



Isolation, *In vitro* Acetylcholinesterase Inhibitory Activity, Molecular Docking, and *In silico* ADME Analysis of compounds from *Crossopteryx febrifuga* (Afzel. ex G. Don) Benth

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ABSTRACT

This study aimed to isolate, characterize, and evaluate the acetylcholinesterase (AChE) inhibitory potential of bioactive compounds from *Crossopteryx febrifuga*, a plant of interest in Alzheimer's disease (AD) research. Crude extracts were obtained from the stem bark and trunk wood using ethanol/water (70:30) maceration. The trunk wood extract was subjected to silica gel column chromatography, leading to the isolation of three pure compounds: β -sitosterol (1), scopoletin (3), and D-mannitol (4) along with a mixture of stigmaterol (2) and β -sitosterol (1). Structural elucidation was performed using 1D and 2D ¹H and ¹³C NMR spectroscopy, along with mass spectrometry (MS). The AChE inhibitory activity of both extracts and isolated compounds was assessed *in vitro* using a modified Ellman's colorimetric method. Additionally, molecular docking simulations were conducted to predict the binding affinities and interactions of the compounds with the AChE active site. *In vitro* assays revealed that the stem bark and trunk wood extracts exhibited moderate AChE inhibition with IC₅₀ values of 424.3 µg/mL and 480.0 µg/mL, respectively. Among the isolated compounds, the mixture of stigmaterol and β -sitosterol showed the highest activity (IC₅₀ = 50.85 µg/mL), followed by β -sitosterol (57.25 µg/mL) and scopoletin (58.97 µg/mL). D-mannitol displayed very weak inhibition (IC₅₀ = 4955.64 µg/mL). Docking results supported these findings, with stigmaterol demonstrating the strongest binding affinity (−10.5 kcal/mol), followed by β -sitosterol (−10.5 kcal/mol), while D-Mannitol showed lower affinity (−5.0 kcal/mol). Despite its moderate inhibition, scopoletin exhibited favorable physicochemical properties, including small molecular size, high solubility, and predicted blood-brain barrier (BBB) permeability. These results suggest that *C. febrifuga* contains promising anti-AChE compounds, particularly phytosterols and coumarins, which may serve as lead candidates for the development of new therapies against Alzheimer's disease.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the gradual decline of memory, cognitive functions, and the ability to perform everyday tasks. AD is the most prevalent form of dementia, accounting for 60–70 % of cases worldwide, and poses a significant social and economic burden (Li-Kai et al., 2023). A key biochemical feature of AD is the reduction of acetylcholine (ACh), a neurotransmitter essential for memory, learning, attention, and cognition. The enzyme acetylcholinesterase (AChE) catalyzes the breakdown of ACh; thus, AChE inhibitors are a major therapeutic target aimed at increasing ACh levels in the brain (Shin-Hua et al., 2011). Currently, drugs such as donepezil, rivastigmine, and galantamine are used to inhibit AChE and alleviate symptoms of AD. However, their use is often limited by serious side effects, highlighting the need to discover new compounds with improved safety profiles.

Crossopteryx febrifuga (Afzel. ex G. Don) Benth., the sole species in the genus *Crossopteryx* of the Rubiaceae family, is a deciduous shrub widely distributed across sub-Saharan Africa. Traditionally, various parts of the plant have been used to treat a range of ailments, including fever, malaria, inflammatory disorders, epilepsy, and pain (Brigitta and Torsten, 2009; Orwa et al., 2009; Burkill, 1997). Notably, its traditional use in managing neurological conditions such as epilepsy, headaches, and convulsions, along with its application in treating fever and inflammation, suggests potential neuroprotective properties.

Phytochemical studies have identified diverse bioactive constituents in *C. febrifuga*, including alkaloids, flavonoids, phenolic compounds, saponins, and triterpenoids (Foresta et al., n.d.; Chouna et al., 2015; Tona et al., 2000; Tamfu et al., 2022). These compounds have been associated with various pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial, and antiparasitic effects (Edeoga et al., 2005; Bealem et al., 2021; Ojewale et al., 2013; Assob et al., 2014; Elufioye and Agbedahunsi, 2004; Halilu et al., 2012; Salawu et al., 2008; Tor-Anyiin and Shimbe, 2012). Given these promising properties and its ethnomedicinal relevance, this study aims to evaluate the potential of *Crossopteryx febrifuga* as a source of neuroprotective agents against Alzheimer's disease.

2. Material and methods

2.1 General experimental procedures

^1H and ^{13}C NMR spectra of the isolated compounds were recorded on Bruker Avance AV-500 and AV-600 spectrometers, using tetramethylsilane (TMS) as the internal reference. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) in hertz (Hz). Electrospray ionization mass spectrometry (ESI-MS) analyses were performed in positive ion mode (scan range: 150–1500 m/z , centroid CV = 20) using a Bruker QTOF spectrometer (Germany) equipped with a ZQ F1 source. Column chromatography (CC) was carried out using silica gel 60 (70–230 mesh, Merck). Thin layer chromatography (TLC) was conducted on silica gel F-254 precoated plates (20 × 20 cm, Merck). Spots were visualized under UV light at 254 and 365 nm, followed by spraying with 10 % H_2SO_4 solution and heating at 105 °C to develop the spots.

2.2 Plant material

The stem bark and trunk wood of *Crossopteryx febrifuga* (Afzel. ex G. Don) Benth. were harvested in July 2020 in the Dang Sub-division, Adamawa Region of Cameroon. The plant was identified by Dr. Fawa, a systematic botanist at the University of Ngaoundere, and a voucher specimen (No. 41,791/HNC) was deposited at the National Herbarium of Cameroon in Yaoundé. The collected plant parts were washed and dried at room temperature in the laboratory for three weeks. Once completely dried, they were ground into a fine powder and stored in airtight containers until use.

