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ORIGINAL ARTICLE

Cholinergic-Enhancing and Antioxidant Effect of *Vigna subterranea* (L.) Verdc. (*Fabaceae*) Landrace Aqueous Extract on Scopolamine-Induced Amnesia in Male Swiss Mice

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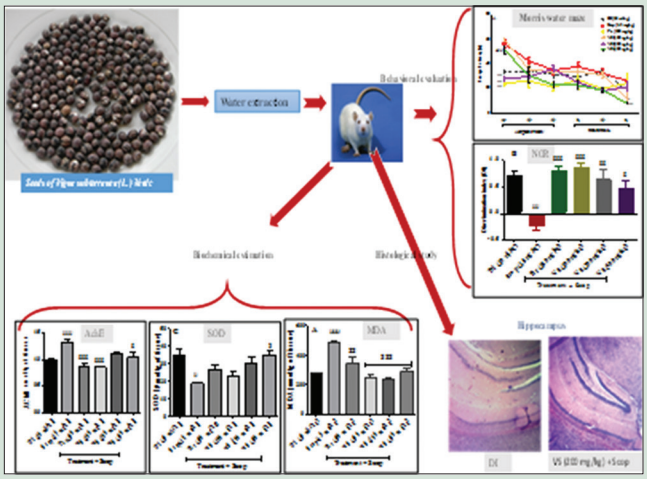
ABSTRACT

Background: Currently, no drug effectively curbs down the progression of Alzheimer's disease (AD), and thus early prevention is important. **Objectives:** The present study was conducted to investigate the protective effects of the aqueous extract of *Vigna subterranea* seed landrace on amnesia induced by scopolamine in mice. **Materials and Methods:** *V. subterranea* aqueous extract (100, 200, and 400 mg/kg BW) was administered by gavage for 9 consecutive days and memory impairment was induced by repeated intraperitoneal injection of scopolamine (1.5 mg/kg). The Y-maze (YM), Morris water maze (MWM), novel object recognition paradigm (NOR), and the T maze™ were used to assess learning, memory, and retention. Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) levels, and acetylcholine esterase activity were also evaluated in the mouse hippocampi homogenates. **Results:** *V. subterranea* aqueous extract (400 mg/kg) significantly increased the percentage of spontaneous alternation in the YM task and decreased escape latency in the MWM. Moreover, this dose brought about a significant improvement in the time spent in the preferred TM arm and discrimination index in the NOR tasks despite repeated scopolamine injection. Additionally, low acetylcholine esterase levels, reduced lipid peroxidation (MDA), but increased antioxidant enzyme (CAT and SOD) activity were observed in hippocampi homogenate of mice pretreated with the extract. A protective action against hippocampal cell damage was also evident. **Conclusion:** This finding suggests that the aqueous extract of *V. subterranea* seed landrace may improve learning and memory.

Key words: Acetylcholine esterase, Alzheimer's disease, antioxidant, memory impairment, scopolamine, *Vigna subterranean*

SUMMARY

- The water extract of *Vigna subterranea* landrace improves learning and memory in scopolamine demented mice in the Y-maze, Morris water maze, novel object recognition paradigms, and the T maze test
- The aqueous extract of *V. subterranea* increased hippocampal antioxidant defence and cholinergic transmission in demented mice
- Neuronal cell death in hippocampi regions associated with repeated scopolamine injection was hindered by the aqueous extract of *V. subterranea*
- This extract could be used as an alternative in the treatment of amnesia.



Abbreviations Used: AD: Alzheimer's disease; CA: Cornu Ammonis; DTNB: 5,5′-Dithiobis-(2-nitrobenzoic acid); *V. subterranea*: *Vigna subterranea*; BW: Body weight; YM: Y-maze; MWM: Morris water maze; NOR: Novel object recognition; TM: T maze; AChE: Acetylcholine esterase; MDA: Malondialdehyde; CAT: Catalase; SOD: Superoxide dismutase.

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INTRODUCTION

The world's population is aging; older persons are increasing in number and make up a growing share of the population in virtually every country.[1] Dementia prevalence is also increasing among the elderly.[2] Alzheimer's disease (AD) is a progressive neurodegenerative age-related disorder. It is the most common form of dementia characterized by a loss of hippocampal and cortical neurons, leading to deterioration of cognitive abilities, memory loss, and reduced learning.[3,4] Stress, genetic predisposition, and some medical conditions are other risk factors related to AD development.[5] Epidemiological data from AD International revealed 46.8 million people with AD among the world's population in

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2016. The number of AD incidence is expected to triple over the next 40 years.[6] These projected increases also have considerable emotional and financial burdens which are estimated to cost about 175 000 dollars per person with AD over their entire life.[7] Alzheimer's-related dementia is also a major health concern in the elderly in Africa due to extended lifespan and growing populations.[8,9]

Two main pathological hallmarks of AD are described to date. Extracellular neuritic amyloid plaques, as amyloid β peptides deposits due to failure of the enzyme β secretase in cleaving the amyloid precursor protein, and intracellular neurofibrillary tangles which set up as a result of hyperphosphorylation of tau protein.[10] Other pathophysiological events consistent with the induction and progression of AD pathology include inflammation, cholinergic system dysfunction, and oxidative stress.[11]

The cholinergic system has been the main target in the development of drugs against AD pathogenesis as low concentration of acetylcholine (ACh) is seen in aging and AD, associated with cholinergic deficit.[12] ACh esterase (AChE) enzyme catalyzes the breakdown of ACh, thereby affecting its bioavailability in the synaptic cleft. Moreover, by binding to amyloid-β-associated proteins, AChE increases the rate of fibrillation and acts as a potent amyloid-promoting factor.[13] In the past years, cholinergic revival therapy through ACh esterase inhibitors has been employed in AD therapy.[14] However, their use is associated with severe side effects, short-life span, limited bioavailability, and efficacy.[15] Medicinal plants provide a rich source of biologically active constituents endowed with a wide range of activities. In the quest for more effective and innovative treatment strategies for subsequent development of innovative therapeutic compounds, herbal medicine is highly solicited. *Vigna subterranea* (L.) Verdc. is commonly known as "Bambara groundnut." It is a legume seed that is easily cultivated and classified under the *Fabaceae* family, Faboidea subfamily, and *Vigna* genus.[16]

