ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.4009588

Validation of *Ganoderma lucidum* against hypercholesterolemia and Alzheimer's disease

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Received: 08 June 2020; **Revised submission:** 10 August 2020; **Accepted:** 27 August 2020

http://www.journals.tmkarpinski.com/index.php/ejbr

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. 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 HC and 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 hypocholesterolemic, lipid profile improving and enhanced cognitive performance of the *G. 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), a neuro-degenerative disorder affects memory and learning abilities and induces behavioral disorder especially in the elderly people [1]. Neurons and neurotransmitters associated with memory, learning and behavioral normalcy become disrupted in the AD patients [1]. Deposition of amyloid beta (Aβ) plaques and/or neurofibrillary tangles (NFTs) in the AD neurons impairs their usual activities [2]. Consequently, AD patients become solely dependent on their family members and care givers and add extra burden as well as negative impact on national and global economy [3,4]. Unfortunately, there is hardly any treatment for more than 40 million AD patients suffering globally [5]. Current COVID-19 epidemic has lessened the attention toward AD medication harshly though the aged people are vulnerable to both AD and COVID-19 [6]. The available therapeutic strategies are aimed at symptom – modifying targets

rather than preventing the neurons from damages [7]. For example, the drugs (donepezil, galantamin, memantine, rivastigmine) approved by the United States Food and Drug Administration (USFDA) can improve AD symptoms only through modulating brain neurotransmitter release [7]. Though AD symptoms appear in late 60s or later, initiation and progression of AD pathogenesis occurs at or during early 40s [8]. Thus, if protective measures could be taken at or before mid-ages of life, AD progression could be withheld, albeit, reduced [8]. 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 [1,5]. Thus, effective management of the co-existing factors of AD has been highly regarded in the most recent recommendation from the Alzheimer's association [1,5]. Thus, time is up for considering AD ameliorating therapeutic approaches that would be easy to reach to the common mass of both the developing and developed nations.

Hypercholesterolemia refers to the elevated level of cholesterol in the blood [9]. Hypercholesterolemia stems from the increased synthesis and/or decreased removal of endogenous cholesterol and also from the excessive supply of dietary cholesterol or cholesterol precursors [10]. Hypercholesterolemia plays pivotal role in the progression of AD [9-11]. Increased brain level of cholesterol has been found during AD progression [9-11]. The role of cholesterol in AD pathogenesis becomes clearer from the opposite relationship between plasma HDL-cholesterol level and dementia [9-12]. HDL is the main lipoprotein in the human brain and can slow down the *in vitro* aggregation of Aβ and subsequent toxicity [9-12]. Some studies have indicated 2-3 times greater risk of late-age dementia and AD for those having mid-life hypercholesterolemia [9-12]. Thus, hypercholesterolemia stands as an early risk factor for AD [13-14]. Similarly, hypercholesterolemia is among the major modifiable risk factors of CVD [13-14]. Cholesterol lowering drugs, statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been reported to lower the risk of death or cardiovascular events in patients with or without CVD as well as protective against the risk of developing AD [15-17]. Statins have been found effective in reducing the risk of AD from 60% even up to 74% [15-17]. Statins can directly withstand the production of Aβ and also facilitate their removal from the brain [15-17].

The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice had suggested life-style and dietary modification as the first line and pharmacological intervention through utilization of lipid lowering drugs as the second line of treatment strategy against hypercholesterolemia [18]. Life style modification involves loss of weight, consumption of diet containing less cholesterol and higher saturated fatty acid, giving up of cigarette smoking and alcohol drinking, intake of unsaturated fatty acids such as DHA and regular physical exercise [18]. However, combination of both life style modification and pharmacological treatment sound better in real life [18].