2.3 Extraction and compounds isolation

The dried trunk wood powder (1000 g) was extracted by maceration at room temperature for 72 h using 6 L of EtOH/ H_2O (70:30, v/v), with occasional shaking. After maceration, the solution was filtered through Whatman No.1 filter paper and concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator, yielding 38.481 g of a dark brown crude extract. The same procedure was applied to the stem bark, yielding 86.235 g of crude extract. A portion of the trunk wood crude extract (30 g) was subjected to silica gel column chromatography, eluted with a gradient of n-hexane/ethyl acetate (Hex/EtOAc), followed by ethyl acetate/methanol (EtOAc/MeOH), and monitored by thin-layer chromatography (TLC). Fractions of 300 mL were collected and concentrated using a rotary evaporator. A total of 285 fractions were collected and pooled into eight major fractions (A–H) based on their TLC profiles. Compound 1 (10 mg), a white amorphous powder, was obtained by washing fraction A (eluted with Hex/EtOAc 95:5) with MeOH. Purification of fraction E on a silica gel column using an isocratic system of Hex/EtOAc (30:70) afforded compound 3 (8 mg).

The same procedure was applied to the stem bark crude extract (30 g). A total of 255 fractions were collected and pooled into seven major fractions (I–O) according to their TLC profiles. Following the same isolation procedure used for compound 1, a white amorphous powder was obtained and further characterized as a mixture of compounds 1 and 2 in a 2:1 ratio. Compound 4 was obtained as a white precipitate from fraction M after washing with ethyl acetate.

β -sitosterol (1): white powder (n-hexane/ethyl acetate 95:5), (ESI) (+): m/z 437.8 $[\text{M}+\text{Na}]^+$ (for $\text{C}_{29}\text{H}_{50}\text{O}_2\text{Na}$) corresponding to the formula $\text{C}_{29}\text{H}_{50}\text{O}$. ^1H NMR (MeOD, 500 MHz): δ_{H} 5.37 (1H, m, H-6), 3.38 (1H, m, H-3), 1.05 (3H, s, H-19), 0.98 (3H, d, J = 6.3 Hz), 0.89 (3H, d, J = 3.05 Hz, H-26), 0.87 (3H, d, J = 2.3 Hz, H-27), 0.86 (3H, s, H-29), 0.75 (3H, s, H-18). ^{13}C NMR (MeOD, 125 MHz): δ_{C} 142.3 (C-5), 122.4 (C-6), 72.5 (C-3), 58.2 (C-14), 57.5 (C-17), 51.7 (C-19), 47.3 (C-24), 43.5 (C-13), 41.2 (C-4), 38.6 (C-12), 37.7 (C-1), 37.4 (C-10), 35.1 (C-20), 33.3 (C-22), 33.0 (C-7), 32.3 (C-8), 30.7 (C-2), 30.4 (C-25), 29.4 (C-16), 27.2 (C-23), 25.3 (C-15), 24.2 (C-28), 22.2 (C-11), 20.2 (C-26), 19.9 (C-19), 19.4 (C-27), 19.3 (C-21), 12.4 (C-29), 12.3 (C-18) (Muhammad et al., 2017; Nyemb et al., 2018).

Stigmasterol (2): appears as white needles (n-Hex/EtOAc 80:20). ^1H NMR (MeOD, 600 MHz): δ_{H} 5.36 (1H, m, H-6), 5.19 (1H, dd, J = 9.15 Hz, H-22), 5.07 (1H, dd, J = 8.15 Hz, H-23), 3.14 (1H, m, H-3), 0.97 (3H, d, J = 6.5 Hz, H-21), 0.90 (3H, s, H-19), 0.89 (3H, d, J = 6.9 Hz, H-26), 0.87 (3H, d, J = 6.9 Hz, H-27), 0.85 (3H, s, H-29), 0.74 (3H, s, H-18). ^{13}C NMR (MeOD, 150 MHz): δ_{C} 140.9 (C-5), 138.4 (C-22), 129.2 (C-23), 121.0 (C-6), 71.0 (C-3), 56.8 (C-14), 56.0 (C-17), 50.3 (C-9), 45.9 (C-24), 42.1 (C-13), 41.6 (C-4), 39.8 (C-12), 37.1 (C-1), 36.3 (C-10), 36.0 (C-20), 30.9 (C-7), 31.6 (C-8), 31.9 (C-2), 28.9 (C-25), 27.9 (C-16), 25.8 (C-11), 23.9 (C-15), 22.8 (C-28), 20.8 (C-21), 18.4 (C-19), 17.9 (C-27), 19.9 (C-26), 10.9 (C-29), 10.8 (C-18) (Munvera et al., 2021).

Scopoletin (3) appears as a yellow crystal (EtOAc/MeOH 90/10). ^{13}C NMR (MeOD, 125 MHz): δ_{C} 164.1 (C-2), 152.5 (C-10), 151.5 (C-7), 147.1 (C-6), 146.1 (C-4), 112.6 (C-3), 112.5 (C-9), 110.0 (C-4), 103.9 (C-8), 56.9 (OMe) (Wisnu et al., 2013).

D-mannitol (4): white solid, m.p. 168–170 °C. ^1H NMR (DMSO- d_6): δ_{H} 4.41 (2H, d, J = 5.5 Hz, HO-2 and 5), 4.33 (2H, t, J = 5.7 Hz, HO-1 and 6), 4.13 (2H, d, J = 7.1 Hz, HO-3 and 4), 3.62 (2H, ddd, J = 10.8, 5.7, 3.5 Hz, H-1a and 6a), 3.56 (2H, t, J = 7.5 Hz, H-3 and 4), 3.47 (2H, m, H-2 and 5) and 3.39 (2H, m, H-1b and 6b). ^{13}C NMR (DMSO- d_6): δ_{C} 63.8 (C-1 and 6), 71.3 (C-2 and 5) and 69.6 (C-3 and 4) (Bealem et al., 2021; Nyemb et al., 2018).