In the Far-North Region of Cameroon, traditional healers systematically use Bambara groundnut in the treatment of mentally deranged persons (madness), indicating that the plant may possess interesting neuropharmacological properties. *V. subterranea* has been reported to possess high quantities of antioxidants and vitamins which could impart many health benefits, thereby reducing the risk of many chronic illnesses such as cancer.[17,18] Andzouana *et al.*[19] reported the presence of flavonoids, phenols, alkaloids, and other nutrients in Bambara groundnut. Moreover, previous studies revealed that *V. subterranea* has antiepileptic properties.[20] Cognitive disorders in epilepsy are now well established. The loss of neuronal cells of the hippocampus due to precipitating aggression (status epilepticus) and recurrent epileptic seizures lead to memory impairment.[21] Beside recurrent seizures in epileptic patients, chronic and persistent dysfunctions of limbic circuits, characteristics of epilepsy, also lead to cognitive decline.[22,23] Screening of *V. subterranea* achieved by Harris *et al.*[24] indicated that black and brown landraces were the richest in flavonoids, with kaemferol being the major representative in whole seeds. Flavonoids can cross the blood–brain barrier, hindering neuronal damage. In a recent study, using STZ-induced AD animal model, Darbandi *et al.*[25] reported that kaempferol increases the expression or activity of antioxidant enzymes and decreases the number of dead cells, especially pyramidal neurons in CA1 hippocampus area.

On the other hand, many species of the "Vigna" genus have been proven to possess potential neurological actions. *Vigna radiata* possess memory improvement activity, *Vigna mungo* has nootropic activity, while *Vigna unguiculata* is endowed with motor coordination activity and antidepressant potentials.[26-29] Despite the promising medicinal profile, there is no known experimental study on the protective effect of *V. subterranea* extract against memory deficit in mice to date. In this context, it appeared reasonable for us to explore the effect of this plant extract on an animal model of neurodegenerative disease. Therefore, the aim of the present study was to evaluate the protective effects of the aqueous extract of *V. subterranea* on scopolamine-induced memory impairment in Swiss mice. Furthermore, we investigated the underlying mechanism by which it could induce its potential neuroprotective effects.

MATERIALS AND METHODS

Animals

Male Swiss mice aged 4 months (20–25 g BW) were used in this study. Mice were purchased from the animal breeding facility of "LANAVET" ("Laboratoire National Vetrinaire," Garoua, Cameroon), acclimatized for 14 days under natural dark-light cycle and provided with water and food *ad libitum*. The present study was approved by the Ethics Committee of the Faculty of Sciences of the University of Maroua (Ref. no. 14/0261/Uma/D/FS/VD-RC), Cameroon. All experiments were accomplished in accordance with the guidelines of Cameroonian bioethics committee (Reg no. FWA-IRB00001954) and NIH-Care and Use of Laboratory Animals manual (8th Edition). Efforts were also made to minimize animal suffering.

Botanical material

Collection and identification

Dry *Vigna subterranea* (L.) Verdc. (*Fabaceae*) seeds [Figure 1] were purchased post harvest in the month of September 2017 from a local market (Doulek; Far North Region, Cameroon). The botanical identification of seeds and authentication was done at the National Herbarium Yaounde Cameroon by comparing to Gerling G N°5121 of the collection of Herbarium N°36574.

Preparation of aqueous extract of Vigna subterranea

The extraction was done as to match the traditional healer's method. Seeds were sorted to remove defective ones. The selected landrace was graded according to size and weighed. 250 g was soaked in water for 8 h and then boiled for 1 h at 100°C. The cooked seeds were crushed using a blender. The paste obtained from crushing was siftered using a 500-µm mesh sift, filtered using Whatman filter paper no. 4, and the solvent was eliminated from the filtrate using a temperature-controlled oven (50°C for 24 h). The powder obtained was weighed and the yield of extraction was determined.

Figure 1: Dry seeds of *Vigna subterranea* landrace

Various doses of extract administered to mice were prepared by mixing a weighed quantity powder with 0.1% TWEEN 80 before addition of water.

Drugs and chemicals

Piracetam was obtained from UCB pharma SA, Braine l'Alleud (Belgium) and scopolamine hydrobromide, trichloroacetic acid, and thiobarbituric acid were purchased from Sigma Chemical, St. Louis, MO (United States). Acetylthiocholine iodide and 5, 50-dithiobis (2-nitrobenzoic acid) (Ellman reagent), TWEEN 80, ethanol, phosphoric acid, sodium hydroxide, hydrogen peroxide, anhydrous sodium, sodium bicarbonate, dichromate acetic acid, sodium citrate, formalin, and sodium pentobarbital were purchased from Biochemica (Shanghai, China).

Experimental design

Mice were randomly divided into two sets of six groups (n = 10) and subjected to the following treatment schedule: normal control group (deionized water 10 ml/kg *p.o*) only; negative control group receiving scopolamine (1.5 mg/kg *i.p*); and positive control group (piracetam 150 mg/kg *p.o*); and test groups (*Vigna subterranea* 100, 200, and 400 mg/kg *p.o*).

All groups were treated for 9 days [Figure 2]. Scopolamine was dissolved in normal saline 0.9% and administered to all groups except the normal control 45 min after treatment. Behavioral tests were launched 30 min after scopolamine injection.

Behavioral studies

All behavioral activities of mice were video tracked and recorded using ANYMAZE trial version 6.03 (Wood Dale, IL, USA, Stoelting Company).

