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Syzygium Cumini (L.) Seed Extract Improves Memory Related Learning Ability of Old Rats in Eight Arm Radial Maze

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Oxidative stress is implicated in age-related deterioration of memory and learning ability. In this study, we evaluated whether oral administration of *Syzygium cumini* seed extract (SE) ameliorates the learning-related memory of old rats by eight-arm radial maze task. After 12-weeks oral administration of SE extract, learning-related memory was ameliorated, concurrently with decreases in the corticohippcampal levels of lipid peroxide (LPO) and increases in the catalase and AChE activities in the cortex. The levels of LPO were positively correlated with the RMEs. In carotid artery occlusion-induced hypoxia study, the SE-fed rats displayed significantly less microglial invasion, cell swelling and rupture in the cortico-hippocampal brain slices, thus suggesting an antioxidative defense of SE. Chemical analyses of SE revealed substantial amounts of polyphenols and free-radical scavenging activities. Finally, our results show that the S. *cumini* seed extract improves learning-related memory in old rats, probably, by instigating the antioxidant defense of the brain

Keyword: Antioxidants, *Syzygium cumini*, Memory, Alzheimer's Disease, Aacetylcholine Esterase and Catalase Enzyme

1. Introduction

Oxidative injury reflects imbalanced equilibrium between reactive oxygen species (ROS) generation and inadequate antioxidative defense of the biological system[1]. According to the free radical theory of aging, an excess generation of ROS relates to age-associated neurodegeneration and related disorder[2]. Neurodegenerative disorders are associated with various degrees of memory impairments[3]. Neuro-behavioral impairments are cumulative effects of oxidative damage to proteins, lipids, and nucleotides[4]. Age-related memory deficits and poor antioxidant defenses can be reversed by foods enriched with the antioxidant[5,6,7]. Therefore much attention is being paid toward dietary antioxidant, particularly those found in fruits and vegetables to prevent age-related deterioration of brain performance such as memory.

Syzygium cumini is a seasonal fruit of Bangladesh and it is considered as a rich source of antioxidative and redical scavenging phytoconstituents[8]. The seeds of this fruit retain extraordinary medicinal properties, which have remained largely undiscovered. We have previously reported that *S. cumini* seed powder possesses anti-diabetes activity[9] and protects the liver against the lipid peroxidation in association with a concurrent amelioration of hepatocellular status of alcoholic rats[10]. *S. cumini* seed extract

also exhibited stronger *in vitro* free radical scavenging activity than that of the synthetic antioxidant, such as butylated hydroxy toluene and natural antioxidant vitamin C. Furthermore, the oral administration of *S. cumini* seed extract restored the levels of lipid peroxides (LPO) in the cerebral cortex of alcoholic rats to those of the normal rats[11]. All these results thus suggest that the *S. cumini* seed extract might play an important role in the oxidation-related deterioration of brain activities. Notably, consumption of alcohol severely impairs memory and confers massive oxidative stress to brain, thus again indicating that *S. cumini* causes an improvement of the brain functions. In addition, we produced *in vivo* hypoxia-induced oxidative stress in the brain. The *S. cumini* seed extract-pretreated rats had an augmented antioxidative defense in the brain. We, therefore, were very much interested to study whether the *S. cumini* seed extract affects the memory-related learning ability of old rats and whether the outcome relates with to the *in vivo* antioxidant activity of *S. cumini* seed extract.

2. Materials and Methods

a. Animals

Twenty ~100 weeks old male rats (210-240g) were used in the present study. The rats were housed in an animal room at 23± 2ºC, under 12 h dark-light cycles (light 8.00-20.00 h; dark 20.00-8.00 h). The rats were randomly divided into two groups: The control group [Control, vehicle (saline) administered rat group]; the *S. cumini* seed extract-administered group (SE). The SE rats were intragastrically administered to seed extract of *S. cumini* at a dose of 400 mg/Kg of body weight per day. Administration of the seed extract was continued until the completion of behavioral study.

