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Full length Research Article

Cajanus cajan (L) Millsp. Seed Extract Ameliorates Scopolamine-Induced Amnesia through Increase in Antioxidant Defense Mechanisms and Cholinergic Neurotransmission

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Summary: Background: Decline in cholinergic function and oxidative/nitrosative stress play a central role in Alzheimer's disease (AD). Previous quantitative HPLC profiling analysis has revealed the presence of Pinostrobin, formononetin, vitexin and other neuroprotective flavonoids in *Cajanus cajan* seed extract. Objective: This study was designed to investigate the protective action of *Cajanus cajan* ethanol seed extract (CC) on learning and memory functions using scopolamine mouse model of amnesia. Materials and methods: Adult mice were pretreated with CC (50, 100, or 200mg/kg, p.o) or vehicle (10ml/kg, p.o) for 16 days consecutively. Scopolamine, a competitive muscarinic cholinergic receptor antagonist (1mg/kg, i.p.) was given an hour after CC pretreatment from days 3 to 16. The mice were subjected to behavioural tests from day 11 (open field test (OFT)/ Y-maze test (YMT) and Morris water maze task (MWM) from days 12-16. Animals were euthanized 1h after behavioral test on day 16 and discrete brain regions isolated for markers of oxidative stress and cholinergic signaling. Molecular docking analysis was undertaken to predict the possible mechanism(s) of CC-induced anti-amnesic action. Results: pre-administration of CC significantly reversed working memory and learning deficits caused by scopolamine in YMT and MWM tests, respectively. Moreover, CC prevented scopolamine-induced oxidative and nitrosative stress radicals in the hippocampus evidenced in significant increase in glutathione (GSH) level, superoxide dismutase (SOD) and catalase (CAT) activities with a marked decrease in malondialdehyde (MDA) production as well as significant inhibition of hippocampal scopolamine-induced increase in acetylcholinesterase activity by CC. The molecular docking analysis showed that out of the 19 compounds, the following had the highest binding affinity; Pinostrobin (-8.7 Kcal/mol), friedeline (-7.5kCal/mol), and lupeol (-8.2 Kcal/mol), respectively, to neuronal muscarinic M1 acetylcholine receptor, α7 nicotinic acetylcholine receptor and amyloid beta peptide binding pockets, which further supports the ability of CC to enhance neuronal cholinergic signaling and possible inhibition of amyloid beta aggregation. Conclusion: this study showed that *Cajanus cajan* seeds extract improved working memory and learning through enhancement of cholinergic signaling, antioxidant capacity and reduction in amyloidogenesis.

Keywords*: amyloid beta peptide; α7 nicotinic acetylcholine receptor; Cajanus cajan; molecular docking; M1 muscarinic acetylcholine receptor; oxidative stress*

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INTRODUCTION

Alzheimer's disease (AD), a progressive and debilitating neurodegenerative disorder is one of the most common causes of dementia in the elderly and more than 40million people are living with AD worldwide (Francis *et al*., 1999; Khurana *et al*., 2021). The main aetiology of AD remains elusive; however, several hypotheses have been postulated to be involved in the pathophysiology of AD amongst which are Proteinopathy, lysosomal-mitophagy dysfunction, oxidative stress, and neuroinflammation (Tonnies and Trushina 2017; Bondi *et al*., 2017), and loss of the cholinergic innervation in the limbic and neocortical region of the brain (Hampel *et al*., 2018; Ishola *et al*., 2020). The cholinergic hypothesis has gained significant attention over the years and several studies have reported significant correlation between extensive depletion of cholinergic neurons in basal forebrain as well as reduced cholinergic fiber network of the cortical mantel and hippocampus and features of AD (Chen and Mobley 2019; Ishola *et al*., 2020; Khurana *et al*., 2021; Llanes *et al*., 2023), thus, resulting in

attention, learning and memory disability. Extracellular formation of senile plaques (comprising of aggregated amyloid-beta peptide (Aβ) and phosphorylated intracellular neurofibrillary tangles (Tau) are the main neuropathological indication of AD. Interestingly, increase in Aβ generation is directly correlated with intracellular neurofibrillary tangles formation, neuronal loss and synaptic dysfunction (Kamat *et al*., 2016).

Oxidative stress plays significant role in the etiopathogenesis of AD due to interaction of metal ions (copper, zinc or iron) with either Aβ peptide or tau peptide catalyze the production of reactive oxygen species (ROS) leading to oxidative damage of surrounding molecule including membrane lipid, protein or nucleic acid) (Tonnies and Trushina 2017; Ishola *et al*., 2017; Cheignon *et al*., 2018). Despite the increasing knowledge about the pathophysiology of AD, the disease condition still remains an unmet medical needs and burden to the healthcare system, economy and family globally. It is worthy of note that the current pharmacological interventions in the management of AD only proffer symptomatic relieve through enhancement of cholinergic neurotransmission and blockade of NMDA receptor (Ishola *et al*., 2019). ROS-induced oxidative damage in the hippocampus and neocortex are well linked with aging and AD development, thus, antioxidants could be a viable option in the management of AD, hence, slowing the progression of AD (Ishola *et al*., 2020). Interestingly, flavonoids are antioxidant and are very ubiquitous in plants with various health benefit including prevention of neurodegenerative diseases (Wan *et al*., 2019; Ishola *et al*., 2020).