Short-term memory and locomotor activity evaluation in the Y-maze test

The Y-maze (YM) test was used to assess short-term memory in mice by assessing the percentage of spontaneous alternation in a single trial on the last day of the experiment. The maze consisted of the following three arms: A, B, and C (35 cm long, 25 cm high, and 10 cm wide) and an equilateral triangular central area. 1 h after treatment and 30 min after scopolamine injection (except for the normal control group), a naive mouse was placed at the end of one arm and allowed to move freely through the maze for 8 min. Behavioral parameters were recorded during the last 5 min of the test. Entry into an arm was considered when all the four paws of a mouse were in an arm. The total number of arm entries was used as a measure of general activity of the animals. Specific arm transition sequences (ABC, BCA, or CAB but not BAB or CAC or CBC) were recorded as spontaneous alternation, which reflects short-term memory. The maze was wiped clean with 70% ethanol between each animal to minimize odor cues.[30]

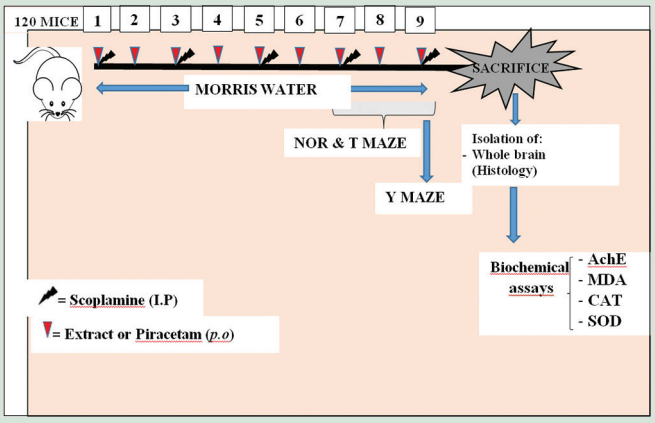

Figure 2: Experimental schedule

Novel object recognition test

This test was performed as earlier described by El-Marasy *et al.*[31] and was used to evaluate recognition in mice. The apparatus consisted of a wooden box painted white. On treatment day 7, each mouse was placed in the apparatus and allowed to freely explore the apparatus for 5 min. On the next day during the "sample" trial (T1), two identical objects were placed in two opposite corners of the apparatus, the animal was placed again in the apparatus, and the exploration time of the objects was recorded for 5 min. The mouse was returned to its home cage and an inter-trial interval of 24 h was given. On the last day of the test (treatment day 9), the "choice" trial (T2) was performed 30 min following scopolamine injection. In T2, a new object (N) replaced one of the objects that were presented in T1, and then the mouse exploration time of the familiar (F) and the new objects (N) was assessed for 5 min. Exploration was defined as follows: directing the nose toward the object at a distance of not more than 2 cm and/or touching the object with the nose. The arena and objects were thoroughly cleaned with 70% ethanol in order to eliminate residual odors. From this measure, a series of variables was then calculated: the total time spent in exploring the two identical objects in T1 and that spent in exploring the two different objects, F and N in T2. The discrimination index (DI) was also determined using the following formula:

$$DI = (N - F)/(N + F).$$

Spatial cognition and retention evaluation in the T-maze task

This task was used to evaluate spatial memory and retention as previously described by Amico *et al.*[32] with slight modifications. The maze was made of a wooden arm (28 cm × 5 cm × 10 cm) at right angle with each other. The test was performed in three phases: the habituation phase consisted of a single-session freechoice trial. The mouse was placed in the start arm and allowed to explore the maze for 5 min. The preferred and discriminated arms of each mouse were noted. During the acquisition phase which was performed the next day, mice were subjected to a forcedchoice trial. The discriminated arm was blocked by a guillotine door. Once the animal was released from the starting arm, it was allowed to explore the maze by entering the preferred arm and returning to the start arm. During retention, all the guillotine doors were opened and the mice were free to explore all the arms. In all the sessions, each mouse was evaluated for 5 min, and the time spent in arms and number of return to start arm were recorded. The floor of the apparatus was cleaned with 70% ethanol between trials to eliminate olfactory cues.

Spatial learning and memory assessment in the Morris water maze

Hippocampal-dependent spatial learning and memory was assessed using a standard Morris water maze (MWM) task as described previously by Harris *et al.*[33] The apparatus consisted of a metallic cylinder (79 cm diameter). Briefly, the mice were placed in the cylindrical tank of opaque water (through the addition of nontoxic paint) in a testing room with various distinct visual cues on the walls. The first phase of testing was visual platform training to verify that performance is not impaired by vision or locomotor problems. In the visual platform phase, mice were trained to swim to a metallic escape platform positioned above the water for 12 trials (4/day). 24 h after visual platform training, the hidden platform phase began. The mice were trained for 12 trials (4/day) to learn the location of the submerged platform. During a given trial, the mouse was slightly placed in the tank at one of the four randomly chosen start points (N, S, E, and W) and allowed 60 s to find the

platform. If the mouse fails to locate the platform within 60 s, it was gently directed to the platform. The platform position remained fixed throughout the hidden platform phase. Escape latency (in seconds) and distance traveled before finding the platform (in meters) were acquired by a video-tracking software. Memory retention was assessed in the probe phase of the MWM which occurred 24 and 48 h after the fifth and final hidden platform trials. During the probe phase, the platform was removed from the tank, and time the mice spent in each quadrant was recorded. The percentage of time spent in the target quadrant, which previously held the hidden platform, was calculated.

Biochemical assay

At the end of the experiment (day 9), mouse euthanasia involved the administration of an overdose of sodium pentobarbital (100 mg/kg b.w., *i.p.*, Sigma). When complete loss of righting reflex and reduction of respiratory rate was achieved, their brains were carefully excised from the skull and dissected for the isolation of hippocampi. Hippocampi were weighed and rinsed in ice-cold isotonic saline solution. Hippocampi tissue samples were then homogenized (1:10) using a mortar and a pestle in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was centrifuged (3000 rpm, 15 min), and the supernatant was used for biochemical analysis.

Estimation of hippocampal acetylcholine esterase enzyme activity

AChE enzyme activity was estimated by Ellman method.[34] After decapitation, hippocampi were quickly dissected out on a Petri dish chilled on crushed ice. Each tissue was weighed and homogenized in 0.1 M phosphate buffer (pH 7.4). Then, 0.4 ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 7.4) and 100 µl of DTNB. The contents of the cuvettes were mixed thoroughly by bubbling air, and absorbance was measured at 412 nm in a spectrophotometer. When absorbance reached a stable value, it was recorded as the basal reading. Further, 20 µl of acetylthiocholine (a substrate) was added, and the change in absorbance was recorded. The change in the absorbance per min was thus determined. The enzyme activity was calculated and expressed as AChE nmol/g of tissue/min.[35]

Estimation of hippocampal oxidative stress biomarkers

Estimation of superoxide dismutase

The superoxide dismutase (SOD) activity in supernatant was measured by the method of Misra and Fridovich.[36] The supernatant (500 µl) was added to 0.8 ml of carbonate buffer (100 mM, pH 10.2) and 100 µl of epinephrine (3 mM). The change in absorbance of each sample was then recorded at 480 nm in a spectrophotometer at 20 s and 80 s. Parallel blanks and standards were run for the determination of SOD activity. One unit of SOD was defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation. The specific activity of SOD was expressed in terms of SOD unit/g of organ.

