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Research report

# β-glucan attenuated scopolamine induced cognitive impairment *via* hippocampal acetylcholinesterase inhibition in rats

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ABSTRACT

β-glucan (polysaccharide) rich diet has been reported to enhance cognition in humans but the mechanism remained elusive. Keeping this in mind, the present study was designed to investigate the interaction of β-glucan with central cholinergic system. Briefly, *in-silico* analysis revealed promising interactions of β-glucan with the catalytic residues of acetylcholinesterase (AChE) enzyme. In line with this outcome, the *in vitro* assay (Ellman's method) also exhibited inhibition of AChE by β-glucan ($IC_{50}$ $=0.68 \pm 0.08$ μg/μl). Furthermore, the *in vivo* study (Morris water maze) showed significant dose dependent reversal of the amnesic effect of scopolamine (2 mg/kg *i.p.*) by β-glucan treatment (5, 25, 50 and 100 mg/kg, *i.p.*). Finally, the hippocampi of aforementioned treated animals also revealed dose dependent inhibition of AChE enzyme. Hence, it can be deduced that β-glucan possesses potential to enhance central cholinergic tone *via* inhibiting AChE enzyme. In conclusion, the present study provides mechanistic insight to the cognition enhancing potential of β-glucan. Keeping in mind its dietary use and abundance in nature, it can be considered as economic therapeutic option against cognitive ailments associated with decline in cholinergic neurotransmission.



## 1. Introduction

Cognition is affected in number of disorders such as Alzheimer's disease (AD). Learning and memory are the important components of the cognitive system. The former is the process of acquiring new information while later is the phenomenon of storing that information for future use. Central cholinergic system has been shown to play an important role in cognitive functions. In this regard, the degeneration of cholinergic neurons have been attributed to the cognitive impairment observed in AD subjects (Schliebs and Arendt, 2011). Furthermore, the antagonism of cholinergic system by scopolamine (a non-selective muscarinic antagonist) was shown to induce deficits in acquisition, retention, consolidation and retrieval of memory (Deiana et al., 2011; Stevens, 1981). Hence, enhancement of the cholinergic tone can presumably revert the cognitive impairment (Anand et al., 2014; Dumas and Newhouse, 2011; Haense et al., 2012).

Keeping in view this hypothesis, several strategies were devised to compensate the cholinergic deficit. This includes the use of ACh precursors and agonists of nicotinic and muscarinic receptors. Unfortunately, none of these showed efficacy because of bioavailability, safety and selectivity issues (Fisher, 2000; Francis et al., 1999; Mangialasche et al., 2010). However, the inhibitors of AChE enzyme appeared to be effective in attaining the desired therapeutic objectives. As a consequence, few inhibitors are developed and being used clinically such as donepezil, rivastigmine, galantamine and tacrine (Hasselmo, 2006; Takada-Takatori et al., 2006). Based on the cost and safety profile of these limited AChE inhibitors, there is a need to identify better candidate molecules.

Morris water maze (MWM) is a behavioural paradigm commonly used to assess spatial learning in rodents (Morris, 1984). Spatial memory is a sub-class of episodic memory, which helps in navigation and stores information in spatiotemporal frame (Burgess et al., 2002). Cognitive map theory proposed that there is a direct relation of spatial memory with the hippocampus (O'Keefe and Nadel, 2011). It is a brain structure, which is considered as cradle of cognition and critical for organization, formation and retrieval of new information (Graves et al., 2012).

The β-glucan is a polysaccharide (*i.e.* a chain of glucose molecules) found abundantly in nature such as oat, barley, rye, wheat, mushroom, fungi and yeast. It was reported to possess

*Abbreviations:* ACh, acetylcholine; AChE, acetylcholinesterase; MWM, Morris water maze; SIL, scopolamine induced locomotion; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus

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neuroprotective and immunomodulatory actions and reduces oxidative stress (Alp et al., 2012; Chan et al., 2009; Goodridge et al., 2009; Kulicke et al., 1997). It is also reported to improve spatial memory deficits in Sprague Dawley rats (Han et al., 2010; Nelson et al., 2012) and preserved memory in a mouse model of vascular dementia (Han et al., 2010). However, the mechanism underlying aforementioned cognition enhancing actions remain elusive. Keeping this in mind, the present study was designed to investigate the interaction of β-glucan with central cholinergic system.

## 2. Results

### 2.1. In-silico analysis

The docking results revealed that hydroxyl groups of middle glucose unit of β-glucan formed hydrogen bonding with SER125, THR83, TYR337 and TYR341 amino acid of active site of AChE. Furthermore, one of the glucose of β-glucan at the terminal end formed a vital interaction with important catalytic residue (SER203) of active site through hydrogen bonding. Thus, β-glucan was found to have very high affinity for active site of AChE enzyme as shown by interaction in Fig. 1(A) and (B).

### 2.2. In-vitro analysis

Donepezil treatment caused significant reduction in AChE enzyme action as compared to control as shown in Fig. 2(A) ($F(1, 4)=2080.546$, $p<0.001$). In similarity, β-glucan exhibited dose dependent inhibition of AChE enzyme as shown by the continuous decrease in the absorbance ($F(6, 14)=3394.258$, $p<0.001$). The $IC_{50}$ value of β-glucan was found to be $0.68 \pm 0.08$ μg/μl (Fig. 2(B)).

