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Acetylcholinesterase inhibition and metal chelating potentials of fractions and compounds isolated from *Cola hispida*

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ABSTRACT

Alzheimer's disease (AD) is an age-related, neurodegenerative disorder characterized by cognitive impairment. Acetylcholinesterase terminates the action of acetylcholine through catalytic hydrolysis. Inhibition of acetylcholinesterase enzymes is considered a promising strategy for AD treatment. In Nigerian ethnomedicine, it was mentioned that the seeds of *Cola hispida* can enhance mental capacity. The present study is therefore aimed at identifying and isolating inhibitors of AChE and metal chelation potential of *C. hispida* seed. *C. hispida* seed was macerated in methanol and partitioned into various solvents of increasing polarity. Ellman colorimetric assay method was used to determine the acetylcholinesterase inhibitory activity *in vitro*. Chromatographic methods were used to isolate and purify bioactive compounds from most active fraction. Their structures were determined by spectroscopic analysis including 1D and 2D NMR. Molecular docking was done using software (MOE 2015.010). The selected PDB was modeled using PDB ID: 10CE (*pacific electric ray*). Chromatographic analyses afforded four compounds identified as Daucosterol, β-sitosterol, 2-hydroxyquinoline-4-carboxylic acid, which are reported for the first time, and 5-hydroxymethylfurfural previously reported. 2-hydroxyquinoline-4-carboxylic acid isolated from ethyl acetate fraction of *C. hispida* seed demonstrated good AChE inhibitory activity (IC_{50}=1.070±0.09 mg/mL) at 1 mg/mL compared to eserine (IC_{50}=0.009±0.00 mg/mL). 5-hydroxymethylfurfural showed good metal chelating potential (IC_{50} value of 0.2951±0.01 mg/mL) at 1 mg compared to EDTA (IC_{50} = 0.045±0.11 mg/mL). Molecular docking reveals hydrophobic and hydrogen bonding interactions. β-sitosterol and 2-hydroxyquinoline-4-carboxylic acid are a possible source of potential lead for new acetylcholinesterase inhibitors.

INTRODUCTION

Neurodegenerative disease is a term applied to a variety of conditions caused by the slow breakdown and deterioration of neurons in the sensory system, specifically the electrical nerve cells in the brain that are responsible for transmitting signals through associations known as synapses (Muddapu et al., 2020). Alzheimer's disease (AD) is a neurons crumbling condition which is portrayed by reformist loss of structure and capacity of neurons at last prompting psychological decrease and decay of practically all intelligent capacities (DeTure and Dickson, 2019). This is due to cholinergic neurons present in each region of the cerebrum, including cortical regions and

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hippocampus. AD happens to be the most common type of dementia that influences a huge number of individuals universally (Singhal et al., 2012).

Neuropathological hallmarks associated with AD are β-amyloid plaques, neurofibrillary tangles, provocative cycles, and disruption of key synapses involved in communication and integration in the CNS (Zhang and Song, 2017). The main clinical components of this disease are psychiatric disorders, memory impairment and transient memory. The most common symptoms associated with AD are memory loss, poor judgment or inability to schedule each day, problems solving problems, problems performing routine tasks, increased misunderstanding of time and place, loss of objects, difficulty understanding visual images. As the condition progresses, additional psychological skills weaken, such as the ability to display visuospatial skills and locomotor apraxia (Neugroschl and Wang, 2011).

There is no cure for AD but there are treatments to control, prevent or stop its progression. New agents for AD in pipeline therapy are β secretion stimulators, γ-secretase inhibitors, α-secretase stimulators, immunotherapy, and TAU inhibitors (Athar et al., 2021). The pathophysiology of AD is diverse and involves several biochemical pathways. Reactive oxygen species (ROS) are continuously produced in cells of all organisms and are part of normal cell function. Nevertheless, excess free radicals derived from exogenous or endogenous sources are responsible for various human diseases. Free radicals cause essential oxidative damage to various macromolecules. A powerful finding implicates ROS in the pathogenesis of AD. Essentially, the physiological role of molecules with antioxidant capacity is to moderate the oxidative chain reaction by scavenging free radical intermediates, which is important for maintaining optimal health (Kurutas, 2016). Therefore, the use of compounds with antioxidant capacity has been investigated to reduce development and neuronal decay (Lee et al., 2020).

Acetylcholine plays a key role in memory and learning processes and is the predominant synaptic deformation seen in AD conditions. In the focal sensory system, acetylcholine incitement of the nicotinic receptors gives off an impression of being connected with scholarly capacity (Houghton et al., 2006). Nevertheless, the availability of the enzyme AChE, which catalyzes degradation, and other choline esters that act as synapses in neurons, in AD patients results in a drastic reduction in the formation of acetylcholine, which has a very short half-life. It reaches the brain by hydrolyzing the ester bond of the ACh atom (Orhan et al., 2004). García-Ayllón et al. (2011) showed that inhibition of acetylcholinesterase further reduced β-amyloid plaque accumulation and neurofibrillary tangle assembly in AD. Subsequently, acetylcholinesterase and butyrylcholinesterase inhibitors are thought to be useful in treating AD by prolonging or increasing the availability of acetylcholine in the brain. Surprisingly, existing anticholinesterase drugs such as galantamine, donepezil, tacrine, and physostigmine have been described to have many limitations (Sharma, 2019).

Medicinal plants have been an excellent source of clinical medicine for many years (Silverman and Holladay, 2014). A vast amount of medicinal plants have been studied for preventive or therapeutic purposes. The therapeutic potential of medicinal plants is attributed to the presence of secondary metabolites or bioactive components such as glycosides, coumarins, flavonoids and alkaloids (Zeeshan et al., 2022). In traditional practice, many medicinal plants found in nature have helped treat central nervous system disorders and provide medicines to improve memory and intellectual function (Elufioye et al., 2013).

C. hispida (Malvaceae) is a shrub or tree up to 40 feet tall found in forests (Figure 1). The leaves are up to 30 cm long and 24 cm wide. The flowers are greenish-yellow inside, with brown hairs on the outside and various reddish-pink insides. It has a fruit that looks like a collection of male goat testicles. Burkill (2000) reported that a decoction of the leaves was used to treat ear infections, lung problems, and coughs. Umenwanne et al. (2021) reported the cardio-protective potential of the leaf extract of *C. hispida* against doxorubicin (Dox)-induced cardiac infarction in male albino rats. Anti-diarrhoeal effect of methanol leaf fraction of *C. hispida* against castor oil-induced looseness of stool in rats was established. This was achieved by the extract inhibiting intestinal peristalsis and hypersecretion of water and electrolytes (Ugochi and Obinna, 2021). The ethanolic extract of the leaf of *C. hispida* has been proven to produce significant inhibition of spontaneous contraction of the uterus and also significantly inhibited both oxytocin and acetylcholine-induced contraction (Nworgu et al., 2009). The crude extract and fractions also showed a significant broad spectrum of antimicrobial activity against the test organisms, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Bacillus substilis*, *Candida albican*, *Aspergillus niger* (Okoro et al., 2022). Nigerian folk medicine states that seeds of *C. hispida* can improve mental abilities, but there is little data to justify these claims and also there are limitations in already existing drugs which necessitated the search for new neurotherapeutic agents. Therefore, the AChE inhibitory activity and metal chelating properties of various fractions and compounds isolated from *C. hispida* were scientifically evaluated to establish their usefulness in traditional medical practice for the management of neurodegenerative diseases such as AD.