In the present study, we evaluated the potential benefits of natural product rich in flavonoids, *Cajanus cajan* (L) Millsp, (Leguminosae) (pigeon pea) is majorly cultivated in tropical and semi tropical regions including Asia (especially south Asia), Africa and Latin America and serves as a major source of dietary protein. Interestingly, our preliminary quantitative chromatographic -spectroscopic analysis revealed the richness of *C. cajan* in flavonoids such as quercetin, cajanin, pinostrobin, cajaninstilbene, cajanolactone, formononetin, Biochanin A and B which is in agreement with previous studies (Hassan *et al*., 2015; Wu *et al*., 2019). Moreso, antioxidant, anti-inflammatory and antimicrobial activities of *C. cajan* have been reported (Zu *et al*., 2010; Hassan *et al*., 2015: Tekale *et al*., 2016). In traditional medicine, *C. cajan* is used in the treatment of neurological disorders, kidney diseases, diabetes, skin irritations, diarrhea, measles, and pain (Ahsan and Islam, 2009; Saxena *et al*., 2010; Pal *et al*., 2011). Hence, this study is designed to investigate the nootropic effect of ethanol seed extract of *C. cajan* (CC) on scopolamine-induced memory dysfunction in mice analogous to what is observed in patients living with dementia. Interestingly, scopolamine induced learning and memory processing impairments are reversed by acetylcholinesterase inhibitors (e.g. physostigmine). Amnesia caused by intraperitoneal injection of scopolamine is a common model of dementia in rodents suggestive of reduced cholinergic function (Ishola *et al*., 2019). Scopolamine, a muscarinic M1 receptor antagonist impairs cholinergic function and mitochondrial function (Klinkenberg and Blokland, 2010), leading to the generation of reactive oxygen radicals in the hippocampus, thus, leads to decline in memory and cognitive functions (Flood and Cherkin, 1986; Liao *et al*., 2020; Ishola *et al*., 2020). M1 Muscarinic acetylcholine receptors (mAChRs) are expressed in the hippocampus and cerebral cortex, where they play a significant role in the aberrant alterations of memory, cognitive processing, and learning, seen among people living with AD (Yousuf *et al*., 2023). Similarly, α7-nicotinic cholinergic receptors play pivotal role in memory modulation, thus, alteration in their function is linked with cognitive deficits. In this study, effort were made to assess the modulatory role of CC on M1 muscarinic acetylcholine receptors and α7- nicotinic cholinergic receptors activities using in silico techniques.

MATERIALS AND METHODS

Laboratory Animals: Adult mice of either sex used in this study (20-25g) were purchased from the Laboratory Animal Centre, College of Medicine, University of Lagos, Lagos, Nigeria. The animals were housed in plastic cages at room temperature and standard environmental conditions. The animals were fed with dried pellet (Livestock meal, Lagos, Nigeria) and clean water daily. The mice were allowed to acclimatize for a period of 7 days before the commencement of the experiment. The animals were properly handled and cared for in accordance with the Health Research and Ethics Committee (HREC) of the College of Medicine, University of Lagos, Nigeria with approval number (CMUL/ HREC/ 01/22/1000).

Drugs and Chemicals: Ethanol, donepezil hydrochloride, scopolamine hydrobromide (Sigma Aldrich St. Louis MO, USA), phosphate buffer 1x (Life Technology, USA).

Extract Preparation: Dried *Cajanus cajan* seeds were purchased from a local herb market in Lagos, Nigeria and pulverized to powder. The powder was then macerated in absolute ethanol for 72hours. It was filtered and filtrate was oven dried to yield a yellowish powder extract.

Experimental Procedure: Mice were randomly divided into seven groups (n=6) as follows; groups 1 and 2 received normal saline (10 ml/kg, p.o.), respectively, group 3-donepezil (1mg/kg; p.o.) and groups 4-7 received graded doses of *C. cajan* (50, 100 or 200mg/kg, p.o., respectively) for sixteen consecutive days. One-hour post-drug administration on day 3, mice in groups 2-6 were given scopolamine (1mg/kg, i.p.) from days 3 to 16 consecutively.

Open field Test (OFT): OFT is a protocol used to assay for locomotion activity, anxiety and readiness to explore in laboratory animals (Owope *et al*., 2016; Ishola *et al*., 2019). The OFT apparatus is a 96cm × 96cm × 45cm box made from wood. The floor of the apparatus is divided into 16 squares (18 × 18cm) by white lines. On day 11, each mouse was placed at the centre point of the apparatus and allowed to acclimatize for 60 seconds. Afterwards the total number of rearing, line crosses and grooming behaviour were recorded for 5mins. After each trial, the maze was cleaned with 10% ethanol and allowed to dry.

Y-Maze Test: The Y maze test is used to evaluate spontaneous exploration behaviour and short-term working memory (Ishola *et al*., 2020). The Y-maze is designed as a

Y shaped wooden apparatus with labelled arms A, B, C. After OFT on day 11, the animal was placed in the centre of the maze, and the total number of arm entries and spontaneous alternations defined as sequence of entries [ABC, BAC, CBA] were observed and recorded by an observer blinded to the treatment groups.

$$\% \text{ Spontaneous alternation} = \frac{\text{Number of alternation}}{\text{Number of entries - 2}} \times 100$$

Morris Water maze Task (MWM): MWM is designed to test spatial learning and memory ability of a rodent. The apparatus consist of a circular black tank (110cm diameter and 60cm height) to a depth of 30 cm filled with water up to 25cm high. The circular tank was divided into four hypothetical quadrants, designated as: N (North), E (East), W (West), S (South). A platform was placed 1.5cm beneath the water surface in the southwest quadrant. The mice were trained to locate the hidden platform within 60s and were allowed to stay on it for 10 s. The time taken for the mouse to locate the escape platform was recorded as escape latency (ELT). In the event that the animal was unable to locate the hidden platform within 60 s, it was gently guided to it and allowed to stay on it for 10 s. Three trials were conducted on each day for four days (days 12-15) (designated as spatial acquisition phase). On day 16, a probe test was carried out to assess retention memory, during which the escape/hidden platform was removed from the pool and the total time spent by the animal within the quadrant of the platform location was recorded within 30s (Ishola *et al*., 2013; Ishola *et al*., 2016; Ishola *et al*., 2019).

