahman. Other authors supervised the experiments, edited and approved the final manuscript.

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