Current Trends in Biotechnology and Pharmacy
Vol. 20(1) 2704-2722 January 2026, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2026.1.1

Development and assessment of a synergy-based combined extracts of *Spondias mombin* L., *Spilanthes filicaulis* (SCHUMACH. & THONN.) C.D. ADAMS and *Piper guineense* THONN. for learning and memory enhancement

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Abstract

Memory challenges and cognitive decline, linked to neurodegenerative illnesses, continue to rise worldwide without effective remedies. Traditional medicinal plants have shown promise in mitigating these conditions. This study evaluated synergy-based aqueous combinations of *Spondias mombin*, *Spilanthes filicaulis*, and *Piper guineense* for learning and memory enhancement. Two formulations, H1 (ratio 2:2:4) and H2 (ratio 2:2:2), were prepared, administered, and assessed using the Morris Water Maze and Y-maze tests, alongside biochemical analyses of oxidative stress markers (MDA, H_2O_2, SOD, GSH, GPx, GST), nitric oxide levels, and acetylcholinesterase activity. Histopathological examination of the hippocampus was also performed. Results showed that scopolamine impaired learning and memory, elevated oxidative stress, and caused hippocampal damage. However, treatment with the combined extracts H1 and H2 significantly improved spatial learning and working memory compared to scopolamine controls, as evidenced by reduced escape latency and increased spontaneous alternation. Additionally, H1 and H2 lowered oxidative stress markers, restored antioxidant enzyme balance, reduced AChE activity, and ameliorated neuronal disruptions. Among the two formulations, H1 consistently demonstrated superior neuroprotective and antioxidant effects across behavioral and biochemical assays. These findings suggest that synergy-based combinations of these plants, particularly H1, may offer a promising approach for memory enhancement and warrant further investigation for development into an herbal therapeutic product.

Keywords: Medicinal plants, Synergistic combination treatment, Neurodegenerative disorders, Antioxidant, Acetylcholinesterase activity.

Introduction

The aging demographic contributes to the escalating prevalence of memory impairment and dementia worldwide (1). Alzheimer's

disease (AD), the primary cause of dementia, progressively impairs intellectual functions centered in the Wernicke areas, affecting learning, memory, language, and personality(2). Elderly individuals are primarily affected by this degenerative neurological ailment, resulting in a decline in cognitive abilities and memory function (3,4). Contrary to normal aging, AD is characterized by its distinct pathological nature (5). According to Alzheimer's Disease International (ADI), global dementia diagnoses surpass 9.9 million yearly, with a startling frequency of one diagnosis every 3.2 seconds(6). Furthermore, the latest data from the World Health Organization (7), positions dementia as the world's seventh leading cause of death, based on the 2019 Global health estimates released in December 2020.

Alzheimer's disease (AD) risk factors encompass genetic elements like the ApoE4 gene and environmental influences such as age, depression, and metabolic conditions like diabetes and hyperlipidemia(8,9)which is characterized by a decline in thinking and independence in personal daily activities. AD is considered a multifactorial disease: two main hypotheses were proposed as a cause for AD, cholinergic and amyloid hypotheses. Additionally, several risk factors such as increasing age, genetic factors, head injuries, vascular diseases, infections, and environmental factors play a role in the disease. Currently, there are only two classes of approved drugs to treat AD, including inhibitors to cholinesterase enzyme and antagonists to N-methyl d-aspartate (NMDA. Recent research highlights multiple contributors to cognitive function impairment, encompassing chemicals, genetic associations, medications, disorders, and the natural aging process (10,11). In addition, studies indicate associations between AD pathologies and oxidative stress, inflammation, hyperhomocysteinemia (10), loss of specific neuronal populations, reduced synaptophysin immunoreactivity, and depletion of cholinergic fibers (12), Although, anticholinesterase drugs are used for neurodegenerative diseases, their limitations; such as low bioavailability, hepatotoxicity, and short action duration; have led to intensified pharmaceutical research focusing on natural acetylcholinesterase (AChE) inhibitors from plants with fewer side effects (10,13–15). This pursuit aims to address cholinergic deficits and enhance neurotransmission, potentially halting or slowing disease progression. A comprehensive, multi-targeted approach appears essential in effectively addressing memory-related disorders.

Traditional African medicine relies on numerous medicinal plants for treating intellectual disorders, including neurodegenerative diseases (1). These plants contain active compounds known as potent acetylcholinesterase (AChE) inhibitors (16), essential for slow-acting chemical communication within the nervous and cholinergic systems (17). Additionally, the combination of active compounds within herbs can produce synergistic pharmacological effects, as demonstrated by studies like Mak et al (3), which highlighted the potential of combining alkaloids from *Coptidis rhizoma* and *Corydalis rhizome*. Similarly, Khan et al (10) reported the synergistic combination of *Withania somnifera* and *Myristica fragrans* effectively inhibiting anticholinesterase activity.

Spondias mombin, also known as hog plum, belongs to the Anacardiaceae family and is originally native to the tropical regions of the Americas. However, it has spread widely and is now found across many parts of Asia and Africa (18). Traditionally, various parts of the plant, including its stem bark, leaves, and roots, have been used in folk medicine to address a range of health conditions (19). cientific studies have highlighted its antimicrobial (20), antioxidant (19,21), and antidiabetic properties (22), supporting its long-standing use in natural healing practices.

Spilanthes filicaulis, commonly called Creeping Spot Flower or African Cress, is widely distributed across tropical and subtropical regions of the world, including Africa, the

Americas, Borneo, India, Sri Lanka, and parts of Asia (23). It is an annual plant from the Asteraceae family, characterized by creeping growth and prostrate stems that root at the nodes (24). In Babungo, located in the Northwest Region of Cameroon, the entire plant is traditionally used to treat ailments such as malaria, gastritis, toothaches, and stomachaches (25). Research has identified several biological activities associated with this species, notably its antimalarial(26), antidiabetic(27), antimicrobial (28), antifungal (29), and antioxidant activities (24,30).