Dissection: After the probe test on day 16, the animals in each group were anaesthetized, then perfused with cold normal saline and brain was rapidly removed, and hippocampus was dissected on iced pack, weighed and kept in 0.1× PBS (pH 7.4) at −20°C until biochemical analysis.

Biochemical Analysis: Malondialdehyde (MDA) is a marker of lipid peroxidation, spectrophotometrically measured using the thiobarbituric acid assay procedure as previously described by Ishola *et al*. (2017). The reduced glutathione (GSH) content of brain tissue as non-protein sulphydryl was estimated according to the method described Sedlak and Lindsay (1968). The activity of superoxide dismutase (SOD) was assayed according to the method described by Nauseef *et al*. (2014). Similarly, we also estimated the nitrite level in mice brain using the Greiss reagent, which served as an indicator of nitric oxide production (Green *et al*., 2005). Catalase activity was also determined according to the method described by Sinha *et al*. (1972). The acetylcholinesterase (AChE) activity in the hippocampal homogenate was quantified using the protocol of Ellman *et al*. (1959).

Molecular Docking

Preparation of target protein: The Protein Data Bank (PDB) database was used to get the crystallographic structure of the target Ligand/receptor of interest (MI muscarinic acetylcholine receptor, α7 nicotinic acetylcholine receptor and amyloid beta42, (PDB ID: 6WJC, 3SQ9 and 3T4G respectively). We selected these structures because they have been used in other molecular docking studies (Li *et al*., 2011; Cheng *et al*., 2012; Maeda *et al*., 2020).

Ligand Preparation: For this study, the ligand structures were obtained from the PubChem database. Nineteen natural compounds identified to be present in *Cajanus cajan* seed from our preliminary study and standard reference drug for the target protein (Table 1).

Table 1:
Natural compounds earlier isolated from *Cajanus cajan* seed extract

S/N	Compound	PUBCHEM ID	IUPAC Name
1	Physcion	10639	1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione
2	Lupeol	259846	(1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol
3	Cajanol	442670	5-hydroxy-3-(4-hydroxy-2-methoxyphenyl)-7-methoxy-2,3-dihydrochromen-4-one
4	Quercetin	5280343	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one
5	Biochanin A	5280373	5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one
6	Formononetin	5280378	7-hydroxy-3-(4-methoxyphenyl)chromen-4-one
7	Vitexin	5280441	5,7-dihydroxy-2-(4-hydroxyphenyl)-8-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one
8	Apigenin	5280443	5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one
9	Luteolin	5280445	2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one
10	Isoquercitrin	5280804	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one
11	Genistein	5280961	5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one
12	Isorhamnetin	5281654	3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one
13	Orientin	5281675	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-8-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one
14	Pinostrobin	73201	(2S)-5-hydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one
15	Friedelin	91472	(4R,4aS,6aS,6aS,6bR,8aR,12aR,14aS,14bS)-4,4a,6a,6b,8a,11,11,14a-octamethyl-2,4,5,6,6a,7,8,9,10,12,12a,13,14,14b-tetradecahydro-1H-picen-3-one
16	Naringenin	932	5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one
17	Cajanin	5281706	3-(2,4-dihydroxyphenyl)-5-hydroxy-7-methoxychromen-4-one
18	Daidzein	5281708	7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one
19	Betulin	72326	(1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a-(hydroxymethyl)-5a,5b,8,8,11a-pentamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol
20	Donepezil	3152	2-(1-benzylpiperidin-4-yl)methyl-5,6-dimethoxy-2,3-dihydroinden-1-one

https://pubchem.ncbi.nlm.nih.gov/ *accessed October, 2022*

Table 2:
Showing the grid centers and Dimension of the binding pockets of each target protein

Target Protein	Grid Center	Dimension (Angstrom)
M1 Muscarinic ACh receptor	X- 21.1150, Y-27.7651, Z- 13.8030	X- 42.9568, Y- 40.4275, Z- 25.000
Alpha-2 nicotinic ACh receptor	X- 18.5315, Y-18.2748, Z-(-)13.1067	X- 38.7118, Y- 34.8079, Z- 25.0000
Amyloid beta	X- 17.4658, Y- 17.3212, Z- 2.7678	X- 25.0000, Y- 25.0000, Z- 25.0000