2.4 In vitro inhibition of AChE

The inhibitory activity of plant extracts and compounds against acetylcholinesterase (AChE) was evaluated using a spectrophotometric method adapted from Ferreira et al. (2020), based on Ellman's

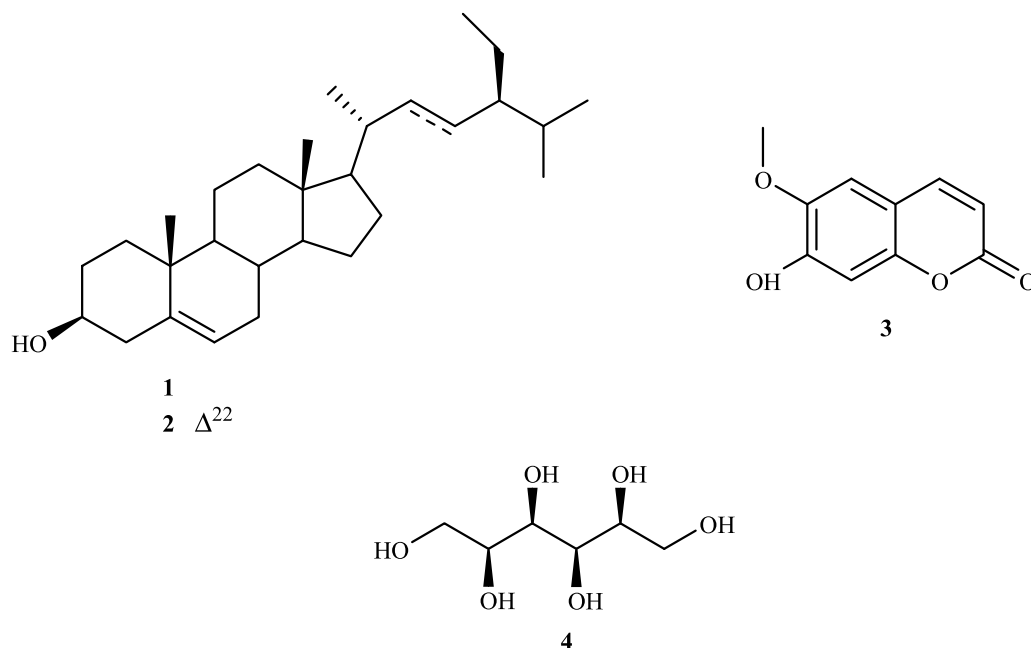


Fig. 1. Chemical structures of the isolated compounds.

colorimetric assay. This method quantifies the yellow-colored product resulting from the reaction between thiocholine, generated by AChE-mediated hydrolysis of acetylthiocholine iodide (AChI), and 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Two types of cuvettes were prepared for the assay. The control cuvette (blank) was used to assess the non-enzymatic hydrolysis of acetylcholine and contained the following: 500 μL of 3 mM DTNB in 0.1 M potassium phosphate buffer (pH 8.0), 100 μL of 15 mM AChI (in distilled water), 275 μL of phosphate buffer, and 100 μL of plant extract at varying concentrations (10, 50, 100, 1000, and 2000 $\mu\text{g/mL}$). For the reaction cuvette, the composition was identical, except that 275 μL of buffer was replaced with 275 μL of AChE enzyme solution (0.16 U/mL). This cuvette was used to assess the enzyme activity in the presence of plant extracts. The reaction mixtures were incubated and monitored spectrophotometrically at 405 nm for 5 min, with absorbance recorded every minute. All assays were performed in triplicate. The reaction rate ($\Delta\text{OD}/\text{min}$) was determined from the slope of absorbance over time. Enzyme activity was then calculated and expressed as nanomoles of thiocholine produced per minute per gram of tissue using the following equation:

$$\text{Enzyme activity} = \frac{\Delta\text{OD}/\text{min} \times V_r}{\epsilon \times V_e} \times \frac{V_{\text{ext}}}{W_{\text{tissue}}} \times P \times 1000$$

Where:

V_r = total reaction volume; V_e = volume of enzyme used; ϵ = extinction coefficient of DTNB-thiocholine complex ($\approx 13\,600\text{ M}^{-1}\text{ cm}^{-1}$); V_{ext} = total volume of enzyme extract; W_{tissue} = fresh weight of tissue (g); P = total percent content.

Percentage inhibition by extracts was determined by comparison of velocities in treated vs. untreated (buffer only) reaction cuvettes:

$$\% \text{Inhibition} = \left(1 - \frac{\vartheta_{\text{sample}}}{\vartheta_{\text{control}}}\right) \times 100$$

Where relevant, the enzyme kinetics were interpreted according to the Michaelis–Menten model:

$$\vartheta = \left(\frac{V_{\text{max}}[A]}{K_m + [A]}\right) \times 100$$

In which V_{max} is the maximum reaction velocity; $[A]$ is substrate concentration; and K_m is the Michaelis constant (substrate concentration

at half-maximal velocity).

3. Molecular docking study

3.1 Preparation of ligands

The 3D molecular structures of compounds were generated by means of Chem3D 15.0 running a windows workstation equipped with an Intel (R) Core (TM) i5–3340 M processor. The optimized 3D structures were saved in .pdb format and imported to the workspace for docking studies.

3.2 Preparation of enzymes

The crystal structure of human acetylcholinesterase (AChE) was retrieved from the Protein Data Bank (PDB ID: 3i6m, resolution: 2.35 Å) and selected as the target protein for molecular docking studies. This structure was chosen based on its high resolution, co-crystallized ligand presence, and relevance to Alzheimer's disease therapeutics, as it represents the human enzyme with a known inhibitor.