Piper guineense, widely known as black pepper, belongs to the Piperaceae family and is distributed across Africa and various other regions around the world, primarily for its culinary importance (31). The plant holds significant value in traditional medicine, with its leaves traditionally used to help regulate menstrual cycles and support the treatment of female infertility (32). Scientific studies have also highlighted its anti-inflammatory, anticonvulsant, and antioxidant properties (33,34).

Additionally, an ethnobotanical survey conducted in Southwest Nigeria highlighted the tree plants for their traditional use as memory enhancers and anti-aging remedies (35,36). Also, each of them has been screened either in vitro to inhibit the acetylcholinesterase activity or in vivo to protect against neurochemical alterations and oxidative stress in the scopolamine model of cognitive dysfunction (30,37–44). Moreover, Hounsou et al (45) demonstrated the promising in vitro antioxidant potential of the combined extract from these three plants. The current study aims at developing a synergy-based combined extract of these plants in different ratios for learning and memory enhancement to suggest the ratio suitable for herbal product formulation based on favorable pharmacological effects and toxicological profiles.

Plant collection, processing and extraction

Samples of *S. mombin* leaves harvested from Ondo Road, Akure; entire *S. filicaulis* plants collected from Ilu abo, Ondo State; and dried *P. guineense* fruits sourced from Bode market, Ibadan, Oyo State, underwent botanical identification and authentication at the Herbarium of the Department of Botany, University of Ibadan, Nigeria. The voucher specimens are UIH-23260 (*Spondias mombin*), UIH-24241 (*Spilanthes filicaulis*), and UIH-23258 (*Piper guineense*). After air-drying and pulverizing the plant materials, the powdered samples were combined. Combination H1 was prepared at a 2:2:4 ratio, while combination H2 utilized a 2:2:2 ratio. These mixtures were macerated with distilled water for 72 hours, with stirring every 24 hours. The filtrate of each combination was freeze-dried, and up until their use, concentrated extracts were kept refrigerated at 4°C.

Procurement, housing, and acclimatization of animals

Ten-week-old male and nulliparous female albino mice (weighing 18-29 g) were procured from the Experimental Animal Unit, University of Ibadan, Nigeria. The selection of the animals was done following the Animal Care and Use Research Ethics Committee (ACUREC), University of Ibadan, Nigeria's approval under the protocol number UI-ACUREC/061-0723/11. Mice, confirmed healthy, were housed in polypropylene cages (10 males or 5 females per cage) with wood shavings, and provided a standard pellet diet, and *ad-libitum* supply of water for two weeks before experimentation.

Acute toxicity and behavioural changes

Following the Organization for Economic Co-operation and Development guideline (46), twenty fasted female mice were administered oral doses of combined extracts H1 and H2 at 300 mg/kg and 2,000 mg/kg to determine their acute toxicity. Post-extract administration, mice were monitored immediately and at 30, 60 minutes, 4 hours, and 24 hours for signs of toxicity. Daily checks over 14 days included monitoring for salivation, defecation, convulsions, skin/fur changes, eye/mucous membrane alterations, respiratory changes, behavior patterns.

Also, weight loss was monitored every three days.

Experimental design and treatment

For the experiment, albino mice were randomly divided into nine groups, each consisting of 10 mice. Following the period of acclimatization, all animals except those in the normal control group (Group 1) were pretreated intraperitoneally with 2 mg/kg of scopolamine for three consecutive days. Starting from day 4 post-scopolamine induction, Group 1 received oral distilled water (0.2 mL/mouse), Group 2 also received oral distilled water and was assigned as the negative control group, while Group 3 was given piracetam orally (200mg/kg) to serve as the positive control. Top of FormGroups 4-6 received oral doses of combined extract H1 (at 150 mg/kg, 100 mg/kg, and 50 mg/kg, respectively), and Groups 7-9 received combined extract H2 (at 150 mg/kg, 100 mg/kg, and 50 mg/kg, respectively). Ten days post-administration, mice underwent three days of Morris water maze training followed by a reference memory test (probe test) on the 14th day, and finally, a Y-Maze test. Dosing continued from the 10th to the 14th day for assessment. The experimental procedure is described in Figure1.

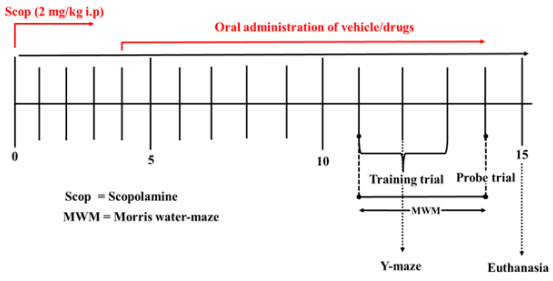

Figure 1: Scheme of the experimental procedure.