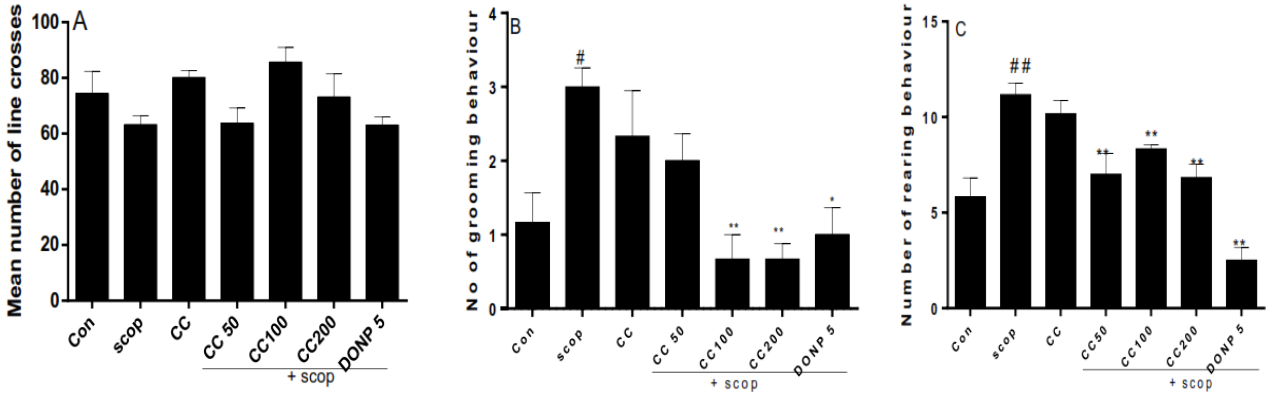

Figure 1 A-C:
Effect of CC and scopolamine on (A) number of line crosses, (B) number of rearing behaviour and (C) grooming behaviour in mice subjected to OFT. Values are expressed as mean±SEM (n=6). $^{*}p< 0.05$, $^{**}p<0.01$;$^{***}p<0.001$ versus scopolamine treated group and $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus control treated group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test.

Docking study: The Biovia Discovery studio 3.0 and Pyrex software were used to conduct the studies on the target proteins. The Biovia discovery studio was used to prepare the protein for docking and post docking analysis while the Pyrex was used to generate binding sites (the box was placed in the catalytic region of the enzyme), the grid center and dimensions of the box in the X-, Y- and Z-axis was noted for the target protein (Table 2). The compounds were docked into the binding sites/pocket of the target protein using Pyrex and their receptor-ligand interactions determined using Biovia Discovery studio (Postdocking analysis).

Statistical Analysis: Data were expressed as mean ±SEM (n=6) and analyzed using one-or two-way ANOVA for repeated measures when appropriate, followed by Tukey post hoc multiple comparison test (whichever is applicable) using Graphpad prism version 6 (Graphpad prism Inc, CA, USA).

RESULTS

C. cajan **increases locomotor activities in open field test**: Administration of scopolamine and CC showed significant effects of treatment on number of line crosses [F(6,35) = 4.97, P< 0.001] (Fig. 1A), number of grooming [F(6,35)=5.595, P=0.0004] (Fig. 1B) and number of rearing behaviour [F(6,35)=14.46, P< 0.0001] (Fig. 1C). Administration of scopolamine and CC caused no significant change in number of line crosses compared to the control treated group. Conversely, scopolamine caused a significant increase in grooming behaviour relative to the control-treated group which was significantly reduced by the pretreatment of mice with CC 100 or 200mg/kg. Furthermore, oral administration of scopolamine increased rearing behaviour in comparison with control group. However, the pre-treatment of mice with CC failed to significantly reverse scopolamine-induced increase in rearing behaviour.

CC prevents scopolamine-induced working memory impairment in YMT: One way ANOVA showed significant effect of treatments on number of arm entries [F (6, 28) = 3.75, P < 0.007] (Fig. 2A) and percent spontaneous alternation [F (6, 28) = 10.46, P<0.0001] (Fig. 2B) in YMT. Post hoc multiple comparisons showed no significant change in mean number of arm entries among all the treatment groups when compared to the control treated group. However, the pretreatment of mice with scopolamine caused significant decrease in percent spontaneous alternation behaviour when compared with vehicle control. In contrast, CC 50 and 100mg/kg caused significant increase in spontaneous alternation behaviour when compared with scopolamine treated group (Figure 2B).

CC prevents scopolamine-induced spatial learning deficit in MWM: Two-way ANOVA showed significant effect of CC and scopolamine treatments on escape latency [F (6, 96) = 6.364, P < 0.0001] (Figure 3A) and probe trial [F (6, 25) = 8.044, P < 0.0001] (Figure. 3B) in MWM task. Scopolamine treated control produced no significant change in escape latency when compared with first session in the

spatial acquisition phase. However, CC pre-administration caused time course and significant decrease in escape latency when compared with first session in the spatial acquisition phase.

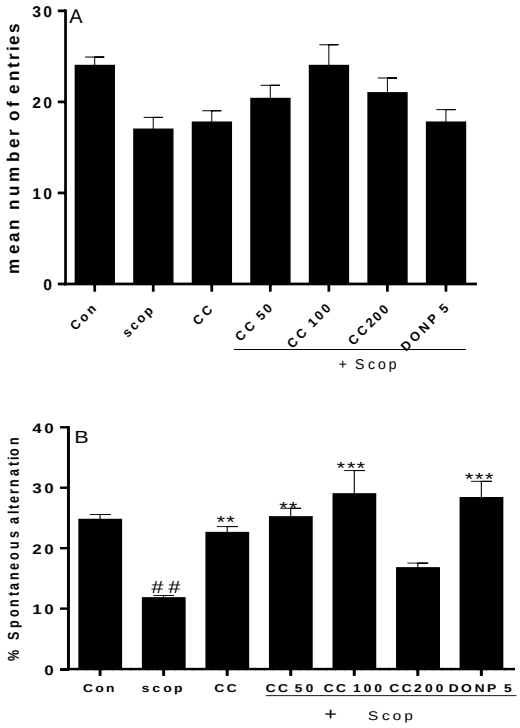

Figure 2A-B:
Effect of CC and scopolamine (A) number of arm entries and (B) percent spontaneous alternation behaviour in mice. Values are expressed as mean±SEM (n=5). $^{*}p< 0.05$, $^{**}p<0.01$,$^{***}p<0.001$ versus SCOP treated group; $^{\#\#}p<0.0001$ versus vehicle control treated group. Statistical level of significance analysis by one way ANOVA followed by Tukey *post hoc* multiple comparison test.