MATERIALS AND METHODS

Reagents/apparatus

n-hexane, dichloromethane, ethyl acetate, methanol,

Figure 1. *Cola hispida* (Malvaceae) fruit and seed.

acetylcholinesterase, acetylthiocholine iodide (ATChI), 5:5-dithiobis-2-nitrobenzoic acid (DTNB), eserine and sodium phosphate buffer salts, FeCl2,. Ferrozin, Ethylenediaminetetraacetic acid (EDTA) and other chemicals were of analytical grade (Sigma Aldrich, USA), measuring cylinder, beakers, conical flask, column silica gel (60-200 mesh size).

Spectroscopic instrument

Electron Impact Mass spectrometry (EI-MS) performed on JEOL MS route and JEOL 600H1, Fast atom bombardment performed on JEOL-600H-2 on the positive and negative mode (FAB-+ve/-ve mode), Proton Nuclear Magnetic Resonance (1H-NMR), 1D and 2D NMR and 13C-NMR were recorded on Bruker Avance Neo 400 and 800 Cryoprobe MHz. Fourier transform infrared spectroscopy (FT-IR) was recorded on Bruker Vector 22 and Ultra violet spectroscopy (UV) was done using Thermo Scientific UV-Visible Spectrophotometer (Evolution 300). Melting point range was also determined to ascertain purity using BUCHI (M-560).

Plant material

C. hispida Brenan & Keay (Mallow) seeds were obtained in March 2017 from Okura Offante, Dekina Municipal Area, Kogi State. The plant is certified by the Forestry Herbarium of Ibadan (FHI) under Forest Research Institute of Nigeria (FRIN) by Mr. Adeyemo, A. and Chukwuma C. Emmanuel, where herbariums voucher specimen is kept (FHI 111321).

Extraction of plant material

Eleven kilograms (11 kg) of powdered dried seeds of *C. hispida* were macerated with methanol for 72 hours. The extract was then filtered and concentrated in vacuo under reduced pressure at an optimum temperature of 40°C. The dried extract was then partitioned between n-hexane, dichloromethane, ethyl acetate and aqueous fractions.

Determination of anticholinesterase activity

AChE inhibitory activity was determined spectrophotometrically using acetylthiocholine iodide (ATChI) as substrate, according to modified method of Ellman et al. (1961). In a 96-well plate was added 240 µL of buffer (50 mM Tris-HCl, pH 8.0,), 20 µL of varying concentrations of the test compounds/extracts (0.03125-1 mg/mL/0.15625-5 mg/mL, respectively), and 20 µL of the enzyme preparation (0.28 U/mL). The reaction mixture was incubated for 30 min at 37°C, after which 20 µL of 10 mM DTNB was added. The reaction was then initiated by the addition of 20 µL of 25 mM ATChI. The rate of hydrolysis of ATChI was determined spectrophotometrically by measuring the change in the absorbance per minute (ΔA/min) due to the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm over a period of 4 min at 30 sec intervals. A solution of buffer was used as negative control. All assays were carried out in

triplicate. Eserine ((-) physostigmine) was used as positive control.

The percentage inhibition (%I) of test sample was obtained using the formula:

$$I\ (\%) = [(V_o \neg\text{-}\ V_i)/\ V_o] * 100$$

Where: I (%) = Percentage inhibition
V_i = enzyme activity in the presence of test sample
V_o = enzyme activity in the absence of test sample

Molecular docking studies

To predict the binding mode of putative acetylcholinesterase inhibitors (Colovic et al., 2013), available PDB ID: 10CE (Physostigmine, an inhibitor of the enzyme from *Tetronarce californica* (Pacific Electric ray) of acetylcholinesterase (E.C. 3.1.1.7) conjugates with analogs) were used to perform molecular docking studies. 8-(cis-2,6-dimethylmorpholino)octylcarbamoylceroline (MF268). The described assay shows the IC_{50} for the following compounds using acetylcholinesterase from *T. californica* (Pacific Electric Ray) (Dvir et al., 2010) compounds were identified using MOE 2015. All connections were energy minimized and subsequently partial charges were added according to the Merck Molecular Force Field (MMFF94). It was later docked using MOE 2015.010. Docking used the MOE Suite's strict default docking protocol. The resulting compound poses were visually examined to understand protein-ligand interactions. Interactions were analyzed using the PLIP web server (https://projects.biotec.tu-dresden.de/plip-web/plip). All visuals are recorded with MOE 2015 Suite.

Fe^{2+} Chelation assay

The ferrous ion chelation (FIC) assay was performed according to the method of Chew et al. (2009) with some modifications. A solution of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine was diluted 20 times. 1 mL of varying concentrations of the test compounds/extracts (0.03125-1 mg/mL/0.15625-5 mg/mL, respectively) were briefly mixed with 1 mL of $FeCl_2 \cdot 4H_2O$.

After 5 min of incubation, 1 ml of Ferrozin was added to initiate the reaction. The mixture was shaken vigorously and after an additional 10 min incubation period the absorbance of the solution was measured spectrophotometrically at 562 nm. Controls were prepared as above, but the extract was replaced with 1 mL of methanol. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. Percent inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Chelating effect \%} = [(A_{control}\text{-}A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ = absorbance of control sample
A_{sample} = absorbance of a tested samples.

The extract concentration providing 50% inhibition (IC_{50}) was calculated was obtained by interpolation from linear regression analysis.

Isolation of bioactive molecules from *C. hispida* seed

A slurry of the concentrated dichloromethane extract (30 g) was subjected to column chromatography over 150 g column silica gel (60-200 mesh size) as adsorbent material using gradient elution of mobile phase in an increasing polarity solvent system starting with n-hexane (100%, each 2000 mL), n-hexane: ethyl acetate (95:5 to 15:85, each 2000 mL), ethyl acetate (100%, each 2000 mL) and ethyl acetate: methanol (98:20 to 350:150, each 2000 mL). The chromatography yielded 151 fractions which were pooled together to 20 sub fractions (labeled as DCH-F1-DCH-F20). Compound 1 was obtained as a white powder from dichloromethane fraction (sub fraction DCH-F14 (pooled fractions 97-103) with eluent ethyl acetate 100% which yielded 20 mg.

A slurry of the ethyl acetate fraction (70 g) was chromatographed using column chromatography on 600 g column silica gel (60-200 mesh size) as adsorbent material using gradient elution of mobile phase in an increasing polarity solvent system starting with n-hexane (100%, each 2000 mL), n-hexane: dichloromethane (9:1 to 1:9, each 2000 mL), dichloromethane (100%, each 2000 mL) and dichloromethane: methanol (99:1 to 1:3, each 2000 mL). The chromatography yielded 389 fractions which were pooled together to 20 sub fractions (labeled as ECH-F1- ECH-F20). Compound 2 was obtained as a white powder from ethyl acetate fraction (sub fraction ECH-F6 (pooled fractions 48-62) in solvent system hexane: dichloromethane (1:7) yielded 23 mg. Compound 3 was obtained as oily substance, isolated from ethyl acetate fraction (Sub fraction ECH-F9 (pooled fractions 96-116) which yielded 125 mg with solvent system hexane: dichloromethane (1:9). Compound 4 was obtained as a white powder isolated from ethyl acetate fraction (Sub fraction ECH-F12 (pooled fractions 138-195) yielded 31 mg with solvent system dichloromethane: methanol (98:2) soluble in pyridine.