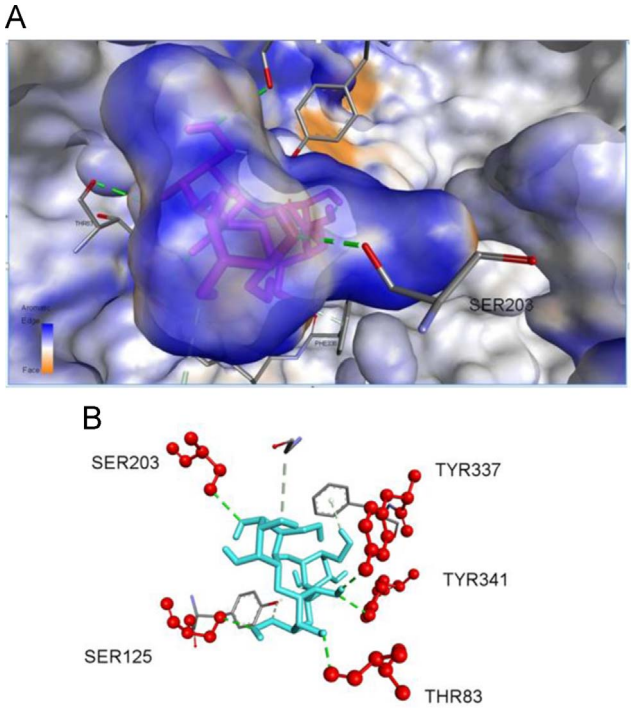



**Fig. 1.** (A) Representation of β-glucan in the active site of the AChE enzyme: Oxygen atom (red) of glucose subunit forms hydrogen bonding (green dotted line) with hydroxyl group (red) of Ser203. (B) Diagram showing chemical interaction of binding residues of β-glucan with AChE enzyme: Middle glucose unit hydroxyl groups of β-glucan made hydrogen bonding with SER 125, THR83, TYR337 and TYR341 of active site, while terminal glucose make interaction with catalytic residue (SER203).

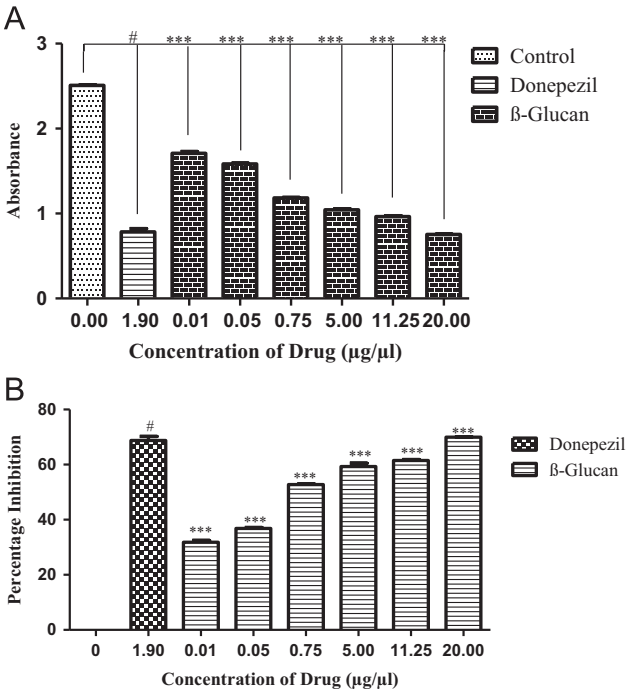



**Fig. 2.** (A) The Bar graph showing effect of β-glucan and donepezil on the absorbance in *in-vitro* AChE assay. The experiments were performed on different concentrations of β-glucan (0.01, 0.05, 0.75, 5.0, 11.25 and 20.0 μg/μl) and donepezil (1.90 μg/μl). The bars represent mean ± SEM of absorbance. Asterisks (#) and (***) suggest $p<0.001$ as compared to control. (B) The Bar graph showing effect of β-glucan and donepezil on the percentage inhibition of AChE enzyme *in-vitro*. The experiments were performed on different concentrations of β-glucan (0.01, 0.05, 0.75, 5.0, 11.25 and 20.0 μg/μl) and donepezil (1.90 μg/μl). The bars represent mean ± SEM of percent inhibition. Asterisks (#) and (***) suggest $p<0.001$ as compared to control.

### 2.3. In-vivo analysis

#### 2.3.1. Training/acquisition trial

All the groups showed significant decline in the escape latencies (time to find platform position) on day 5 as compared to respective day 1, as shown in Fig. 3.1(A)–(G).

#### 2.3.2. Probe trial

*2.3.2.1. Time spent in platform quadrant.* The rats treated with scopolamine showed significant impairment in time spent in the platform quadrant as compared to the normal saline treated rats as shown in Fig. 3.2 ($p<0.001$, ($F(1, 14)=7.889$)). The donepezil treatment has significantly reversed this effect by increasing the time spent in platform quadrant as compared to scopolamine treated rats ($p<0.001$, $F(2, 21)=4.405$). In similar manner, the β-glucan treatment also caused dose dependent increase in the time spent in the platform quadrant ($F(5, 42)=2.648$).

*2.3.2.2. Latency to find previous platform position.* The scopolamine treated rats showed significant impairment in latency to find previous platform position as compared to the rats treated with normal saline as shown in Fig. 3.3 ($p<0.001$, ($F(1, 14)=13.081$)). The treatment with donepezil significantly antagonized this effect by decreasing the latency time as compared to scopolamine treated rats ($p<0.001$, ($F(2, 21)=9.627$)). In similarity with standard, the β-glucan treatment also caused dose dependent decrease in latency time to find previous platform quadrant ($F(5, 42)=6.514$).

