



Cholinergic Inhibition and Antioxidant Potential of *Gongronema latifolium* Benth Leaf in Neurodegeneration: Experimental and In Silico Study

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Abstract

The use of *Gongronema latifolium* for the management of various forms of neurological disorders has generated a lot of interest in the need to further investigate its neurotherapeutic constituents. This work, therefore, focused on assessing the inhibitory potential of selected bioactive components derived from *G. latifolium* against key neurotherapeutic targets and oxidant species associated with neurodegeneration using in vitro analysis and biomolecular modelling. *G. latifolium* methanol extract (GLME), solvent partition, chromatographic fractions (A-F) of GLME and pregnane compounds (Iloneoside and marsectohexol) derived from fraction-B with the highest activity were investigated for in vitro acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and monoamine oxidase (MAO) inhibition in addition to their in vitro antioxidant activities. The interactions of iloneoside, marsectohexol, and reference drugs with human acetylcholinesterase, butyrylcholinesterase, and β -secretase (BACE-1) were further assessed using molecular docking, binding free energy calculations, cluster analysis, and molecular dynamics simulations. The GLME and fractions inhibited the activities of both acetylcholinesterase and butyrylcholinesterase in a dose-dependent manner. Iloneoside and marsectohexol exhibited in vitro concentration-dependent inhibitory activities against acetylcholinesterase ($IC_{50} = 19.28, 184.9 \mu M$, respectively) and butyrylcholinesterase ($IC_{50} = 30.75, 43.4 \mu M$, respectively). These compounds also possess ferric ion-reducing, hydroxyl, and superoxide radical-scavenging activities. Iloneoside had the highest docking scores of $-9.8, -9.9 -9.4$ Kcal for AChE, BChE, and BACE1, respectively. The stability of the interaction of the bioactive compounds with the catalytic residues of the protein targets was preserved in a 100 ns molecular dynamics simulation. Iloneoside, a rare pregnane glycoside, was identified as a neurotherapeutic constituent of *G. latifolium* leaf. Further studies are suggested to investigate the neurotherapeutic potential in animal models.

Keywords *G. latifolium* · Acetylcholinesterase · Antioxidants · Pregnanes · Iloneoside · Molecular docking and dynamic simulation

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Introduction

Neurodegenerative disorders (NDDs) are a group of illnesses characterized by a steady deterioration of the structure and function of the neurological systems in humans, with a resultant decline in the brain function, decrease in the quality of life of the patients and ultimately leading to death [1]. Dementia, Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease are some of the most significant NDDs that need to be addressed worldwide, particularly in older people [1].

Targeting many etiological actors of NDDs has been a recent method for the development of new medicines for

NDDs [2]. Neurotransmitter reduction in the brain, oxidative stress, inflammation, mitochondrial malfunction, apoptosis, hereditary factors, and misfolding of proteins that tend to aggregate—all of which can contribute to neuronal degeneration—are examples of pathogenic processes implicated in neuronal degeneration [3]. The neurodegenerative alterations linked to NDDs are thought to be caused by a decrease in neurotransmitters such as acetylcholine, butyrylcholine, and neuroactive amine in the brain. The absence of acetylcholine is a distinguishing characteristics of AD, for this reason, there are available treatments that enhances cholinergic transmission [4]. Cholinesterase inhibitors are necessary because increased cholinesterase activity, which catalyses the breakdown of acetylcholine and butyrylcholine, may worsen cholinergic deficiency and cognitive decline. By increasing the amount of acetylcholine and butyrylcholine in the synaptic cleft, these inhibitory techniques will enhance neuronal transmission inside the brain. In this regard, a number of cholinesterase inhibitors have been developed as treatments for the management of NDDs [5]. These cholinesterase inhibitors are also used to treat non-degenerative diseases (NDDs), such as dementia and lewy bodies, which are characterized by a decrease in cholinergic transmission [6]. Parkinson's disease is characterized by the loss of dopamine in the significant nigra pars compacta and dopaminergic neuron [7]. Additionally, some of the symptoms of Parkinson's disease (PD) and other NDDs can be relieved by taking inhibitors of enzymes such monoamine oxidase (MAO), which catalyses the oxidative deamination of dopamine [8].

Additionally, another aetiology cause for AD and PD is the aggregation of misfolded proteins, which leads to the deadening of neurons and the stultification of synaptic function [9]. The β -secretase enzyme (BACE1), is primarily responsible for cleaving the transmembrane amyloid protein to generate the β -stubs. This process is thought to be the rate-limiting catalytic step that generates A β [10]. Therefore, it is possible that BACE1 enzyme inhibitors will provide a therapeutic alternative for AD management and therapy.