Statistical Analysis

All data will be expressed as mean ± S.D. and of triplicate parallel measurements. Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using Microsoft Excel.

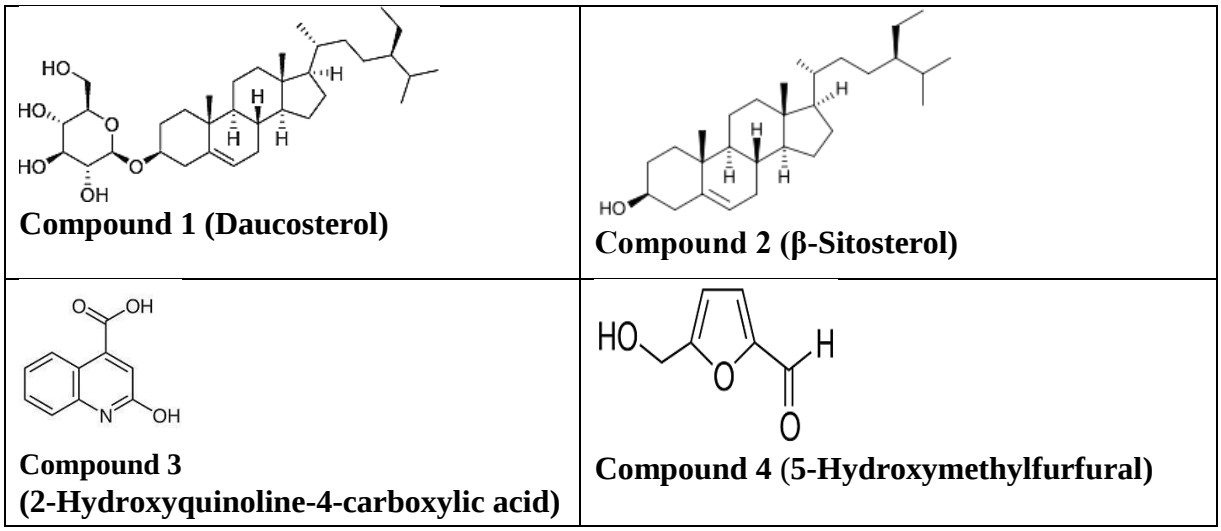

Figure 2. Compounds isolated from the seed of *Cola hispida*.

RESULTS AND DISCUSSION

Characterization of compounds isolated from *C. hispida* seed

Compound 1

It has retardation factor (R_f) value of 0.84 in solvent system ethyl acetate: methanol (1:1), Melting point (M.p): 295.5°C-303.7°C, its low resolution electron impact (EI) mass spectrum showed the existence of a sterol skeleton and a molecular ion peak at m/z 414 [M+]. The exact mass was detected on FAB on the positive mode, Mass (m/z): 577.85 g/mol [M+], IR (γ_{max} ^{KBr} cm^{-1}): 3386.2, 2959.3, 2931.1, 2873.0, 1647.5, 1463.7, 1374.7, 1070.3, 1024.5, 668.2. Molecular formula ($C_{35}H_{60}O_6$) and structure has been assigned to the substance named Daucosterol (β-sitosterol-3-*O*-β-D-glucoside) (Figure 2) based on the physical data and spectroscopic analysis (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR) and by comparison with reported literature (Flamini et al., 2001).

Daucosterol, a steroid saponin was obtained as a white powder. The ^{1}H-NMR spectrum of daucosterol reveals an olefinic proton signal of H-6 at δ 5.34 (br d, 1H, *J* = 4 Hz) and methyl signals at δ 0.66 (s) and δ 0.99 (s). The signal at δ 5.06 (d, *J* = 7.6 Hz) suggested the presence of one anomeric proton. The chemical shift and coupling constant of this proton suggested axial-axial coupling thus showing that the sugar moiety was β-linked to the aglycone. The ^{13}C-NMR (δ, AVANCE NEO 400 MHz C5D5N) showed the presence of thirty five carbons. It also reveals that the olefinic proton resonate at δ 121.9 while the C-3 (β-linkage) signal resonate at δ 78.64 suggesting that the sugar moiety was linked to the oxygen at C-3 of the aglycone. The presence of a sugar moiety was further confirmed by the signal at δ 102.6 which was assigned to the anomeric carbon. The signals for the other sugar carbons were observed at δ 75.37, 78.64, 71.74 and 62.88. These chemical shifts confirmed that the sugar moiety was glucose. Daucosterol is reported for the first time from the species *C. hispida*. Daucosterol is a saponin present in various natural sources has been explored for its various biological activities such as antioxidant, antidiabetic, hypolipidemic, anti-inflammatory, immunomodulatory, neuroprotective, and anticancer (El Omari et al., 2022).

Compound 2

The hexane:chloroform:ethyl acetate (4:1:1.5) solvent system has a retardation factor (R_f) value of 0.6. Melting point (M.p): 134.4°C-135.1°C, Mass (m/z): 414.3 g/mol [M+]. UV (MeOH) λ/nm (log ε): 219.00 nm (A 0.627), 270.00 nm (A 1.404) (190-600 nm) IR (γ_{max} ^{KBr} cm^{-1}): 3446.3, 2936.5, 1642.3, 1462.4, 1376.6, 1058.6, 961.7, 591.7. The molecular formula ($C_{29}H_{50}O$) and structure, based on physical data and spectroscopic analysis (UV, IR, MS, ^{1}H-NMR, and ^{13}C-NMR), were compared with those published to be β-sitosterol (Figure 2) (Wright et al., 1978). β-Sitosterol are a class of organic stigmastane compound. The ^{1}H NMR spectrum of β-sitosterol showed the presence of six methyl signals appearing as two methyl singlets at δ 0.66 and δ 0.98. Three methyl doublets appearing at δ 0.80, 0.82, and 0.90. The ^{1}H NMR spectrum of also showed an olefinic proton at δ 5.32. The ^{1}H NMR spectrum also showed a proton corresponding to the proton attached to the C-3 hydroxy group appearing as a

multiplet at δ3.50. ^{13}C NMR shows that the aromatic/olefinic protons are attached to carbon at δ 138.3/121.72, while the protons associated with the C-3 hydroxy group resonate at δ 71.81. This indicates the presence of double bonds. ^{13}C NMR with COZY, HMQC and HMBC revealed 29 carbon signals including 6 methyls, 11 methylenes, 10 methane and 3 quaternary carbons. This compound was first described in *C. hispida*.

