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Effects of *Syzygium cumini* Seed Extract on the Memory Loss of Alzheimer's Disease Model Rats

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Abstract

Alzheimer's disease (AD) is the most prominent dementia-related disease and characterized by the presence of insoluble amyloid beta peptide (Aβ) fibers in or around the brain neurons of the affected person. Therefore, agent(s) capable of inhibiting brain amyloid deposition might delay the occurrence or retard the progress forwards of AD and related neurobehavioral symptoms. Here, we report whether, chronic oral administration of *Syzygium cumini* (locally known as Jam)-seed extract exerts protection against the progressive cognitive decline in the A$\beta_{1\text{-}40}$-infused AD model rats. After 12 weeks of feeding with *S. cumini* seed extract (at 300 mg/kg BW), we evaluated the learning-related memory of the rats by 8-arm radial maze task, where we determined two types of memory errors, namely reference memory errors (RMEs) and working memory errors (WMEs). After completion of memory tests, rats were sacrificed and the levels of lipid peroxide (LPO), the A$\beta_{1\text{-}40}$-burden, A$\beta_{1\text{-}40}$-oligomers, proinflammatory TNFα, brain derived neurotrophic factor (BDNF), Tyrosine-kinase B (TrkB), postsynaptic-density protein 95 (PSD-95) and Synapse-associated protein (SNAP-25) were determined in the cortico-hippocampal tissues of the brain. In addition, *in vitro* antioxidative effects of *S. cumini* seed extract were evaluated. The oral administration of *S. cumini* extract significantly increased the memory-related learning ability of the AD model rats, concomitantly with reductions in the levels of corticohippocampal A$\beta_{1\text{-}40}$-burden and A$\beta_{1\text{-}40}$-oligomers. Furthermore, the extract suppressed the levels of TNFα and LPO in the corticohippocampal tissues of the AD rats and also the later in the plasma, suggesting an anti-oxidative and anti-inflammatory activities of the *S. cumini* extract in the brains of AD model rats. *S. cumini* extract also increased the levels of brain cognition and memory-related proteins, including BDNF, TrKB, PSD-95 and SNAP-25. We thus

suggest that *S. cumini*-seed extract could be used in neurobehavioral deficits and associated pathogenesis of Alzheimer's disease.

Keywords

Alzheimer's Disease, *S. cumini*, Memory, Amyloid Peptide, Lipid Peroxide, BDNF, TrKB, PSD-95 and SNAP-25

1. Introduction

The pathological hallmarks of Alzheimer disease (AD) brain include amyloid neuritic plaques and neurofibrillar tangles, concurrently with progressive loss of neurons and memory-related learning ability of the patients [1]. Once it is cleaved and released from membrane-bound amyloid precursor protein (APP), amyloid beta peptide (Aβ) transforms into insoluble amyloid fibers and deposits in the brains of the affected persons [2] [3]. The predominant amyloid species found in the deposits of AD brains are A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$. It is thus conceivable that learning-related memory impairments could also be induced by the infusion of these short stretches of A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ peptides into the cerebral ventricle of rats [4] [5] [6]. In this investigation, we infused A$\beta_{1\text{-}40}$ into rat ventricles and prepared the AD model rats. AD is also an age-related neurodegenerative disease and associated with decline in memory-related learning ability [7] [8]. Numerous research conducted on AD over the past several decades have concentrated on oxidative stress and its impacts on the pathogenesis [9]. The free radicals cause oxidation of lipids [10] [11], proteins [12] [13] and DNA/RNA [14] [15] of the neurons, which become very vulnerable to death in AD. In this regard, it is thus plausible that antioxidant therapy would provide an important beneficial effect on the memory decline of AD patients. Accordingly, several studies have focused that vitamin E attenuates the toxic effects of amyloid peptide and improves cognitive performance, with a concurrent suppression of lipid peroxidation [16]-[22].

S. cumini is a seasonal fruit of Bangladesh and can act as a rich source of antioxidative and radical scavenging phytoconstituents [23]. The seeds of this fruit retain extraordinary medicinal properties, which have remained largely undiscovered. The genus *Syzygium* is one of the genera of the myrtle family Myrtaceae. Plants of this family have a rich history of uses both as edibles and as traditional medicines in divergent ethnobotanical practices throughout the tropical and subtropical world [24] [25]. The plants have beneficial effects on digestive disorders, diuresis [26], enteric disorders [27] and diarrhoea [28]. Since unknown time, *Syzygium* is also used as traditional medicine in numerous ailments, such as cough, dysenteria, ringworm, piles, pimples and liver tonic [25] [29]. We have previously reported that *S. cumini* seed powder possesses anti-diabetic activity [30] and protects the liver against the lipid peroxidation with a concurrent amelioration of hepatocellular anatomical disintigrity

of alcoholic rats [31]. *S. cumini* seed extract also exhibited stronger *in vitro* radical scavenging activity than that of the artificial antioxidant, such as butylatedhydroxy toluene (BHT). Furthermore, the oral administration of *S. cumini* seed extract restored the oxidative stress in the brain cerebral cortex of alcoholic rats [32]. We have also reported that *S. cumini* seed extract protects embryofoetal brains against intrauterine oxidative toxicity in rats during hypoxia-reperfusion injury [33]. Recently, we also have also reported that *S. cumini* seed extract improves memory-related lerarning ability of old rats [34]. In all of these contexts [23]-[34], there is substantial evidence to support the view that *S. cumini* extract might also have positive effects on the Alzheimer's disease, which is frequently seen associated with increased oxidative stress. Accordingly, we pre-administered the rats with *S. cumini* extract and infused $A\beta_{1\text{-}40}$ into rat brain ventricles to generate AD model rats. Afterwards, we evaluated whether the oral administration of *S. cumini* extract affects the memory-impairments of AD model rats. We also determined if the levels of important neuronal proteins, such as, brain-derived neurotrophic factor (BDNF), tyrosine kinase B (TrkB), post-synaptic density protein (PSD-95) and synapse-associated protein-25 (SNAP-25), essential for the maintenance of brain cognition and learning-related memory ability of the AD model rats, are affected by the oral administration of *S. cumini* seed extract.

2. Materials and Methods

Syzygium cumini (Jam) was purchased from local market. Their seeds were separated, sun-dried and powdered by grinding. The grinded seed powder was extracted by using methanol (**Figure 1**). After evaporation by rotary evaporator, the extract was suspended in distilled water and orally administered to rats and/or used in *in vitro* studies.