Neurobehavioural test

Morris water maze test

Applying the technique described by Khan et al (10), the Morris water maze task was performed to assess mice's spatial learning in a sizable setting. The maze was made of a circular tank (125 cm diameter, 34 cm height) held water (15 cm height), made opaque by the addition of evaporated milk. A Tracking system (ANY-maze 7.20), connected to a camera was used to facilitate the recording of the swimming pattern of mice. The tank was divided into four quadrants, hiding a submerged platform (14 cm height, 1 cm below water level) in one quadrant. Over four days, comprising three training days and one probe trial, mice underwent four of 120-second trials each day from different starting positions. The escape latency (i.e., the time taken by the mice to locate the hidden platform) was recorded. Mice that were unable to locate the platform within a 2-minute timeframe were subsequently positioned on it for a duration of 15 seconds. Up to 15–20 minutes gap separated the trial sessions. On the fourth day, the hidden platform was taken away, and during a 120-second trial, entries over the previous platform location and time spent in the target quadrant were recorded to evaluate memory retention and spatial recall. This methodology allowed for efficient assessment of mice's spatial navigation abilities without manual data recording, ensuring a comprehensive understanding of their learning capabilities in the maze environment.

Y- Maze test

In assessing short-term memory in mice via the Y maze test as described by Kraeuter et al (54), a wooden maze with three arms A, B, and C, oriented at an angle of 120 ℃ to each other was used. Mice were positioned in the maze center and given 5 minutes to explore the arms, and their arm entries and alternations were recorded. Cleaning of the maze with 70% ethanol before and between tests was performed to maintain consistency of conditions. An entry was noted when the animal went into an arm with all its paws, while an alternation occurred if the mouse consecutively entered all three arms in a sequence (e.g., ABC, CAB). This rigorous protocol ensured standardized testing conditions, enabling accurate assessment of mice's

short-term memory performance. The total arm entries and proportion of spontaneous alternations were determined by applying the equation below:

%Alternation = (Number of Alternations / [Total number of arm entries – 2]) x 100

Tissue Processing

On the final day, euthanasia via cervical dislocation on ice was performed on the animals. From each group of 10 mice, the brains of 4 were dissected entirely, with the hippocampus sectioned for biochemical tests. Similarly, brains from 4 mice in each group underwent preservation in phosphate formalin buffer after cardiac perfusion using normal saline and 10% phosphate formalin buffer for histopathology as previously outlined by Olopade et al (72). The isolated hippocampus tissues were rinsed, weighed, homogenized in ice-cold homogenizing buffer (0.1 M phosphate buffer, pH 7.4), then centrifuged at −4 °C (12,000 rpm for 10 minutes). The resulting post-mitochondrial fraction (PMF) was collected for biochemical parameter assessments according to Oyagbemi et al (73).

Biochemical assays
Biomarkers of oxidative stress
Estimation of MDA level

Hippocampal lipid peroxidation was carried out by measuring thiobarbituric acid reactive products using the procedure reported by Varshney and Kale (74). Briefly, 200 **µ**L of supernatant was added to Tris-KCl buffer (800 **µ**L, 0.15M, pH 7.4), Trichloroacetic acid (500 **µ**L, 30%), and Thiobarituric acid (500 **µ**L, 0.7%). The solution was mixed thoroughly and heated in water bath at 80°C for 45 minutes. It was then cooled and centrifuged at 400 rpm for 10 minutes. The absorbance of the supernatant was read at a wavelength of 532nm, and findings were denoted as Units/mg protein.

Estimation of H_2O_2 level

The hydrogen peroxide (H_2O_2) generation was determined according to the procedure reported by Wolff (75). Basically, 1mL of buffer, ammonium ferrous sulphate (100 ***µ***L), sorbitol (40 ***µ***L), xylenol orange (40 ***µ***L), H_2SO_4 (20 ***µ***L) were added to 40 ***µ***L of sample (supernatant). The solution was mixed thoroughly and incubated at 25°C for 30min. The absorbance was read at a wavelength of 560nm and H_2O_2 generated was extrapolated from H_2O_2 standard curve.

Measurement of acetylcholinesterase (AChE) activity

The acetylcholinesterase activity was assayed spectrophotometrically in mice's hippocampus as reported by Turner et al (76). Then, a reaction mixture containing 1 mL of buffered Ellman's reagent and 300 ***µ***L of acetylthiocholine iodide solution was added 200 ***µ***L of supernatant. The absorbance was then monitored at 412 nm over a period of 3 minutes at 30 seconds interval using UV-Vis spectrophotometer. Activities were expressed as mmole of substrate/min/mg protein**.**

Hippocampal antioxidant defense system
Determination of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity in homogenates was assessed based on the procedure of Fridovich (77) with minor modifications by Oyagbemi et al (78). An aliquot of 100 µL of supernatant was added to carbonate buffer (20mL, pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of freshly prepaered adrenaline (300 µL, 0.3mM) to the mixture wich was quickly mixed by inversion. The increase in absorbance at a wavelength of 480nm was monitored every 30 seconds for 150 seconds. 1 unit of SOD activity was given as the amount of SOD to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute time frame.

Determination of reduced glutathione (GSH)

The measurement of reduced glutathione (GSH) activity was carried out in accordance with Ellman's methodology (79). To 100

µL of sample was added 1 mL of sulfosalicylic acid. The solution was mixed thoroughly and centrifuged at 4000 rmp for 50min.500 **µ**L of Ellman's reagent was then added to 1mL of supernatant the absorbance was read at a wavelength of 412nm using water as blank. The GSH activity was extrapolated from standard curve.

Determination of Glutathione Peroxidase (GPx)

The activity of glutathione peroxidase was assessed using the procedure reported by Beutler (80). Each mixture for the reaction is consisted of 1 mL of potassium phosphate buffer, 200 ***µ***L of sodium azide, 400 ***µ***L of GSH, 20 ***µ***L of H_2O_2, 100 ***µ***L of tissue sample, 120 ***µ***L of distilled water, and 200 ***µ***L of TCA. After a 5-minute incubation at room temperature, the mixture underwent centrifugation at 3000 rpm for 5 minutes. Subsequently, 1 mL of supernatant was supplemented with 500 ***µ***L of 0.3 K_2PHO_4 followed by 500 ***µ***L of Ellman's reagent. The new mixture was then read at a wavelength of 412 nm using spectrophotometer and findings were denoted as Units/mg protein.