Compound 3

It has retardation factor (R_f) value of 0.93 in solvent system methanol: water (3:2), Mass (m/z): 189.1 g/mol [M+]. IR (γ_{max} ^{KBr} cm^{-1}): 3415.4, 3108.8, 2965.1, 2842.2, 2757.9, 1680.0, 1590.1, 1385.7, 1346.0, 1311.4, 827.5. Molecular formula ($C_{10}H_7NO_3$) and structure has been assigned to the substance 2-hydroxyquinoline-4-carboxylic acid (Figure 2) based on the physical data and spectroscopic analysis (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR) and by comparison with reported literature (Zhiwei et al., 2017). 2-hydroxyquinoline-4-carboxylic acid is a quinolinemonocarboxylic acid. It is a conjugate acid of a 2-oxo-1,2-dihydroquinoline-4-carboxylate. Its low resolution electron impact (EI) mass spectrum on JEOL 600H-1 instrument showed major fragmentation at m/z (%): 189.1 (100.0), 172.1 (2.6), 161.1 (26.7), 144.1 (55.0), 132.1 (4.5), 117.1 (50.7), 104.0 (2.7), 89.0 (19.4), 75.0 (3.9), 63.0 (9.3), 50.9 (3.7), Its IR spectrum revealed several broad peaks in the range of 3415.4-3108.8 cm^{-1} characteristic of bonded N-H or O-H stretching. ^{1}H- NMR reveals the position of the acid which resonate at δ 13.2 (-COOH), the aromatic protons resonate around δ 7.66 as broad singlet, δ 7.55-7.48 as multiplets, δ 7.25-7.21 multiplets and at δ 8.82 as doublet with coupling constant of 8 Hz which is a characteristics of quinoline ring. ^{13}C-NMR-(δ, AV-III-HD 800 MHz Cryo-Probe CD5D5N) showed the presence of ten carbons. ^{13}C-NMR also revealed that the acid is attached to carbon at δ 169.05. DEPT-HSQC reveals that proton at 8.82 ppm is attached to carbon at 127.73 ppm, proton at 7.66 ppm is attached to carbon at 124.88, proton at 7.55-7.48 ppm is attached to carbon at δ 115.95, δ 130.85 and proton at 7.25-7.21 ppm is attached to carbon at 122.32 ppm. This compound is reported for the first time in the species *C. hispida*.

Compound 4

This molecule consists of a furan ring containing both aldehyde and alcohol functional groups. 5-Hydroxymethylfurfural is a member of the furan class, i.e. furans substituted with formyl and hydroxymethyl substituents at the 2- and 5-positions, respectively. The molecular formula ($C_6H_6O_3$) and structure are based on physical data and spectroscopic analysis (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR) and by comparison with the published literature, the substance 5-hydroxymethylfurfural (Figure 2) was assigned (Onoja and Rukesh, 2021).

Anti-cholinesterase inhibitory activities of fractions and isolated compounds of *C. hispida* seed

Plants have been used in traditional medical systems since ancient times to treat memory impairment. Medicinal plants are rich in bioactive compounds that can be used to treat a variety of ailments that threaten human health. Diseases such as AD are now essential for new drugs that can improve memory and learning or slow neurodegenerative processes. Because natural products are multi-targeted and multi-functional in nature, they offer additional benefits such as synergistic and additive effects that are typically required for the treatment of complex diseases such as AD, thereby enhancing learning and memory. Studies conducted in some species have identified compounds that are currently in clinical use or have served as templates for other drug discovery. Galantamine, an alkaloid isolated from *Galanthus nivalis* L. (Amaryllidaceae). Galantamine was approved by the FDA in 2001 for use as an acetylcholinesterase inhibitor in the treatment of AD. Rivastigmine was synthesized from the lead compound physostigmine from *Physostigma venenosum* and approved by the US FDA in 2000 (Konrath et al., 2013), Huperzine A, an alkaloid isolated from *Huperzia serrata*, is also marketed in China as a dietary supplement to improve memory and treat symptoms of AD (Xing et al., 2014). Acetylcholinesterase is a biologically important enzyme that hydrolyzes acetylcholine, a key neurotransmitter in the brain thought to play a role in the pathology of AD (McHardy et al., 2017). One of the most urgent tactics to treat this disease is to use AChE inhibitors to increase brain acetylcholine levels. Therefore, inhibition of acetylcholinesterase, a key enzyme in the breakdown of acetylcholine, is considered one of the therapeutic strategies for several neurological disorders, including Alzheimer's disease (Marucci et al., 2020).

In this study, methanolic extracts of *C. hispida* seeds and their derivative fractions (n-hexane, dichloromethane, ethyl acetate, and aqueous methanol) at various concentrations were used for anticholinesterase inhibition in vitro using ATChI and DTNB tested for activity. This principle involves measuring the rate of production of thiocholine when acetylthiocholine is hydrolyzed. This is accomplished by the sequential reaction of thiol groups with DTNB to generate the yellow anion of 5-thio-2-nitrobenzoic acid (TNB). All tested samples showed concentration dependent cholinesterase inhibition. The smaller the IC50 value, the higher the enzyme inhibitory activity (Garcia-Molina et al., 2022). The AChE inhibition potency of ethyl acetate fraction of *C. hispida* seed at 5 mg/mL was most promising with IC_{50} values of 0.656±0.24

mg/mL followed by dichloromethane fraction (IC_{50} = 0.788±0.16 mg/mL) when compared to eserine at 0.1 mg/mL (IC_{50} = 0.009±0.00 mg/mL) (Table 1). The results imply that the ethyl acetate fraction of *C. hispida* can inhibit acetylcholinesterase, which causes the degradation of acetylcholine, thus prolonging the half-life of acetylcholine in the brain and thereby possibly improving learning and memory.

Many studies describe alkaloids as the main compounds that can inhibit the AChE enzyme (Ortega et al., 2004). Studies have pointed to several new classes of secondary metabolites as potent inhibitors of the AChE enzyme, including flavonoids (Hillhouse et al., 2004), flavones (Sawasdee et al., 2009), steroids, terpenoids, oils and other phenolic compounds (Ji and Zhang, 2008). In this study, the compounds isolated were evaluated for their acetylcholinesterase (AChE) inhibitory activities. 2-hydroxyquinoline-4-carboxylic acid isolated from ethyl acetate fraction of *C. hispida* seed exhibited the highest AChE inhibitory activity (IC_{50} = 1.070±0.09 mg/mL) followed by β-sitosterol (IC_{50} = 1.251±0.29 mg/mL) and 5-hydroxymethylfurfural (IC_{50} = 1.605±0.37 mg/mL) at 1 mg/mL compared to eserine (IC_{50} = 0.009±0.00 mg/mL) at 0.1 mg/mL (Table 2).

This indicates that the compounds may increase the half-life of acetylcholine in the brain and improve learning and memory. This study is further supported by the study carried out by Ayaz et al. (2017) who reported the anti-alzheimer's potential of β-sitosterol isolated from *Polygonum hydropiper*. The authors assessed the potential of β -sitosterol against several pathological targets of AD. *In vitro* and *in vivo* studies revealed that β-sitosterol possesses strong anticholinesterase and antioxidant potentials in the frontal cortex (FC) and hippocampus (HC). The inhibitory activity of 2-hydroxyquinoline-4-carboxylic acid on acetylcholinesterase could be due to their Quinoline core which is regarded a very important moiety because of their wide biological range as a pharmacophore in drug discovery. Quinolines moiety are very interesting due to their broad spectrum as anti-malarial, anticancer, antimicrobial, antibacterial, antifungal and use as an inhibiting agent (Dhaval et al., 2017).

Molecular docking study on Anticholinesterase

A molecular modeling study was performed to investigate the binding mode of a putative inhibitor to acetylcholinesterase (AChE) from *T. californica*. In this context, a PDB (PDB ID: 10CE) complexed with the inhibitor MF268 (the physostigmine analogue 8-(cis-2,6-dimethylmorpholino)octylcarbamoylceroline) was chosen and the compound was docked using the coordinates of the cognate ligand (Fukuto, 1990). Several compounds has been reported to show binding with pacific electric ray. Geissospermine from Geissospermum vellosii exhibits hydrogen bonding, hydrophobic interactions and p–p stacking (Araújo et al., 2011), and berberine isolated from dried rhizomes of *Rhizoma coptidis* is a promising cholinesterase inhibitor with predominantly hydrophobic interactions (Ji and Shen, 2012), an infructopurine isolated from *Cortinarius infractus* preferentially binds to the oxyanion-hole of the AChE enzyme through p–p interactions with aromatic residues (Geissler et al., 2010).