2.1. Animals and Diet

Twenty male *Wistar* rats (50-weeks-old) were purchased and housed in a room under controlled temperature (23˚C ± 2˚C), relative humidity (50% ± 10%) and light-dark cycles (light: 08:00 to 20:00 h: dark: 20:00 to 08:00 h), and provided with a standard pellet diet. Then they were randomly divided into 2 groups: $A\beta_{1\text{-}40}$-infused Alzheimer's disease (AD) model rats [control group (n = 10)] and

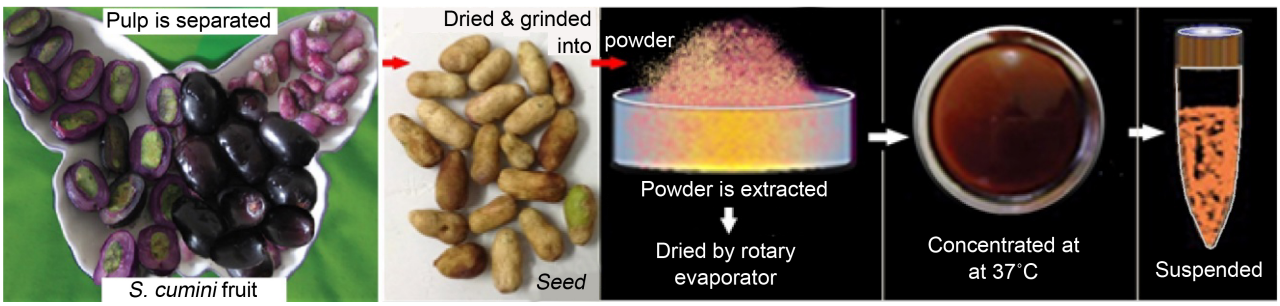

Figure 1. Collection of *S. cumini* (Jam) seed from ripe fruit and extraction from the seed powder.

S. cummini-pre-administered AD model rats [Test group (n = 10)]. Test group was administered with the extract of *S. cumini* seed powder (300 mg/kg BW) dissolved in 0.9% saline and control rats (AD) received the saline only. After 8 weeks of oral pre-administration, the rats from both groups were subjected to surgical procedures to generate AD model rats (**Figure 2**). After complete recovery from surgical stress they were subjected to eight-arm radial maze task (for memory test) and extract feeding was continued (for another 5 weeks) until the end of behavioral experiments.

Surgery

The rats were anaesthetized lightly with sodium pentobarbital (50 mg/kg BW i.p.). Before surgery, the hair of the skull was removed; the skin above the skull was fissured by using a surgical blade in aseptic condition. The rats were then placed in the sterotaxis frame and the Bregma was first identified. The surgical techniques used were essentially the same as those described previously by Hashimoto *et al.* (2002) [4] (**Figure 2**). Two holes (right and left, relative to Bregma; 0.8 mm posterior, 1.4 mm lateral) were drilled onto the exposed skull

Figure 2. Surgical procedures for Alzheimer's disease (AD) model rats. 1: Rat before surgery. 2: Hair is shaved and antiseptic liquid is treated on the shaved region. 3: Rat is placed on the stereotaxic frame. 4: Skull is opened and cleaned. 5: Bregma is identified. 6: Right and left ventricles are identified. Ventricles are 0.8 mm posterior and 1.2 mm lateral to the Bergsma. 7: Two pinholes are drilled by using microdriller. 8: Two holes are seen onto skull just above the brain's left and right ventricles. 9: Infusion of $AlCl_3$ into the right ventricle is being carried out. 10: Infusion of $A\beta_{1\text{-}40}$ is being carried out into the left ventricle. 11: After the infusion (of $AlCl_3$ and $A\beta_{1\text{-}40}$) is completed, the holes of the skull are closed by bone-cement. 12: After stitching, the rats are kept at 37˚C in the hotplate incubator. 13: Incision has been recovered (healed) after surgery. 14: The skull's condition after 5 - 7-days of surgery, indicating a complete recovery from surgery.

according to the Atlas of Paxinos and Watson (1986) [35] using a stereotaxic frame (Narishige, Tokyo, Japan). About 8.0 nmol of $A\beta_{1\text{-}40}$ (in 5 µl solvent) was infused 3.5 mm deep into the left ventricle of the brain for about 5 min with a cannula by using a 10-µl Hamilton syringe. Then, 0.5 µg $AlCl_3$ (in 5 µl volume) was infused 3.5 mm deep into the right ventricle of all rats using a Hamilton syringe according to Oka *et al.* (1999) [36] with slight modification. After complete recovery from surgery, the AD model rats were fed either with saline or *S. cumini* extract (**Figure 3**).

2.2. Behavioral Analysis

2.2.1. Adaptation Trials of the Rats

The animals were habituated with the experimenter and radial maze (once per day for 7 days prior to the memory experiment). An eight-arm radial maze used for the estimation of learning ability and set at 60 cm elevation above floor level, consisted of an octagonal center platform surrounded by eight equally spaced radial arms (50 cm long and 11 cm wide). Food cups, 1.5 cm deep and 2.5 cm in diameter, were located at the end of each arm. Then, for five days, the rats were familiarized with the apparatus, where the reward pellets were scattered through-out the maze. In this way, the rats were habituated to explore the reward (food pellet) placed in food cups at the end of the radial arm.

2.2.2. Data Collection for Learning Ability

After familiarization of the 8-arm maze, the rats were subjected to test trials, as previously described [37]. A trial consisted of baiting only four arms with reward pellet and placing the rat in the center platform facing a randomly selected arm. Here, the same four arms were baited consistently for any one animal. The testing continued for five weeks and involved two parameters of memory function:

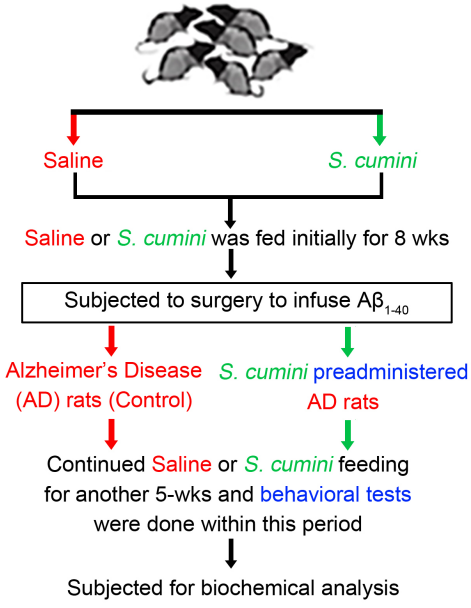

Figure 3. Experimental design.

reference memory error (RME), entry into unbaited arms; and working memory error (WME), repeated entry into arms that had already been visited within a trial. A 5 minutes trial to collect the pellets was allowed for each rat. The experimenter maintained a constant position beside the maze and observed the behavior of the rats.