Determination of glutathione S-transferase (GST)

GST activity was assessed using the method described by Habig et al (81). To achieve this, 1 mL of buffer was added to 100 **µ**L of the processed sample followed by 50 **µ**L of Reduced Glutathione (GSH) solution. 500 **µ**L of 1- chloro 2, 4, - di nitrobenzene (CDNB) solution was further added, and the mixture was read spectrophotometrically at a wavelength of 480 nm and monitored every 60 secs for 150 secs. The results were reported as **µ**mole/min/mg protein.

Serum marker of inflammation

The assessment of nitrite levels, serving as an indicator of nitric oxide (NO) production in the hippocampus of mice, was conducted following the procedure outlined by Olaleye (82). The short-lived nature of nitric oxide (NO) results in its rapid conversion into stable compounds, namely nitrate (NO_3), and nitrite (NO_2^-) (Ishola *et al.*, 2018). Briefly, 100 ***µ***L of sample was added to 1mL of Griess solution. The solution was mixed thoroughly and incubated at 25°C for 30min. The absorbance was read at a wavelength of 542nm and nitite concentration was extrapolated from NO standard curve.

Histopathology

For the histopathology study, brain samples underwent a routine procedure of paraffin embedding. Using a microtome (Microm GmbH. D-6900 Heidelberg, West Germany), 5-mm thick sections were produced and stained with Haematoxylin and Eosin (H&E) for examination of the general histology (83). Every stained slide was visualized using a microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Utilizing Graphpad Prism 9.5.1, the statistical assessment was conducted with a confidence limit established at 95%. The representation of data values was in the format of mean ± standard error of the mean (SEM). Comparisons were made between the means of individual groups and the control, while groups receiving combined synergistic treatments were compared against the group receiving scopolamine only. ANOVA (one- or two-way) was applied to the data, and then the Tukey post hoc multiple comparison test was performed. At the five percentile ($P \leq 0.05$), differences in means were deemed statistically significant.

Results

Acute toxicity test

After 14 days of post-administration of H1 and H2 for the acute toxicity testing, there were no obvious signs of toxicity at all doses studied (300 mg/kg; 2000 mg/kg). No deaths were also recorded. There was no evidence of tremors, diarrhea, convulsions, or salivation. Skin, nose, eyes, and fur were observed to have normal morphological features, however, strange behaviors like walking backward and self-mutilation were not present. Furthermore,

the body weight of the treated mice gradually increased during the duration of the study.

Neurobehavioural tests
Morris water maze tasks

Results showed that the combined extract H1 and H2, as well as piracetam, reversed scopolamine-induced cognitive impairment and memory deficit in mice as indicated by a significant reduction [F (8, 173) = 25.69, P<0.0001] in escape latency times throughout the trials for acquisition conducted on days 1 to 3, an increase [F (8, 62) = 10.52, P<0.0001] in crossing rates of the target quadrant, and an increase [F (8, 55) = 10.00, P<0.0001] in the length of time in the quadrant of interest (Figure 2).

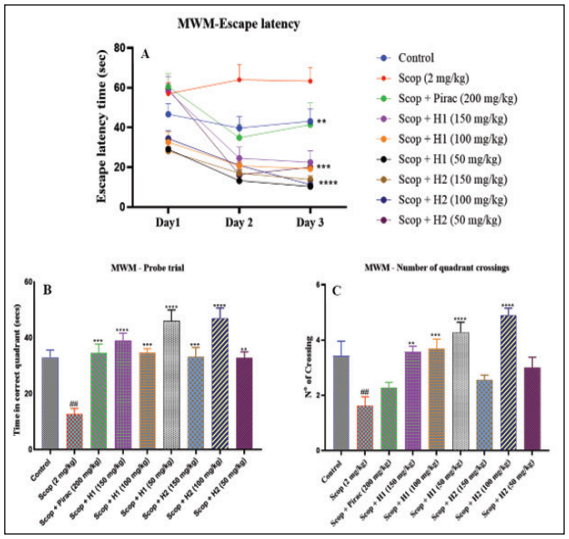

Figure 2: Effects of combinations H1 and H2 on the escape latency time (A), time spent in target quadrant (B), number target quadrant crossing (C) in Morris water maze. Each line in the plot shows the average of 4 trials per day for each animal (A). Values were presented as mean ± SEM (n=10) through one-way ANOVA analysis. Two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze escape latency time. Markers represent the differences ## p<0.01 when compared to control. **p<0.01, ***p < 0.001, ****p<0.001 when compared to Scop group.

Y-maze test

A one-way ANOVA revealed that the treatment of mice with the combined extracts H1 and H2 improved the scopolamine-induced cognitive deficits in mice by significantly augmenting [F (8, 59) = 4.151, P=0.0006] the spontaneous alternation percentage of mice during the Y-maze task. However, the number of arm entries did not significantly differ [F (8, 71) = 0.9536, P=0.4790] between any of the groups (Figure 3)

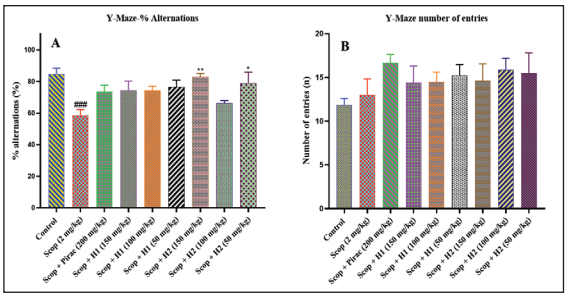

Figure 3: Effect of H1 and H2 on spontaneous alternation percentage (A) and total number of arm entries (B) in Y-maze. Values were presented as mean ± standard error of the mean (n=10). #p<0.05, ###p<0.001 vs. Control group. *p<0.05, **p<0.01 vs. scopolamine-treated group. One-way ANOVA was applied to the data, and then the Tukey post hoc multiple comparison test was performed