As shown in Figure 3, the top ranked docking pose of β-sitosterol has hydrophobic interaction with TRP279, PHE 290, PHE 330, PHE 331, and TRY 334 (estimated binding free energy (ΔG) -7.42 kcal/mol). This is in agreement with a study conducted to determine the binding affinity of β-sitosterol on protein databank (PDB) PDB ID: 1ACL and 4BOP for AChE and BChE, respectively. The most favorable docking poses were observed inside the binding pockets of the two proteins with proper orientation in term of docking score -5.3168602 Kcal/mol (AChE) and -6.75507879 Kcal/mol (BChE). Such lower values indicate good fitness of the compound in the binding pocket of the protein and stable β-sitosterol-protein interaction (Ayaz et al., 2017).

The pose of 5-hydroxymethylfurfural is shown in Figure 4. This compound forms a pi-stacking interaction with TRP 84 (estimated binding free energy (ΔG) -4.45 kcal/mol). Top ranked docking poses of 2-hydroxyquinoline-4-carboxylic acid form a series of hydrophobic and hydrogen bonding interactions with GLU199 (3.00) and TYR 130 (2.86) and pi-stacking interactions with TRP84 and PHE330 (estimated free energy of binding (Δ G) -5.27 kcal/mol) (Figure 5) (Table 3).

This indicates that the compounds tested can improve memory and learning, or slow down neurodegenerative processes involved in diseases such as AD, due to their ability to bind to catalytic/inhibitor sites with moderate energy and thus proposedly mediate competitive inhibition of the enzyme.

Metal chelating activity of fractions and isolated compounds of Cola hispida seed

Metal ions have been shown to accumulate abnormally in the brain with aging and during the course of several neurodegenerative diseases and AD (Wang et al., 2020). In particular, the interactions between metal-protein interactions and oxidative stress have recently been highlighted by several laboratories (Sayre et al., 2001). Therefore, metal chelation therapy can now be considered a promising clinical approach for AD treatment (Fulgenzi et al., 2020). As the demand for new, more effective drugs to treat AD continues to grow, pharmacological strategies aimed at lowering metal ions in the brain and targeting Aβ/metal ion interactions include chelation. Neurotoxic heavy metals such as Pb and Cd are also known to disrupt

Table 1. *In vitro* acetylcholinesterase inhibitory and metal chelating activity of crude extract and fractions of *C. hispida* at 5 mg/mL.

Plant material	Crude and fraction(s)	AChE inhibition IC_{50}±SD (mg/mL)	Fe^{2+} chelating activity IC_{50}±SD (mg/mL)
C. hispida seed	n-Hexane	6.875±0.59	4.124±0.26
	Dichloromethane	0.788±0.16	1.622±0.02
	Ethyl acetate	0.656±0.24	0.624±0.05
	Aqueous methanol	1.698±0.92	2.447±0.20
	Crude	0.871±0.43	2.092±0.02
	Eserine (STD)	0.009±0.00	NA
	Vitamin C	NA	0.019±0.00

NA, not applicable; Values are presented as mean ± standard deviation (n=3).

Table 2. Acetylcholinesterase inhibitory and metal chelating activity of compounds isolated from *C. hispida* seed at 1 mg.

Compounds	AChE inhibition IC_{50}±SD (mg/mL)	Fe^{2+} chelating activity IC_{50}±SD (mg/mL)
β-sitosterol	1.251±0.29	0.334±0.14
2-hydroxyquinoline-4-carboxylic acid	1.070±0.09	0.960±0.02
5-hydroxymethylfurfural	1.605±0.37	0.295±0.01
Eserine (Standard)	0.009±0.00	NA
EDTA (Standard)	NA	0.045±0.11

NA, not applicable; Values are presented as mean ± standard deviation (n=3).

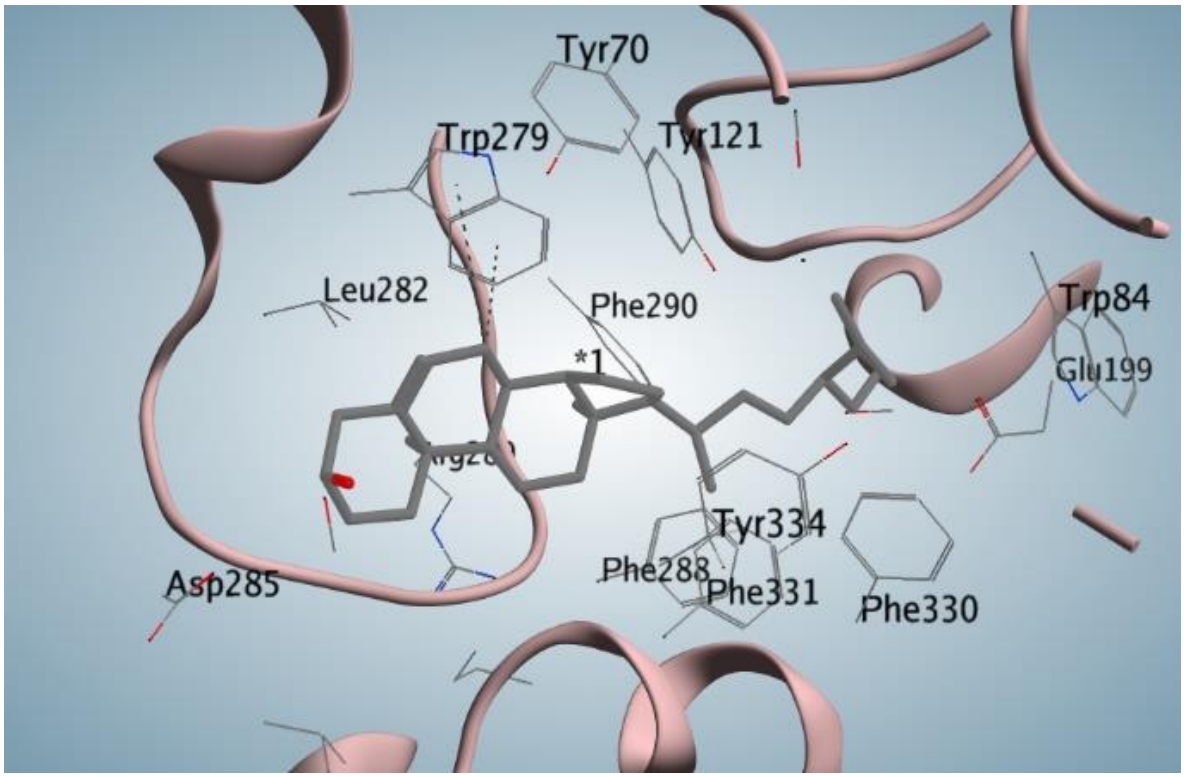

Figure 3. The simulated poses of the compound β-sistosterol. Hydrogen bonds are presented in blue lines. Grey sticks show the ligand while the acetylcholinesterase residues are shown as pink ribbons. The images were rendered using MOE 2015.01.08.