2.3. Brain Tissue Preparation

After the behavioral studies for memory evaluation were completed, the rats were anesthetized 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 [37]. Tissues were either first stored at −80˚C or immediately homogenized in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 2 mM EDTA, and 0.2 mM phenylmethylsufonyl fluoride using a Polytron homogenizer (PCU 2-110; Kinematica GmbH, Steinhofhale, Switzerland). The homogenates were also centrifuged to prepare cytosolic fraction, which was stored at −80˚C until analyses were performed.

2.4. Lipid Peroxide (LPO) Assay

The oxidative stress in the plasma and brain tissues was evaluated by determining the levels of lipid peroxide (LPO). LPO was determined by estimating the thiobarbituric acid reactive substances (TBARs) according to the method Hashimoto *et al*., (2002) [4]. The samples (plasma and/or whole homogenate, 0.1 ml) were added to 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 0.1 ml distilled water. Each tube was tightly capped and heated at 95˚C for one hour. After cooling, 2 ml of n-butanol-pyridine (15:1, v/v) was added to tube and shaken vigorously for about 10 min. The tubes were then centrifuged at 1200 ×g for 10 min at room temperature (Digital centrifuge; DSC-1512SD). The absorbance of the supernatant fraction was measured at 532 nm. TEP (1,1,3,3-tetraethoxypropane) was utilized as standard. The levels of LPO were expressed as nmol/mg of protein.

2.5. ELISA for $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}40}$ Oligomers

The levels of $A\beta_{1\text{-}40}$ were determined by following the methods of Hashimoto *et al*., (2005) [38]. The levels of $A\beta_{1\text{-}40}$ oligomers were determined as previously described by Hossain *et al*. (2009) [5]. The level of $A\beta_{1\text{-}40}$ was determined from the detergent-insoluble membrane fraction (DIF) by indirect ELISA with beta amyloid 1 - 40 rabbit recombinant oligoclonal antibody [Novex by life Technologies, Catalog # 710174]. The levels of $A\beta_{1\text{-}40}$ oligomers were determined from the cytosolic fraction by the conformation-specific antibody [Invitrogen, rabbit polyclonal anti-oligomer antibody (A11), Cat # AHB0052] that recognizes the amino acid sequence-independent oligomers of the peptides. In brief, the multiwell plates were coated with detergent-insoluble fraction (DIF) or cytosolic fraction in a volume of 100 µl/well of carbonate buffer (pH 9.6) and incubated overnight

at 4˚C. After three washes, the wells were blocked with 100 µl/well 3% BSA in Tris-buffered saline (TBS) at room temperature for 1 h. Following three washes with TBS, primary antibodies, each of which was diluted at a ratio of 1:1000, were added to respective wells and incubated overnight at 4˚C. After three washes with TBS, the plates were incubated with 100 µl of HRP-conjugated anti-rabbit antibody (Biosource International, Inc., Camarillo, CA, USA) for 1 h at room temperature. After final three washes with TBS, the reaction was developed with tetramethylbenzidine for 30 min and stopped with HCl and absorbance was recorded at 450 nm. The absorbance of the samples was 4 - 5-fold higher than that of the background (blank). $A\beta_{1\text{-}40}$ concentrations in the cortex or hippocampus were determined by comparison with the $A\beta_{1\text{-}40}$ standard curve and expressed as picograms of $A\beta_{1\text{-}40}$ per milligram of total protein. However, the relative concentrations of $A\beta_{1\text{-}40}$ oligomers in the $A\beta_{1\text{-}40}$ + *S. cumini* samples were calculated by considering the absorbance of the control ($A\beta_{1\text{-}40}$-alone) well as 100%. All values were normalized to total protein concentrations.

2.6. TNFα, PSD-95, SNAP-25, BDNF and TrkB Assay

The levels of TNFα, PSD-95, SNAP-25, BDNF and TrkB proteins were determined by using indirect ELISA as previously described [39]. In brief, the multiwell plates were coated with 50 µg of cytosolic protein in a volume of 100 µl/well of carbonate buffer (pH 9.6) and incubated overnight at 4˚C. After three washes, the wells were blocked with 1% BSA in Tris-buffered saline (TBS) at room temperature for 1 h. Following three washes with TBS, primary anti-TNFα-antibody, primary anti-PSD-95 antibody, primary anti-SNAP-25 antibody, primary anti-BDNF antibody and primary anti-TrkB antibody, each of which was diluted at a ratio of 1:1000, were added to respective wells and incubated at 4˚C overnight. Again, after three washes with TBS, the plates were incubated with 100 µl of HRP-conjugated anti-rabbit secondary antibody for 1 h at room temperature. After final three washes with TBS, the color was developed with tetramethylbenzidine for 30 min and the reaction was stopped with HCl, and absorbance was recorded at 450 nm (ErbaLisascan II, Mannheim, Germany). The absorbance of the samples was 4 - 5-fold higher than that of the background (blank).However, the relative concentrations of TNFα, PSD-95, SNAP-25, BDNF and TrkB in the $A\beta_{1\text{-}40}$ + *S. cumini* samples were calculated by considering the absorbance of the control ($A\beta_{1\text{-}40}$-alone) well as 100%. Finally, all absorbance values were normalized to per mg of protein.

2.7. *In Vitro* Anti-LPO (Lipid Peroxide) Study

The brain tissues from normal old rats were homogenized in phosphate buffer (50 mM, pH 7.4) using Dounce glass homogenizer (the USA). The homogenates were adjusted to a final concentration of 100 mg of tissue/ml of buffer. Briefly, 100 µl whole homogenate containing 100 µg of protein from each brain was treated with Fenton's reagent [H_2O_2 (45 mM) + $FeSO_4$ (2.0 mM)] in the absence

or presence of *S. cumini* seed extract. Controls and extract-alone homogenates were treated at identical conditions without the addition of Fenton's reagents. The reaction mixtures were then incubated at room temperature for 4 hours. At the end of incubation, the levels of LPO were determined as described above for those of the plasma [4].

2.8. *In Vitro* Free Radical Scavenging Activity of *S. cumini* Seed Extract

Antioxdative power of the *S. cumini* seed extract also was analyzed by its DPPH radical scavenging activity, as described previously [40]. Briefly, the antioxidant activity of the extract was studied at several concentrations (0 - 1.0 mM GAE, gallic acid equivalent). Aliquots of DPPH solution in ethanol (0.4 mM, 100 µl) and each extract solution (100 µl) or standard solutions of BHT (0 - 1.0 mM, 100 µl) and quercetin (0 - 1.0 mM, 100 µl) were mixed in the wells of 96-well plate. The plate was then sealed, shaken for 10 s and placed in the dark for 30 min. Afterwards, the plate was carried out at room temperature, again shaken for 10 s, and then absorbance was taken at 515 nm. Data were reported as the average of three measurements. The IC_{50} value was expressed as the concentration of extract or standards (BHT and quercetin) required to decrease the absorbance of DPPH (0.2 mM, final concentration) by 50%. The value can be determined graphically by plotting the absorbance against the used extract or standard concentrations.