AutoDockTools (ADT) was employed to prepare both ligand and receptor structures by adding appropriate Gasteiger and Kollman charges and identifying ligand rotatable bonds. Water molecules were removed from the enzyme structure to minimize non-specific interactions during docking calculations. The binding site was defined based on the co-crystallized ligand position with the following center coordinates: $x = 2.470$, $y = 65.903$, $z = 64.982$. It encompasses two main subsites: the catalytic anionic site (CAS) containing the catalytic triad (Ser200, His440, Glu327) and the peripheral anionic site (PAS) including aromatic residues Trp84, Trp279, Tyr70, Tyr121, and Phe330. The gorge connecting these sites has a depth of approximately 20 Å.

Molecular docking simulations were performed using AutoDock 4.2 with the Lamarckian genetic algorithm. The docking parameters were set as follows: population size of 150, maximum number of evaluations of 27,000, maximum number of generations of 2500,000, mutation rate of 0.02, crossover rate of 0.8, the number of docking runs of 100 per ligand and exhaustiveness of 8. Grid maps were generated using Auto-Grid with a spacing of 0.375 Å, and the grid box was centered on the active site with dimensions $30 \times 30 \times 30$ Å, sufficient to encompass the entire binding pocket. Docking validation was performed by re-docking the co-crystallized ligand, achieving an RMSD of <2.0 Å from the

experimental binding pose.

All molecular docking calculations were performed in triplicate to ensure reproducibility. For each compound-enzyme complex, 100 independent docking runs were conducted, and the binding energies are reported as mean $\pm$ standard deviation. The most favorable binding pose from each run was selected based on the lowest binding energy and cluster analysis.

3.3 Physicochemical and pharmacokinetic characteristics

The physicochemical and pharmacokinetic properties of the isolated derivatives were predicted using SwissADME (<http://www.swissadme.ch/>). This tool facilitated a comprehensive assessment of several pharmacokinetic parameters, notably absorption, distribution, metabolism, and excretion (ADME) (Fu et al. 2024). Using the BOILED-Egg model, additional predictions were made regarding blood–brain barrier (BBB) permeability, passive gastrointestinal absorption (HIA), and interaction with permeability glycoprotein (P-gp), identifying compounds as potential substrates or non-substrates (Fu et al., 2024).

4. Results and discussion

4.1 Structure of isolated compounds

After column chromatographic fractionation of the crude extract on silica gel, three pure compounds and one mixture were obtained. The structures of these compounds were elucidated by the mean of 1D-NMR (^1H -NMR, ^{13}C -NMR, and DEPT) and 2D-NMR (HSQC and HMBC) analysis, and therefore identified by comparison with reported data in the literature. Thus, pure compounds were identified as β -sitosterol (1) (Chouna et al. 2015), scopoletin (3) (Wisnu et al. 2013) and D-mannitol (4) (Bealem et al. 2021) (Fig. 1). Furthermore, the mixture has been identified as β -sitosterol (1) and stigmasterol (2) (Nyemb et al., 2018), two quite inseparable steroids, in the ratio 2:1. It is important to note that, to the best of our knowledge, it is the first report of stigmasterol (2) and scopoletin (4) from this plant species. However, β -sitosterol (1) has already been reported in the stem bark of this plant (Chouna et al. 2015) while Bealem et al. (2021) reported the isolation of D-mannitol (4) from the $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1:1) crude extract of the leaves of the plant, collected in Chad.

The primary aim of this study was to evaluate the acetylcholinesterase (AChE) inhibitory activity of compounds isolated from *Crossopteryx febrifuga*, a plant species with limited phytochemical investigation but promising pharmacological potential. The crude extracts and the isolated compounds, β -sitosterol, stigmasterol, scopoletin, and D-mannitol were assessed through *in vitro* AChE inhibition assays, and their potential molecular interactions with AChE were further explored via molecular docking.

The *in vitro* results revealed that the crude extracts of stem bark and trunk wood exhibited moderate inhibitory activity, with IC_{50} values of 424.3 $\mu\text{g/mL}$ and 480.0 $\mu\text{g/mL}$, respectively. Among the isolated compounds, the mixture of β -sitosterol and stigmasterol showed the most potent activity ($\text{IC}_{50} = 50.85 \mu\text{g/mL}$), followed by β -sitosterol ($\text{IC}_{50} = 57.25 \mu\text{g/mL}$) and scopoletin ($\text{IC}_{50} = 58.97 \mu\text{g/mL}$). D-mannitol demonstrated negligible inhibition ($\text{IC}_{50} = 4955.64 \mu\text{g/mL}$), consistent with its lack of reported anticholinesterase activity in the literature. All those compounds presented very low activities comparing to galantamine ($\text{IC}_{50} = 8.30 \mu\text{g/mL}$) use here as positive control. The IC_{50} value obtained for β -sitosterol is in good agreement with previous findings. For instance, Ahmad et al. (2016) reported an IC_{50} of approximately 55 $\mu\text{g/mL}$ for β -sitosterol isolated from *Polygonum hydropiper*, indicating reproducible moderate activity against AChE. This consistency supports the reliability of the current experimental approach for this compound. Stigmasterol isolated from *Phormidium retzii* exhibited an IC_{50} of 0.214 μM ($\sim 0.088 \mu\text{g/mL}$), as reported by Mansour et al. (2021), indicating much stronger inhibitory potential. The mixture of β -sitosterol and

Table 1

Results of score value.