Furthermore, oxidative stress, especially those associated with accumulation of metal ions in the brain has been linked to NDDs [3]. Despite the fact that iron is an essential component for many physiological processes, including the production of proteins and cofactors, too much free iron can lead to oxidative stress, particularly in the brain, where antioxidative defense is comparatively compromised [11, 12].

Several culinary herbs and medicinal plants, which have long been used to treat Alzheimer's disease and other NDDs, are well known for their neuroprotective and antioxidant properties [13], including *Gongronema latifolium* Benth [14].

The usage of *G. latifolium* leaf in ethnobotanical medicine to treat a variety of neurological conditions by native of African descent has been established [15–17], together with experimental data from several reported ethnopharmacological activity [18–20].

Quick in vitro and in silico techniques are useful for determining out how the bioactive components of these plants interact with certain disease targets in order to identify therapeutic medicinal products and preventative nutraceuticals. Molecular docking, molecular dynamics modelling, and quantitative structure-activity relationships (QSARs) are a few examples of computational techniques that have proven useful in the accurate prediction of bioactive components found in medical and culinary herbs [21, 22]. In silico computer examination of natural molecules has led to the suggestion of several anti-Alzheimer's drugs [23, 24].

Green leafy vegetables like *G. latifolium*, which are popular in Nigeria and other parts of Africa, have been shown to have antioxidant and neuroprotective qualities [25–27]. However, prior research, including computational screening, has indicated that this GL may have polypharmacological potential in NDDs [24, 27]. Information on the investigation that involves isolated compounds and bioactive fractions from *G. latifolium* on is scarce. Therefore, combining computational research and in vitro testing might yield further empirical support for this plant's ethnopharmacological applications. The aim of the present study is to combine both computational and invitro analysis to identify the neurotherapeutic constituent in *G. latifolium* leaf. The current work employs in vitro techniques to examine the inhibitory potential of different solvent fractions, chromatographic fractions, extracts, and pregnane compounds derived from *G. latifolium* on the in vitro activities of antioxidants, monoamine oxidase, and cholinesterase. The molecular interactions of the isolated pregnanes and pregnane glycosides with human acetylcholinesterase, butyrylcholinesterase, and BACE-1 proteins, were also analysed using molecular docking, binding free energy calculations, cluster analysis, and molecular dynamics simulations.

Materials and Methods

Plant Material

The leaves of *G. latifolium* were collected from Okuku, Osun State, in the south-western region of Nigeria. It lies between longitude 04 °E and 05°5 and latitude 05° 55'8 N and 08° 07' W. Mr. Bolu Ajayi of the Plant Biology department at the University of Ilorin in Nigeria, identified

the leaves with a deposited voucher sample (UILH/002/1176).

Extraction, solvent partitioning and chromatographic fractionation

The 80% methanol leaves extract of *G. latifolium* was obtained according to the method described by Harbone [28]. The comprehensive methods for the extraction, solvent portioning, chromatographic separation, isolation and characterization of pregnanes has been previous reported in our previous manuscript [29]. Extracts, fractions and pregnanes obtained from our previous study, was used in this study. In a summary of these procedure, the column chromatographic fractionation of the ethyl acetate extract on silica gel, using different solvent (*n*-Hex-CHCl₃, EtOAc, acetone, and MeOH) gave 9 fractions (Fr. 1 - 9). However, in this study the fractions were further pulled together to give 6 fractions (Fr. A – F). The methanol extract, solvent fractions (ethyl acetate and methanol), chromatographic fractions (Fr. A – F) and pregnane compounds (iloneoside and marsectohexol) were then evaluated for the various activities.

In Vitro Analysis

Determination of cholinesterase inhibitory activities

A spectrophotometric method based on the Ellman method [30] modified with modification [31] was employed to evaluate the inhibitory activities of the methanol extracts, solvent fractions, chromatographic fractions, and pregnanes on AChE and BChE. Acetylcholine iodide and butyrylcholine iodide were used as substrates.

The inhibitory activities of the methanol extracts, solvent fractions, chromatographic fractions and pregnanes on AChE and BChE were assessed using spectrophotometric method based on Ellman method [30] as modified with modification [31], using Acetylcholine iodide and butyrylcholine iodide as substrates. Micro plates containing 50, 100, 150, 200, and 250 µg/mL of the extract, partition, and chromatographic fractions, as well as 100, 80, 60, 40, and 20 µM of the pure compounds made by serial dilution, were filled with 500 µL of 0.1 M phosphate buffer (PH 8.0), 200 µL of AChE enzyme solution, and 100 µL solution of 3.3 mM 5,5-dithio-bis (2-nitrobenzoic) acid. The mixture was then incubated for 20 minutes at 25 °C.