Both edible and medicinal mushrooms lower blood total cholesterol (TC), very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in rats [19-28]. Investigations involving the potentiality of mushrooms as the inhibitor of cholesterol biosynthetic key enzyme 3-hydroxy 3-methyl glutaryl co-enzyme A reductase (HMGCo-AR) have yielded promising outcome [28]. *Ganoderma lucidum* has been hailed for its immense medicinal values [19, 21-23]. Among them, its antioxidative, anticancer, antibiotic, antiviral, hepatoprotective and neuroprotective effects are most notable [19, 21-23]. Medicinal feat of *G. lucidum* could be attributed to its content of polysaccharides, polyphenols and triterpenes [23-25]. Despite vast expanse of research concerning *G. lucidum,* there is hardly any study addressing combined hypocholesterolemic and AD ameliorating effect of this mushroom, though both of these pathophysiologies

have some common links. Thus, the present study has been designed to elucidate the effect of *G. lucidum* on experimentally induced hypercholestorlemic and AD model animals.

2. MATERIALS AND METHODS

2.1. Preparation of hot water extract of *G. lucidum*

Purchased from a local farm in Selangor, Malaysia, the fruiting bodies of *G. lucidum* were identified and authenticated by experts via DNA sequencing by Mushroom Research Centre, University of Malaya herbarium. A voucher specimen was deposited in the University of Malaya herbarium (KLU-M1233). Powdered fruiting bodies of *G. lucidum* were boiled in distilled water at the ratio of 1: 20 (w/v) for 45 minutes. 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. Preparation of AD and HC model animals

2.2.1. Animals, maintenance and dosage of treatment

Forty eight wistar male rats (weight range 120 ± 5 gm) were divided into six groups: control (C), *G. lucidum* HWE fed control (CE), hypercholesterolemic (H), *G. lucidum* HWE fed hypercholesterolemic (HE), Alzheimer's disease (A) and *G. lucidum* HWE fed Alzheimer's disease (AE) each group containing 8 rats. The extract fed groups (CE, HE and AE) received 200 mg/kbw *G. lucidum* HWE. Animals had been housed in a 12 hr day night cycle at 25±2°C temperature. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)].

2.2.2. Induction of hypercholesterolemia

Hypercholesterolemia to the H rats was evoked by adding 1% cholesterol and 1% cholic acid (for intestinal better absorption of cholesterol) with the basal diet of the rats [23].

2.2.3. Preparation of AD model rats

Alzheimer's disease model rats (A) were prepared by infusing $A\beta_{1\text{-}42}$ (ab120959, Abcam, USA) to the cerebral ventricles following the method of Abdullah et al. [29].

2.3. AD studies

2.3.1. Barnes maze study

Memory and learning related behavioral performance of the rats was evaluated using Barnes maze. A Barnes maze made of stainless steel and having a diameter of 150 cm was used (Fig. 1). It contained 20 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 is 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 was cleaned 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.

2.4. Sacrifice of the animals

Twenty four hours following the last treatment and test, the rats were kept fasting overnight. Then, the rats were anesthesized with intra-peritoneal injection of sodium pentobarbital (35 mg/kbw) and sacrificed.

2.5. Statistical analyses

Conducting all the experiments in triplicate, data have been presented as mean±SEM. Using SPSS version 16, ANOVA was performed following least significance difference at 95% level.

3. RESULTS AND DISCUSSION

3.1. Hypocholesterolemic effect of *G. lucidum* HWE

3.1.1. Effect of G. lucidum HWE on body weight change

Gradual increase in body weight (ranging from 140 ± 5 g up to 280 ±2 g) of the rats of all the groups was noticed throughout the experimental period (Fig. 1). However, the hypercholesterolemic rats (H) gained the maximum weight and their rate of becoming weighty surpassed that of the others. Weight gain tendency of the normo-cholesterolemic rats was moderate and feeding of *G. lucidum* HWE resulted in decreased weight gain as time passed by. Similarly, *G. lucidum* HWE fed AD rats experienced relatively lower body weight growth than their non-fed counterparts. These findings indicate towards body weight lowering potencies of the *G. lucidum* HWE up on the experimental animals.