*2.3.2.3. Number of crossing through platform position.* Rats treated with scopolamine showed significant impairment in number of crossings through platform position as compared to rats treated

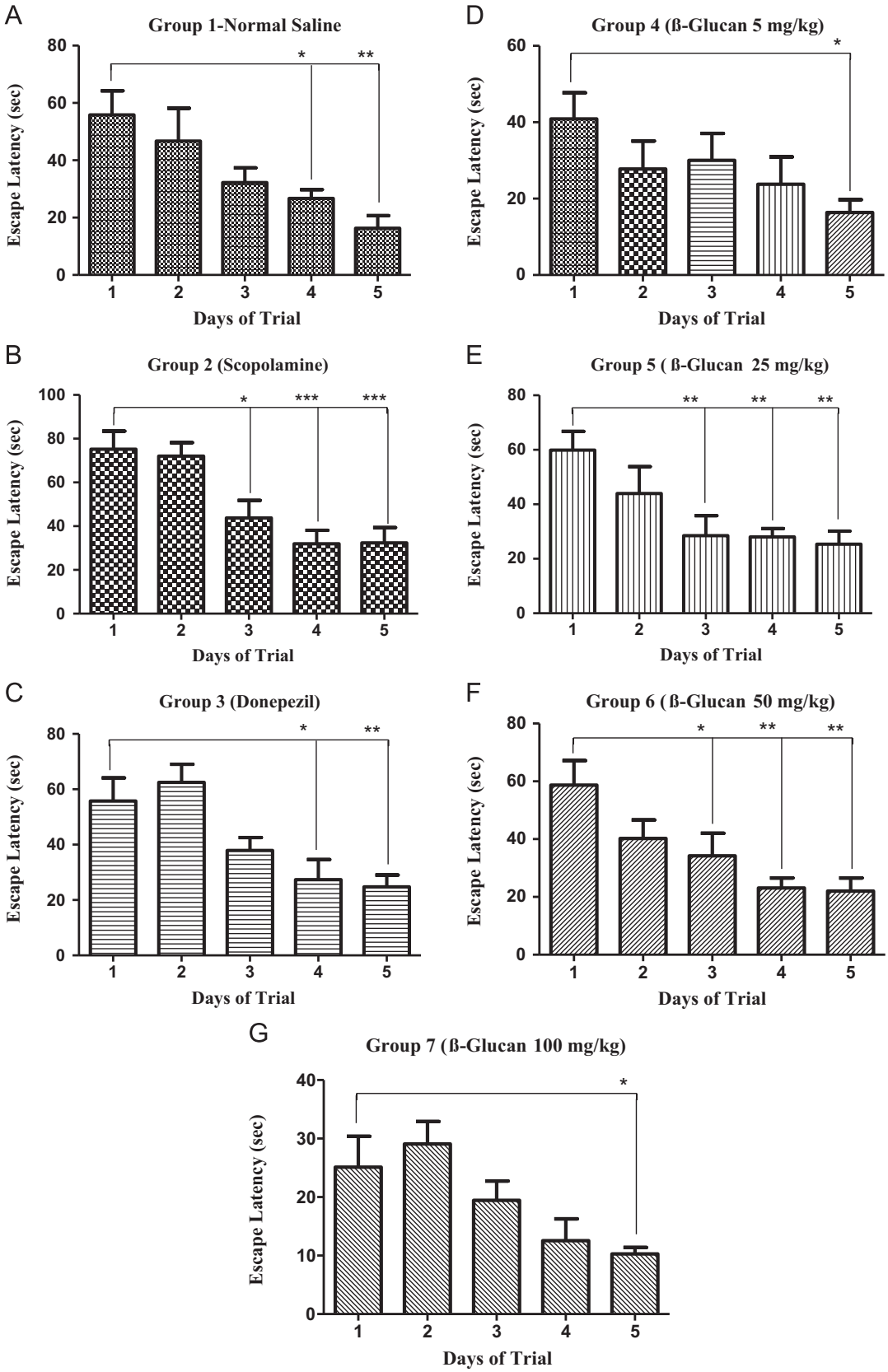



**Fig. 3.1.** (A) Bar diagram showing escape latency of various group rats in familiarization /acquisition trials of MWM. *$p < 0.05$ and **$p < 0.01$, suggests significant decline in escape latency as compared with day 1 (n=8). (B) Bar diagram showing escape latency of scopolamine group rats in MWM. *$p < 0.05$ and ***$p < 0.001$ represent significant decline in escape latency as compared with day 1 (n=8). (C) Bar diagram showing escape latency of donepezil group rats in MWM. *$p < 0.05$ and **$p < 0.01$ suggests significant decline in escape latency as compared with day 1 (n=8). (D) Bar diagram showing escape latency of beta-glucan (5 mg/kg) group rats in MWM. *$p < 0.05$ suggests significant decline in escape latency as compared with day 1 (n=8). (E) Bar diagram showing escape latency of beta-glucan (25 mg/kg) group rats in MWM. **$p < 0.01$ suggests significant decline in escape latency as compared with day 1 (n=8). (F) Bar diagram showing escape latency of beta-glucan (50 mg/kg) in MWM. *$p < 0.05$ and **$p < 0.01$ suggests significant decline in escape latency as compared with day 1 (n=8). (G) Bar diagram showing escape latency of beta-glucan (100 mg/kg) in MWM. *$p < 0.05$ suggests significant decline in escape latency as compared with day 1 (n=8).