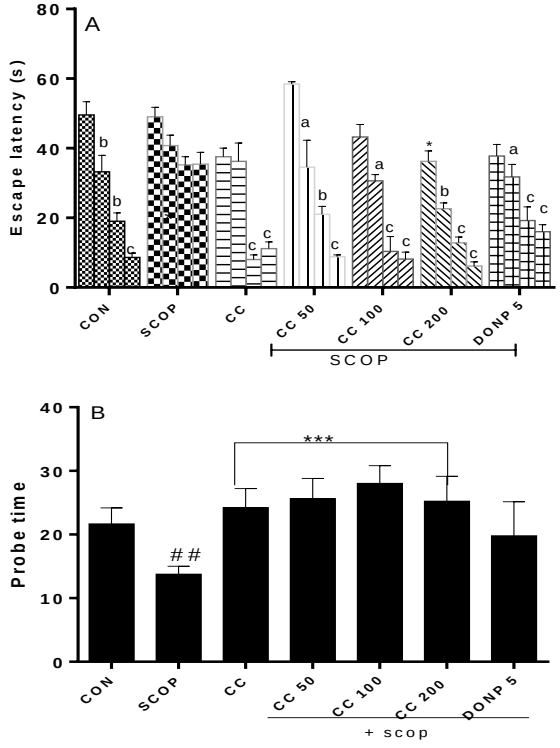

Figure 3A-B:
Effect of CC and scopolamine on (A) escape latency time and (B) probe trial in MWM task. Values are expressed as mean±SEM (n=5). $^{a}p< 0.05$, $^{b}p< 0.01$ $^{c}p< 0.001$ versus day 1., $^{\#\#}p<0.01$ versus vehicle control; $^{***}p<0.05$ versus SCOP-control treated group. Statistical level of significance analysis by one- or two-way ANOVA followed by Tukey *post hoc* multiple comparison test.

Moreso, in the probe trial, post hoc multiple comparison test showed significant reduction in time spent in the hidden platform location area by scopolamine treated when compared to the control group. Moreso, CC caused significant increase in time spent by the animal within the quadrant location when compared with scopolamine treated control.

CC attenuates scopolamine-induced MDA and nitrite generation: Scopolamine treatment caused significant increase in malondialdehyde (MDA) [F (6, 28) = 3.884, P = 0.0060], and nitrites [F (6,28)=6.308,P = 0.0003] generation in the hippocampus. Post hoc analysis showed that the pretreatment of mice with CC (50, 100 and 200mg/kg) significantly attenuated MDA and nitrite generation induced by scopolamine, with peak effect observed at CC 200mg/kg as shown in Figure 4A and B.

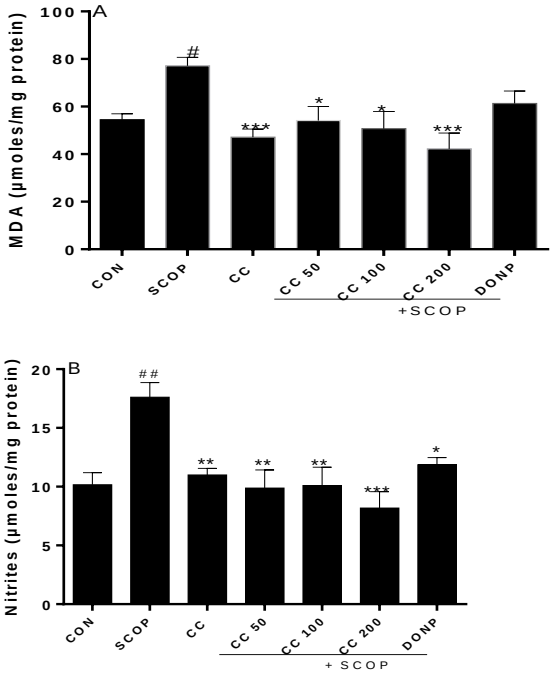

Figure 4 A-B:
Effect of CC and scopolamine (A) MDA and (B) nitrites. Values are expressed as mean ±SEM (n=5). $^{\#}P<0.05$, $^{\#\#}P< 0.01$versus vehicle-control treated group and $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, versus vehicle-scopolamine treated group. Statistical analysis was done using one way ANOVA followed by Dunnet multiple comparison test.

CC reversed scopolamine-induced hippocampal antioxidant enzymes deficits: One way ANOVA showed significant effect of subacute exposure to scopolamine and CC evidenced in a significant decrease in GSH level [F (6,35) = 13.06,P<0.001] (Fig. 5A), SOD [F (6, 35)=14.95, P < 0.0001] (Fig. 5B), and catalase [F (6, 28) = 17.51, P < 0.0001] (Fig. 5C) activities in the hippocampus. Tukey post hoc multiple comparison test showed that subacute administration of scopolamine significantly reduced GSH level (3.2 folds), SOD (2 folds) and catalase (1.5 folds)

when compared to normal control. However, the pretreatment of mice with CC 200mg/kg significantly reversed scopolamine-induced GSH level (3 folds), SOD (1.5 folds) and catalase (2 folds) when compared with scopolamine-vehicle treated group (Figure 5A-C). In another experiment, the administration of scopolamine significantly increased [F(6,28)=5.637,P=0.0006] acetylcholinesterase activity in the hippocampus relative to the control group. However, post hoc multiple comparison revealed that the pretreatment of mice with CC (100 or 200mg/kg) significantly inhibited this effect. Also, both CC 100 and 200mg/kg produced similar activity compared to the standard drug (donepezil) treated group as shown in Figure 5D.