Biochemical analysis
Biomarkers of oxidative stress

Our findings showed statistically significant (p<0.05) in the level of the hippocampal markers of oxidative stress ([F (8, 63) = 105.8, P<0.0001] MDA level and [F (8, 63) = 10.26, P<0.0016] of AChE activity in the groups treated with the combined extracts H1 and H2 initially increased by scopolamine. However, scopolamine-alone treated mice showed a decrease in hippocampus NO level, with significant increases [F (8, 63) = 15.43, P<0.0001] observed in groups treated with H1 and H2 (Table 1).

Table 1: Effects of H1 and H2 on hippocampus indices of oxidative stress

Parameter	MDA	H_2O_2	AChE	NO
Control	2.6722 ± 0.515	3.5229 ± 0.625	18.0015 ± 3.637	25.6638 ± 2.569
Scop (2 mg/kg)	5.2793 ± 0.505####	4.5646 ± 0.88⁴#	20.0016 ± 1.979	25.3672 ± 2.001
Scop + Pirac (200 mg/kg)	3.4039 ± 1.110****	3.9396 ± 0.589	18.8587 ± 2.425	27.1473 ± 6.294
Scop + H1 (150 mg/kg)	0.8879 ± 0.025****	3.0021 ± 0.120****	17.1443 ± 2.969	53.0633 ± 14.519****
Scop + H1 (100 mg/kg)	0.8223 ± 0.075****	2.6896 ± 0.170****	15.4298 ± 2.176*	50.0964 ± 12.511****
Scop + H1 (50 mg/kg)	0.9472 ± 0.017****	2.8979 ± 0.295****	16.7157 ± 3.534	46.5732± 6.6145****
Scop + H2 (150 mg/kg)	0.8845 ± 0.112****	2.7938 ± 0.496****	19.4302 ± 1.979	44.3480 ± 4.485***
Scop + H2 (100 mg/kg)	0.8875 ± 0.037****	3.2625 ± 0.874***	14.7628 ± 4.125**	43.2354 ± 5.503***
Scop + H2 (50 mg/kg)	0.6667 ± 0.066****	2.8458 ± 0.313****	19.2873 ± 1.641	44.3480 ± 6.453***

Data were presented as Mean ± Standard deviation (n= 4). #<0.05, ### P<0.01 vs. control group and ***p<0.001 ****P<0.001 vs. scopolamine group using one-way ANOVA following by Tukey's multiple comparisons test. MDA: malondialdehyde (**µmol** of MDA formed/mg protein); H_2O_2: hydrogen peroxide generation (**µmol**/min/mg protein); AChE: acetylcholinesterase (**µmol**/min/mg protein); NO: nitric oxide (**µmol**/mg protein)

Antioxidant defense system

The treatment of mice with both combined extracts H1 and H2 significantly increased [F (8, 63) = 49.72, P<0.0001] SOD and [F (8, 63) = 123.3, P<0.0001] GSH level in mice hippocampus initially decreased in scopolamine induction. However, GPx and GST activities were found to increase in scopolamine-alone treated mice with significant decreases observed in mice post-treated with H1 and H2 (Table 2).

Table 2: Effects of H1 and H2 on hippocampus antioxidant defense system

Parameter	SOD	GSH	GPx	GST
Control	6.6975 ±0.7124	9.5319 ± 0.903	6.6392 ± 0.977	1.57 ± 0.839
Scop (2 mg/kg)	1.6864 ± 1.185####	4.9574 ± 0.752#	8.5428 ± 0.795####	4.94 ± 0.56####
Scop + Pirac (200 mg/kg)	2.6823 ± 0.762	9.3191 ± 0.601****	6.2491 ± 1.070****	0.815 ± 0.101****
Scop + H1 (150 mg/kg)	1.7120 ± 0.759	37.1915 ± 4.343****	3.0459 ± 0.061****	2.01 ± 0.980****
Scop + H1 (100 mg/kg)	5.1649 ± 0.949****	41.7128 ± 9.701****	2.9985 ± 0.271****	0.41 ± 0.05****
Scop + H1 (50 mg/kg)	2.9709 ± 0.493*	35.5957 ± 1.209****	3.1724 ± 0.243****	0.882 ± 0.089****
Scop + H2 (150 mg/kg)	1.2896 ± 0.037	38.2021 ± 4.159****	3.0107 ± 0.108****	1.43 ± 0.193****
Scop + H2 (100 mg/kg)	2.0294 ± 0.680	39.3723 ± 1.567****	2.9526 ± 0.038****	0.947 ± 1.100****
Scop + H2 (50 mg/kg)	2.4400 ± 0.169	38.2553 ± 1.401****	2.8880 ± 0.142****	1.72 ± 0.77****

Data were presented as mean ±SEM (n= 4). #P<0.05 vs. control group and *P<0.05, **P<0.01, ****P<0.0001 vs. scopolamine group using one-way ANOVA following by Tukey's multiple comparisons test. SOD: superoxide dismutase (units/mg protein); GSH: reduced glutathione (**µmol**/mg protein); GPx: glutathione peroxidase (units/mg protein); GST: glutathione S-transferase (mmol 1-chloro-2,4-dinitrobenzene–GSH complex formed/min/mg protein)