In addition, to directly visualize the antioxidant activity of the extract, aliquots of 8 µl 0.4 mM DPPH solution were subjected to thin layer chromatographic (TLC) plate. After air drying, 8 µl of the extract and standards (BHT and quercetin) were re-applied onto the DPPH spots. After 30 min of incubation, the spots were photographed and analyzed by Image J.

2.9. Total Polyphenol/Flavonoid Contents of *S. cumini* Extract

To estimate total polyphenol content of *S. cumini* extract gallic acid was used as standard, as described previously by Hossain *et al.* (2012) [32]. Briefly, the calibration curve was generated using different concentrations of gallic acid (0 - 0.2 mM) in the reaction mixture of Folin-Ciocalteu's reagent (FCR) in total volume 200 µl in micro well plate. These solutions were incubated at room temperature for 40 min. The absorbance was recorded at 710 nm using ELISA plate reader. Total polyphenol content was expressed as µmol of gallic acid equivalents per 100 mg of dry powder (GAE/100 mg of dry powder). Total flavonoid content of the *S. cumini* extract was estimated following aluminum chloride colorimetric assay described by Chang *et al.* (2001) [41]. Quercetin was used as standard. Total flavonoid content was expressed as µmol of quercetin equivalents per 100 mg of dry powder (µmol of QE/100 mg of dry powder).

2.10. Statistical Analysis

Results were expressed as mean ± SEM (standard error of means). Behavioral

data were analyzed by a 2-factor (group and session) randomized block factorial ANOVA (expressed as mean ± SD (standard deviations), and all other parameters were analyzed for intergroup differences by unpaired students *t*-test and/or one-way ANOVA. 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.

3. Results

3.1. Effects of *S. cumini* Administration on the BW of A*β*-Infused AD Model Rats

No significant differences were observed in body weight or food intake, measured after 12 wks, between Control ($A\beta_{1\text{-}40}$ + Vehicle-administered) and *S. cumini* seed extract-administered AD model rats ($P < 0.05$). The body weight of $A\beta_{1\text{-}40}$ infused rats was increased to the extent of 139% as compared to 135.5% increase in weight of $A\beta_{1\text{-}40}$ + *S. cumini* seed extract-administered rats. Average daily food intake in Control was 15.60 gm/rat and in *S. cumini* seed extract rats was 16.13 gm/rat.

3.2. Effects of *S. cumini* Administration on the Memory of A*β*-Infused AD Model Rats

Figure 4(A) shows the effect of chronic administration of *S. cumini* seed extract on reference memory-related learning ability in AD rats. The score is expressed as mean number of reference memory errors (REMs) with data averaged over blocks of six trials.

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 (F5, 41 = 5.96; $P < 0.0001$) and group (F1, 5 = 17.3; $P < 0.0002$) on the

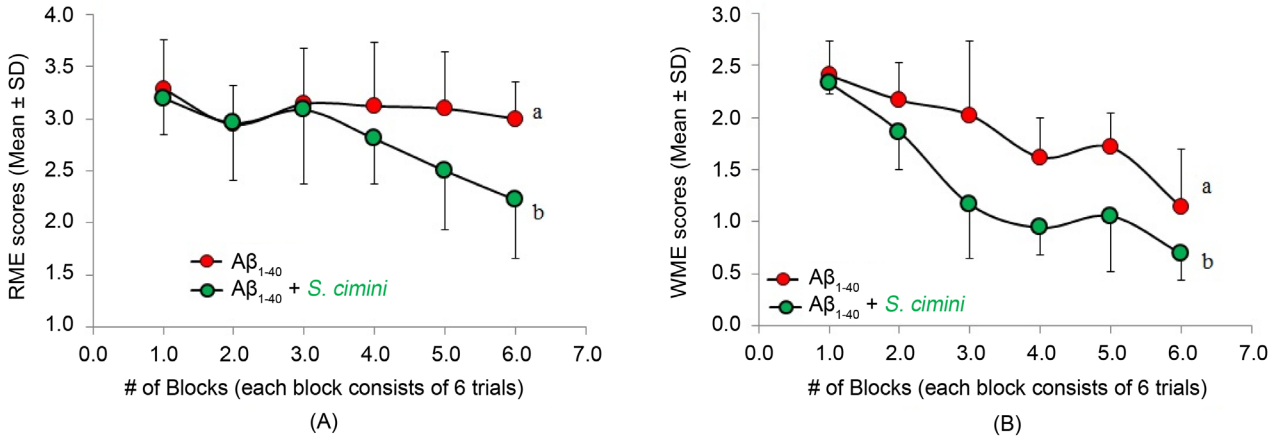

Figure 4. Effects of oral administration of *S. cumuni* extract on reference memory error (RME) (A) and working memory error (B). Data are the mean ± SD for each block of six trials showing the number of reference memory error until the rat acquired all the rewards. Data were analyzed by randomized block two-way (block and group) ANOVA.

number of RME. The analysis also revealed a significant block-group interaction (F5, 205 = 2.403; $P = 0.038$).

The effect of oral administration of *S. cumini* on working memory related learning abilities of the AD rats is shown in Figure 4(B). 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 (F5, 41 = 10.76; $P < 0.0001$) and groups (F1, 5 = 17.3; $P < 0.0001$) on the number of WME. The analysis did not reveal a significant block-group interaction (F5, 205 = 1.02; $P = 0.40$).

3.3. Effects of *S. cumini* Administration on the Plasma and Brain LPO of AD Model Rats

The levels of LPO significantly decreased by ~28% in the plasma of the *S. cumini*-fed ($A\beta_{1\text{-}40}$ + *S. cumini*) rats, when compared to those of $A\beta_{1\text{-}40}$ alone infused AD control rats. The levels LPO also decreased both in the cerebral cortex (by~26.5%) and hippocampus (by~33%) of the *S. cumini* seed extract-administered AD model ($A\beta_{1\text{-}40}$ + *S. cumini*) rats (Figure 5), as compared to those of the $A\beta_{1\text{-}40}$-infused AD control rats.