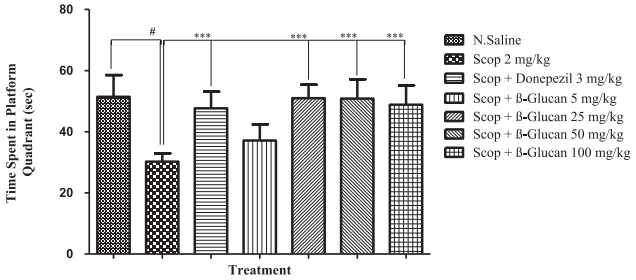



**Fig. 3.2.** Effect of β-glucan treatment on the time spent in platform quadrant. The bars represent mean ± SEM of time spent in the platform quadrant of MWM (n=8). The scopolamine (2 mg/kg; *i.p.*) treatment significantly reduced the time spent in the platform quadrant. However, the donepezil (3 mg/kg; *i.p.*) and β-glucan (25, 50 and 100 mg/kg; *i.p.*) treatments significantly antagonize the changes induced by scopolamine. #$p < 0.001$ and ***$p < 0.001$ represent significant differences as compared to normal saline and scopolamine treated groups respectively.

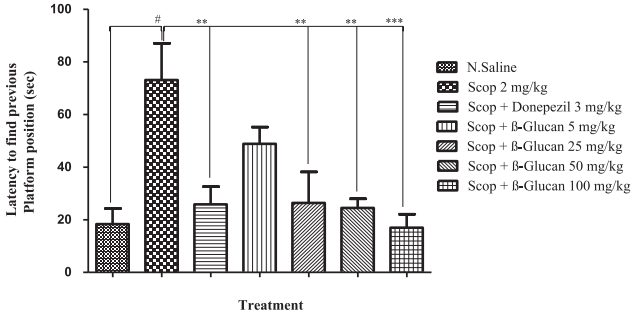



**Fig. 3.3.** Bar diagram showing the effect of β-glucan on latency to find previous platform position in MWM. Control group was administered normal saline (5 ml/kg; *i.p.*). Scopolamine (2 mg/kg; *i.p.*) significantly increased the latency time as compared to saline-treated group. The donepezil (3 mg/kg; *i.p.*) and β-glucan (25, 50 and 100 mg/kg; *i.p.*) significantly reversed the changes induced by scopolamine. Error bars represent mean ± SEM of latency time. #$p < 0.001$ treatment was significantly different as compared to normal saline group, ***$p < 0.001$, **$p < 0.01$ treatment was significantly different as compared with scopolamine-treated group (n=8).

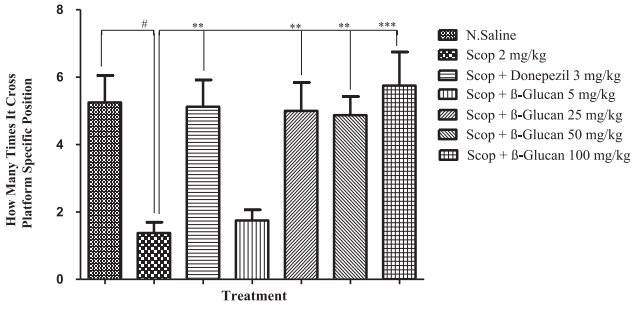



**Fig. 3.4.** Bar diagram showing the effect of β-glucan on the number of times rat crosses that specific point at which platform was placed on training trial days. The scopolamine (2 mg/kg; *i.p.*) treatment significantly decline the number of crossing donepezil (3 mg/kg; *i.p.*) and β-glucan (25, 50 and 100 mg/kg; *i.p.*) treatments significantly reversed the changes induced by scopolamine. Error bars represent mean ± SEM of number of crossings. #$p < 0.001$ treatment was significantly different as compared to normal saline group, ***$p < 0.001$, **$p < 0.01$ treatment was significantly different as compared with scopolamine-treated group (n=8).

with normal saline ($p < 0.001$, (F (1, 14)=20.323)). The treatment with donepezil significantly antagonized this effect by increasing number of crossings through platform position as compared to scopolamine treated rats ($p < 0.001$, (F (2, 21)=10.684)). In similarity with standard, the β-glucan treatment also caused dose dependent increase in number of crossings through platform position (F (5, 42)=7.741) as shown in Fig. 3.4.

*2.3.2.4. Movement patterns.* The movement patterns of the animals showed that the normal saline treated rats spent most of the time in platform quadrant as shown in Fig. 3.5(A). However, the scopolamine (2 mg/kg) treated rats showed arbitrary (diffused) movements in the MWM (Fig. 3.5(B)). The donepezil (3 mg/kg, Fig. 3.5(C)) and β-glucan (5, 25, 50 and 100 mg/kg, Fig. 3.5(D)–(G)) treatments have also confined the animals in platform quadrant.

### 2.4. Locomotor activity test

The scopolamine treatment has significantly increased the locomotion of rats as compared to normal saline group as shown in Fig. 4($p < 0.001$, F (1, 14)=22.123). The donepezil (3 mg/kg) caused significant reduction in locomotion as compared to scopolamine treated rats ($p < 0.05$, F (2, 21)=11.099). In similar manner, β-glucan (5, 25, 50 and 100 mg/kg) also caused significant reduction in locomotion as compared to scopolamine treated rats ($p < 0.05$, F (5, 42)=4.834).