Estimation of catalase activity

Catalase (CAT) activity was assayed following the method of Sinha.[37] The reaction mixture consisted of 750 µl phosphate buffer (0.01 M, pH 7.5), 50 µl supernatant. Reaction was started by adding 200 µl H_2O_2 50 mM, incubated at 37°C for 1 min, and the reaction was stopped by the addition of 2000 µl of dichromate acetic acid reagent. The tubes were immediately kept in a boiling water bath for 10 min, and absorbance was read at 570 nm on a spectrophotometer. Control tubes, devoid of enzyme, were also processed in parallel. The difference in absorbance per unit was used as the measure of CAT activity.

Estimation of lipid peroxidation

The level of lipid peroxides was estimated by thiobarbituric acid reaction method described by Wilbur *et al.*[38] 250 µl of the supernatant was added to and briefly mixed with 125 µl of 20% trichloroacetic acid in 250 µl 0.67% thiobarbituric acid. After the vortex mixing, the samples were maintained at 95°C for 15 min. Furthermore, the samples were centrifuged at 3000 rpm for 15 min, and the supernatants were read at 532 nm using a spectrophotometer (Uviline 6600, Alès, France). A calibration curve was constructed using malondialdehyde (MDA) as standard, and the results were expressed as nmol/g protein.

Histopathological studies

Two mice from each group were subjected to transcardiac perfusion and decapitated, and their brains were collected and fixed in 10% formalin for a week. Fifty millimeters of coronal sections were made from the brain in the hippocampus region using the mouse brain Atlas with the following coordinate (anterior/posterior D 2.0 mm, medial/lateral D 1.5 mm, and dorsal/ventral AP D 2.0 mm).[39] The hippocampi sections were collected in 9-well plates. The dehydration of these sections consisted in introducing tissues in ascending concentration of ethanol followed by immersion in xylol and then embedding in paraffin. Paraffin sections of the brain were deparaffinized and rehydrated through washes in descending concentration series of alcohol. Hippocampi were then stained using hematoxylin and eosin stains. The brain sections were thereafter photographed, and the images were captured using a digital camera attached to a light microscope (Scientico, Haryana, India).

Statistical analysis

Statistical analysis was done using the software GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA). The differences among groups were analyzed using one-way and two-ways analysis of variance. $P < 0.05$ was considered statistically significant. Bonferroni posttest was used for multiple comparisons. Pearson's correlation coefficient and regression analysis were used to investigate the association between behavioral and biochemical parameters.

RESULTS

Effect of *Vigna subterranea* on spontaneous alternation and locomotion of

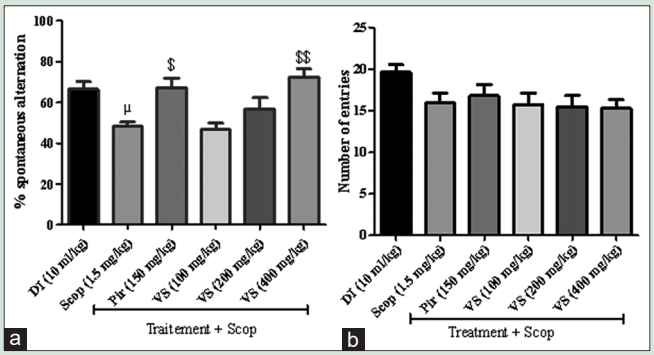

Figure 3: Effect of *Vigna subterranea* on scopolamine-induced memory impairments in the Y-maze test. Each column represents mean ± standard error of the mean of the spontaneous alternation percentage (a) and the numbers of arm entries (b) of ten mice. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $^{\$\$\$}P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: Scopolamine; Pir: Piracetam

scopolamine-demented mice in the Y-maze test

The impulsive memory functioning and probing behavior of mice was investigated by recording the spontaneous alternation using the Y maze test. The result of spontaneous alternation shows that there are differences in the percentages of spontaneous alternation between all the groups [Figure 3a]. Scopolamine administration decreased this percentage. Pretreatment of mice with the extract at the dose of 400 mg/kg statistically significantly ($P < 0.001$) reversed scopolamine-induced decrease in the percentage of spontaneous alternation. Repeated administration of the reference drug piracetam (150 mg/kg) also significantly increased spontaneous alternation percentage. The different doses of *V. subterranea* aqueous extract did not enhance or impair the locomotor activity of scopolamine-demented mice expressed as the number of entries in YM arms [Figure 3b].

Effect of *Vigna subterranea* on recognition memory of scopolamine-induced cognitive deficit in mice

All mice that received repeated administration of different doses of *V. subterranea* as well as piracetam spent more time exploring the novel object as compared to the familiar object [Figure 4a]. However, this was not the case with mice that received scopolamine injections only, as they spent more time exploring the novel object instead. The highest dose of the extract (400 mg/kg) brought the least statistically significant ($P < 0.05$) increase in novel object exploration time as compared to the scopolamine-treated mice. Discrimination index was statistically significantly ($P < 0.001$) diminished in scopolamine-demented rats that received the vehicle as compared to normal mice. Repeated treatment of mice with *V. subterranea* at all doses as well as piracetam induced an increase in DI. A statistically significant ($P < 0.0001$) increase in discrimination was achieved with the lowest dose of the extract (100 mg/kg) just like the standard drug piracetam, between the two objects [Figure 4b].