After 20 minutes, the mixture was mixed with 100 µL of 0.05 mM acetylthiocholine iodide, and the AChE activity was measured at 412 nm within 3 minutes at room temperature.

For measuring BChE activity, the same methodology was used with butyrylthiocholine as the substrate [31]. The

enzyme's inhibitory activity was calculated and shown as a percentage of inhibition. Methanol served as the negative control, and duplicates of the experiment were performed.

$$\text{Inhibition \%} = [(\text{Abs}_{\text{cont}} - \text{Ab}_{\text{sam}}) / \text{Abs}_{\text{cont}}] \times 100 \quad (1)$$

where Abs_{cont} is the change in absorbance of the negative control, and Ab_{sam} is the change in absorbance of the sample.

Determination of monoamine oxidase inhibitory (MOA) activities

The extracts' and partition fractions' monoamine oxidase inhibitory activity were ascertained using a slightly modified version of a previously published procedure [32]. The reaction mixture contained 250, 200, 150, 100, and 50 µg/mL solutions of water-dissolved test samples, semi-carbazide (12.5 mM), benzylamine (10 mM at pH 7), and 75 µL of enzyme (MAO). After 30 minutes, 250 µL of acetic acid was added, and the mixture was centrifuged after boiling for three minutes in a water bath. Before being incubated for 10 minutes at room temperature, the resulting supernatant (1 mL), 0.05% 2, 4-DNPH (1.25 mL), and 1.25 mL benzene were combined. The separated benzene layer of the combination above and equal quantities of sodium hydroxide (1 N) were combined, and the mixture was heated for 10 minutes at 80 °C. After the alkaline layer was decanted, duplicate measurements of the absorbance at 450 nm were made [33]. The inhibition in percentage was calculated as follows:

$$\text{Inhibition \%} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Samples}}) / \text{Abs}_{\text{Control}}] \times 100 \quad (2)$$

Antioxidant activities determination

Ferric ion reducing power The ferric ion reducing power of the best chromatographic and pregnanes (iloneoside and marsectohexol) was measured following previously reported method with slight modifications [34]. 0.7 mL of various concentrations (100, 80, 60, 40, 20 and 0 µM) of pregnanes solution and (0, 20, 40, 60, 80 and 100 µg/mL) of fraction B were mixed with 0.7 mL phosphate buffer (50 mM; pH 7.0) followed by 0.5 mL of (1% w/v K₃Fe(CN)₆). The mixture was incubated for 20 minutes at 50°C and centrifuged at 3000 rpm for 10 min after the addition of trichloroacetic acid (10% w/v). Distilled water (0.5 mL) and 0.1 mL of FeCl₃ (0.1% w/v) were added to 0.5 mL of the separated upper layer. The absorbance of the resultant mixture was measured in duplicates at 700 nm against blank.

Superoxide radical scavenging activity The superoxide anion scavenging activity of the best chromatographic and pregnanes (iloneoside and marsectohexol) was assessed following the method described by Robak and Gryglewski [35]. To generate superoxide radicals, the oxidation of NADH in a PMS/NADH system was adopted and assayed through reduction of nitro blue tetrazolium (NBT) to a purple formazan. A constitutive mixture of phosphate buffer (20 mM; pH 7.4), NADH (73 μ M), NBT (50 μ M), PMS (15 μ M) and solution of various concentrations (0, 20, 40, 60, 80 and 100 μ M) of pregnanes and fraction B (100, 80, 60, 40, 20 and 0 μ g/mL) were used for the assay. The absorbance was recorded at 562 nm, after incubation at room temperature for 5 minutes. Ascorbic acid was used as positive control.

The superoxide radical scavenging activities (%) was computed as:

$$[(Abs_{control} - Abs_{sample}) / Abs_{control} \times 100] \quad (3)$$

Determination of hydroxyl radical scavenging activity The hydroxyl radical-scavenging activity of the best chromatographic fraction and pregnanes was evaluated using the method described by Smirnoff and Cumbe [36]. A 3.0 mL constitutive reaction mixture containing 0.3 mL sodium salicylate (20 mM), 0.7 mL hydrogen peroxide (6 mM), 1.0 mL $FeSO_4$ (1.5 mM), and 1.0 mL of various concentration (100, 80, 60, 40, 20 and 0 μ g/mL) of pregnanes and Fr. B (0, 20, 40, 60, 80 and 100 μ M) were prepared. The various reaction mixtures were incubated for 60 min at 37 °C and the absorbance of the hydroxylated salicylate complex formed was read at 562 nm.