Compounds	Docking score values (kcal/mol)	Cluster size	Interacting residues
L0	-12.1 ± 0.3	85/100	van der Waals - A chain: GLY119 Conventional Hydrogen Bond - A chain: SER200, GLU199 Carbon Hydrogen Bond - A chain: HIS440, TYR121 Pi-Sigma - A chain: TRP84, TYR334 Amide-Pi Stacked - A chain: GLY118 Pi-Alkyl - A chain: TRP233, PHE288, PHE290, PHE330, PHE331
Galantamine	-8.8 ± 0.2	72/100	Conventional Hydrogen Bond - A chain: GLY119, SER200, TYR121, SER122 Carbon Hydrogen Bond - A chain: PHE330, TYR70 Pi-Alkyl - A chain: PHE331 Pi-Sigma - A chain: TRP84, PHE330, PHE331 Pi-Alkyl - A chain: TYR334, HIS440
β -Sitosterol	-10.4 ± 0.3	71/100	Conventional Hydrogen Bond - A chain: TYR130 Carbon Hydrogen Bond - A chain: SER122 Pi-Pi Stacked - A chain: TRP84 Conventional Hydrogen Bond - A chain: ARG289 Pi-Sigma - A chain: TRP84, PHE331, TYR334 Pi-Alkyl - A chain: PHE330, TRP279
Scopoletin	-7.3 ± 0.5	45/100	Conventional Hydrogen Bond - A chain: TYR130 Carbon Hydrogen Bond - A chain: SER122 Pi-Pi Stacked - A chain: TRP84 Conventional Hydrogen Bond - A chain: ARG289 Pi-Sigma - A chain: TRP84, PHE331, TYR334 Pi-Alkyl - A chain: PHE330, TRP279
Stigmasterol	-10.5 ± 0.4	68/100	Conventional Hydrogen Bond - A chain: TYR70, ASN85, SER122, TYR334
D-Mannitol	-5.0 ± 0.6	23/100	

stigmasterol exhibited the most potent acetylcholinesterase (AChE) inhibitory activity among all tested samples, with an IC_{50} value of 50.85 $\mu\text{g mL}^{-1}$. This enhanced activity may be attributed to a possible synergistic effect between the two structurally similar phytosterols. Both β -sitosterol and stigmasterol share a sterol backbone with minor differences in side-chain unsaturation, which can influence their interaction with the active or peripheral anionic site of AChE. The stronger inhibition observed for the mixture, compared to each compound alone, suggests that co-occurrence may enhance binding affinity or modulate enzyme conformation more effectively than individual components. Previous reports have shown moderate AChE inhibitory activity for β -sitosterol (Ahmad et al., 2016) and a more potent effect for stigmasterol under specific assay conditions (Mansour et al., 2021). However, studies focusing on their combined activity remain limited. The present findings highlight the importance of evaluating natural compound mixtures, as they may better reflect the bioactive potential of crude plant extracts, where such compounds naturally co-exist. This observation supports the hypothesis that phytosterol combinations may contribute significantly to the overall cholinesterase-inhibitory profile of plant-based therapeutics and underscores their potential as lead compounds for further development.

Scopoletin, a coumarin derivative, showed an IC_{50} of 58.97 $\mu\text{g/mL}$ (approximately 307 μM) in our assay. However, previous studies reported significantly lower IC_{50} values for scopoletin. Zheng et al. (2016) recorded an IC_{50} of $10.18 \pm 0.68 \mu\text{M}$, suggesting higher potency. The lower activity observed here may be due to solubility limitations, compound stability under assay conditions, or matrix effects such as interaction with residual non-active components. The weak activity of D-mannitol confirmed the specificity of the assay. Its high IC_{50} value

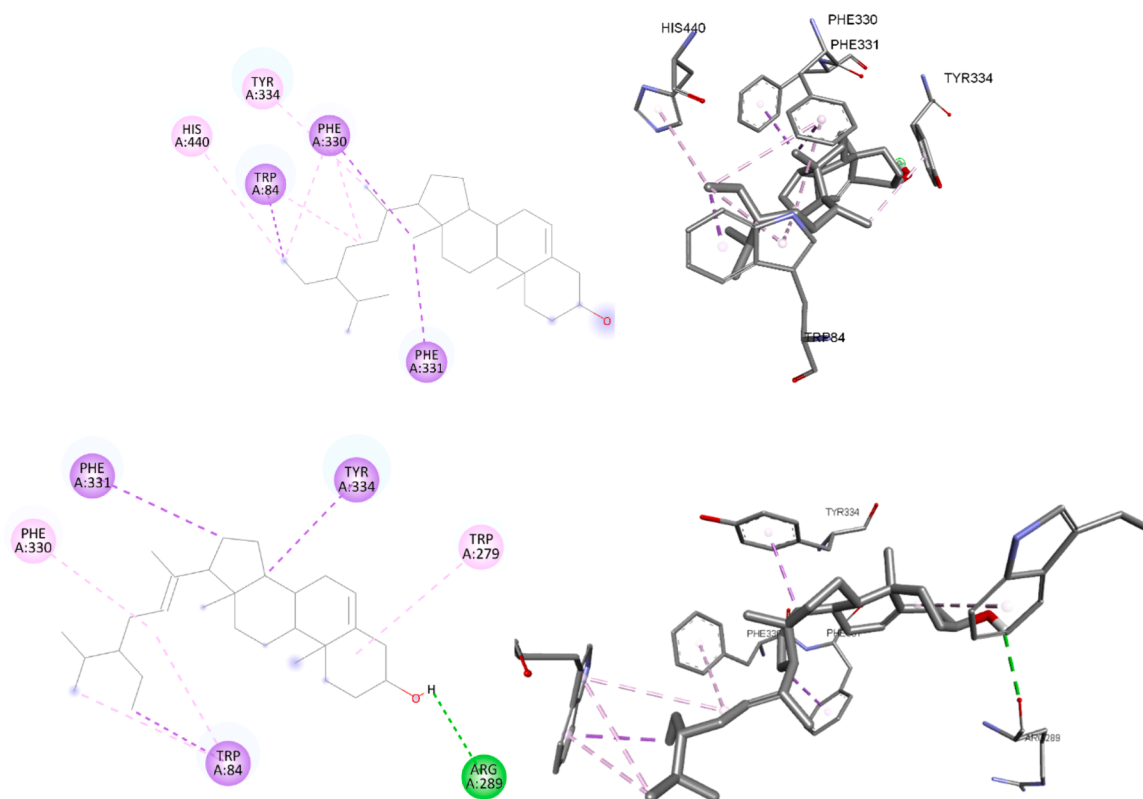


Fig. 2. Pharmacophore model of AChE inhibitors.

aligns with previous reports indicating a lack of AChE inhibitory activity (Sánchez-Rodríguez et al., 2021).