b. Eight- Arm Radial Maze Tusk

Learning-related behavior was assessed by four-arm baited eight-arm radial maze paradigm using standard eight-arm radial maze as described previously[12]. The radial maze is an octagonal central platform surrounded by eight equally spaced radial arms with a food cup at the end of each arm. The maze was placed in a closed room decorated with some fixed visual cues. The rats were first adapted to radial maze. Rats were then trained to collect food reward at the end of each of four arms of the eight-arm radial maze under food deprivation (~10-15%) schedule. A trial was terminated after either all the bait was consumed or after 4 min had elapsed, whatever occurred first. The performance in this situation involved two parameters (i) reference memory errors (RME), entry into unbaited arms; and (ii) working memory errors (WME), repeated entry into arms that had already been visited and obtaining the rewards within a trail. The testing continued for six days weak. Each rat was given one daily trial for six days/week for a total of 10 weeks.

c. Preparation of Brain Tissue

After the behavioral studies were completed, the rats were anaesthetized with sodium pentobarbital (65 mg/kg BW, i.p.) to collect blood, and the hippocampus and cerebral cortex were separated on ice as described previously[12]. Tissues were either first stored at −30 ºC or immediately homogenized in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 2 mM EDTA, protease inhibitor cocktails and 0.2 mM phenylmethylsufonyl fluoride using a Polytron homogenizer (PCU 2–110; Kinematica GmbH, Steinhofhale, Switzerland). The homogenates were immediately subjected to the assays described below or stored at −80 ºC.

d. Measurement of Acetylcholine Esterase Activity of Cerebral Cortex

Acetylcholine esterase activity was measured by the method of Ellman et al. (1961) [13] at 25ºC. The method is based on the hydrolysis of the substrate acetylthiocholine. The standard 1.0 ml reaction mixture for the assay contained 100 mM phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 0.50 mM acetylthiocholine, 0.125 mM 5,5'-dithiobis-2-nitrobenzoic acid and 100 □g homogenate protein. The blank consisted of solutions without the protein. The reaction was recorded at 412 nm by using a Hitachi U-2000 spectrophotometer and the rate was calculated as □moles of substrate

hydrolyzed per min per mg protein. Acetylcholine esterase activity was expressed as U/mg of protein. One unit was the amount of enzyme that hydrolyzes 1 µmol of acetylthiocholineiodide/min/mg protein.

e. Measurement of Catalase Activity of Cerebral Cortex

Catalase activity was assayed according to the method of Claiborne (1985)[14]. Briefly, the assay mixture consisted of 1.90 ml of phosphate buffer (50 mM, pH 7.0), 1 ml of H_2O_2 (0.02 M) and 0.1 ml of cortex homogenate in a final volume of 3 ml. Control cuvette contained all the components except substrate. Change in absorbance was recorded at 240 nm. Catalase activity was expressed as U/mg protein by using molar absorption coefficient of H_2O_2 as 43.6 M-1cm-1 (one unit was the amount of enzyme that utilized 1 µmol of H2O2/ min/mg protein).

f. Lipid Peroxidation (LPO) Assay

Lipid peroxide content (LPO) was estimated by the thiobarbituric acid reactive substances (TBARS) test of Ohkawa et al. (1979)[15]. Tissue homogenate (0.1 ml) was mixed with 0.1 ml of 8.1% (w/v) sodium dodecylsulphate, 2 ml of 0.4% thiobarbituric acid in 20% acetic acid (pH 3.5) and 0.1ml distilled water. Each tube was tightly capped and heated in a heating block at 95 °C for 1h. After cooling the tubes with tape water, 1.5 ml of n-butanol-pyridine (15:1, v/v) was added and shaken vigorously for 10 min. The tubes were then centrifuged at 2500 rpm for 10 minutes at room temperature and the absorbance of the supernatant fraction was measured at 532 nm. The levels of LPO were expressed as nmol/mg of protein of the tissue homogenate against 1, 1, 3, 3-tetraethoxypropane as standard. Total protein was estimated by the method of Lowry *et al.* (1951)[16].

g. Surgery for Unilateral Common Carotid Artery Occlusion to Produce Hypoxia-Induced Oxidative Stress

In parallel set of experiments, hypoxia was induced by unilateral occlusion of the common carotid arteries of both the *S. cumini* seed extract-pretreated and control rats. Under pentobarbital anesthesia (0.2 ml for induction, 0.1ml for maintenance), a midline neck incision was made and the left common carotid artery was occluded with surgical thread, the incision was closed and left for 4h on a heating pad (35°C to 37°C) for recovery. After 4h, the carotid arterial blocks were released and allowed further 1h in order to allow re-perfusion. Afterwards, the rats were killed and their hippocampus and cortex were separated from the skull for oxidative stress assay and/or perfused with saline followed by 10% formaldehyde for brain histology.