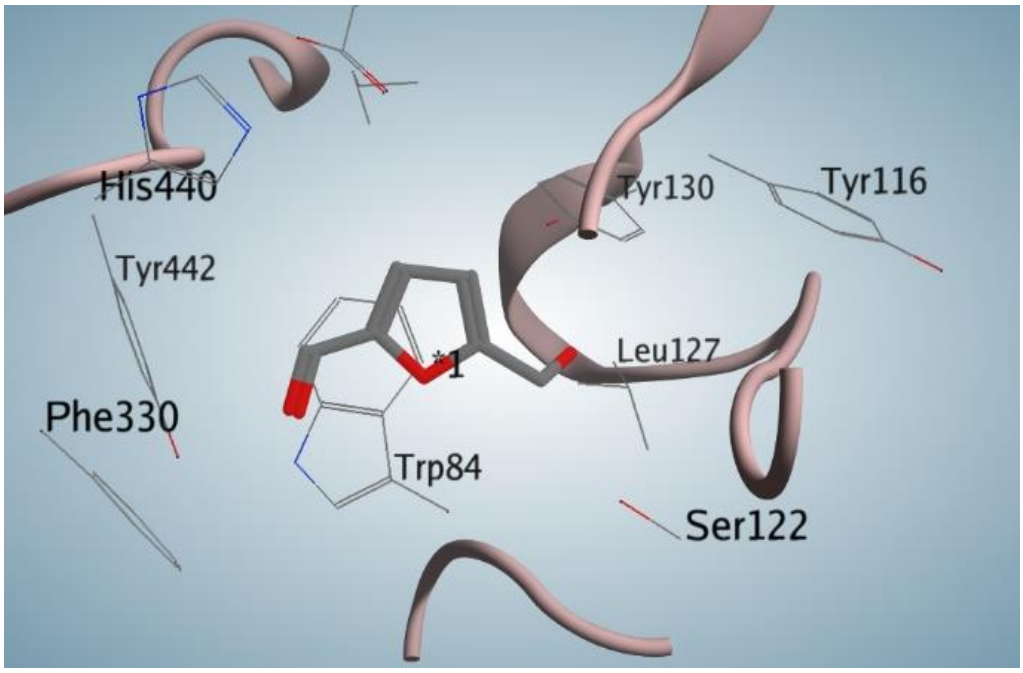

Figure 4. The simulated poses of the compound 5-hydroxymethylfurfural. Hydrogen bonds are presented in blue lines. Grey sticks show the ligand while the acetylcholinesterase residues are shown as pink ribbons. The images were rendered using MOE 2015.01.08.

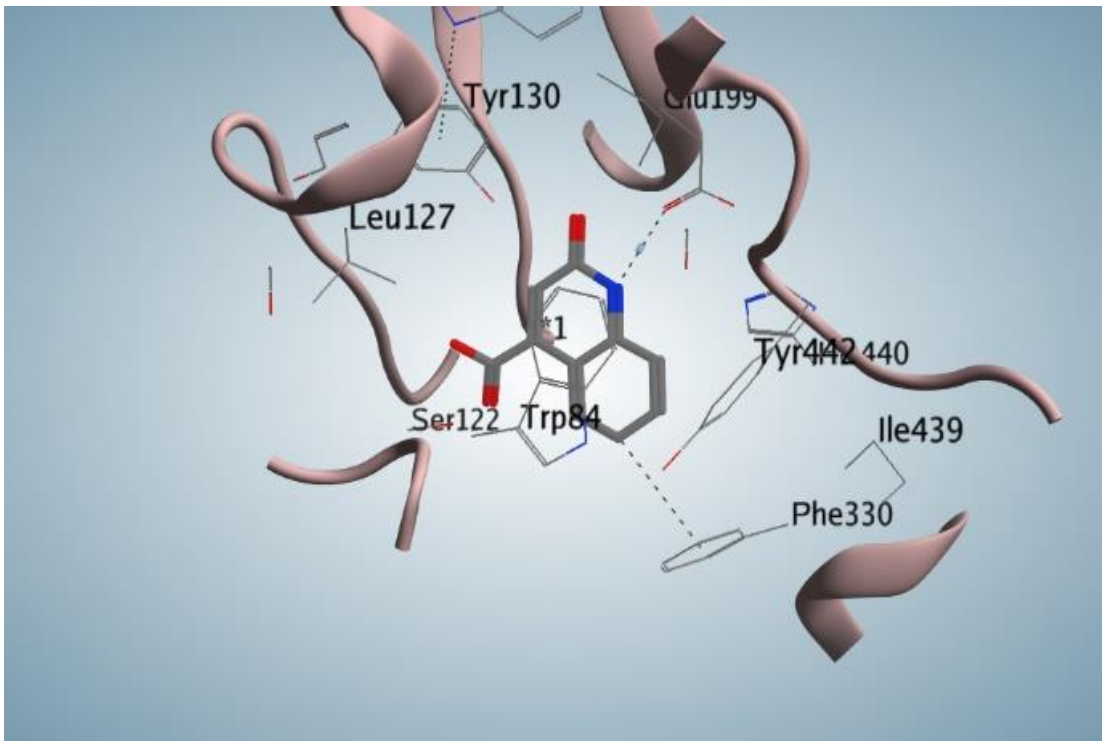

Figure 5. The simulated poses of the compound 2-hydroxyquinoline-4-carboxylic acid. Hydrogen bonds are presented in blue lines. Grey sticks show the ligand while the acetylcholinesterase residues are shown as pink ribbons. The images were rendered using MOE 2015.01.08.

Table 3. Docking finding of isolated compounds (- Log FBE (kcal/mol) and Residues with interaction.

Compounds	(- Log FBE (kcal/mol)	Residue interaction
β-sitosterol	-7.42	hydrophobic interactions with TRP279, PHE 290, PHE 330, PHE 331 and TRY 334
2-hydroxyquinoline-4-carboxylic acid	-5.27	hydrophobic interactions and hydrogen bonds with GLU199 (3.00) and TYR 130 (2.86) and Pi-Stacking interactions with TRP84 and PHE330
5-hydroxymethylfurfural	-4.45	Pi Stacking interaction with TRP 84

PDB ID:10CE (Pacific electric ray) AChE.

structural features of cells in this region of the brain (Zhu et al., 2014). Studies suggest that curcumin significantly reduces her Pb- and Cd-induced neurotoxicity in rat hippocampal neurons (Dairam et al., 2007) and increases hippocampal neurogenesis in chronically stressed rats (Xu et al., 2007). The use of phytochemicals as an alternative strategy to improve neurotoxic mechanisms has received much attention recently (Libro et al., 2016). Ferrozine quantitatively forms a complex with ferrous iron, giving it a red color. However, in the presence of chelating agents, complex formation is disrupted and the red color is reduced. Color measurements provide an estimate of the binding affinity of coexisting chelators. In this study 5 mg/mL of the dichloromethane and ethyl acetate fractions of *C. hispida* seed showed good metal chelating activity by inhibition of ferrozine–Fe^{+2} complex formations with IC_{50} values of 1.622±0.02 mg/mL and 0.624±0.05 mg/mL, respectively as compared to vitamin C at 0.1 mg/mL (IC_{50} = 0.019±0.00 mg/mL) (Table 1). The dichloromethane and ethyl acetate fractions, due to their antioxidant properties, may lower metal ions in the brain and demonstrate the ability to target Aβ/metal ion interactions, offering great potential for chelation therapy.