Histopathology

Histopathological examination of the brain hippocampus by photomicroscopy demonstrated that scopolamine is associated with deleterious effects as evidenced by multiple apoptotic deaths of neurons and distortion of neuronal morphology along with a reduction

of neuronal cells in the *cornus ammonis* 1 (CA1) region as compared to normal control group. In addition, the dentate gyrus (DG) of the hippocampus in the scopolamine group exhibited multiple deformed neurons (Figure 4.1 A-F). Regarding the piracetam-treated group, a normal histological structure of the brain hippocampus was observed with few lightly stained nuclei unlike the scopolamine group (Figure 4.1 D-I). The treatment of the mice with the combinations H1 and H2 at the doses 150 mg/kg for H1 and 100-150 mg/kg for H2 exhibited significant reversal of scopolamine-induced alterations in the brain hippocampus and showed relatively normal cellular architecture in the CA1 region and DG as shown in Figure 4.2 B-C and Figure 4.3 B-F.

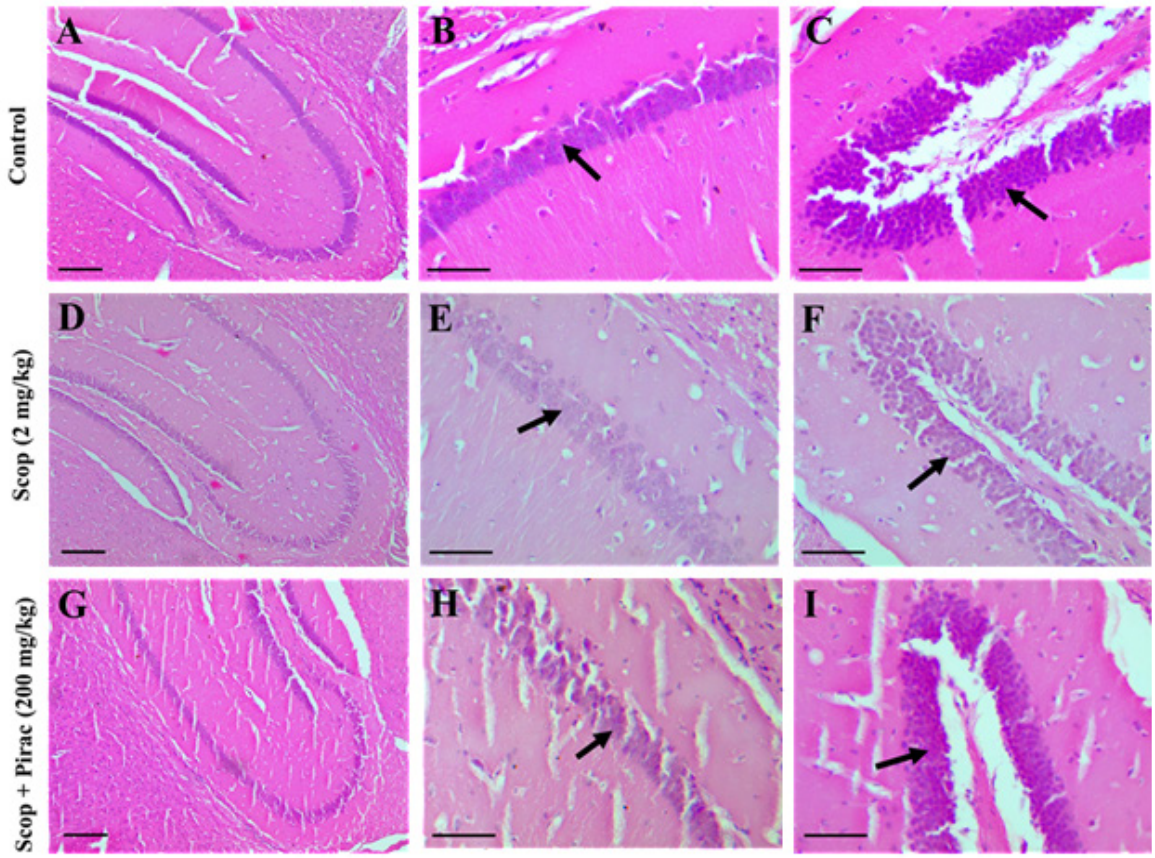

Figure 4.1: Micrographs showing the longitudinal section of hippocampus in mice (H&E; Magnification: A, D&G 10x, B, C, E, F, H, I 40x; Scale bars: A, D&G 100µm, B, C, E, F, H, I 50µm).

(A) Lower magnification showing a normal hippocampus. **(B&C)** Higher magnification showing normal *cornus ammonis* 1 (CA1) region and dentate gyrus of the hippocampus. **(D)** Lower magnification showing the scopolamine-induced mice hippocampus. **(E)** Scopolamine treated mice revealed multiple apoptotic death neurons and the neuronal morphology appear distorted with reduced neuronal cells in the CA1 region. **(F)** The dentate gyrus in the scop group exhibiting multiple deformed neurons (black arrow). **(G)** Lower magnification showing normal hippocampus. (**H&I)** Piracetam treated mice rvealed a normal CA1 region with few lightly stained nuclei and a normal dentate gyrus.