h. Histology

The brains were paraffin processed and excised for coronal blocks from the cortex and hippocampus tissue. Sections with a thickness of 10 m were cut and stained with hematoxylin and eosin for examining the overall morphology (Leica CM1850, Leica Microsystems GmbH, Wetzlar, Germany). Sections were mounted on gelatin–chrome–alum coated slides. The microscopic observation was done by fluorescent microscope normal spectra (Nikon eclipse E200) in 100X and 10X. The picture was taken by digital camera attached to it.

i. Total Polyphenol Contents and In *Vitro* Free Radical Scavenging Activity of *S. cumini* Seed Extract

To estimate the polyphenolic content by modified Folin- Ciocalteu's method[17] gallic acid was used as an analytical standard, as described previously[11], but with slight modification. Briefly, the calibration curve was generated using different concentrations of gallic acid (0 - 0.2 mol) in the reaction mixture of Folin-Ciocalteu's phenol (FCP) reagent in total volume 200 l in microwell plate. These solutions were incubated at room temperature for 40 min. The absorbance was recorded at 710 nm using ELISA plate reader.

Antioxdative power of the *S. cumini* seed extract was analyzed by its DPPH radical scavenging activity, as described previously[11]. The free radical scavenging effect was compared with that

of the quercetin and BHT using equimolar concentrations with equal reaction volumes. Antioxidant activity was expressed as the concentration of the seed extract (IC50) required to decrease the absorbance of the DPPH by 50%.

To directly visualize the antioxidant activity of the extract, aliquots of 8 µL 4 mM DPPH solution were subjected to thin layer chromotagraphic (TLC) plate. After air dry, 8 µL of the extract and reference standard was re-applied onto the DPPH spots. After 30 min of incubation, the spots were photographed and analyzed by ImageJ. The references were equimolar concentrations of vitamin C and BHT.

3. Statistical Analysis

Results are expressed as mean ± SEM (standard error of means). Behavioral data were analyzed by a 2-factor (group and session) randomized block factorial ANOVA, and all other parameters were analyzed for intergroup differences by unpaired student's *t*-test. Correlation was determined by simple regression analysis. Statistical programs used were GB-STAT™ 6.5.4 (Dynamic Microsystems, Inc., Silver Spring, MD, USA), and STATVIEW v4.01 (MindVision Software, Abacus Concepts, Inc., Berkeley, CA, USA). A level of $P < 0.05$ was considered statistically significant.

4. Results

4.1 Body Weight and Food Intake

No significant differences were observed in body weight or food intake, measured after 12 wk, between control and *S. cumini* seed extract groups.

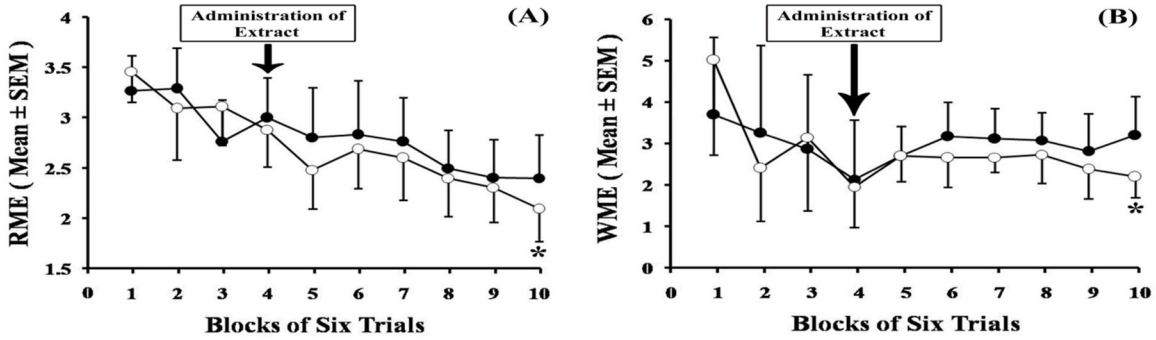

Fig 1. Effects of oral administration of S. *cumuni* extract on reference memory error (RME) (A) and working memory error (B). Data are the mean ± SEM for each block of six trials showing the number of reference memory error until the rat acquired all the rewards. Data were analyzed by randomized block two-way (block and group) ANOVA.