3.4. Effects of *S. cumini* Administration on the Levels of $A\beta_{1\text{-}40}$ in the Brains of AD Model Rats

The levels of $A\beta$ peptide$_{1\text{–}40}$, as determined by indirect ELISA, significantly decreased in the detergent-insoluble fraction (DIF) of cortex (~18%) and hippocampus (>20%) homogenates of ($A\beta_{1\text{-}40}$ + *S. cumini*) rats. This suggest that *S. cumini* extract, by somehow decreased the amyloid burden in the brains of Alzheimer's disease model rats (Figure 6).

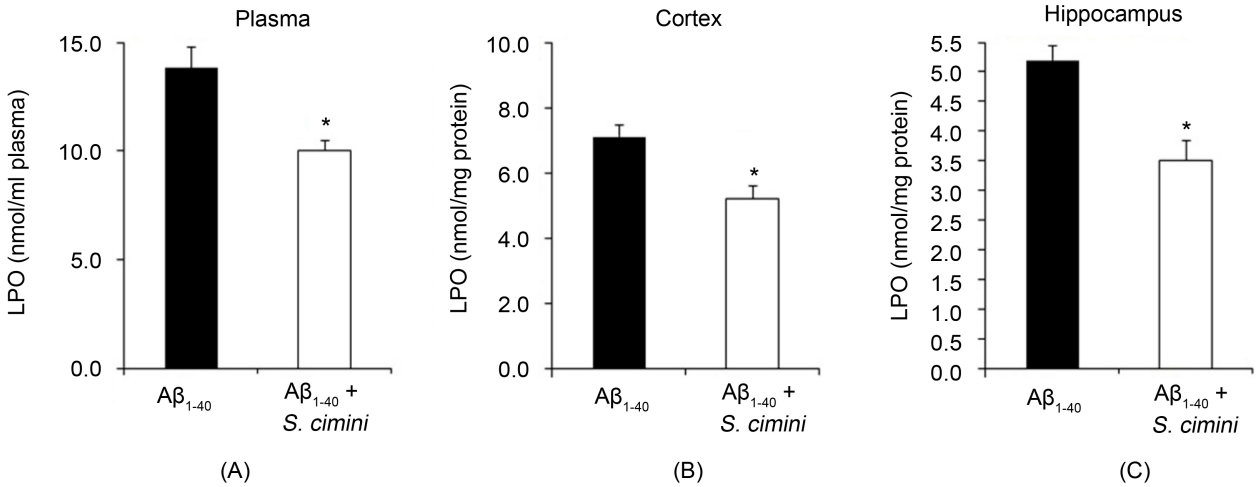

Figure 5. Effects of oral administration of *S. cumini* seed extract (SC) on the plasma (A), cerebral cortex (B) and hippocampus (C) lipid peroxide (LPO) levels of the $A\beta_{1\text{-}40}$-infused Alzheimer's disease (AD) model rats. Results are mean ± SEM for 7-9 rats each with duplicate determinations. Bars with different notations are significantly different at $P < 0.05$.

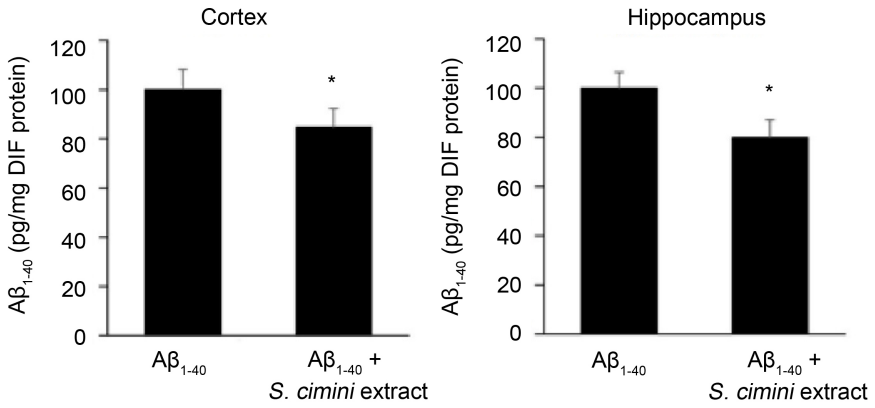

Figure 6. Effects of oral administration of *S. cumini* seed extract on the levels of A$\beta_{1\text{-}40}$ in the detergent-insoluble membrane fractions (DIF) of cortex and hippocampus. DIF was prepared as described previously [38]. Each bar represents the mean ± SEM (n = 7 - 8). The data were analyzed by unpaired student's *t*-test. **P* < 0.005.

3.5. Effects of *S. cumini* Administration on the Levels of A$\beta_{1\text{-}40}$ Oligomers in the Brains of AD Model Rats

The relative levels of Aβ oligomers, the most toxic species of A$\beta_{1\text{-}40}$ also significantly decreased in the cortex of *S. cumini*-fed Alzheimer's disease (AD) model (A$\beta_{1\text{-}40}$ + *S. cumini*) rats (**Figure 7**). This suggests that the components in *S. cumini* might have reduced the brainamyloid A$\beta_{1\text{-}40}$ burden by inhibiting at the stages of oligomer formation.

3.6. Effects of *S. cumini* on the TNFα, BDNF, TrKB, PSD-95, SNAP-25 of AD Model Rats

The oral administration of *S. cumini* decreased the levels of proinflammatory TNFα and invariably increased the levels of the brain cognition/synaptic plasticity-related proteins (**Figure 8**). The levels of pro-inflammatory TNFα decreased by 18%, BDNF increased by 37%, TrKB increased by 14%, PSD-95 increased by 31% and SNAP-25 increased by 44% in the cortex of *S. cumini*-administered AD model rats, as compared those of the A$\beta_{1\text{-}40}$-infusedalone AD rats. (**Figure 8**, upper panel).

The levels of pro-inflammatory TNFα decreased by 17%, BDNF increased by 27%, TrKB increased by 24%, PSD-95 increased by 45% and SNAP-25increased by 28% in the hippocampus of *S. cumini*-administered AD model (A$\beta_{1\text{-}40}$ + *S. cumini*) rats, as compared those of the A$\beta_{1\text{-}40}$-alone infused AD rats. (**Figure 8**, lower panel).