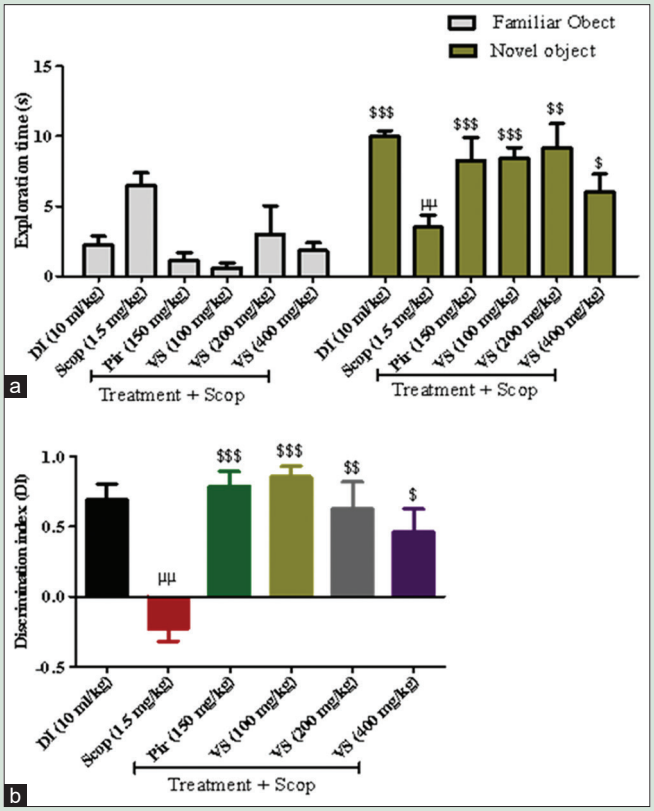

Figure 4: Effect of *Vigna subterranea* on scopolamine-induced memory impairment on the exploration time of the familiar versus novel object (a) and discrimination index (b) in object recognition test. Each column represents mean ± standard error of the mean of ten animals. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: Scopolamine; Pir: Piracetam

Effect of *Vigna subterranea* on retention in the T-maze task

Repeated intraperitoneal administration of scopolamine (1.5 mg/kg) brought about a decrement in the time spent by mice in the preferred arm associated with an increment in the number of entries in the discriminated arm [Figure 5a and b]. The standard drug (piracetam 150 mg/kg) reversed this trend with a statistically significant ($P < 0.0001$) increase in the time spent in the preferred arm. Nine-day treatment with the extract resulted in an increase in the time spent in the preferred arm. This increment was significant with the medium dose of the extract (200 mg/kg) and corresponds to about two-folds of the distilled water–scopolamine-treated mice's score. All the doses of the extract reduced the number of entries in the discriminated arm. The most statistically significant ($P < 0.0001$) decrement was obtained with the highest dose of the extract.

Related to the variable "return into initial arm," all the doses of the aqueous extract of *V. subterranea* statistically significantly ($P < 0.0001$) decreased the number of initial T-maze arm return. This performance was quite similar to that of the standard drug piracetam, a known antiamnesic drug [Figure 5c].

Effect of *Vigna subterranea* on visuo-spatial learning

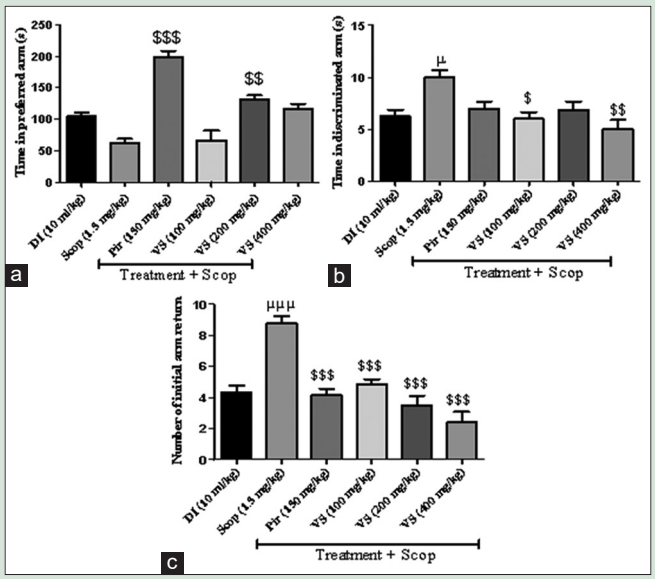

Figure 5: Effect of *Vigna subterranea* on scopolamine-induced memory impairments in the T-maze test. The time spent in the preferred arm (a), discriminated arm entries, (b) and initial arm return (c). Each column represents mean ± standard error of the mean of ten mice. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $^{\$\$\$}P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: Scopolamine; Pir: Piracetam

and memory in the Morris water maze

The time taken to find the hidden platform by scopolamine-demented mice decreased throughout the acquisition and the reversal phases with the administration of all doses of the extract as compared to those that received scopolamine solely [Figure 6a]. A statistically significant decrease ($P < 0.0001$) in escape latency was noted with the highest dose of the extract (400 mg/kg) on both the last day of acquisition and reversal training (days 3 and 6, respectively). Further, the extract and the standard drug reversed the decrease in speed related to repeated scopolamine injection. Mice that were treated with 400 mg/kg of extract had a statistically significantly ($P < 0.0001$) increased swim speed in the pool [Figure 6b].

24 h after the reversal trial, the platform was removed from the maze during the probe trial. Scopolamine administration statistically significantly ($P < 0.0001$) shortened the time spent by mice in the quadrant that contained the escape platform the previous day [Figure 6c] as compared to the control. However, piracetam-treated mice, as well as those treated with the different doses of the aqueous extract of *V. subterranea*, spent more time searching the platform in the target quadrant during the probe trial. This increment in time was statistically significant ($P < 0.0001$).

The visible platform (cued version) task, ran on the last day of the test, revealed a statistically significant ($P < 0.0001$) increase in the escape latency with the distilled water–scopolamine-treated mice as compared to the normal control mice. Mice that received the different doses of the extract took less time to escape from the water. The reference drug piracetam statistically significantly ($P < 0.0001$) shortened the escape latency as did the extract at the dose of 200 mg/kg during this phase [Figure 6d].