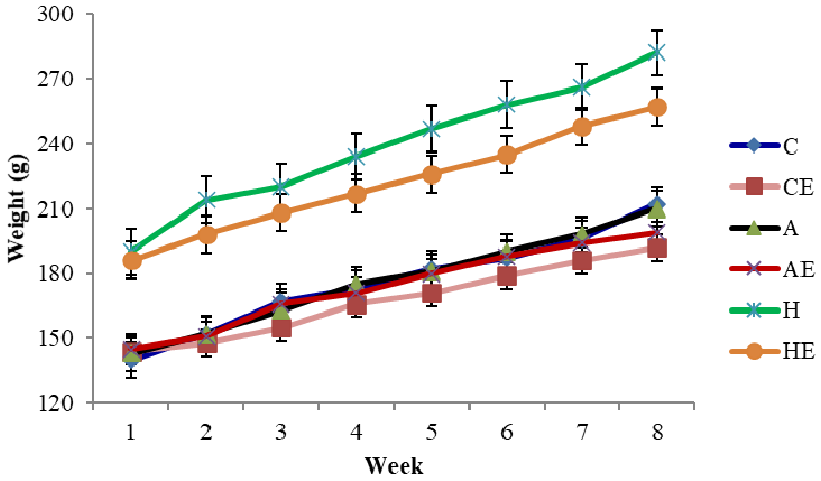

Figure 1. Effect of *G. lucidum* HWE upon body weight change of the rats.

Data are expressed as mean±SEM of triplicate measurements. Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Where, 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 (n=8 for every group).

Current findings are in agreement with several others who reported a gradual increased body weight in the cholesterol fed rats [30]. As the present experimental animals were almost matured from their beginning of the experiment, decreased body weight gain of the *G. lucidum* HWE fed rats might occur due to decreased

fat (triacylglycerol) deposition, decreased cholesterol biosynthesis or increased lipolysis and/or the combined effect of all.

3.1.2. Effect on plasma TG level

Feeding of hypercholesterolemic diet to the rats resulted in their increased plasma TG levels (Table 1). The hypercholesterolemic (H) rats had 1.88 times higher plasma TG level compared to the controlled (C) rats indicating the increased atherogenic propensity of the H rats. Feeding of *G. lucidum* HWE lowered plasma TG level in both the control, hypercholesterolemic and AD rats. Plasma TG lowering effect of the *G. lucidum* HWE was in the order of controlled (20%) > hypercholesterolemic (16%) > AD (11%) rats (Table 1). TG lowering effect of *G. lucidum* HWE was statistically significant in each group compared to their respective controls (Table 1). Increased TG levels in the hypercholesterolemic rats might be due to the decreased clearance of TG owing to the lowered lipoprotein lipase (LPL) activity or due to increased deposition of LDL-C [31]. TG lowering effect of the HWE *G. lucidum* might be mediated through increased inhibition of the HMGR by the phenolics content of the HWE of *G. lucidum* [23, 28].

Table 1. Lipid profile of the experimental animals.

Plasma parameter (mg/dL)	C	CE	H	HE	A	AE
TG	120.73 ± 0.63^{a}	100.93 ± 0.85^{b}	197.0 ± 0.880^{c}	160.33 ± 0.90^{d}	145.93 ± 0.70^{e}	135.33 ± 0.52^{f}
TC	94.6 ± 0.74^{a}	85.9 ± 0.91^{b}	139.2 ± 0.84^{c}	110.4 ± 1.0^{d}	118.67 ± 0.85^{e}	103.87 ± 0.56^{f}
HDL-C	28.06 ± 0.42^{a}	34.47 ± 0.55^{b}	27.94 ± 0.58^{c,e}	34.33 ± 0.40^{d,f}	27.73 ± 0.40^{c,e}	33.4 ± 1.35^{d,f}
LDL-C	37.97 ± 0.58^{a}	25.68 ± 0.10^{b}	68.86 ± 0.90^{c}	44.60 ± 1.34^{d,f}	57.75 ± 0.91^{e}	40.8 ± 0.57^{d,f}
VLDL-C	25.54 ± 0.13^{a}	21.78 ± 0.17^{b}	39.40 ± 0.18^{c}	32.47 ± 0.18^{d}	29.19 ± 0.14^{e}	25.67 ± 0.10^{f}