3.7. Effects of *S. cumini* Extract on the Fenton's Reagent-Induced Oxidative Stress of the Brain Tissues Homogenates (*in Vitro* Anti-LPO Effects)

The levels of LPO significantly incresed (>70%) in the homogenates of Fenton's reagents (FR)-incubated brain samples. This indicates a successful induction of oxidative stress (OS) in the FR+ homogenate samples, when compared to those

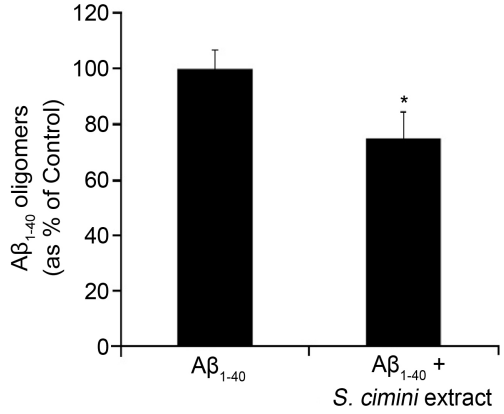

Figure 7. Effects of oral administration of *S. cumini* seed extract on the levels of A$\beta_{1\text{-}40}$ oligomers of in the cortex of AD model rats. The mean absorbance value, normalized to one mg of protein, of the control (A$\beta_{1\text{-}40}$-alone) samples was considered as 100%. Each bar represents the mean ± SEM (n = 7 - 8). The data were analyzed by unpaired student's *t*-test. **P* < 0.005.

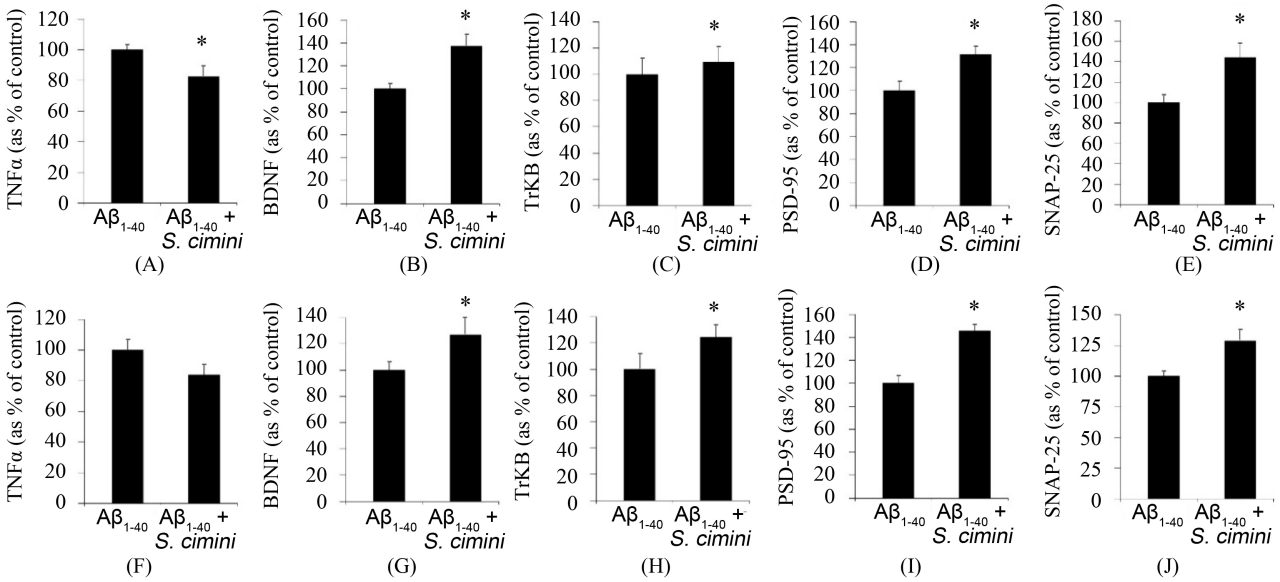

Figure 8. Upper panel: Effects of oral administration of *S. cumini* seed extract on the levels of (A) TNF*α*, (B) BDNF, (C) TrKB, (D) PSD-95 and (E) SNAP-25 in the cortex of AD model rats. Lower panel: Effects of oral administration of *S. cumini* seed extract on the levels of (F) TNF*α*, (G) BDNF, (H) TrKB, (I) PSD-95 and (J) SNAP-25 in the hippocampus of AD model rats. Each bar represents the mean ± SEM (n = 7 - 8). The data were analyzed by unpaired student's *t*-test. **P* < 0.05.

of the control homogenate alone (**Figure 9**). However, if the oxidative stress was instigated in the presence of *S. cumini*, the levels of LPO were significantly suppressed (28% as compared to those of the control). This suggests that *S. cumini* extract significantly withstood the oxidative stress, as indicated by the reductions of the levels of LPO in the *S. cumini*-incubated brain homogenates (**Figure 9**). *S. cumini* alone also decreased the levels of LPO.

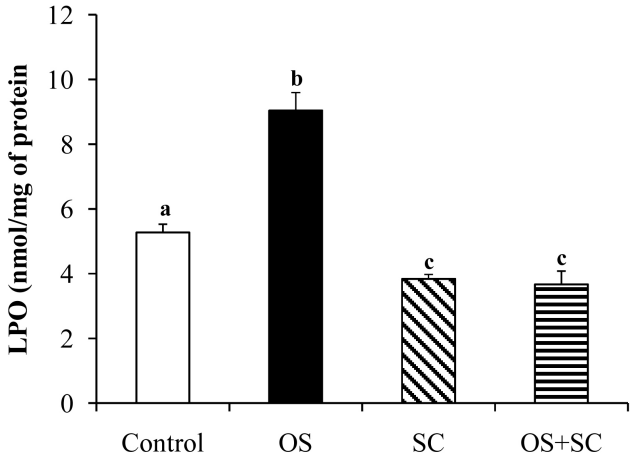

Figure 9. *In vitro* effects of seed extract on the oxidatve stress (OS)-induced production of lipid peroxide (LPO) in the brain tissues of the rats. Results are expressed as mean ± SEM. Bars that do not share a common alphabet are significantly different at $P < 0.05$ (Two-way ANOVA). Con = Control homogenate. OS = Oxidative stress was induced in the homogenate samples by Fenton's reagents. SC = Homogenate samples incubated with the *S. cumini* extract alone. OS + SC = OS was induced in the *S. cumini* extract-preincubated samples.

3.8. *In Vitro* Free-Radical Scavenging Effects of *S. cumini* Seed Extract

The DPPH-scavenging activities of *S. cumini* seed extract versus BHT and quercetin are shown in the Figure 10. The non-linear analyses revealed IC50 of 0.12 ± 0.03 mM, 0.014 ± 0.001 mM and 0.006 ± 0.0.001 mM GAE, respectively, for BHT, quercetin and the *S. cumini* seed extract (Figure 10(a)). The seed extract or standards dose-dependently (0 - 0.1 mM) decolorized the DPPH spots (Figure 10(b)). Therefore, the intensity, as evaluated by ImajeJ, of the DPPH spots gradually decreased with the concentrations of the extract and standards (Figure 10(c)). This indicates an anti-oxidative potential of the *S. cumini* extract.