4.2 Eight- Arm Radial Maze Task

Figure 1A shows the effect of chronic administration of DHA on reference memory-related learning ability in aged rats. The score is expressed as mean number of reference memory errors (REMs) with data averaged over blocks of six days. A randomized block two-way ANOVA (block and group) was conducted based on the scores. The analysis revealed a significant main effect of blocks of trials ($F_{9,59} = 2.81$; $P<0.0085$) and group ($F_{1,9} = 7.68$; $P<0.022$) on the number of RME. The analysis also revealed a significant block-group interaction ($F_{9,59} = 2.86$; $P < 0.0075$). The effect of oral administration of *S. cuminii* on working memory related learning abilities of the old rats is shown in Figure 1B. The score is expressed as mean number of working memory errors (WMEs) with data averaged over blocks of six days. A randomized block two-way ANOVA (block and group) was conducted based on the scores. The analysis revealed a significant main effect of blocks of trials ($F_{9,59} = 5.59$; $P < 0.0001$)

on the number of WME but not of group ($F_{1,9}$ = 5.57; P<0.043). The analysis also reveals a significant block-group interaction ($F_{9,59}$ = 2.11; P < 0.048).

4.3 Brain Lipid Peroxidation

The levels of LPO significantly decreased both in the cerebral cortex and hippocampus of the *S. cumini* seed extract-administered rats (Table 1), as compared to those of the control rats.

Table 1. Effects of oral administration of *S. cuminii* extract on the levels of LPO in cerebral cortex and hippocampus

	Lipid Peroxidation (nmol/mg of protein)	
	Cerebral cortex	**Hippocampus**
Control	3.10 ± 0.10 [a]	2.58 ± 0.20 [a]
S. cumini	1.60 ± 0.35 [b]	1.55 ± 0.10 [b]

Data are the mean ± SEM, n = 5. Values in the same column that do not share common superscripts are significantly different at P < 0.05. Data were analyzed by unpaired student's *t*-test.

4.4 Enzyme Activity of Cerebral Cortex

Acetylcholine esterase activity of cerebral cortex of *S. cumini* seed extract-administered rats increased significantly, as compared to those of the control rats (Fig 2A). Catalase activity also significantly increased in the *S. cumini* seed extract-administered rats as a result of oral administration of *S. cumini* extract (Fig 2B).

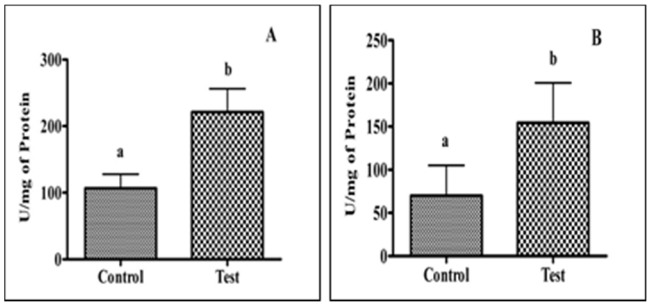

Fig 2. Effects of oral administration of *S. cumini* seed extract on cerebral cortex acetylcholine esterase (A) and catalase (B) activities. Results are mean ± SEM for six rats each with duplicate determinations. Bars with different alphabets are significantly different at P < 0.05.

4.5 Correlation Between Learning Ability Vs. The Corticohippocampal Levels of LPO

Regression analysis showed a significant positive relationship between the corticohippocampal levels of LPO and mean number of RMEs in the final block (Fig 3). Regression analysis also revealed significant negative relationship between acetylcholine esterase activities in the cerebral cortex and mean number of RMEs in the final block (r = - 0.63; P < 0.05).