Data are expressed as mean±SEM of triplicate measurements each (n=8). Mean values containing different lower case superscripts are statistically significant at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test of every triplicate data. Where, TG = triacylglycerol; TC = total cholesterol; HDL-C = high density lipoprotein-cholesterol; LDL-C = low density lipoprotein cholesterol; VLDL-C = very low density lipoprotein cholesterol; 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 (n=8, in every group), respectively.

3.1.3. Effect on plasma TC level

Feeding of hypercholesterolemic diet to the rats resulted in 1.62 times increased plasma TC levels (Table 1). Later on, treating the rats with *G. lucidum* HWE lowered plasma TC level significantly (P≤0.05) in all the rat groups. TC lowering effect of *G. lucidum* might be mediated by competitive inhibition of HMG Co-A reductase, the rate limiting step of cholesterol biosynthesis, or through inhibition of 14α demethylase by the ganoderic acids or by impaired intestinal absorption by β-D glucan present in the *G. lucidum* HWE [24].

3.1.4. Effect on plasma HDL-C level

In the present study, the hypercholesterolemic and AD rats had lowered plasma HDL levels compared to the normo-cholesterolemic controls (Table 1). Feeding of *G. lucidum* HWE increased plasma HDL-C levels significantly (P≤0.05) in all the rat groups. However, the rate of increasing was highest in the

normocholesterolemics and the increasing trend was C (27.5%) > H (22.80%) > A (20.20%) rats (Table 1). Lowered level of HDL-C in the hypercholesterolemic rats of the present study might be due to the accelerated clearance of apo A1 from the plasma following hypercholesterolemia in the H and A rats [32]. On the other hand, significantly increased ($P \leq 0.05$) plasma HDL-C level in the *G. lucidum* HWE fed rats indicates increased clearance of TC from the peripheral tissue to the liver for excretion that points towards CVD ameliorating effect of *G. lucidum* HWE.

3.1.5. Effect on plasma LDL-C and VLDL-C level

Plasma LDL-C level increased 1.92 times in the hypercholesterolemic and 1.42 times in the AD rats, compared to the respective controls (Table 1). Similarly, plasma VLDL-C level increased by 1.76 times in the H and by 1.65 times in the A rats. Increased levels of LDL-C and VLDL-C in the hypercholesterolemic rats might arise from their intake of hypercholesterolemic diet that might have downregulated the hepatic LDL receptors of the H rats [32]. *Ganoderma lucidum* HWE supplementation caused significant lowering effect upon plasma level of both LDL-C and VLDL-C in all the rat groups (Table 1). *Ganoderma lucidum* HWE caused significantly lowering effect upon the plasma LDL-C level of both H and AD rats as compared to the controlled. *Ganoderma lucidum* HWE mediated decreased cholesterol absorption and biosynthesis might cause decreased availability of hepatic cholesterol for lipo-protein biosynthesis in the extract fed rats (CE, HE, AE). As a consequence, decreased VLDL secretion in the plasma along with decreased conversion of VLDL into LDL may end into lowered plasma LDL level [32]. Mechanistically, *G. lucidum* HWE induced increased LDL receptor in the rat hepatocytes may contribute to the LDL lowering effect that resulted in lowered secretion of LDL in the rat plasma [32]. Increased clearance of LDL from the blood of the mushroom-fed rats may also have been involved [32].

3.2. AD ameliorating effects of *G. lucidum* HWE

3.2.1. Barnes maze study

Compared with the respective controls, the *G. lucidum* HWE fed rats made less mistake in finding the escape box (Fig. 2). This observation corresponds towards enhanced memory and learning abilities of the *G. lucidum* HWE fed rats. To the best of our knowledge, ours is the first report concerning Barnes maze study of *G. lucidum* HWE fed rats and thus comparative discussion could not be furthered. Ganocomponents of different structure and function might have imparted AD ameliorating effect observed in this behavioral study [20-24].