4.2 Docking study

This study performed molecular docking to investigate the potential of four compounds as inhibitors of AChE, a key enzyme in the cholinergic system, targeted for the treatment of AD. The crystal structure of AChE (RCSB ID: 3i6m) was used for docking simulations. Galantamine, a known AChE inhibitor, and the co-crystallized ligand (L0) served as references for evaluating the binding affinity and interaction patterns of the tested compounds.

The docking results expressed as binding scores (kcal/mol) and interacting residues are summarized in Table 1 and provide a basis for understanding the observed binding affinities.

L0, expectedly, exhibited the strongest binding affinity (−12.1 kcal/mol), forming a comprehensive network of interactions involving van der Waals forces, conventional hydrogen bonds with SER200 and GLU199, carbon-hydrogen bonds with HIS440 and TYR121, pi-sigma interactions with TRP84 and TYR334, an amide-pi stacked interaction with GLY118, and pi-alkyl interactions with TRP233, PHE288, PHE290, PHE330, and PHE331. This diverse range of interactions highlights the importance of these residues within the active site for effective AChE inhibition.

Galantamine, used as a positive control, showed a good binding score of −8.8 kcal/mol, primarily interacting with the catalytic site through conventional hydrogen bonds with GLY119, SER200, TYR121, and SER122. Additional interactions included carbon-hydrogen bonds with PHE330 and TYR70, and pi-alkyl interactions with PHE331.

Among the docked compounds, two of them demonstrated promising binding affinities comparable to or exceeding that of galantamine (Fig. 2). It is the case of stigmasterol (2) that exhibited the highest binding score (−10.5 kcal/mol), followed by β -sitosterol (1) (−10.4 kcal/mol), suggesting significant potential for AChE inhibition. These

highest binding affinities were attributed to interactions with key residues including ARG289, TRP84, PHE331, TYR334, PHE330 and TRP279 for stigmasterol (2); TYR334, HIS440, PHE330, PHE331 and TRP84 for β -sitosterol, through conventional hydrogen bonds, and a diverse range of other interactions such as carbon-hydrogen bonds, pi-donor hydrogen bonds, pi-sigma, pi-pi stacked, and pi-alkyl interactions.

While binding scores provide a relative measure of affinity, the specific interactions between the ligands and the enzyme are crucial for understanding the inhibitory mechanism. Analysis of the interacting residues revealed common interaction points amongst the high-affinity binders. Residues such as TRP84, TYR334, PHE330, and PHE331 appeared frequently involved in pi-sigma and pi-alkyl interactions, suggesting their importance in stabilizing ligand binding within the active site.

Notably, scopoletin (−7.3 kcal/mol) exhibited comparatively lower binding affinities. This could be attributed to fewer interactions with key residues within the active site or unfavorable binding poses. Further investigation of these compounds' structures and interactions could provide insights for future optimization.

4.3 ADME study

ADME analysis is very essential in the evaluation of the pharmacodynamic activities of a drug candidate. ADME properties constitute the pharmacokinetic profile of a drug molecule. According to the results obtained from this analysis (Table 2), scopoletin (3) stands out significantly in several properties when compared to the other molecules in the dataset. With a molecular weight of 192.17, it is the smallest molecule among the four, and its lower number of heavy atoms (14) and aromatic heavy atoms (10) suggests that it has a simpler structure than the other molecules, particularly β -sitosterol (1) and stigmasterol (2), which both have more complex structures and higher molecular weights as given in Table 2. The fraction of sp^3 hybridized carbons (0.1) is much lower in scopoletin (3) compared to β -sitosterol (1) and stigmasterol (2),

Table 2
ADME properties of isolated derivatives (1–4).

Molecule	1	2	3	4
MW	414.71	412.69	192.17	182.17
#Heavy atoms	30	30	14	12
#Aromatic heavy atoms	0	0	10	0
Fraction Csp3	0.93	0.86	0.1	1
#Rotatable bonds	6	5	1	5
#H-bond acceptors	1	1	4	6
#H-bond donors	1	1	1	6
MR	133.23	132.75	51	37.93
TPSA	20.23	20.23	59.67	121.38
iLOGP	5.05	5.08	1.95	0.52
XLOGP3	9.34	8.56	1.38	−3.1
WLOGP	8.02	7.8	1.51	−3.59
MLOGP	6.73	6.62	0.76	−2.77
Silicos-IT Log P	7.04	6.86	1.94	−1.91
Consensus Log P	7.24	6.98	1.51	−2.17
ESOL Log S	−7.9	−7.46	−2.36	1.31
ESOL Solubility (mg/ml)	5.23E-06	1.43E-05	8.32E-01	3.75E+03
ESOL Solubility (mol/l)	1.26E-08	3.46E-08	4.33E-03	2.06E+01
ESOL Class	Poorly soluble	Poorly soluble	Soluble	Highly soluble
Ali Log S	−9.67	−8.86	−2.24	1.12
Ali Solubility (mg/ml)	8.90E-08	5.71E-07	1.12E+00	2.38E+03
Ali Solubility (mol/l)	2.15E-10	1.38E-09	5.80E-03	1.31E+01
Ali Class	Poorly soluble	Poorly soluble	Soluble	Highly soluble
Silicos-IT LogSw	−6.19	−5.47	−3.17	2.57
Silicos-IT Solubility (mg/ml)	2.69E-04	1.40E-03	1.31E-01	6.71E+04
Silicos-IT Solubility (mol/l)	6.49E-07	3.39E-06	6.81E-04	3.68E+02
Silicos-IT class	Poorly soluble	Moderately soluble	Soluble	Soluble
GI absorption	Low	Low	High	Low
BBB permeant	No	No	Yes	No
Pgp substrate	No	No	No	No
CYP1A2 inhibitor	No	No	Yes	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	Yes	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No
log Kp (cm/s)	−2.2	−2.74	−6.49	−9.61
Lipinski #violations	1	1	0	1
Ghose #violations	3	3	0	2
Veber #violations	0	0	0	0
Egan #violations	1	1	0	0
Muegge #violations	2	2	1	3
Bioavailability Score	0.55	0.55	0.55	0.55
PAINS #alerts	0	0	0	0
Brenk #alerts	1	1	1	0
Leadlikeness	2	2	1	1
#violations				
Synthetic Accessibility	6.3	6.21	2.65	3.3