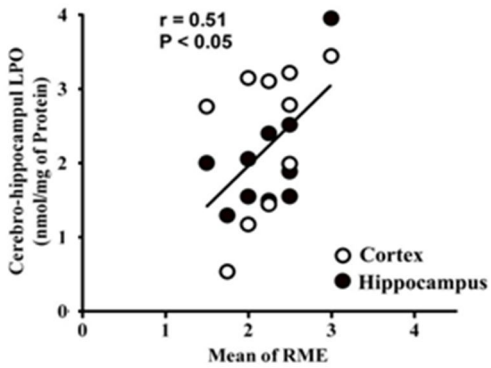

Fig 3. Correlation between learning ability and the levels of lipid peroxide in cortex and hippocampus. Learning ability is expressed as the mean of reference memory error (RME) in the final block. Data were subjected to linear simple regression analysis.

4.6 Histopathology

In hypoxic rat brain cortex we found cell swelling characteristic in most of the cells and many cell membrane was found broken in contrast with those in the non-hypoxic control brain slice. Normal characteristic of the CA region of the hippocampus also was found damaged in the hypoxic rats. The pyramidal layer cell layer was scattered and showed high inflammation of that region due to the presence of many inflammatory cells. In the hypoxic group treated with *S. cumini* extract, the pyramidal cell layer of the hippocampus was visually normal and there was much less inflammation than that of the untreated control rats. Therefore, these results suggest that carotid artery occlusion caused hypoxia in the brain tissues and consequently oxidative stress-mediated brain cell damage. Moreover, the hypoxia-induced oxidative stress was evident by the increased levels of LPO both in the cortex and hippocampus (data not shown). However, seed extracted-pretreated hypoxic rats treated with seed extract exhibited very few damaged cells and more over cells with less-swelling characteristics. Most of the cells retained their normal cellular structure, thus demonstrating that an extract-mediated protective effects against cellular damages in the corticohippocampal brain tissues.

4.7 Antioxidant Properties of *S. cumini* Extract

Since fruits grown in different seasons vary in its antioxidant capacity, we re-determined the total polyphenol contents of *S. cumini* seed extract. This method revealed that *S. cumini* seed extract contained ~11 µmol GAE/100 mg of dry powder, which was slightly higher than that our previous result (~ 8 µmol GAE/100 mg of dry powder). The result is consistent with the seasonal variation of the antioxidants in the seed powder of *S. cumini* fruits. Accordingly, the IC_{50} values were: 9.0, ~ 200 and ~ 7.0 µM GAE, respectively, for quercetin, BHT and *S. cumini* seed extract. The results were also consistent with the dose dependent disappearance of the DPPH's purple color spots of the thin layer chromatographic plate in the presence of *S. cumini* seed extract (Fig. 1B).

5. Discussion

The present study demonstrates that the oral administration of S. *cumini* seed extract improves radial arm performance in old rats concomitantly with the amelioration of the levels of LPO in the cerebral cortex and hippocampus. Radial maze behavior allows the simultaneous measurement of reference memory and working memory without any harmful effects on the rats while most widely used to study the spatial memory performance[12].

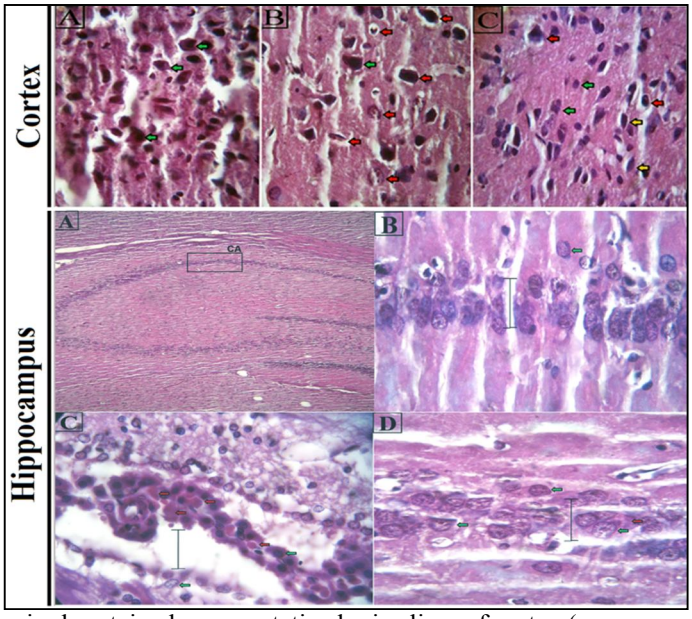

Fig 4. Hematoxylene and eosin dye stained representative brain slices of cortex (upper panel) and hippocampus (lower panel). **Cortex**: Normal (A), hypoxic (B) and hypoxic treated with seed extract (C). **Hippocampus**: Normal (A, B), hypoxic (C) and hypoxic rats treated with seed extract (D). Here, green arrows showed normal cell with normal physiological characteristics, red arrows showed cell swelling and cell rupture, yellow arrows showed cells with less damage showing improvement in characteristics. Picture (A) (B) (C) was taken at 100×magnification.