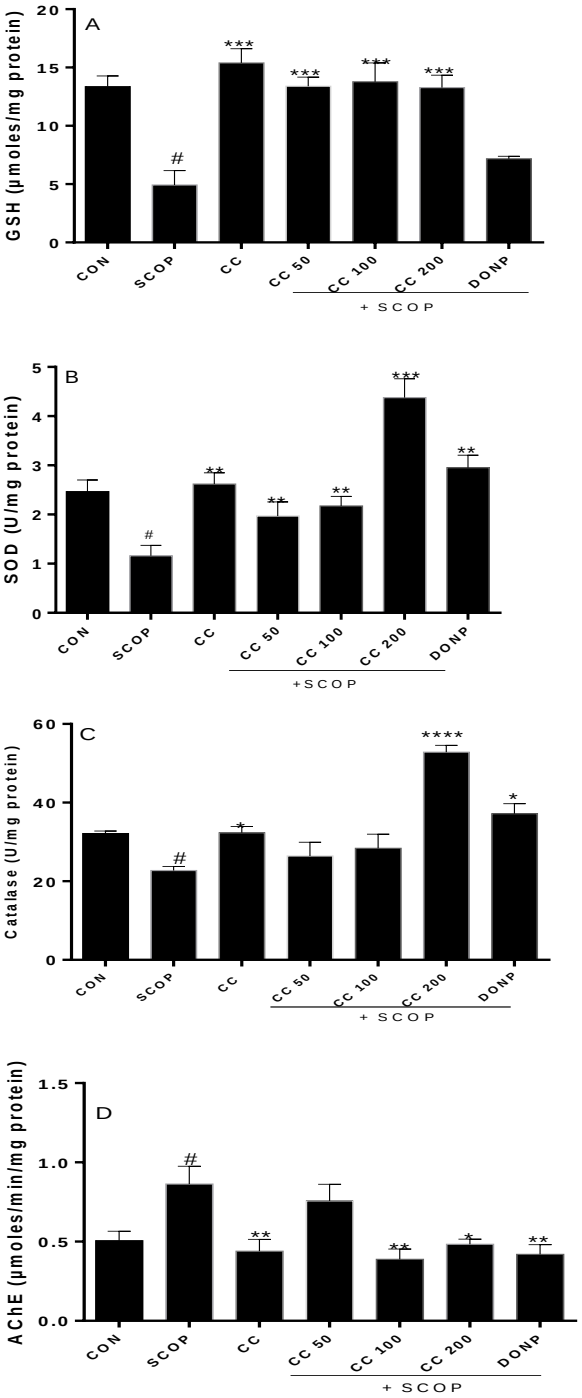

Figure 5A-D: Effect of CC and scopolamine on (A) GSH level, (B) SOD activity, (C) catalase activity and (D) acetylcholinesterase activity in the hippocampus. Values are expressed as Mean ±SEM (n=5). $^{\#}P<0.05$ versus vehicle-control treated group; $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ versus scopolamine treated group. Statistical analysis was done using One-way ANOVA followed by Tukey *post hoc* multiple comparison test.

Receptor-ligand Interactions

Receptor-ligand Interaction at Amyloid beta receptor site: Results from our molecular simulation showed that ligands such as friedeline (-7.5Kcal/mol), lupeol (-6.7Kcal/mol), apigenin and luteolin (-6.5Kcal/mol) binding affinity with amyloid beta active site compared with donepezil (acetylcholinesterase inhibitor, -5.8Kcal/mol) (Table 3). Also, these ligands interacted with amino acid residues: Lys9, Val7 and Leu5 while donepezil reacted with Ile2.

Table 3:
Binding Energy of ligands at Amyloid beta receptor site

S/N	Ligand	Chem ID	Binding Affinity ((Kcal/Mol)	rmsd /ub	rmsd /lb
1	Friedeline	91472	-7.5	0	0
2	Lupeol	259846	-6.7	0	0
3	Apigenin	5280443	-6.5	0	0
4	Luteolin	5280445	-6.5	0	0
5	Betulin	72326	-6.4	0	0
6	Physcion	10639	-6.3	0	0
7	Isorhamnetin	5281654	-6.3	0	0
9	Daidzein	5281708	-6.2	0	0
10	Biochanin A	5280373	-6.2	0	0
11	Pinostrobin	73201	-6.1	0	0
12	Cajanol	442670	-6	0	0
13	Naringenin	932	-6	0	0
14	Genistein	5280961	-5.9	0	0
15	Cajanin	5281706	-5.9	0	0
16	Quercetin	5280343	-5.9	0	0
17	Donepezil	3152	-5.8	0	0
18	Vitexin	5280441	-5.8	0	0
19	Orientin	5281675	-5.7	0	0
20	Formononetin	5280378	-5.7	0	0
21	Isoquercitrin	5280804	-5.6	0	0

Analysis of the mode of docking of the ligands at M1 Muscarinic ACh receptor: Results obtained from the docking study showed that pinostrobin (-8.7Kcal/mol), friedeline (-8.3Kcal/mol), formononetin and vitexin (-7.6Kcal/mol) binding affinity with the M1 muscarinic ACh active site with binding affinity better than donepezil (standard acetylcholinesterase inhibitor) as shown in Table 4. Showed good interaction with Pro97, Leu89, Ala93, Trp145 via pi-pi alkyl bond.