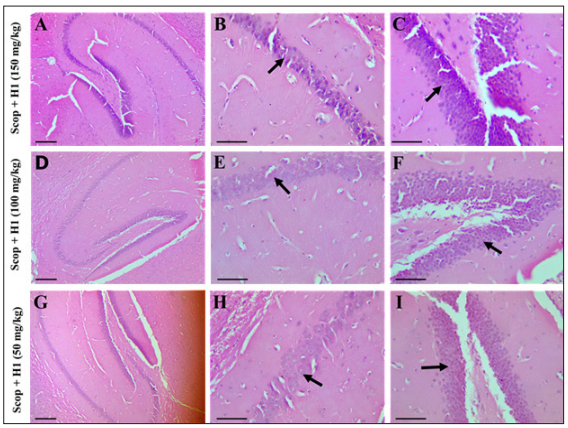

Figure 4.2: Micrographs showing the longitudinal section of hippocampus in mice (H&E; Magnification: A, D&G 10x, B, C, E, F, H, I 40x; Scale bars: A, D&G 100µm, B, C, E, F, H, I 50µm). **(A)** Lower magnification of a normal hippocampus showing restoration of the cellular architecture. (**B&C**) Higher magnification showing relatively normal cellular architecture in the *cornus ammonis* 1 (CA1) region and dentate gyrus. (**D&G**) Lower magnification showing relatively normal hippocampus. (**E, F, H&I)** Higher mignfication**,** few apoptotic death and distorted appearance of neuronal cells were observed in the CA1 and dentate gyrus.

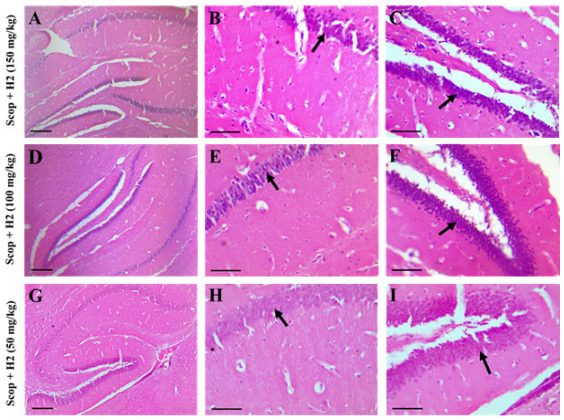

Figure 4.3: Micrographs showing the longitudinal section of hippocampus in mice (H&E; Magnification: A,D&G 10x, B,C,E,F,H,I 40x; Scale bars: A,D&G 100µm, B,C,E,F,H,I 50µm). **(A&D)** Lower magnification of a normal hippocampus showing restoration of the histomorphology. (**B, C, E&F)** Higher magnification showing normal *cornus ammonis* 1 (CA1) region and dentate gyrus of the hippocampus**. (G)** Lower magnification showing relatively normal hippocampus. (**H&I**) Higher magnification showing multiple apoptotic death and distorted appearance of neuronal cells were observed in the CA1 and dentate gyrus.

Discussion

This study focused on assessing the cognitive and antioxidative effects of combined extracts from Spondias mombin, Spilanthes filicaulis, and Piper guineense against scopolamine-induced memory impairment in mice. These plants have been reported in literature from ethnobotanical surveys conducted in some parts of Southwest Nigeria as memory-enhancing and anti-aging agents (35,36). Also, each of them has been screened either in vitro to inhibit the acetylcholinesterase activity or in vivo to protect against neurochemical alterations and oxidative stress in the scopolamine model of cognitive dysfunction (30,37–41,43,44). After extraction with distilled water, the concentrated combined extracts H1 and H2 were subjected to biological investigations.

First of all, to assess safety for human use, the acute toxicity of the combined extracts was investigated following the OECD guideline (46). As per Al-Kadmi (47), to determine whether drugs and medicinal plant products are safe for human consumption, a toxicological evaluation must be done to determine toxicity and to determine the dosage that can be used safely. Throughout this study, doses of up to 2000 mg/kg administered orally to female mice revealed no apparent signs of toxicity. There were no observable tremors, diarrhea, convulsions, or abnormalities in behavior, maintaining normal external features of skin, nose, eyes, and fur. Unusual behaviors like walking backward or self-mutilation were notably absent. Importantly, no deaths were reported at the 2000 mg/kg dose, signifying an LD50 value exceeding 2000 mg/kg (48).

Scopolamine, functioning as a muscarinic receptor antagonist, disrupts cholinergic neuronal pathways and memory circuits, resulting in significant deficits in learning, memory, and short-term memory (49). Widely used model to simulate dementia-related conditions, it induces cognitive deficits by hampering the central cholinergic system crucial for learning and memory 8,43-44. In this study, the Morris water maze and Y-maze tests were employed to assess the potential of the combined extracts H1 and H2 in alleviating scopolamine-induced memory impairment in mice, evaluating their impact on memory function.

Various experiments have used the Morris water maze test to investigate rodent spatial learning and drug effects on cognitive abilities (52,53). In this research, scopolamine-treated mice displayed cognitive impairment, evidenced by increased escape latency, reduced crossing rates in the target quadrant, and decreased time spent there. Conversely, the administration of synergistic combinations notably improved these deficits across all doses, decreasing escape latency and increasing both quadrant crossings and time spent in the target quadrant in comparison to the scopolamine group. These enhancements indicated improved learning during acquisition trials and better retention of the target quadrant area. Consequently, the synergistic combination notably ameliorated spatial learning and memory in scopolamine-treated mice, particularly combinations H1 (50 mg/kg) and H2 (100 mg/kg), which exhibited the most notable enhancements in learning and memory.

Evaluating rodent spatial memory with the Y maze task relies on their natural exploration behavior (54). Scopolamine in the current study impairs this, reducing spontaneous alternation, a sign of spatial memory decline (38,55–57). In contrast, treating mice with the combined extracts H1 and H2 notably enhanced spatial working memory, evident in increased spontaneous alterations compared to scopolamine-treated mice. Importantly, locomotor activity remained unaffected by scopolamine or the combined extract treatments. These findings indicate the potential of these synergistic combinations to enhance short-term memory, with H2 at 100 mg/kg showing the most promising results.