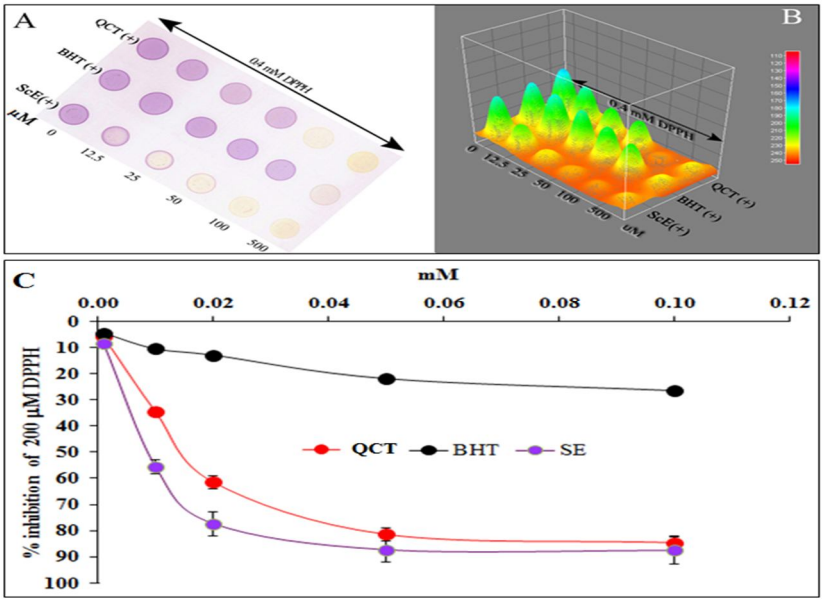

Fig 4. **A**: DPPH staining of TLC plate (A) in the absence or presence of quercetin (QCT), butylated hydroxyl tolune (BHT) and *S. cumini* seed extract (SE).The scavenging effects were dose dependent. **B**: The color of the DPPH spot was 3-D digitized by using ImageJ analyzer. **C**: Scavenging of stable DPPH free radicals by varying concentrations of *S. cumini* seed extract (SE), QCT and BHT. Each symbol represents the mean ± SEM of triplicate determinations. Data were subjected to nonlinear regression analysis with the hyperbola) equiation [$Y = B_{max}*X=/(K_i + X]$, where B_{max} is the maximal inhibition and K_i is the concentrations required to reach half maximal inhibition (IC50).

Prior to oral administration of S. *cumini* seed extract, the rats did not show any significant changes in the RME and WME scores between the control and the test rat groups. However, administration of S. *cumini* seed extract significantly decreased the numbers of RME and WME scores, thus demonstrating the improvement of spatial memory related-learning ability in SE-administered old rats (Fig 4). There was a significant positive correlation between the cerebrohippocampal levels of LPO and RME scores.

Hippocampus is considered as the key structure for the memory formation[18]. Furthermore, impairment of cognitive performance in older rats relates with increases of oxidative stress[19]. It is thus speculated that the improvement of memory-related learning ability was due to the ameliorating effect of seed extract on the oxidative stress, i.e. lipid peroxide. We previously demonstrated that the production of ROS exceeds the capacity of the cellular antioxidant defense system during aging[20]. Excessive ROS causes oxidative stress that leads to cellular damage and subsequent cell death mainly by apoptosis in neurodegeneration because the ROS oxidize vital cellular components such as lipids, proteins, and DNA[21,22]. In the present study, the administration of SE significantly decreased oxidative stress on cerebral cortex and hippocampus, as indicated by the significant reductions in the levels of LPO in these brain regions of the S. *cumini* seed extract-fed rats than those of the control rats. Regarding this, we also observed a significant augmentation of the cortical catalase activity in the test group rats than that of the control rats. Catalase activity of cerebral cortex is known to undergo an age-dependent decrement that may make polyunsaturated fatty acids (PUFA)-rich brain more susceptible to oxidative damage by free radicals in aging[1,23]. Malondialdehyde is a major product of free radical attack to membrane polyunsaturated fatty acids and it is, probably, the most widely used biomarker of lipid peroxidation[24]. Thus, the level of LPO rises as aging marker. The reductions in the levels of LPO in cerebral cortex and hippocampus of the S. *cumini* seed extract-administered rats were, thus, at least partially, attributable to the increased catalase activity.