### 2.5. Ex-vivo AChE enzyme analysis

No significant change in absorbance (activity) between normal saline and scopolamine treated group was observed as depicted in Fig. 5(A). The donepezil (3 mg/kg) treatment caused significant decline in the activity when compared to scopolamine treated group as shown in Fig. 5(A) and (B) respectively ($p < 0.001$, F (2, 6)=112.965). In similar manner, β-glucan treatment also caused significant reduction in AChE activity ($p < 0.001$, F (5, 12)=12.315). Data is rectified at 0.29 g/dl protein concentration as constant.

## 3. Discussion

Acetylcholine (ACh) is the principal neurotransmitter in the central nervous system, which plays a vital role in cognitive functions *via* interacting with its nicotinic and muscarinic receptors (Reis et al., 2009; Drever et al., 2011). The deficit in this cholinergic neurotransmission has been linked with several cognitive disorders such as AD (Schliebs and Arendt, 2011; Sims et al., 1983). Presumably, enhancement of cholinergic tone has been an important therapeutic target, which has led to the development of drugs classified as AChE inhibitors. This enzyme is the serine hydrolase, which degrades the ACh leading to decrease in its levels (Francis et al., 1999). In pursuit of the proof of concept of β-glucan as cognition enhancer, the preliminary virtual screening was performed. Our data revealed that β-glucan holds potential to interact with important catalytic residues of AChE enzyme (Fig. 1(A) and (B)). Briefly, the hydroxyl group of β-glucan was seen forming hydrogen bonding with an important Ser203. This residue is known to degrade ACh *via* making a nucleophilic attack at its carbonyl group (Kryger et al., 1999). Additionally β-glucan made hydrogen bonding with other residues like Ser125, Thr83, and Tyr341, which is suggestive of the firm anchoring of ligand in active site. The aforementioned results exhibited that the binding of β-glucan appears to be promising in establishing its AChE inhibitory potential. In conformity with these results, the β-glucan was found to inhibit the enzyme *in vitro* with an $IC_{50}$ value of 0.68 ± 0.08 μg/μl (Fig. 2(a)and (b)).

In order to assess the effectiveness of β-glucan *in vivo*, MWM was used to assess the spatial learning in rodents. In similarity with existing reports (Bromley-Brits et al., 2011), our data showed decrease in escape latency during familiarization/acquisition trials in MWM which is suggestive of learning in rats as shown in Fig. 3.1 (A)–(G). Cholinergic deficit was induced by using scopolamine