Multiple studies link oxidative stress to neurodegenerative diseases (58–60). Conversely, research findings support that antioxidants can prevent damage caused by oxidative stress and enhance memory in animal models of Alzheimer's disease (61). This study revealed that scopolamine- induced hippocampal oxidative stress, evident in elevated MDA and H2O2 levels. Physiologically, hydrogen peroxide can generate the highly toxic hydroxyl radical (OH-) in cells via the Fe2+-dependent Fenton reaction (62,63). Simultaneously, increased MDA signifies polyunsaturated fatty acid membrane damage due to lipid peroxidation (64,65). Consequently, the surge in lipid oxidation products likely arises from hydrogen peroxide and its resultant compounds, contributing to the observed hippocampal oxidative stress. In addition, scopolamine-treated mice exhibited elevated oxidative stress biomarkers alongside reduced SOD activity and GSH content, coupled with increased AChE activity, indicating that brain tissues were exposed to oxidative stress. According to Oyagbemi et al (66), SOD acts as a primary defense against tissue damage caused by oxidation, while GSH plays a pivotal role in cellular defense against oxidative injury(67). Conversely, AChE regulates acetylcholine, a neurotransmitter in synapses, disrupting its function (68). As a consequence of this study, treatment with the combined extracts H1 and H2 significantly reversed these alterations, decreasing MDA and H2O2 levels while increasing SOD activity and content of GSH, affirming their antioxidant potential. This confirmed our previous findings where we demonstrated the in vitro antioxidant capacity of the combined extracts H1 and H2 (45).

Furthermore,Scopolamine-treated mice exhibited notably increased hippocampal

glutathione peroxidase (GPx) and glutathione S-transferase (GST) activity, potentially indicating an adaptive response. Moreover, nitric oxide's role in learning and memory is well-documented in animal studies(69–71). Remarkably, administering the combined extracts H1 and H2 to scopolamine-pretreated mice significantly raised hippocampal NO levels, indicating their potential to enhance spatial learning and memory. Additionally, the histopathological examination in this study displayed substantial cellular repair in the hippocampus CA1 region and dentate gyrus in mice treated with H1 and H2 (Figure 4), supporting their efficacy in enhancing learning and memory, consistent with biochemical markers.

In the rank of all the experiments and assays carried out in this study, H1 consistently displayed remarkable potential for cognitive improvement and oxidative stress reduction. As determined using the Morris water maze, the results highlighted its efficacy in enhancing learning and memory. From the biochemical analysis, it was evident that combination H1 significantly modulated key biomarkers. This included a marked decrease in oxidative stress markers (MDA and H2O2) and significant elevations in the activity of SOD, GSH, and NO, all of which signify enhanced antioxidant defenses. Furthermore, H1 was well-tolerated within a therapeutic range and positively impacted brain structure and cellular integrity based on acute toxicity assessments and histopathological examination of brain tissues. Thus, the combined extract of S. mombin, S. filicaulis, and P. guineense at a 2:2:4 ratio effectively enhanced cognitive function, mitigated oxidative stress, and reversed scopolamine-induced effects within the hippocampal region of the brain

Conclusion

This study employed a comprehensive screening strategy to identify the most effective synergistic combination derived from the aqueous extracts of *Spondias mombin* leaves, the whole plant **of** *Spilanthes filicaulis*, and *Piper guineense* fruits. The formulated blend was evaluated for its neuroprotective potential, specifically its ability to enhance cognitive performance, mitigate oxidative stress, and counteract scopolamine-induced impairments in the hippocampus of mouse brains.

Our findings provide compelling evidence that the combined extracts of *S. mombin*, *S. filicaulis*, and *P. guineense* exhibit significant synergistic effects, contributing to improved learning and memory functions. These results underscore the therapeutic promise of this phytochemical blend as a natural intervention for cognitive enhancement and neuroprotection.

However, while the observed outcomes are promising, further investigations are essential to fully understand the molecular and biochemical mechanisms driving the synergistic interactions among the plant constituents. Future studies should also explore dose optimization, long-term safety, and efficacy in diverse animal models and, eventually, in human clinical trials. Such research will be critical in validating the practical applications of this combination in the development of novel treatments for neurodegenerative disorders and cognitive decline.

Acknowledgement

The authors sincerely thank the African Union Commission for funding this research. We also extend our special appreciation to all the laboratories involved in any part of this work for their invaluable support.

Authors' contributions

K.E.H. and M.D.A. conducted the experiment, wrote the original draft of the manuscript, and reviewed the manuscript. M.A.S. and T.O.E. designed the study, supervised the work, and finalized the manuscript. A.A.O. and F.E.O. assisted with the experiments and finalized the manuscript. All authors contributed to this study and revised the article critically for important intellectual content.

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Funding

Funding for this research was provided by the African Union Commission through the Pan African University Life and Earth Sciences Institute (including Health and Agriculture), Ibadan, Nigeria.

Data availability statement

Access to the data supporting the study findings is available through contacting the corresponding author in reasonable manner.

List of Abbreviations

AD: Alzheimer's disease
ADI: According to Alzheimer's Disease International
AChE: Acetylcholinesterase
ACUREC: Animal Care and Use Research Ethics Committee
OECD: Organization for Economic Co-operation and Development
MWM: Morris water maze
PMF: Post-mitochondrial fraction
MDA: Malondialdehyde
H_2O_2 : Hydrogen peroxide
SOD: Superoxide dismutase
GSH: Reduced glutathione
GPx: Glutathione peroxidase
GST: Glutathione S-transferase
NO: Nitric oxide
CA1: Cornus ammonis 1.

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