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

3.2.2.1. BDNF

Brain derived neurotrophic factor (BDNF), a neuroprotectin group of growth factor, is involved in neuronal survival and functioning. Its pivotal role in in non-neuronal cells such as in smooth muscle and endothelial cells have also been documented [34]. Though BDNF has been regarded as a pro-atherogenic factor, recent studies have reported its ameliorating effects on CVD [35]. Elevated BDNF levels have been reported to be associated with lowered CVD morbidity and mortality [35].

In the present study, we observed significantly reduced level of BDNF in the AD rats compared to those of the controls (Fig. 3). However, BDNF level increased in the *G. lucidum* HWE fed rats: in the HC rats,

the level increased much than those of the AD rats. Thus, increased BDNF level in the *G. lucidum* HWE fed rats indicate both AD and hypercholesterolemia ameliorating effects. In this regard, our findings coincide with those of Kaess et al. [34]. Observed memory and learning related behavioral attainment of the *G. lucidum* HWE fed rats are also substantiated by this marker [35-38]. Tri-terpenoids and phenolics present in *G. lucidum* HWE might have imparted BDNF and memory enhancing as well as hypocholesterolemic effects [39].

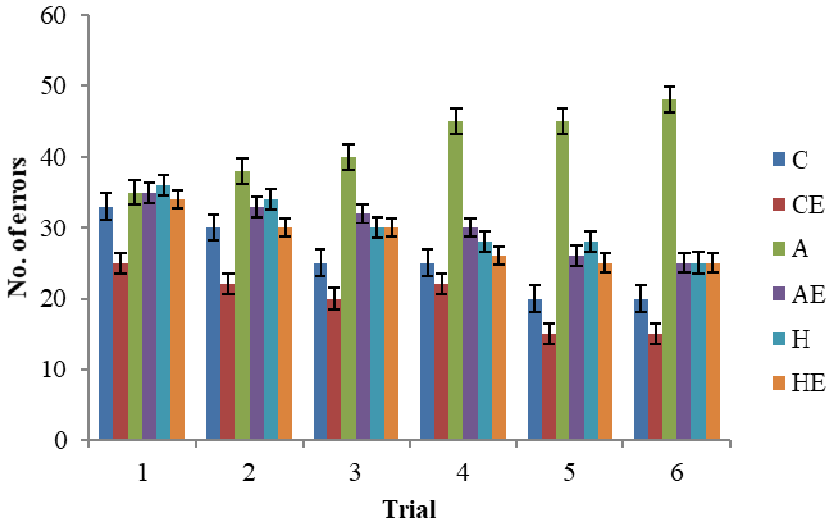

Figure 2. Total errors of the rats in finding escape cage.

Data are expressed as mean±SEM (n=8; each trial is the average of six sessions). Data were analyzed with one-way ANOVA and *post-hoc* Tukey's HSD test (P≤0.05). Here, C = Control, CE = *G. lucidum* HWE fed control, A = AD model rats, AE = *G. lucidum* HWE fed AD rats, H=hypercholesterolemic and HE = *G. lucidum* HWE fed hypercholesterolemic rats, respectively.