3.9. Total Polyphenol and Flavonoid Contents of *S. cumini* Extract

We determined the total polyphenol/flavonoid contents of *S. cumini* seed extract. *S. cumini* seed contained 8.11 ± 0.10 μmol GAE/100 mg of dry powder. Total flavonoid content was 2.3 ± 0.07 μmol QE/100 mg of dry powder.

4. Discussion

The aim of the present study was to investigate whether the chronic oral pre-administration of *S. cumini* seed extract improves the memory-related learning impairments of Alzheimer's disease (AD) model rats, suppresses the levels of oxidative stress, pro-inflammatory TNFα and elevates important brain cognition-related proteins, such as BDNF, TrKB, PSD-95 and SNAP-25. On the basis of quantitative lipid peroxidation assays, amyloid peptide assays (for amyloid

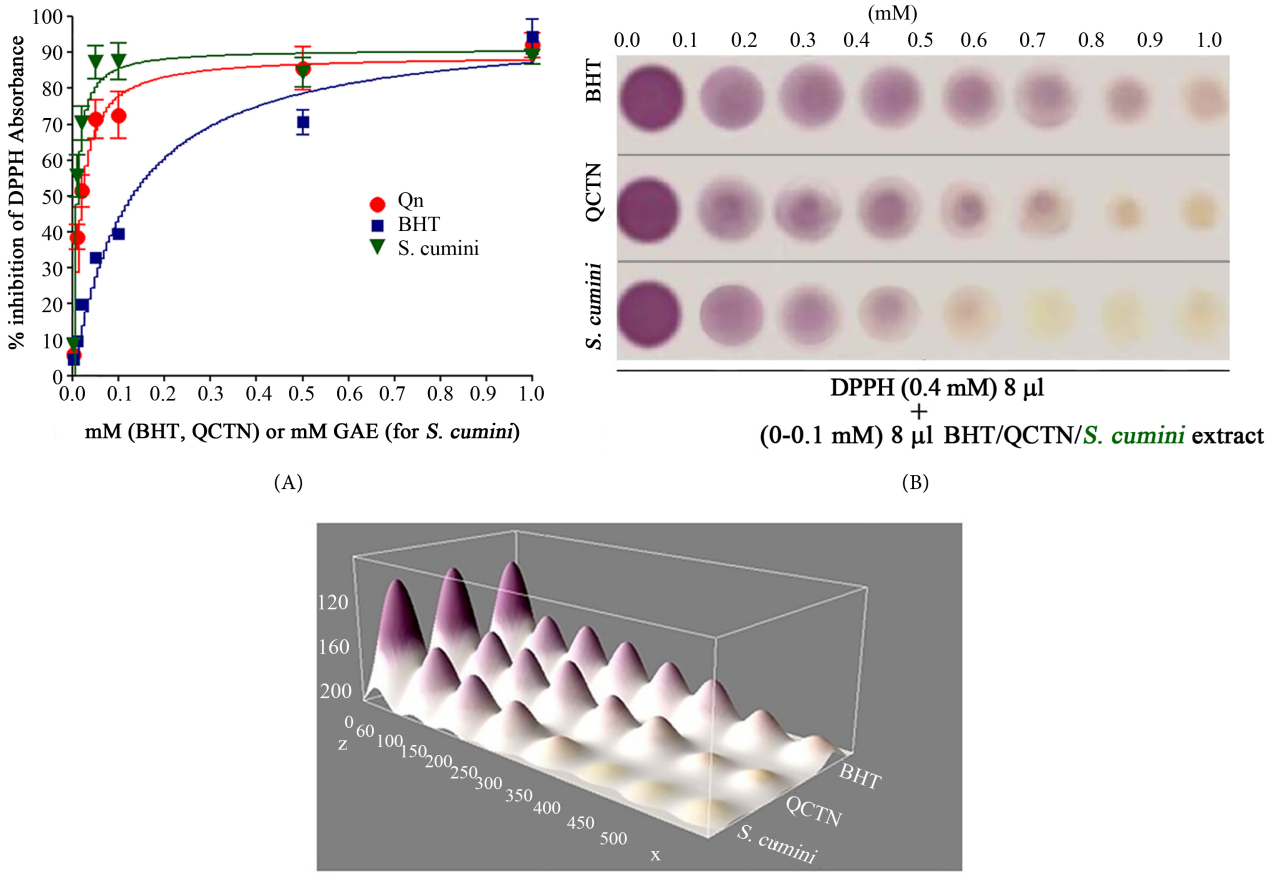

Figure 10. (A): DPPH-free radical scavenging effects of butylated hydroxytoluene (BHT), quercetin (QCTN) and *S. cumini* seed extract. Each symbol represents the mean ± SEM of triplicate determinations. Data were subjected to nonlinear regression analysis with the hyperbola equiation [$Y = B_{max}*X = /(K_d + X)$], where B_{max} is the maximal inhibition and K_d is the concentrations required to reach half maximal inhibition (IC_{50}). (B): DPPH staining of TLC plate in the absence or presence of BHT, QCTN and *S. cumini* seed extract. The scavenging effects were dose dependent. (C): The intensity of the color of the DPPH-spots of Figure (B) was 3-D digitized by using ImageJ analyzer.

A$\beta_{1\text{-}40}$ burden and amyloid oligomer levels in the brains), our study clearly demonstrates that the oral administration of *S. cumini* seed extract improves the memory impairments of the AD model rats. The improvements might relate to the decreases in the amyloid burdens in the brains of *S. cumini*-administered AD model rats, as indicated by the decreased levels of A$\beta_{1\text{-}40}$ fibers and A$\beta_{1\text{-}40}$ oligomers in these rats. Amyloid burden associated with theneuritic plaques and tau-hyperphosphorylatedneurofibrillar tangles is the pathological hall mark of Alzheimer's brains. Aβ passes through diverse stages, including α-helix to β-sheet transformation, nucleation of β-sheet into oligomers, beading of oligomers into fibrills (fibrillation) and coalescence of fibrils into larger fibers, leading to matured amyloid aggregates [2]. These molecular transitions are typically associated with neuronal loss and cognitive deficits in AD. Therefore, the principal