Several lipophilic metal chelators, such as clioquinol and its derivative PBT2, are currently undergoing clinical trials and have shown promising results in some AD patients (Faux et al., 2010). Among the compound evaluated for their metal chelating potentials, 5-hydroxymethylfurfural isolated from ethyl acetate fraction gave good metal chelating activity with IC_{50} of 0.295±0.01 mg/mL alongside β-sitosterol (IC_{50} of 0.334±0.14 mg/mL) and 2-hydroxyquinoline-4-carboxylic acid (IC_{50} of 0.960±0.02 mg/mL) at 1 mg as compared to Ethylene diaminetetraacetic Acid (EDTA) with IC_{50} of 0.045±0.11 mg/mL (Table 2). Overall, the results indicate that 5-hydroxymethylfurfural can effectively attenuate ROS-mediated neuronal cell death in AD due to its antioxidant properties. 5-hydroxymethylfurfural (5-HMF) has been found to exhibit novel antioxidant activity by scavenging the 2,2-azinobis-3-ethylbenzothiazolin-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picryhydrazyl (DPPH) free radicals and also inhibited the 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) induced hemolysis in a dose-dependent manner which demonstrated that 5-HMF could prevent the peroxidation from the source to protect the erythrocytes (Ling et al., 2013). In another study, 5-HMF isolated from *Laurencia undulata* displayed its potential antioxidant character on the molecular, cellular, and gene levels. 5-HMF can effectively scavenge free radicals (DPPH, hydroxyl, alkyl, and superoxide anion) and cellular ROS, but it also protects the cell membrane from oxidation stress, as well as inhibits the activity of the oxidant enzyme (MPO). 5-HMF can also significantly increase the expressions of antioxidant enzymes (GSH and SOD), which are antioxidant enzymes that play important roles in defense against oxidation damage from reactive free radicals (Li et al., 2009).

Conclusion

β-sitosterol and 2-hydroxyquinoline-4-carboxylic acid can improve memory and learning or slow neurodegenerative processes involved in diseases such as Alzheimer's disease. This is due to their ability to bind and possibly mediate at the catalytic/inhibitor site with modest energies. These two biomolecules could serve as potential lead compounds for developing new drugs to treat Alzheimer's disease.

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LIST OF ABBREVIATIONS

AD, Alzheimer’s disease; **NMR**, Nuclear Magnetic

Resonance; **PDB**, Protein Data Bank; **MOE**, Molecular Operating Environment; **AChE**, Acetylcholinesterase; **EDTA**, Ethylenediaminetetraacetic acid; **CNS**, Central Nervous System; **ROS**, Reactive oxygen species; **ACh**, Acetylcholine; **ATChI**, Acetylthiocholine iodide; **DTNB**, 5,5-dithiobis-2-nitrobenzoic acid; **EI-MS**, Electron Impact Mass spectrometry; **UV**, Ultra violet spectroscopy; **FT-IR**, Fourier transform infrared spectroscopy; **DCH**, Dichloromethane fraction of *Cola hispida*; **FBE**, Free binding energy; **Pb**, Lead; **Cd**, Cadmium.

REFERENCES

Araújo J. Q., Lima J. A., Angelo da C. P., Ricardo B. A. & Albuquerque M. G. (2011). Docking of the alkaloid geissospermine into acetylcholinesterase: A natural scaffold targeting the treatment of Alzheimer's disease. J. Mol. Model. 17:1401–1412.

Athar T., A. l. Balushi K. & Khan S. A. (2021). Recent advances on drug development and emerging therapeutic agents for Alzheimer's disease. Mol. Biol. Rep. 48(7):5629-5645.

Ayaz M., Junaid M., Ullah F., Subhan F., Sadiq A., Ali G., Ovais M., Shahid M., Ahmad A., Wadood A., El-Shazly M., Ahmad N. & Ahmad S. (2017) Anti-Alzheimer's studies on β-Sitosterol isolated from *Polygonum hydropiper* L. Front. Pharmacol. 8:697.

Burkill H. M. (2000). The useful plants of west tropical Africa. Vol. 5. 2nd ed. Royal Botanic Gardens, Kew, 686.

Chew Y. L., Goh J. K. & Lim Y. Y. (2009). Assessment of *in-vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. Food. Chem. 116:13–18.

Colovic M. B., Krstic D. Z., Lazarevic-Pasti T. D., Bondzic A. M. & Vasic V. M. (2013). Acetylcholinesterase inhibitors: Pharmacology and toxicology. Curr. Neuropharmacol. 11(3):315-355.

Dairam A., Limson J. L., Watkins G. M., Antunes E. & Daya S. (2007). Curcuminoids, curcumin, and demethoxycurcumin reduce lead-induced memory deficits in male Wistar rats. J. Agric. Food. Chem. 55:1039–1044.

DeTure M. A. & Dickson D.W. (2019). The neuropathological diagnosis of Alzheimer's disease. Mol. Neurodegener. 14, 32.

Dhaval B., Patel R. H. V., Kinjal D. P., Neelam P. P., Mahesh S. V., & Hitesh D. P. (2017). Recent advances in synthesis of quinoline-4-carboxylic acid and their biological evaluation: A review. J. Chem. Pharmaceut. Res. 9(2):216-230.

Dvir H., Silman I., Harel M., Rosenberry T. L. & Sussman J. L. (2010). Acetylcholinesterase: From 3D structure to function. Chemico-Biological Interactions. 187(1-3):10-22.

El Omari N., Jaouadi I., Lahyaoui M., Benali T., Taha D., Bakrim S., El Menyiy N., El Kamari F., Zengin G., Bangar S. P., Lorenzo J. M., Gallo M., Montesano D. & Bouyahya A. (2022). Natural sources, pharmacological properties, and health benefits of daucosterol: Versatility of actions. Appl. Sci. 12:5779.

Ellman G. L., Courtney K. D., Andres V. & Featherstone R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95.

Elufioye T. O., Obuotor E. M., Agbedahunsi J. M. & Adesanya S. A. (2013). Acetyl andbutyryl cholinesterase inhibitory effect of *Peltophorum pterocarpum* (DC) Backerex K. Heyne (family Legumonosae). J. Pharmacogn. Phytother. 5:77–82.

Faux N. G., Ritchie C. W., Gunn A., Rembach A., Tsatsanis A., Bedo J. (2010). PBT2 rapidly improves cognition in Alzheimer's disease: Additional phase II analyses. J. Alzheimer's Dis. 20:509–16.

Flamini G., Antognoli E. & Morelli I. (2001). Two flavonoids and other compounds from the aerial part of *Centaurea bracteata* from Italy. Phytochemistry. 57:559-564.

Fukuto T. R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. In: Environmental Health Perspectives. 87:245-254.

Fulgenzi A., Vietti D., Ferrero M.E. (2020). EDTA chelation therapy in the treatment of neurodegenerative diseases: An update. Biomedicines. 3;8(8):269.

García-Ayllón M. S., Small D. H., Avila J. & Sáez-Valero J. (2011). Revisiting the role of Acetylcholinesterase in Alzheimer's Disease: Cross-talk with P-tau and β-Amyloid. Front Mol. Neurosci. 13;4:22.

Garcia-Molina P., Garcia-Molina F., Teruel-Puche J. A., Rodriguez-Lopez J. N., Garcia-Canovas F., Muñoz-Muñoz J. L. (2022). The relationship between the IC_{50} values and the apparent inhibition constant in the study of inhibitors of tyrosinase diphenolase activity helps confirm the mechanism of inhibition. Molecules. 13;27(10):3141.

Geissler T., Brandt W., Porzel A., Schlenzig D., Kehlen A., Wessjohann L. & Arnold N. (2010). Acetylcholinesterase inhibitors from the toadstool *Cortinarius infractus*. Bioorg. Med. Chem. 18:2173–2177.