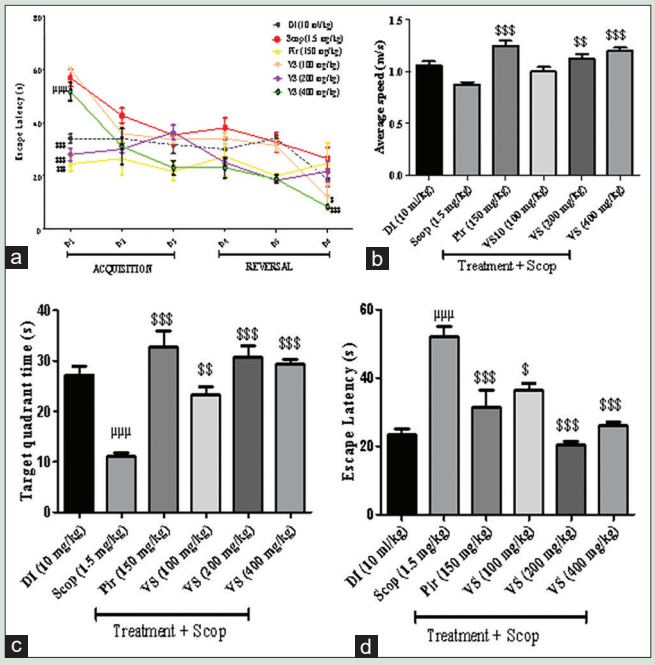

Figure 6: Effect of *Vigna subterranea* on scopolamine-induced memory impairment on retention parameters in the Morris water maze. Escape latency existing platform (a), average swim speed (b), time spent in the target quadrant during probe trial inexistent platform, (c) and visible platform escape latency (d). Each column represents mean ± standard error of the mean of ten animals. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $^{\$\$\$}P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: scopolamine; Pir: Piracetam

Effect of *Vigna subterranea* on cholinergic impairment, lipid peroxidation, and oxidative stress

Acetylcholine esterase activity in the hippocampus

AChE is a pivotal neurostransmitter in the cholinergic system whose main role is to breakdown ACh. Scopolamine-administered mice statistically significantly ($P < 0.0001$) increased the concentration of AChE in hippocampi as compared to the control group [Figure 7]. *V. subterranea*-treated mice showed a decrease in hippocampal AChE activity as compared to the scopolamine-treated group. The lowest dose of the extract of *V. subterranea* (100 mg/kg) showed a statistically significantly ($P < 0.0001$) higher suppression of AChE activity, which is in line with that of the piracetam-treated group.

Lipid peroxidation and oxidative stress in the hippocampus

There is a close connection between scopolamine-induced amnesia and the development of oxidative stress in brain tissues. Figure 8a shows that lipid peroxidation was statistically significantly ($P < 0.0001$) higher in scopolamine-administered mice as compared to the control group. This level was statistically significantly ($P < 0.001$) decreased in scopolamine-administered mice that received the standard drug piracetam. The aqueous extract at all doses exhibited a more statistically significant ($P < 0.0001$) reduction of lipid peroxidation in hippocampal tissue despite repeated scopolamine administration. Figure 8b and c shows that repeated administration of scopolamine decreased antioxidant enzyme (CAT and SOD) activity. However, pretreatment with different doses of extract inhibited scopolamine-induced oxidative stress by increasing CAT and SOD activities. However, statistical significance was not achieved. These increases in antioxidant enzyme activity were similar to that obtained by piracetam, the standard drug. On the other hand, linear regressions determined revealed statistically significant negative correlation between MDA and time spent in the target quadrant ($r = 0.704$, $P < 0.0001$) [Figure 9a] and statistically significant negative correlation between DI and AChE activity ($r = 0.590$, $P < 0.0006$) [Figure 9b].

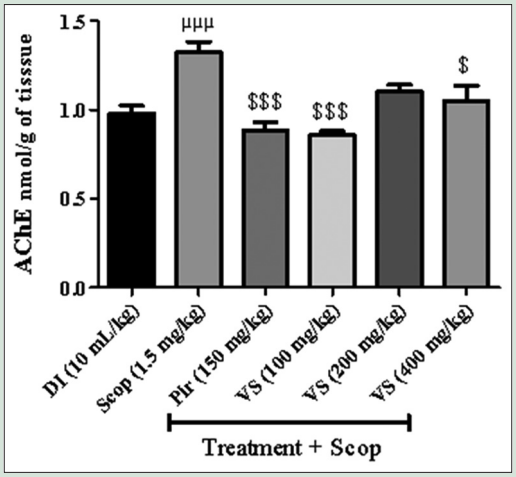

Figure 7: Effects of *Vigna subterranea* on acetylcholinesterase activity of scopolamine-treated mice. Each column represents mean ± standard error of the mean of ten animals. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $^{\$\$\$}P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: Scopolamine; Pir: Piracetam

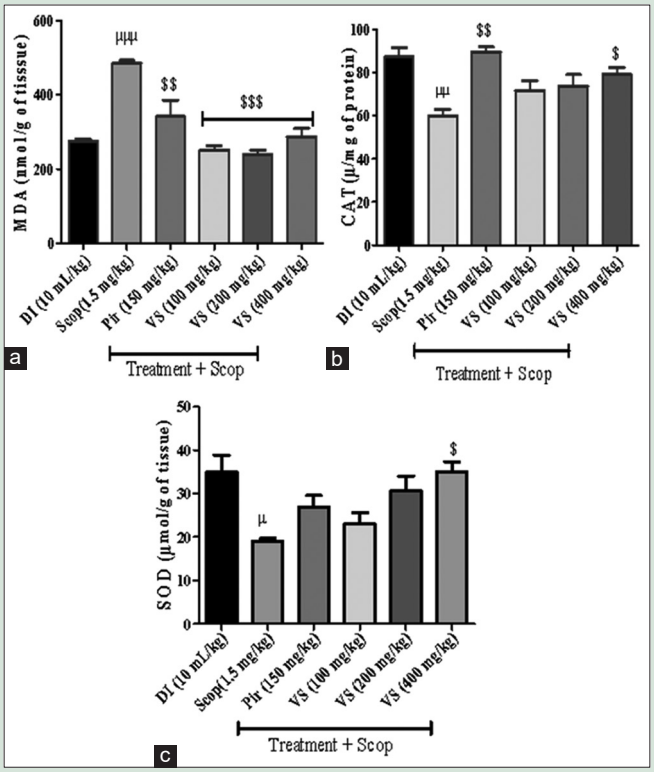

Figure 8: Effects of *Vigna subterranea* on malondialdehyde level (a), catalase, (b) and superoxide dismutase (c) activities in hippocampi tissue of scopolamine-treated mice. Each column represents mean ± standard error of the mean of ten animals. $^{\$}P < 0.05$, $^{\$\$}P < 0.001$, $^{\$\$\$}P < 0.0001$ versus scopolamine-treated group (DI + Scop); $^{\mu}P < 0.001$ versus deionized water group. DI: Deionized water; VS: *Vigna subterranea*; Scop: Scopolamine; Pir: Piracetam