Analysis of the mode of docking of the ligands at alpha-7 nicotinic receptor: Lupeol (-8.2Kcal/mol), friedeline (-8.1Kcal/mol), botulin (-7.8Kcal/mol), orientin and vitexin (-7.6Kcal/mol) binding energy which was than that of the standard drug, donepezil (-7.2Kcal/mol) at the alpha-7 nicotinic acetylcholine binding pocket (Table 5). Post docking analysis showed that the compounds with better binding affinity interacted with Tyr115, Gln121 and Ala90

via hydrogen bond and Pro97 through alkyl bond. Other interactions were also formed with Ala89, leu88 amino acid residues.

Table 4:
Binding Energy of ligands at M_1 muscarinic ACh receptor site

S/N	Ligand	Chem ID	Binding Affinity (Kcal/mol)	rmsd/ ub	rmsd/ lb
1	Pinostrobin	73201	-8.7	0	0
2	Friedeline	91472	-8.3	0	0
3	Formononet	5280378	-7.6	0	0
4	Vitexin	5280441	-7.6	0	0
5	Apigenin	5280443	-7.6	0	0
6	Isorhamnetin	5281654	-7.5	0	0
7	Cajanin	5281706	-7.5	0	0
8	Daidzein	5281708	-7.5	0	0
9	Biochanin A	5280373	-7.4	0	0
10	Luteolin	5280445	-7.4	0	0
11	Genistein	5280961	-7.3	0	0
12	Physcion	10639	-7.2	0	0
13	Quercetin	5280343	-7.1	0	0
14	Naringenin	932	-7	0	0
15	Donepezil	3152	-7	0	0
16	Isoquercitrin	5280804	-7	0	0
17	Cajanol	442670	-6.8	0	0
18	Betulin	72326	-6.6	0	0
19	Orientin	5281675	-6.1	0	0
20	Muscarine	9308	-5.2	0	0

Table 5:
Binding Energy of ligands at alpha-7 nicotinic receptor site.

/N	Ligand	Chem ID	Binding Affinity (Kcal/mol)	rmsd /ub	rmsd /lb
1	Lupeol	259846	-8.2	0	0
2	Friedelin	91472	-8.1	0	0
3	Betulin	72326	-7.8	0	0
4	Orientin	5281675	-7.6	0	0
5	Vitexin	5280441	-7.3	0	0
6	Isoquercitrin	5280804	-7.2	0	0
7	Donepezil	3152	-7.2	0	0
8	Cajanin	5281706	-6.9	0	0
9	Pinostrobin	73201	-6.9	0	0
10	Physcion	10639	-6.8	0	0
11	Cajanol	442670	-6.8	0	0
12	Quercetin	5280343	-6.8	0	0
13	Isorhamnetin	5281654	-6.8	0	0
14	Luteolin	5280445	-6.7	0	0
15	Genistein	5280961	-6.7	0	0
16	Daidzein	5281708	-6.7	0	0
17	Naringenin	932	-6.6	0	0
18	Apigenin	5280443	-6.4	0	0
19	Biochanin A	5280373	-6.1	0	0
20	Formononeti	5280378	-5.7	0	0

DISCUSSION

Findings from this study revealed that scopolamine caused spatial learning and memory deficits as well as cholinergic dysfunction and oxidative stress in the hippocampus (Ishola *et al*., 2020; Liao *et al*., 2020), however, *C. cajan* seed extract attenuated scopolamine-induced cognition, antioxidant system and cholinergic neurotransmission deficits in mice. Moreso, *C. cajan* phytochemicals such as friedeline, Pinostrobin and lupeol showed significant interaction with neuronal M1 muscarinic cholinergic receptor, α7 nicotinic cholinergic receptor and amyloid-beta peptide indicative of their ability to improve cholinergic signaling as well as preventing amyloid-beta aggregation. Several reports have shown that acetylcholine plays pivotal role in the regulation of adult hippocampal neurogenesis (implicated in learning and memory), as the dentate gyrus receives afferent cholinergic inputs from the basal forebrain (Li *et al*., 2022). Moreso, the current treatment option for AD involves synaptic elevation of acetylcholine level through inhibition of acetylcholinesterase enzymes activity. Interestingly, inhibitors of acetylcholinesterase enzymes activity promote neurogenesis. In addition, acetylcholine signaling has been shown to strengthen the associations between environmental cues and reward availability, thus, improves learning and memory (Ishola *et al*., 2017).

Scopolamine, a muscarinic receptor antagonist, widely used to model cognitive decline in rodents as seen in AD (Ishola *et al*., 2020). Salimi *et al*. (2022) posited that scopolamine-induced memory and learning impairment is associated with mitochondrial dysfunction, neuroinflammation and oxidative stress which have also been linked with AD pathology. In this study, we evaluated the effect of scopolamine on spatial recognition memory in Y-maze (Ishola *et al*., 2016). Y-maze is a behavioural test used to investigate spatial recognition as well as spontaneous alternation behaviour in rodents (Ishola *et al*., 2020). Animals treated with scopolamine had increased spontaneous motor activity with no significant change in spontaneous alternation. In the present study, scopolamine reduced percent spontaneous alternation movement suggestive working memory impairment. In contrast, the pretreatment of mice with CC caused significant improvement in percent alternation behaviour indicative of enhanced spatial memory (Ishola *et al*., 2020). To ascertain the impact of CC on acquisition spatial learning and retention memory, the MWM task was carried out.