strategy for preventing AD is to inhibit the amyloid deposition in the brains of the AD patients. Although the accumulation of amyloid fibrous deposits is one of the important characteristic features of AD, the pathological significance of fibrillar plaques is still a matter of great debate [42] [43]. Rather, the levels of amyloid oligomers better correlate with the status of dementia in the AD patients [44]. Klyubin *et al.* (2005) [45] reported that oligomer decreases learning and memory by inhibiting the long-term potentiation. Oral administration of *S. cumini* extract decreased the amyloid burden in the brains of the AD model rats (**Figure 6**), however, the mechanism through which *S. cumini* decreased the amyloid burden from the AD rats remains to be known clearly. We speculate that the components of the *S. cumini* extract might have inhibited the amyloid oligomers, hence the amount of $A\beta_{1\text{-}40}$ deposits in the brains. Otherwise, the levels of $A\beta_{1\text{-}40}$ could have not been reduced (**Figure 6**) in the brains of *S. cumini*-fed AD rats. Infusion of Aβ into the brain ventricle reduced the learning-related memory of AD model rats, when compared to that of the control rats [4] [38], thus suggesting a clear evidence of the detrimental effect of infused-Aβ on the memory of AD model rats. In the present investigation, we thus infer that *S. cumini* ameliorated the cognitive deficits of AD rats by decreasing the amyloid burden/oligomerconcentrations from the brains of AD rats. The results are also consistent with our previous reports [34] that the *S. cumini* not only ameliorates the memory dficts of eldely rats but also those of the Alzheimer's disease model rats.

The increases in the oxidative stress, as indicated by the increased levels of lipid peroxide, are attributed to the neurodegenerative loss of neurons and related cognitive loss in the Alzheimers's disease [46] [47]. In our investigation, the memory-deficits in the AD rats were also associated with increases in the levels of LPO both in the plasma and brain cortico-hippocampal tissues (**Figure 5**). The oral administration of *S. cumini* significantly reduced the levels of LPO both in the plasma and brain tissues (**Figure 5**). Therefore it is conceiveable that *S. cumini*-induced decreases in the oxidative stress contributed to the amelioration of the cognitive deficits of AD model rats. The speculation is consistrent with the *in vitro* anti-LPO effect, DPPH-free radical scavenging effect, concurrently with the presence of subtantial amounts of total polyphenols/flavonoids in the extract of *S. cumini* seeds.

Neurodegeneration of Alzheimer's disease is also characterized by inflammation in the brain. Pathophysiology and associated inflammation of the AD brain thus might help to elucidate potential therapeutic and preventative options [48]. Neuro-inflammation driven by glial activation contributes to the damage of neurons [49]. These reports are well consistent with the increased levels of TNFα in the AD model rats of the present study. Whatever the mechanism is, our results provide a clear evidence that the induction of AD through ventricular infusion of Aβ in the rat brains resulted in an increase of the level of proinflammatory TNFα. Furthermore, the prescription of non-steroidal anti-inflmmatory (NSAID) drugs have been shown to benefit the AD symptoms [50] [51]. *S. cumini*-induced

decreases in the levels of TNFα (Figure 8) are thus consistent with these reports and that the ameoliration of proinflammatory TNFα might have contributed to the improvement of memory impairments of AD model rats in the present investigation.

Next, we evaluated whether the oral administration of *S. cumini* extract could affect the brain synaptogenesis-marker proteins, namely PSD-95 and SNAP-25. PSD-95 binds to and co-localizes with NMDA receptor (NMDAR) at postsynaptic sites for downstream signal transductions. Intraventricular infusion of Aβ peptides leads to memory impairments in rats, with a corresponding lower levels of synaptic transmission and plasticity-related proteins like BDNF and PSD-95 [52] [53] [54]. Our resuts are thus consistent with these reports. The levels of brain-derived neurotrophic factor (BDNF) and TrkB (the receptor of BDNF) decreased significantly inour A$\beta_{1\text{-}40}$-infused AD rats. BDNF mediates neuroprotective activities through various intracellular signaling pathways triggered by activating the TrkB. BDNF and its receptor TrkB are critical regulators of neuronal survival, synapse formation, neuronal plasticity of brain [55] [56]. The levels of BDNF in cortex and hippocampus also decreased in post-mortem AD patients [35] [36]. Therefore, the decreased levels of these proteins might impair the functions such as memory in the A$\beta_{1\text{-}40}$-infused AD rats, which after feeding with *S. cumini* exhibited a better memory performance.

The reduction in the level of PSD-95 in the brains of the A$\beta_{1\text{-}40}$-infusedAD model rats would be expected to alter the PSD95-NMDAR complex formation at post-synaptic membrane sites and related downstream signals, and finally disturb brain cognitions. Furthermore, the presyanptic protein SNAP-25, a component of the SNARE complex, is linked to the triggering of calcium-mediated exocytosis [57] and plays an important role in presynaptic neuro-transmitter release [58]. The levels of SNAP-25 proteins also decreased in the brains of the AD model rats. Therefore, the reductions in the levels of both PSD-95 and SNAP-25 proteins might affect the release and synaptic transmission to the postsynaptic neurons in the brains of the AD rats. The increased levels of PSD95 and SNAP-25 are thus consistent with the positive effects of *S. cumini* extract on the neurotransmitter packaging/release/synaptic connectivity, and finally on amelioration of memory *S. cumini*-fed AD rats.

5. Conclusion

The oral administration of *S. cumini* seed extract significantly ameliorated the learning-related memory impairments of A$\beta_{1\text{-}40}$-infused AD model rats. The ameliorative effects were associated with suppressions of the levels of LPO both in the brains and plasma of A$\beta_{1\text{-}40}$ + *S. cumini* rats. The effects could be attributed to antioxidative potentials of *S. cumini* seed extract. The levels of proinflammatory TNFα decreased and the levels of brain cognition/synaptic transmission-related proteins, including BDNF, TrKB, PSD-95 and SNAP-25 rose after oral administration of *S. cumini* to AD model rats. Finally, it is

concluded that *S. cumini* could be used as prophylaxis in the neurodegenerative diseases, such as Alzheimer's disease. However, the exact mechanism of action(s) of *S. cumini* extract on the BDNF, TrKB, PSD-95 and SNAP-25 proteins remains to be investigated. Thus, further experimentation is a must.

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Disclosure Statement

The authors declare no conflict of interest.

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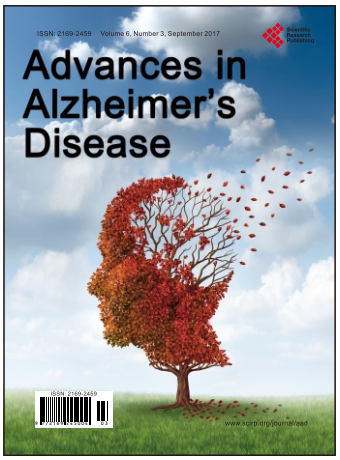

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