Hillhouse B. J., Ming D. S., French C. J. & Towers G. H. N. (2004). Acetylcholine esterase inhibitors in *Rhodiola rosea*. Pharmaceut. Biol. 42(1):68–72.

Houghton P. J., Ren Y., Howes M. J. (2006). Acetylcholinesterase inhibitors from plants and fungi. Nat. Product. Report. 23:181-199.

Ji H. F. & Shen L. (2012). Molecular basis of inhibitory activities of berberine against pathogenic enzymes in Alzheimer's disease. The Scientific World Journal. 823201.

Ji H. F. & Zhang H. Y. (2008). Multipotent natural agents to combat Alzheimer's disease. Functional spectrum and structural features. Acta Pharmacologica Sinica. 29(2):143–151.

Konrath E. L., Passos C. S., Klein-Júnior L. C. & Henriques A. T. (2013). Alkaloids as a source of potential anticholinesterase inhibitors for the treatment of Alzheimer's disease. J. Pharm. Pharmacol. 65(12):1701–1725.

Kurutas E.B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr. J. 25;15(1):71.

Lee K. H., Cha M. & Lee B. H. (2020). Neuroprotective effect of antioxidants in the brain. Int. J. Mol. Sci. 28;21(19):7152.

Li Y.-X., Yong L., Zhong-Ji Q., Moon-Moo K. & Se-Kwon K. (2009). *In vitro* antioxidant activity of 5-HMF isolated from marine red alga *Laurencia undulata* in free radical mediated oxidative systems. J. Microbiol. Biotechnol. 19(11):1319–1327.

Libro R., Giacoppo S., Soundara R. T., Bramanti P. & Mazzon E. (2016). Natural phytochemicals in the treatment and prevention of dementia: An overview. Molecules. 21(4):518.

Ling Z., Jianping C., Jianyu S., Lin L., Songqing H., Bing L., Xia Z., Zhenbo X., Tianfeng C. (2013). *In vitro* antioxidant and antiproliferative activities of 5-Hydroxymethylfurfural. J. Agric. Food. Chem. 61:10604−10611.

Marucci G., Buccioni M., Ben D. D., Lambertucci C., Volpini R., Amenta F. (2020). Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease. Neuropharmacology. 190:108352.

McHardy S. F., Wang H. L., McCowen S. V. & Valdez M. C. (2017). Recent advances in acetylcholinesterase inhibitors and reactivators: an update on the patent literature (2012-2015). Expert. Opin. Ther. Pat. 27(4):455-476.

Muddapu V. R., Dharshini S. A. P., Chakravarthy V. S. & Gromiha M. M. (2020). Neurodegenerative diseases â is metabolic deficiency the root cause?. Front. Neurosci. 14:213.

Neugroschl J. & Wang S. (2011). Alzheimer's disease: diagnosis and treatment across the spectrum of disease severity. Mt. Sinai. J. Med. 78(4):596-612.

Nworgu Z. A. M., Owolabi O. J., Amaechina F. C., Falodun A. & Ojo O. D. (2009). *In vitro* inhibitory evaluation of *Cola hispida*, Brennan and Keay (Sterculiaceae) on the isolated rat uterus. Can. J. Pure. Appl. Sci. 3(2):809-811.

Okoro F. C., Omachi D. O., Samuel A. E., Ufuoma S. O., Agbo M., Augustina O. I. & Efeturi A. O. (2022). Phytochemical and antimicrobial studies of the leaf extract of *Cola hispida* Brenan & Keay Sterculiaceae, FUW Trends in Science & Technology Journal. 2022: 7(3):175–179.

Onoja O. J., Rukesh M. (2021). Effects of 5-hydroxymethylfurfural isolated from *Cola hispida* on oral adenosquamous carcinoma and

MDR *Staphylococcus aureus*. J. Med. Plant Herb. Ther. Res. 8:1-7.
Orhan I., Sener B., Choudhary M. I. & Khalid A. (2004). Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J. Ethnopharmacol. 91:57-60.
Ortega M. G., Agnese A. M. & Cabrera J. L. (2004). Anticholinesterase activity in an alkaloid extract of *Huperzia saururus*. Phytomedicine. 11(6):539–543.
Sawasdee P., Sabphon C., Sitthiwongwanit D. & Kokpol U. (2009). Anticholinesterase activity of 7-methoxyflavones isolated from *Kaempferia parviflora*. Phytother. Res. 23(12):1792–1794.
Sayre L. M., Smith M. A. & Perry G. (2001). Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr. Med. Chem. 8:721-738.
Sharma K. (2019). Cholinesterase inhibitors as Alzheimer's therapeutics (Review). Mol. Med. Report. 20:1479-1487.
Silverman R. B. & Holladay M. W. (2014). The organic chemistry of drug design and drug action. Academic press. 2–4.
Singhal A. K., Naithani V. & Bangar O. P. (2012). Medicinal plants with a potential to treat Alzheimer and associated symptoms. Int. J. Nutr. Pharmacol. Neurol. Dis. 2(2):84-91.
Ugochi O. N. & Obinna M. O. (2021). Anti-diarrhoeal effect of methanol leaf fraction of *Cola hispida* Brenan and Keay in rats. Trop. J. Pharmaceut. Res. 20 (7):1455-1462.
Umenwanne C., Ogugofor M. O. & Njoku O. U. (2021). Ethyl acetate fraction of *Cola hispida* leaf protects against doxorubicin-induced myocardial injury in male albino rats. Future J. Pharmaceut. Sci. 7:61.
Wang L., Yin Y. L., Liu X. Z., Shen P., Zheng Y. G., Lan X. R., Lu C. B., & Wang J. Z. (2020). Current understanding of metal ions in the pathogenesis of Alzheimer's disease. Transl. Neurodegener. 9:10.
Wright J. L. C., McInnes A. G., Shimizu S., Smith D. G. & Walter J. A. (1978). Identification of C-24 alkyl epimers of marine sterols by 13C nuclear magnetic resonance spectroscopy. Can. J. Chem. 56:1898-1903.
Xing S. H., Zhu C. X., Zhang R. & An L. (2014). Huperzine a in the treatment of Alzheimer's disease and vascular dementia: A meta-analysis. Evidence-Based Compl. Altern. Med. 2014:363985.
Xu Y., Ku B., Cui L., Li X., Barish P. A., Foster T. C. & Ogle W. O. (2007). Curcumin reverses impaired hippocampal neurogenesis and increases serotonin receptor 1A mRNA and brain-derived neurotrophic factor expression in chronically stressed rats. Brain Res. 1162:9–18.
Zeeshan B. M., Ismail H. & Khan K. W. (2022). Plant secondary metabolites: Therapeutic potential and pharmacological properties. Secondary metabolites-trends and reviews. doi: 10.5772/intechopen.103698.
Zhang Y. & Song W. (2017). Islet amyloid polypeptide: another key molecule in Alzheimer's pathogenesis? Prog. Neurobiol. 153:100–20.
Zhiwei S., Zhonghui M., Li Y., Buming L. & Sheng-Xiang Q. (2017). N-containing phytochemicals from the seeds of *Brucea javanica*. Chemistry of natural compounds. 53(4):799-801.
Zhu H., Jia Y., Cao H., Meng F. & Liu X. (2014). Biochemical and histopathological effects of subchronic oral exposure of rats to a mixture of five toxic elements. Food. Chem. Toxicol. 71:166–175.