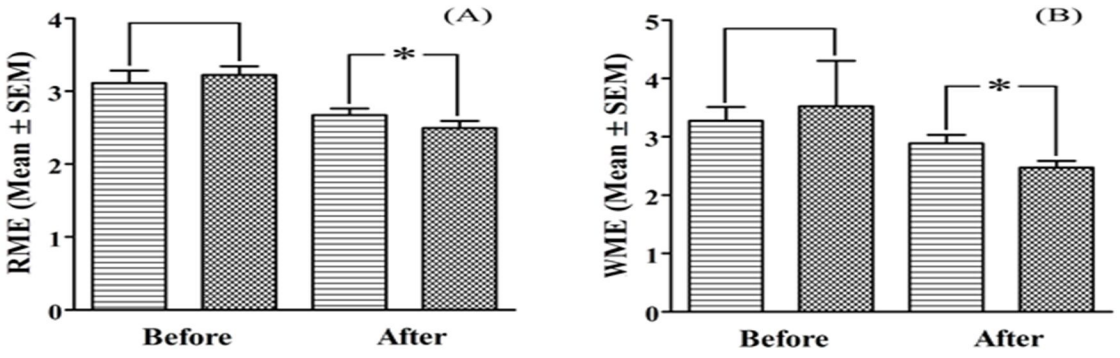

Fig 4. Effect of *S. cumini* seed extracts on of RME (A) score and WME (B) before and after oral administration of SE. Data are the mean ± SEM, n = 5. Bars with different notions are significantly different at P<0.05. Data were analyzed by student's *t*-test.

Aging is associated with a decline in cognitive function that can, in part, be explained by neurotransmission dysfunction that leads to degeneration of the cholinergic neurons in the hippocampus and cortex[25]. Hippocampus is crucially involved in spatial memory while the cortex is necessary for working memory and executive function[17,26]. In the present study, the

activity level of acetylcholine esterase enzyme significantly increased in the seed extract-administered rats than that of the control rats.

Aging is associated with changes in term of both morphological atrophy and reduction of the number of synapses that could affect the mechanism of plasticity[17,27,28]. Increased level of acetylcholine esterase activity passively indicates increased synthesis and/or release of acetylcholine. A rise in the acetylcholine esterase activity corresponds to modulation of the dendritic branching pattern or dendritic aborization and thus functionally reflects plasticity related synaptogenesis[29,30,31]. It was indirectly speculated that seed extract-induced amelioration in the cognitive function was either due to increased or restoration of age-related decline of the cholinergic system or the slowdown of the cholinergic degeneration. Long lasting change in synaptic efficacy (e.g. long-term potentiating; LTP) is the cellular basis of the memory[32]. Age-related alterations in hippocampal synaptic efficacy correlate with impairments in hippocampal-dependent learning and memory tasks[33]. Declining of spatial memory in aged rats, such as RME in the present study, is due to a decline of the synaptic efficacy of the hippocampus[34].

6. Conclusion

Finally, the results of the present investigation indicate that the memory enhancement occurs in the *S. cumini*-administered rats. Antioxidants such as vitamin E, vitamin C have are amply reported to improve the cognitive impairments of Alzheimer's disease model rats. Accordingly, we have been testing whether the oral administration of S. *cumini* seed extract to the amyloid β peptide-infused Alzheimer's disease model rats ameliorate the cognitive memory. However, further investigations are required to evaluate the mechanism(s), such as, what component(s) of S. *cumini* seed extract provide the exact beneficial effects on the learning-related memory of the rats.

7. Acknowledgement

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