The Morris water maze test is a common behavioural model used to evaluate spatial learning and memory function (Saba *et al*., 2017; Lee *et al*., 2018; Ishola *et al*., 2020). In this study, mice in the control group quickly acquired spatial learning evidenced by the decrease in time course of escape latency following the different acquisition spatial learning trials and an increased time spent in the escape target quadrant location during the probe test. Similar to previous studies, scopolamine treated group showed an increased time swimming and lesser time in the escape target quadrant in the probe test indicative of spatial learning and retentive memory deficits (Saba *et al*., 2017; Lee *et al*., 2018; Ishola *et al*., 2020; Khurana, *et al*., 2021). However, pretreatment of animals with CC showed time course decrease in latency and increased time spent quadrant of hidden platform location which depict improvement in hippocampal spatial learning and retentive memory (Ishola *et al*., 2020).

Literature findings have reported that oxidative stress plays a key role in the pathogenesis and exacerbation of neurodegenerative disorders like AD and it is characterized

by increased production of reactive oxygen/nitrogen species (ROS/RNS) leading to decreased superoxide dismutase, and glutathione level, with a concomitant increase in malondialdehyde production (Chen and Zhong, 2014; Souza Ferreira *et al*., 2015; Tsikas, 2017). In AD, oxidative stress disrupts synaptic activity and neuronal signaling resulting in memory impairment and it has also been linked with mitochondrial dysfunction as well as accumulation of amyloid beta (hallmark of AD) which could worsen the disease prognosis (Tönnies and Trushina, 2017; Ishola 2019). In this present study, we observed a significant decrease in antioxidant enzymes; GSH, SOD and catalase activities as well as significant increase in hippocampal MDA and nitrite levels following subacute exposure of mice to scopolamine indicative of oxidative and nitrosative stress. However, the pretreatment of mice with CC reversed scopolamine-induced lipid peroxidation and nitrative stress through significant increase in antioxidant enzymes activity in the hippocampus supporting the earlier reported antioxidant properties of CC (Hassan *et al*., 2015).

In another experiment, the effect of CC on scopolamine-induced acetylcholine hydrolysis was investigated. It is well known that an increase in acetylcholinesterase activity would invariably result in acetylcholine hydrolysis into choline and acetic acid leading to loss or reduced cholinergic function. The central cholinergic system contributes to learning and memory functions. Moreso, acetylcholine is one of the important neurotransmitters that plays a critical role in regulating cognitive performances as well as learning and memory processes (Haam and Yakel, 2015; Ishola *et al*., 2020; Liao *et al*., 2020). In line with previous studies, scopolamine-induced an increase in acetylcholinesterase activities in the hippocampus of mice (Ishola *et al*., 2017; 2020). However, scopolamine induced increase in acetylcholinesterase activity was inhibited by CC subacute administration which further lend credence to the ability of CCt to enhance hippocampal cholinergic signaling.

To further validate the effect of *C. cajan* on cognition, molecular docking simulations were used to explore the potential interaction of the secondary metabolites with cholinergic receptors and amyloid-beta peptides. Our earlier reported compounds present in *C. cajan* were docked with M1 muscarinic acetylcholine receptor, alpha-7 nicotinic cholinergic receptor and amyloid beta. Muscarinic acetylcholine receptors plays distinct roles in the regulation of learning and memory processes, such as encoding cue-reward associations and consolidating these associations in long-term memory. Similarly, the α7 nicotinic acetylcholine receptors are highly ubiquitous in the hippocampus cortex where they play a pivotal role in memory formation, as such considered a potential therapeutic agents target (Jerusalinsky *et al*., 2000; Servent *et al*., 2011). Lastly, amyloid-beta is one of the key molecules in the pathogenesis of AD. Our results showed that phytochemicals from *C. cajan* seed had favorable receptor-ligand complex via pi-alkyl bonds with Pro97, Ala93, Leu89, Pro117 and Trp 145 similar to the interactions formed by donepezil, a cholinesterase inhibitor. Docking results also showed that phytocompounds from CC formed favourable receptor-ligand complex with alpha-7 nicotinic receptor, indicative of significant role for neuronal nicotinic cholinergic signaling. In silico study also showed that phytochemicals present in CC including Pinostrobin, friedeline, formononetin and vitexin showed better binding affinity for M1 muscarinic acetylcholine receptor active sites indicative of the involvement of M1 muscarinic ACh in cognition enhancing-like activity of CC. in addition, several reports of neuroprotective and cognitive enhancing action of formononetin, vitexin and friedeline have been reported (Fei *et al*., 2018; Fu *et al*., 2019; Sandhu *et al*., 2022). It is well known that M1 muscarinic acetylcholine receptors are largely found in the hippocampus and is said to have precognitive effects (Green *et al*., 2000; Zhao *et al*., 2018). Moreso, molecular docking simulation of the natural compounds with amyloid beta sheet revealed better interaction with the binding site/pocket of the target protein indicative of their ability to reverse amyloid-beta aggregation formation. Previous study has also shown the ability of friedelin to improve neuronal synapse and reversed scopolamine-induced memory impairment through inhibition of β-secretase enzyme (BACE-1) to halt amyloidogenic pathways of amyloid-β production (Sandhu *et al*., 2022).

In conclusion, our observations from this study showed that C.cajan seed extract prevents scopolamine-induced learning and memory deficits, oxidative stress and cholinergic deficit through enhancement of antioxidant defense mechanisms and cholinergic signaling.

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