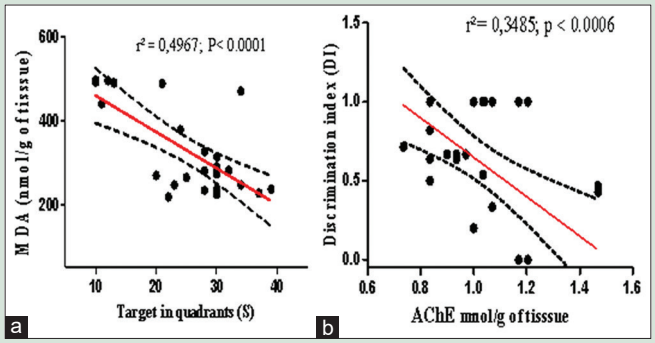

Figure 9: Pearson's correlation between malondialdehyde and time spent in target quadrants (a) Discrimination index versus acetylcholine esterase (b)

Effect of Vigna subterranea on hippocampi cell architecture

Hematoxylin and eosin staining was used to assess hippocampi cell alteration. Repeated scopolamine injection resulted in disorganization, decrease in thickness, and regression of the density of pyramidal cells in the CA1 and CA3 sub regions and granular cells of the DG hippocampal sub regions [Figure 10b] as compared to the control group [Figure 10a]. On the other hand, 200 and 400 mg/kg doses of the aqueous extract of *V. subterranea* reversed scopolamine-induced alteration and maintained a normal architecture [Figure 10e and f] of these regions evidenced by intact pyramidal cell thickness, which is comparable to what is seen in the section of hippocampi of mice treated with the standard drug piracetam [Figure 10c]. The lowest dose of the extract (100 mg/kg) also had a similar but lesser effect [Figure 10d].

DISCUSSION

AD is a multifactorial neurodegenerative disorder that appears in aging adults, inflicting serious behavioral and cognitive damages. Some clinical symptoms of AD include memory loss, apathy, and language disorder.[40] Scopolamine is an alkaloid which impairs learning and short-term and long-term memory in rodents and humans. It selectively blocks muscarinic receptors (M1) and hinders cholinergic transmission, leading to the development of oxidative stress status[41] and subsequent neurodegeneration associated with the deposit of tau protein and β amyloid.[42] Scopolamine also significantly decreases ACh level and increases AChE activity,[43] the reason why scopolamine-mediated memory impairment can be used as a valid model for investigations on molecules that may reverse AD symptoms.[44] In the present study, the potentials of *V. subterranea* aqueous extract (100, 200, and 400 mg/kg) in inhibiting behavioral and cognitive impairment associated with repeated scopolamine injection was tested in the YM, novel object recognition (NOR), T-maze, and MWM. Each test highlighted a specific cognitive aspect.

The YM and the MWM are believed to be excellent tools for the evaluation of hippocampal-dependent memory.[45] Rodents' ability to explore new environments is highlighted in the YM task. Spatial working memory was assessed by scoring the percentage of spontaneous alternation.[46] Nine-day treatment of mice with *V. subterranea* aqueous extract resulted in an increase in the percentage of spontaneous alternation in the YM. Krishna *et al.*[26] reported that an increase in spontaneous alternation corresponds to an improvement in short-term memory, whereas a decrease corresponds to decreased working memory. The aqueous extract of *V. subterranea* thus contributed in recovery from scopolamine-induced spatial short-term or working memory impairment. Moreover, neither scopolamine nor the different doses of the aqueous extract of *V. subterranea* affected the number of arm entries in the YM, cancelling any possible psychostimulant effect. To confirm the spatial memory-enhancing potential of the extract, the MWM was used. This test also evaluates the spatial and related forms of learning and memories.[47] Different doses of *V. subterranea* aqueous extract decreased the escape latency in the MWM. This result evidence the efficacy of the extract in ameliorating spatial learning and memory across the acquisition and retention phases of the MWM tasks. It has been reported that the decrement in escape latency of the first trial of each day is a representation of reference memory, whereas a decrease in this parameter from one trial to the next is an icon of working memory.[48,49] Treatment with the aqueous extract facilitated recall of the platform position in the MWM, confirming its spatial memory effect observed in the YM. Spatial navigation is very important to rodents and many other species, and this depends on their ability in learning and remembering locations. Deficit in short-term episodic memory, attention, and spatial orientation have been reported as early symptoms of AD;[50,51] as spatial information gotten from sensory inputs (neocortex) are encoded by hippocampal and parahippocampal place and grid cells.[52] Hence, *V. subterranea* aqueous extract's spatial learning and memory-ameliorating action might be related to its potential in reversing the marked scopolamine-induced cognitive decline.

In addition, retention which is an index of long-term memory was first assessed by scoring mouse spatial bias for the platform location in the probe trial of the MWM. The extract increased the motivation of mice in searching the absent platform as they spent more time in

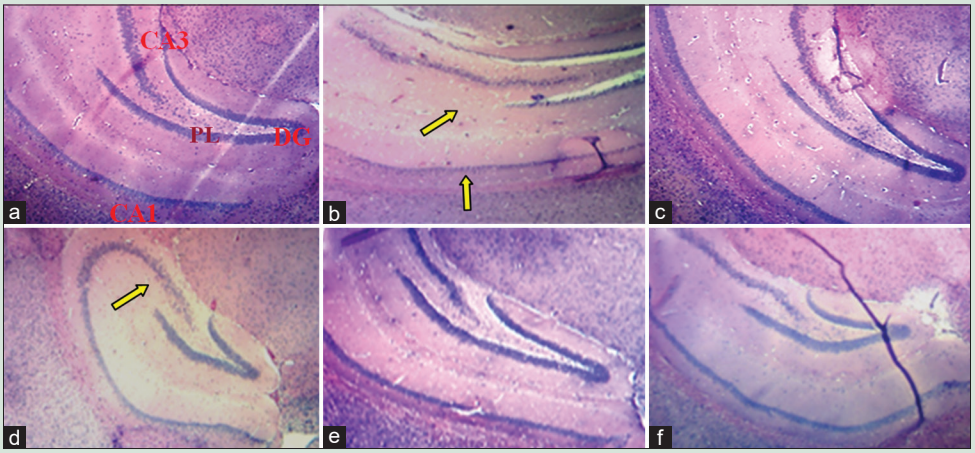

Figure 10: Effect of *Vigna subterranea* on scopolamine-induced neuronal cell loss in the CA1 and CA3 hippocampal subregions. Deionized water (a), Scopolamine (b), Piracetam (c), *Vigna subterranea* 100 mg/kg (d), 200 mg/kg (e), and 400 mg/kg (f). CA: Cornu ammonis; DG: Dentate gyrus; PL: Pyramidal layer. Representative photomicrographs were taken at magnification ×400 after hematoxylin and eosin staining