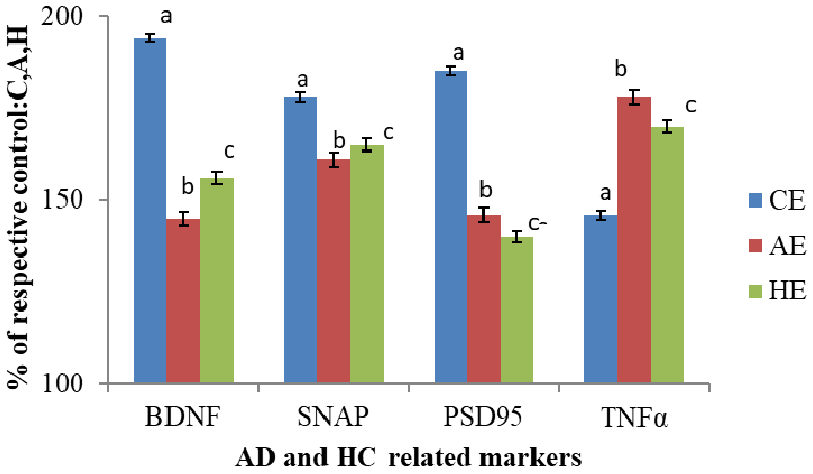

Figure 3. Effect of *G. lucidum* HWE on memory and learning related markers.

Mean values containing different lower case superscripts are statistically significant at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=8). Here, BDNF = Brain derived neurotrophic factor, SNAP = Synaptosomal associated protein, PSD95 = Post-synaptic density protein 95 KD, TNFα = Tumor necrosis factor alpha, 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.

3.2.2.2. SNAP 25

Synaptosomal-associated protein 25 KD (SNAP 25), a pre-synaptic membrane protein plays important role in maintaining long-term potentiation (LTP) and working memory [40-41]. Reduced level of SNAP25 had been reported in AD brains [40-41]. Similar trend was observed in the hippocampi of the AD rats in the present study (Fig. 3). Interestingly, *G. lucidum* HWE fed rats showed significantly ($P \leq 0.05$) soared level of SNAP 25 in their hippocampi that might impart improved cognitive performance of the *G. lucidum* HWE fed rats. SNAP25 has also been implicated in elevating serum triacylglycerol level and weight increment [42]. Thus, the increased SNAP25 level in the HC rats might have been due to elevated expression of SNAP25 in those rats.

3.2.2.3. PSD 95

Post-synaptic density and maturation of the excitatory synapses are maintained by the post-synaptic density protein 95 KD (PSD95) [43]. Inverse relationship between Aβ and PSD95 level has been reported in the AD hippocampi [44]. In line with this, lowered level of PSD95 has been observed in the AD rats hippocampi, compared with those of the normal (Figure 3). There was an increment of PSD95 level in the *G. lucidum* HWE treated rats' hippocampi that correspond to their improved memory and learning abilities (Fig. 3). Besides, level of PSD95 had been reported to be increased in hypercholesterolemic rats [45]. Elevated level of PSD95 in the HC rats of the present study coincide with the reported findings [45].

3.2.2.4. TNFα

Tumor necrosis factor alpha (TNFα), a neurotoxin, acts as pro-AD signaling agent, promotes atherogenesis and disrupts lipid metabolism [46-48]. Increased level of TNFα in the AD and HC rats correspond towards disrupted cognitive performance and dyslipidemia of the respective rat groups (Fig. 3). These AD hallmarks have been ameliorated with *G. lucidum* HWE treatment that might be attributed towards its content of phenolics, triterpenoids and polysaccharides [19-23].

3.2.2.5. VAchT

Cholinergic neurotransmission is regulated by the vesicular acetylcholine transporter (VAchT) and its reduced level impairs cognitive performance, another hallmark of AD [49]. AD and HC rats of the present study showed lower level of VAchT while those of the *G. lucidum* HWE treated showed enhanced level (Fig. 3). Thus, AD and HC rats had suffered from disrupted cholinergic neuronal activities while *G. lucidum* HWE treatment could ameliorate this disruption [50-51].

4. CONCLUSIONS

Infusion of soluble $A\beta_{1-42}$ 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.

Authors' Contributions: NAb, NAm and SH planned and supervised the research, edited the manuscript. MAR conducted the research, statistical analyses and prepared the manuscript. The final manuscript has been read and approved by all authors.

Conflict of Interest: The author has no conflict of interest to declare.

Acknowledgment: The authors gratefully thank University of Malaya and the Ministry of Higher Education, Malaysia for the HIR-MOHE Research Grant F000002-21001 funding and Mohammad Azizur Rahman is grateful to Jahangirnagar University and the University Grants Commission of Bangladesh.

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