indicating it may be less flexible in terms of molecular rotation. As regards solubility, scopoletin (3) has excellent characteristics, being categorized as ‘soluble’ across different solubility models, such as ESOL, Ali, and Silicos-IT, with solubility values much higher than those of β -sitosterol (1) and stigmasterol (2), which are ‘poorly soluble’. This contrasts with the highly soluble D-mannitol (4), though scopoletin (3) is still significantly more soluble than the other molecules. Additionally, the bioavailability score of scopoletin (3) (0.55) is consistent with β -sitosterol (1) and stigmasterol (2), despite its low gastrointestinal absorption rate (classified as high).

In terms of pharmacokinetics, scopoletin is the only one among the four to be blood-brain barrier (BBB) permeant, which is a notable feature, especially when considering its potential for neurological applications. The ability of scopoletin to inhibit CYP1A2, along with its

solubility and moderate toxicity profile, also sets it apart. Despite these advantages, scopoletin still has some significant challenges in its synthetic accessibility (rated 2.65, indicating moderate ease of synthesis), and its higher number of violations in the Ghose and Muegge rules (0 and 1 violations, respectively) indicate certain areas of optimization are needed for ideal drug development.

Overall, scopoletin presents a unique blend of favourable properties, especially its solubility and BBB permeability, but some issues related to its synthetic accessibility and minor rule violations require additional optimization compared to the other molecules in the group.

In Fig. 3, each plot visualizes the relative magnitude of the physicochemical properties for each molecule, with red lines indicating their respective values. β -Sitosterol (1) exhibited high lipophilicity and moderate flexibility, while scopoletin (3) demonstrated lower values in flexibility and solubility, suggesting a simpler molecular structure. These radar plots provide a comparative analysis of the molecules based on their key physicochemical characteristics.

Fig. 4 illustrates the relationship between WLOGP (lipophilicity) and TPSA (topological polar surface area) for various molecules, with the aim of assessing their potential for blood- BBB permeability. Scopoletin is positioned within the yellow-shaded region, signifying its potential to cross the BBB. In contrast, β -sitosterol and stigmasterol lie outside the BBB permeability range, suggesting these molecules cannot effectively target the brain. The plot also indicates that one molecule falls outside the acceptable range, as noted in the remarks section. Additionally, the presence of PGP+ and PGP- symbols identifies whether the molecules are likely to be substrates of the P-glycoprotein pump, which impacts their absorption and distribution. The graph serves as a visual tool for evaluating the pharmacokinetic properties of the molecules under study.

5. Conclusion

The phytochemical investigation of crude extracts from the stem bark and trunk wood of *C. febrifuga* led to the isolation and identification of four compounds: β -sitosterol (1), a mixture of β -sitosterol (1) and stigmasterol (2), scopoletin (3), and D-mannitol (4). Notably, scopoletin is reported here for the first time in this plant species. The *in vitro* acetylcholinesterase (AChE) inhibition assays revealed that the crude extracts exhibited moderate activity, with IC₅₀ values of 424.3 μ g/mL (stem bark) and 480.0 μ g/mL (trunk wood). Among the isolated compounds, the mixture of β -sitosterol and stigmasterol demonstrated the strongest inhibitory activity (IC₅₀ = 50.85 μ g/mL), followed by β -sitosterol (IC₅₀ = 57.25 μ g/mL) and scopoletin (IC₅₀ = 58.97 μ g/mL). D-mannitol showed negligible inhibition (IC₅₀ = 4955.64 μ g/mL), indicating minimal interaction with the target enzyme. These experimental results were consistent with *in silico* molecular docking studies. Stigmasterol and β -sitosterol showed favorable binding affinities toward AChE, comparable to the reference inhibitor galanthamine, whereas D-mannitol had the lowest binding score. Scopoletin, despite its smaller size and simple structure, also showed a meaningful binding interaction. Additionally, scopoletin stood out for its excellent solubility and blood brain barrier (BBB) permeability, unique among the four isolated compounds—making it a particularly attractive candidate for neurological applications. Taken together, scopoletin (3) demonstrates a compelling balance of drug-like properties and bioactivity, highlighting its potential as a lead compound for the development of novel AChE inhibitors. Future efforts should include structure-activity relationship (SAR) studies to improve potency and selectivity. This study illustrates the value of integrating experimental and computational approaches to identify and validate natural compounds as potential therapeutics for neurodegenerative diseases such as Alzheimer’s disease.

Disclosure statement

The authors report no competing interests to declare.