the target quadrant during the probe trial. Memory retention was investigated further using the NOR test.[53] This test measures nonspatial working memory based on the natural exploratory propensity of a rodents. Scopolamine-treated mice could not discriminate the novel object from the familiar object. *V. subterranea* aqueous extract pretreatment protected against recognition memory deficit as indicated by significantly higher DI, a cue of good recognition memory. This result is consistent with the time spent in the target quadrant of the MWM, supporting the long-term memory-enhancing potential of the extract. To confirm the potential of *V. subterranea* aqueous extract on both scopolamine-induced retention and spatial short-term memory deficits, the T-maze test, a well-known sensitive tool for the detection of hippocampal-dependent dysfunction, was used.[54] Treatment with the aqueous extract could increase the time spent in the preferred arm associated with a decrease in the time spent in the discriminated arm and number of initial arm return. Storage and retrieval of newly acquired information are ensured by a complex circuit consisting of numerous cognitive projections extending from Meynert's nucleus basilis to the hippocampus and amygdala and through the cortex.[55] In a nutshell, the cognitive behavioral tests revealed that the aqueous extract of *V. subterranea* improves scopolamine-induced short- and long-term memory deficits.

Cholinergic function governs vital aspects of memory.[56] The amygdala, hippocampus, and cortices are the main brain regions known to be involved in the learning and memory processes through cholinergic transmission. In the neurochemical analysis to elucidate the mechanism of memory-enhancing effect of *V. subterranea* through the cholinergic system, hippocampal ACh level was measured. The hippocampus has long been known as the key structure involved in cognitive map formation.[57] Lesions, long-term potentiation saturation, pharmacological inhibition, and the loss-of-function of receptors within the hippocampus may affect spatial learning and memory.[58-61] Cognitive disorder has been associated with constant Ach deficiency which results from the overexpression of AchE activity.[62] Hence, inhibition of AChE can be a potential source of treatment for AD-related cognitive impairment. We found that repeated scopolamine injection brought about a significant increase in the AChE in mouse hippocampi homogenates. However, treatment with *V. subterranea* aqueous extract recovered the increment in the AChE hippocampal homogenate of scopolamine-treated mice. Therefore, our results suggest that the aqueous extract of *V. subterranea* has the potential to treat cognitive dysfunction in scopolamine-demented mice possibly through AChE inhibition. There exist similar reports[63,64] on the potential of other plant extracts in enhancing the availability of ACh within synapses. Besides AChE increment, there is evidence that reactive oxygen species generated by oxidative stress potentially plays a vital role in the development of AD among elderly persons.[65] Lipid peroxidation is one of the most important indicators of neurodegeneration occurrence in the brain. The major structural and components of cell membrane; lipids, and proteins are vulnerable to oxidation by free radicals.[66] Unlike other membranes, neuronal membranes' key constituents are polyunsaturated fatty acids involved in signal transfer. A third of the inhaled oxygen is consumed by the brain, making it a favorable site for lipid peroxidation.[67] In this study, scopolamine administration induced oxidative stress by significantly elevating MDA level, indicating lipid peroxidation. All the doses of *V. subterranea* aqueous extract significantly suppressed this increment. Lipid peroxidation may be enhanced due to decreased levels of the key antioxidant enzyme CAT and SOD.[68] H_2O_2 release is associated with the formation of other oxidant species. CAT is endowed with the ability to detoxify H_2O_2.[69] Similarly, SOD plays a role in detoxifying superoxide anions which damage cell membrane macromolecules. Repeated injection of scopolamine was found to decrease the level of CAT and SOD in hippocampi homogenates. Pretreatment with *V. subterranea* aqueous extract preserved the activity of these antioxidant enzymes. The antioxidant effect of the aqueous extract of *V. subterranea* might be related to its antioxidant properties. The findings of Krishna *et al.*[26] also revealed the nootropic potential of *Vigna mungo* through antioxidant activity. Moreover, there is evidence that adult hippocampal neurogenesis is very important in memory consolidation in the brain.[70] Scopolamine injection resulted in the death of small and large pyramidal cells of the CA1 and CA3 regions and the dentate gyrus granular cells. *V. subterranea* aqueous extract protected the hippocampus from cell death associated with repeated scopolamine injection, thereby maintaining an intact cell architecture. The observed effects of *V. subterranea* in rescuing the deficits in hippocampal neurons may be attributed to the redox imbalance which altered hippocampal neurons, resulting in learning and memory deficits. Although compounds responsible for the *V. subterranea* aqueous extract neuroprotectory, learning, and memory-improving effects were not evaluated in this study, there is evidence that its seeds contain alkaloids, saponins, and many polyphenolic compounds

mainly from the class of flavonoids.[18,19,71] The ability of the extract to inhibit oxidative stress could thus be linked to the antioxidant nature of compounds found in the extract.

In addition, when linear regression was determined, we found significant negative correlation between MDA and time spent in the target quadrant and between DI and AChE activity. The increment in behavioral scores in the MWM and NOR tests alongside with decrease in MDA content and a decrease in AChE activity could be correlated with the involvement of the aqueous extract of *V. subterranea* in neuroprotection against lipid peroxidation AChE expression induced by scopolamine in mouse hippocampi.

Given that our investigation does not outline the core pathology of AD, we therefore intend to assess the anti-inflammatory effects of the extract using a model of neuroinflammation, in order to delineate the mechanism of action of the extract in neuroprotection.

CONCLUSION

The aqueous extract of Bambara groundnut enhances hippocampal-dependent learning and memory. This effect may be attributed to its AChE inhibitory activity and antioxidant potential that were observed in this study. Hence, dietary usage of Bambara groundnut could be beneficial and can also be associated with the existing therapies as an adjuvant in the treatment of amnesia.

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Conflicts of interest

There are no conflicts of interest.

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