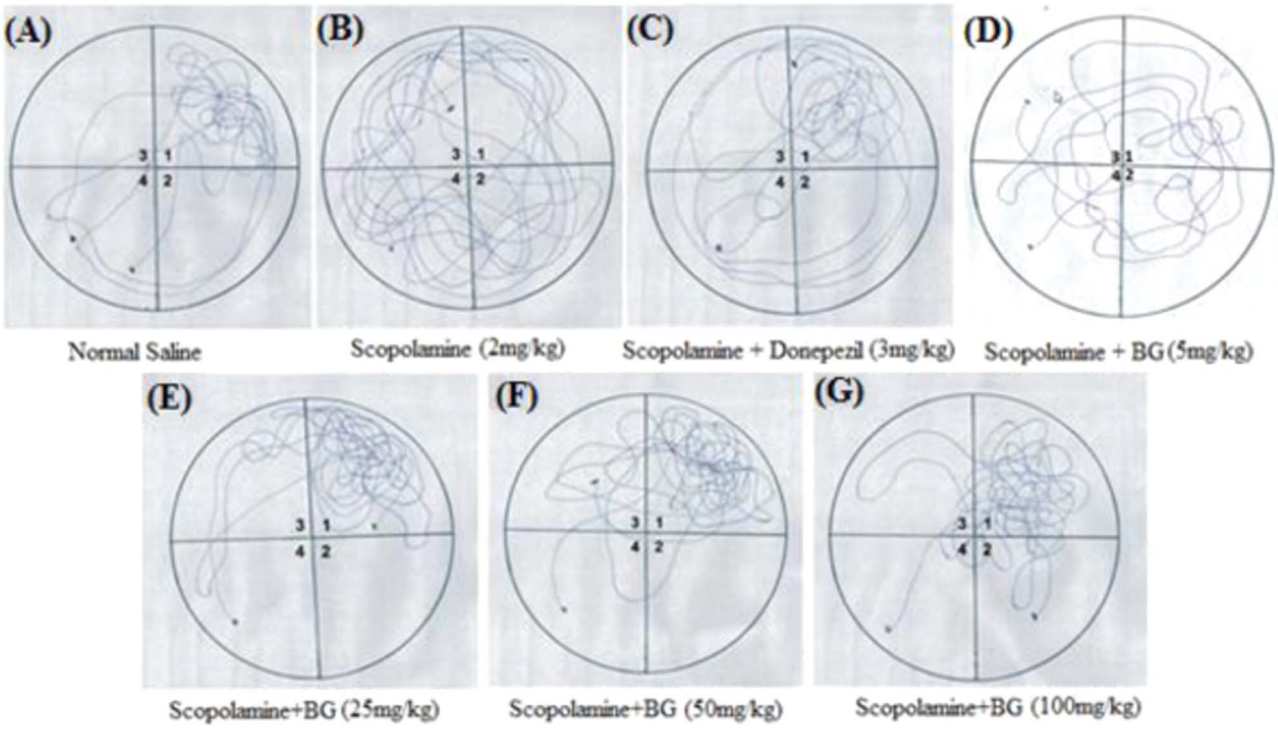



**Fig. 3.5.** Effect of β-glucan treatment on the movement patterns of rats in MWM. Quadrant labelled as 1 was platform quadrant while the quadrant 4 was starting position. The diagrams show the movement pattern of (a) normal saline group, (b) scopolamine (2 mg/kg; *i.p.*), (c) donepezil (3 mg/kg; *i.p.*), (d) scopolamine + β-glucan (5 mg/kg; *i.p.*), (e) scopolamine + β-glucan (25 mg/kg; *i.p.*) and (f) scopolamine + β-glucan (100 mg/kg; *i.p.*). BG indicates β-glucan. The normal saline treated rats showed confined movements in the platform quadrant, which was diffused by scopolamine treatment. The donepezil and β-glucan treatments have again restricted the movements in the platform quadrant.

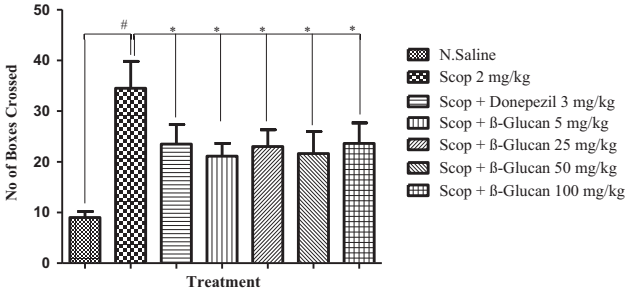



**Fig. 4.** Effect of β-glucan treatment on the locomotor activity of rats. The bar represents mean ± SEM of number of boxes crossed in 5 min time interval. #$p < 0.001$ and *$p < 0.05$ shows significant difference as compared to normal saline and scopolamine-treated group, respectively (n = 8).

(2 mg/kg), a muscarinic antagonist with amnesic action (Blokland, 1995). In similarity with earlier reports (Beatty and Bierley, 1985; Stevens, 1981), our data revealed poor spatial learning by scopolamine treated rats. The animals showed decreased time spent in the platform quadrant (Fig. 3.2), increased latency to find platform position (Fig. 3.3) and number of crossing through platform position (Fig. 3.4). The disrupted spatial reference memory under scopolamine treatment can also be observed in the movement pattern (Fig. 3.5(B)) which revealed that rats did not spend most of time in platform quadrant. It is important to note that all these scopolamine induced deficits were significantly reversed by both standard drug, Donepezil and β-glucan 25, 50 and 100 mg/kg (Fig. 3.2–3.4). They also promoted spatial strategy used by rat on the basis of spatial cues (Fig. 3.5(C)–(G)). Hence, it can be deduced

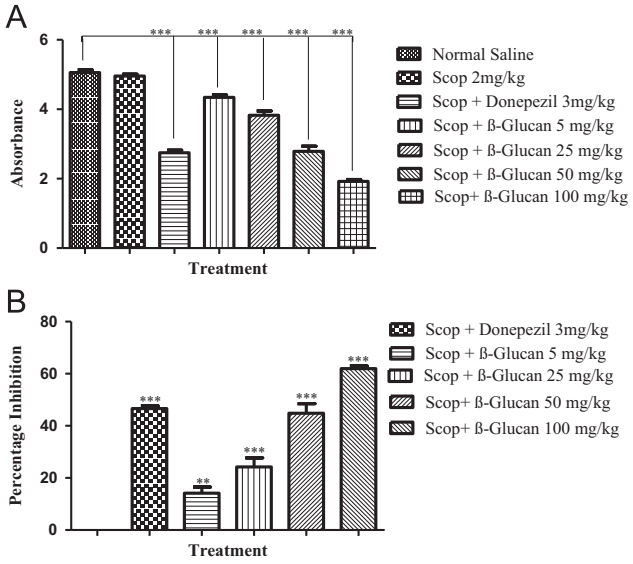



**Fig. 5.** (A) Bar diagram shows the effect of β-glucan treatment on the activity of hippocampal AChE enzyme. The bars represent mean ± S.E.M of the absorbance of product, which co-relates with the activity of AChE enzyme in Ellman's reaction. Donepezil (3 mg/kg) was used as a positive control whereas β-glucan was used in the doses of 5, 25, 50, 100 mg/kg. ***$p < 0.001$ suggest significant difference with scopolamine-treated group (n = 8). (B) Bar diagram shows the effect of β-glucan treatment on the activity of hippocampal AChE enzyme. The bars represent mean ± S.E.M of percentage inhibition of AChE enzyme in hippocampus after rectification of data at 0.29 g/dl as constant. Donepezil (3 mg/kg) was used as a positive control whereas β-glucan was used in the doses of 5, 25, 50, 100 mg/kg. ***$p < 0.001$, *$p < 0.05$ suggest significant difference in percent inhibition with normal saline treated group (n = 8).

the β-glucan possesses potential to attenuate the cognitive deficits induced by scopolamine.