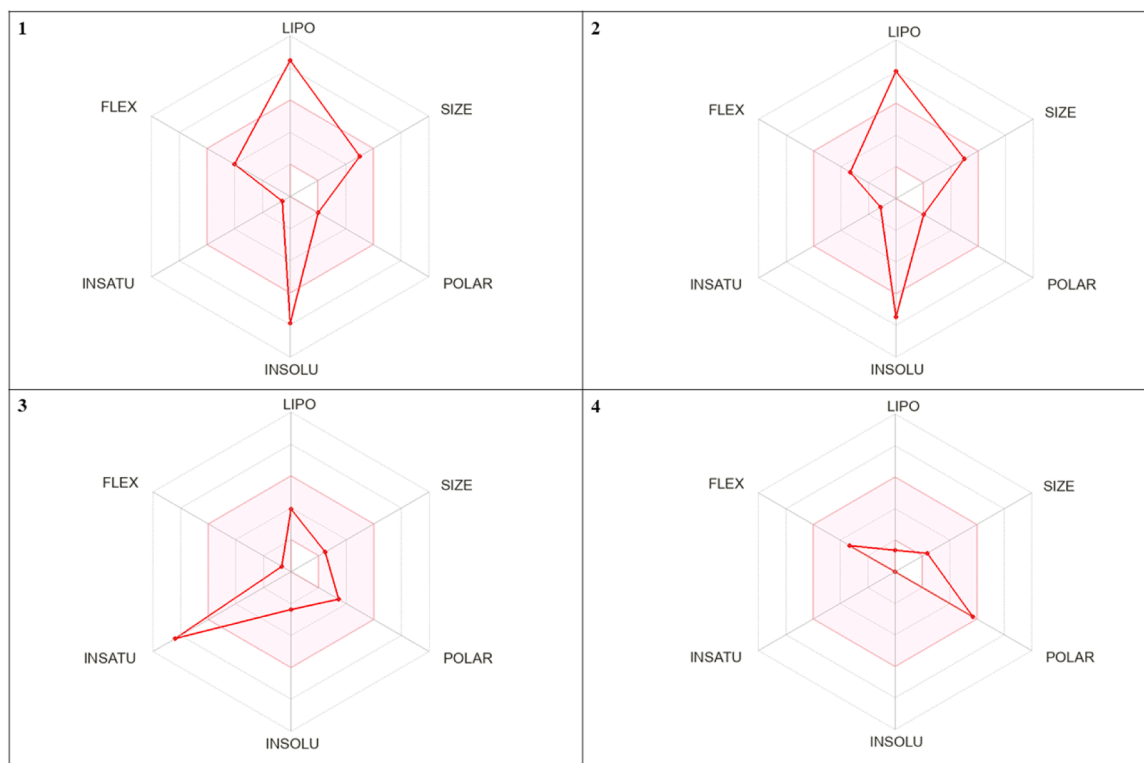


Fig. 3. Radar chart presentation of isolated derivatives (1–4).

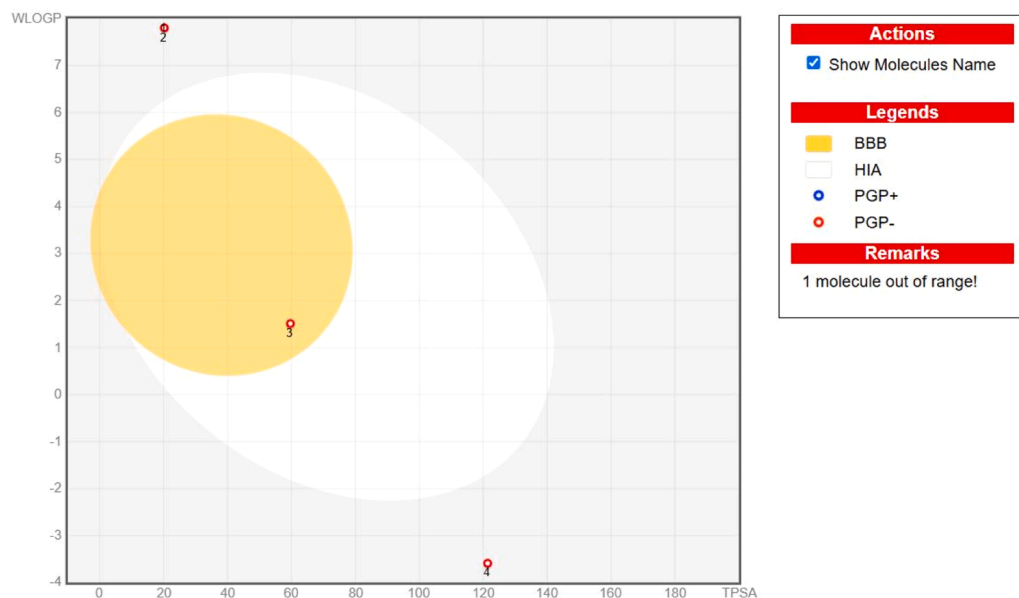


Fig. 4. Boiled egg presentation of isolated derivatives (1–4).

CRedit authorship contribution statement

Samuelson Martin Luther King Boum Bindebe: Writing – review & editing, Software, Methodology, Investigation, Data curation, Conceptualization. **Jean Noël Nyemb:** Writing – review & editing, Supervision, Software, Conceptualization. **Joël Abel Gbaweng Yaya:** Writing – original draft, Methodology, Investigation, Data curation. **Hervé Landry Ketsemen:** Writing – original draft, Methodology, Investigation, Data curation. **Syeda Abida Ejaz:** Writing – original draft, Methodology, Investigation, Data curation. **Hafiz Muhammad**

Attaullah: Writing – original draft, Methodology, Investigation, Data curation. **Céline Henoumont:** Writing – original draft, Methodology, Investigation, Data curation. **Sophie Laurent:** Writing – original draft, Methodology, Investigation, Data curation. **Emmanuel Talla:** Writing – original draft, Methodology, Investigation, Data curation. **Elisabeth Ngo Bum:** Writing – original draft, Methodology, Investigation, Data curation. **Marcello Iriti:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.sajb.2025.10.042](https://doi.org/10.1016/j.sajb.2025.10.042).

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