In MWM, locomotion of rat is another critical factor to be considered. In similarity with earlier reports (Day et al., 1991; Riedel et al., 2009), our data depicted hyper-locomotion in scopolamine treated rats (Fig. 4). In conformity with existing literature, the donepezil (Cachard-Chastel et al., 2008), as well as β-glucan decreased the hyper-locomotion induced by scopolamine. There are two possible explanations for this outcome. Firstly, the stimulants may elicit false positive results in the MWM due to increased locomotion, which can help locating the platform in short period of time. Our results showed that β-glucan has no motor stimulatory action therefore the mnemonic effect observed in MWM was not an artefact. Secondly, the decline in scopolamine-induced locomotion (SIL) was also suggestive of the cholinergic modulation by β-glucan. In this regard, it was previously reported that mesopontine cholinergic neurons consists of two major nucleuses *i.e*. laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT). These nuclei are reported to play an important role in scopolamine-induced locomotion (Chintoh et al., 2003; Laviolette et al., 2000). They lie adjacent to the mesencephalic locomotion region of the caudal midbrain and make synapses with dendrites and soma of dopaminergic cells. It has been assumed that the SIL is mediated by cholinergic projections of LDT and PPT (Chintoh et al., 2003; Laviolette et al., 2000). Therefore, the decrease in locomotion by β-glucan implicates its intervention in the cholinergic pathway. This strengthens our aforementioned results, which suggests interaction of β-glucan with AChE enzyme.

Hippocampus is the brain structure, which is hypothesized to play important role in cognitive functions including learning and memory (Graves et al., 2012). Therefore, hippocampi of treated animals were immediately dissected out, following behavioural assay, for measurement of AChE activity. In similarity with existing reports (Geerts et al., 2005), donepezil showed significant reduction in AChE activity in hippocampi. In line with aforementioned outcomes, β-glucan also showed significant reduction in AChE activity in hippocampi (Fig. 5(a) and (b)).

### 3.1. Short conclusion

In conclusion, the present study showed the reversal of scopolamine induced cognitive deficits by β-glucan. This action can be attributed to AChE enzyme inhibitory potential. Hence, the aforementioned conclusion and ample availability in nature supports the consideration of β-glucan as economic therapeutic option for cognitive ailments linked with cholinergic dysfunction.

## 4. Experimental procedure

### 4.1. Animals

Male Sprague Dawley rats (120–170 g) were obtained from Animal care facility of COMSATS Institute of Information and Technology, Abbottabad, Pakistan. They were kept under standard conditions including temperature (25 ± 1 °C), 12-h light/dark cycle (lights on 8:00 a.m–8:00 p.m) and free access to food and water. All experiments were performed according to the guidelines of the ethical committee of CIIT and Animal Scientific Procedure Act 1986 (UK).

### 4.2. Chemicals

Acetylthiocholine iodide (ATCI), β-glucan (*Saccharomyces cerevisiae*), scopolamine, donepezil and 5,5′-dithiobis 2-nitro benzoic acid (DTNB) were obtained from Santa Cruz, USA. Sodium dihydrogen phosphate ($NaH_2PO_4$) and di-sodium hydrogen phosphate ($Na_2HPO_4$) were purchased from Sigma Aldrich, USA. The Triton X-100 was provided by Amresco, USA.

### 4.3. In-silico study

For computational analysis, the AChE protein crystal structure (PDB: 4EY7) was obtained from protein data base bank. The enzyme crystallizes as a dimeric protein so it was dissected to obtain monomeric molecule. Ligand and associated water molecules were removed from the protein with the help of discovery studio software. Furthermore, the polar hydrogens were added using autodock tools followed by saving the file in pdbqt format. Protein grid was selected and ligand was drawn in Chemdraw and also converted to pdbqt format for docking. All information regarding protein, ligand and grid (active site) location were put in the configuration file followed by docking. Docked structures were analyzed in pymol and interactions of ligand with protein residues were checked in discovery studio. Energy values, site of docked molecules and interaction with active site residues were some important parameters used to analyze the results.

### 4.4. In-vitro AChE enzyme inhibition assay

The membrane of human red blood cells (Erythrocytes) was used as a source of AChE enzyme, while inhibition assay was performed using Ellman's method. The erythrocyte membrane preparation and Ellman's method are as follows:

#### 4.4.1. Human erythrocytes membrane preparation

After informed consent, the phlebotomist drew blood (15 ml) from adult male (26 years) by single intravenous puncture. The blood was collected in syringe, transferred into the vacutainer (containing anti-coagulant heparin) and used for erythrocytes membrane preparation as follows: The erythrocytes were separated from the blood by centrifugation (800 g for 15 min) followed by washing thrice with Tris–HCl buffer (10 mM Tris–HCl buffer in 0.9% NaCl, pH 7.4). The washing was performed by suspending isolated erythrocytes in 1 ml of Tris–HCl buffer followed by centrifugation (1000 g for 15 min). After third wash, the supernatant was removed and cell were subjected to hypotonic buffer (5 mM Tris–HCl and 1 mM EDTA, pH 7.4) and stored at −20 °C for overnight. After thawing and centrifugation (12,000 *g* and 30 min), the supernatant was discarded and cells were subjected to hypertonic solution (50 mM Tris–HCl, 1 mM EDTA, 500 mM NaCl, pH 7.4). This process ruptured the cells leading to release of haemoglobin. After centrifugation (12,000 g and 30 min), supernatant containing haemoglobin was discarded and erythrocytes membrane pellet were obtained at the bottom of centrifuged tubes. The AChE is a membrane bound enzyme and was therefore extracted by solubilising membrane in non-ionic detergent *i.e*. 0.1% Triton X-100 (v/v) by gentle shaking and incubating at 4–6 °C for 30 min. The extract was further diluted with 50 mM $Na_2HPO_4/NaH_2PO_4$ (pH 7.4) and stored at −20 °C for AChE enzyme inhibition assay using Ellman's method (Srivastava et al., 2012).

#### 4.4.2. Ellman's method

The AChE enzyme inhibition assay was performed using Ellman's method (Ellman et al., 1961). The total assay volume was 2 ml, which contained 20 μl enzyme, different volume (5, 10, 50, 100, 150 or 200 μl) of test compound solution, 40 μl DTNB (10 mM, 3.96 mg/ml) and remaining volume was filled with phosphate buffer (0.1 M, pH 8.0). This mixture was incubated for 15 min. Finally, 150 μl of ATCI (100 mM, 28.92 mg/ml) was added followed

(10 min) by measuring the absorbance at 412 nm. The donepezil (100 μM) was used as positive control.

Percent AChE enzyme inhibition was calculated using following formula:

$$\text{Percentage inhibition in absorbance} = 100 - \frac{\text{Absorbance of the test compound}}{\text{Absorbance without test compound}} \times 100$$

### 4.5. Morris water maze

The effectiveness of test substance was assessed *in vivo* using MWM (Morris, 1984) tank. This maze constituted of a water filled circular pool (black color having diameter and depth of 180 cm and 50 cm, respectively). The lines were drawn on the surface of pool to divide it into 4 equal quadrants. A platform (black with diameter and height of 10 cm and 20 cm, respectively) was placed in one of the quadrant. The position of platform was kept constant throughout the course of experiment. High contrast spatial cues were affixed along the wall of maze. The pool was filled with water ($25 \pm 2$ °C) till 20 cm. The entire experiments were video recorded with Handycam (DCR-SX40f, Japan) which were subsequently analyzed to measure different parameters. The experiment consisted of three different types of trials (familiarization, acquisition and probe trial) as follows:

#### 4.5.1. Familiarization/acquisition trials

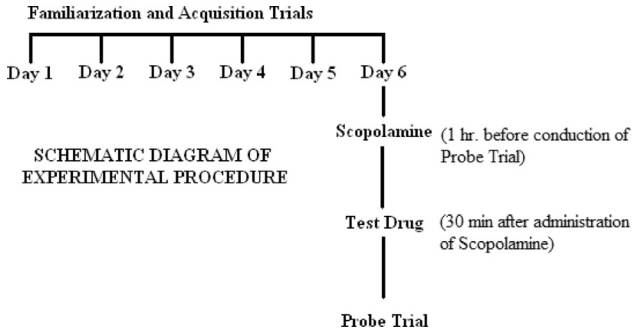
In familiarization trial (day 1), the platform was kept 1 cm above the water level. During acquisition trials from day 2nd–5th, the platform was hidden *i.e.* it was placed 1 cm below water level. Both of these trials were performed five times once daily by placing the animals in all four quadrants and center and allowed to find the platform. The maximum trial time was 120 s and inter-trial time was 60 s. Upon finding the platform, the rat was allowed to stay on platform for 5 s. In case the animal could not find the platform (cut off time = 120 s), it was gently guided to platform and allowed to stay for 30 s. The video recordings with Handycam were used to measure escape latency (time to find platform position).

#### 4.5.2. Probe trial

On day 6th, probe trial was conducted. In this trial, no platform was placed in the pool. The animals were treated (*i.p.*) before conduction of probe trial as follows:

Group 1: Normal saline (5 ml/kg), Group 2: Scopolamine (2 mg/kg for induction of cholinergic deficit), Group 3: Scopolamine (2 mg/kg) was administered 30 min before administration of donepezil (3 mg/kg) and Group 4/5/6/7: In group 4/5/6/7, animals were administered scopolamine (2 mg/kg) 30 min before administration of 5, 25, 50 or 100 mg/kg of β-glucan.

In probe trial, the animals were allowed to swim in the pool for 120 s. The recordings with Handycam were subsequently used for analysis of different parameters (latency to find previous platform position, time spent in platform quadrant and number of crossing through platform position) and also for drawing of movement patterns with the help of trace paper. The schematic diagram below is given below for the ease of understanding the experimental procedure.




### 4.6. Locomotor activity test

The activity cages were used to assess the effect of test substance on locomotor activity of animals. This cage consisted of wooden box ($18 \times 18$ in.) divided it into four equal halves. After performing the probe trial, rats were individually placed in activity cage for six minutes. The entire experiment was video recorded with Handycam. The number of boxes crossed by animals were counted for last five minutes, provided first minute for acclimatization (Cassel et al., 1998) by these recordings.

### 4.7. Ex-vivo AChE enzyme inhibition assay

After locomotor activity test, the rats were sacrificed to dissect out hippocampi and kept in ice cold phosphate buffer saline (0.1 M, pH 8.0). After homogenization and centrifugation (1000 g at 4 °C for 15 min), the supernatant obtained was used as a source of AChE enzyme. The Ellman's assay was performed to measure the enzyme activity as described above (Ellman et al., 1961). The protein estimation was also performed to rectify/harmonize the data.

### 4.8. Statistical analysis

The data was expressed as mean ± standard error of the mean (SEM) of n = 8/group in behavioural assays. The enzyme inhibition data was shown in both absorbance and percentage inhibition. The results were statistically analyzed by One-way ANOVA using SPSS software. The minimum level of significance was $p < 0.05$.

## Conflicts of interest

The authors declare no conflicts of interest.

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