Calculation: The percentage activity was computed as:

$$\% \text{ Hydroxyl radical scavenging activity} = \left[1 - \frac{(A_1 - A_2)}{A_0} \right] \times 100 \quad (4)$$

Where : A_0 = absorbance (control)
 A_1 = absorbance (test compounds)
 A_2 = absorbance (without sodium salicylate)

Statistical analysis

The Data were analyzed by one-way ANOVA, Duncan's post hoc test was used for multiple comparisons, in IBM SPSS Statistics version 23.0 (IBM Corp., Armonk, N.Y., USA). Difference at $p < 0.05$ was considered significant. Graphs were created using Microsoft excel for windows 2016.

In silico studies

Retrieval and preparation of proteins The protein data bank with the website (<http://www.rcsb.org>) provided the 3D structures of human butyrylcholinesterase (hBChE) in complex with propidium (PDBID: 6ESJ), human acetylcholinesterase (hAChE) in complex with donepezil (PDBID: 4EY7), and human β -secretase (BACE-1) in complex with aminoquinoline pH (PDBID: 5I3V). The native ligand and all the water molecules that accompanied the crystalized protein were removed, and MGL-AutoDockTools (ADT, v1.5.6) was used to add the missing hydrogen atoms. The partial atomic charge was added along with the Kollam charges. [37]. This procedure was applied to all the proteins.

Ligands preparation The two pregnancies (iloneoside and marsectohexol) and the co-crystallized and/or reference inhibitors (Donepezil and Propidium) were evaluated using the Structure Data Format (SDF) available from the PubChem database at www.pubchem.ncbi.nlm.nih.gov. ChemDraw 19.0 (CambridgeSoft, Cambridge, MA, USA) was used to sketch structures that were not present in the database. The chemicals that were drawn were stored in SDF format. Openbabel was used to convert the ligands' SDF format (downloaded and drawn) to PDB (protein data base) format [38]. To convert the ligands to PDBQT format, PyRx 0.8's AutoDock function tools were utilised [39]. To convert the ligands from PDB to PDBQT in PyRX, we use the "convert to autodock format" command.

Targeted molecular docking *Validation of Docking Protocol:* Using BIOVIA Discovery Studio Visualizer 20.1.0, San Diego, CA, USA 2020, the co-crystallized reference compounds were extracted from their corresponding crystal structures and saved in PDB format. The ligands that were retrieved from the proteins were docked into the reference active site of the corresponding protein. Using the software, the docked conformation with the least minimum energy was chosen and superimposed on the native ligand that had been retrieved. The same software was used for other analysis [40, 41].

After validation of the docking protocol, the isolated pregnane compounds and reference inhibitors (donepezil and propidium) were docked into the active site of (hAChE) and (hBChE) using the AutoDock Vina function incorporated in PyRx 0.8 [39]. The ligands were imported and minimized with OpenBabel in PyRx 0.8 [38]. Universal Force Field (UFF) was used as the energy reduction parameter, while conjugate gradient descent was used as the optimisation technique. The grid box around the amino acids that interacted with the co-crystallized compounds in

the binding pocket was used to define the catalytic pockets of each enzyme. A grid box with the size and centre shown in Table S1 defines the mapped-out area. The Discovery Studio Visualizer version 16 was used to view the molecular interactions.

Molecular dynamics The apo enzymes (4EY7, 6ESJ and 5I3V), and the complexes of the iloneoside with the enzymes were evaluated for structural stability using Molecular Dynamic (MD) simulation for 100 ns on GROMACS 2019.2 employing the GROMOS96 43a1 forcefield on the WebGRO [42–44]. For the docking process, the same techniques as described in our earlier publication were employed [45–47]. The CHARMM36m force field was used to determine the parameters for ions, water molecules, and amino acids in proteins. Conversely, the small molecules were parameterized using the CHARMM general force field (CGenFF) tool, which is part of CHARMM-GUI [48–50]. GROMACS 2020.3 was employed for the simulation [42]. Using the TIP3P water model with a 1 nm padding, the systems were solvated in a cubic box. NaCl ions were then added to the system at a concentration of 0.154 M. An MD package was used to carry out the simulation, applying periodic boundary conditions (PBC) in three directions. Initially, the maximum permitted force of 100 KJ.mol⁻¹.nm⁻¹ and the number of steps set to 100,000 minimization steps were utilised to minimise the system's potential energy and remove atomic collisions using the steepest descent technique. We began with an NVT ensemble, in which the volume, temperature, and number of atoms are all held constant. We then used the V-rescale approach to equilibrate the temperature to 310 K, while maintaining an atmospheric pressure of one by the use of a Berendsen barostat. [51]. Lastly, an NVT ensemble production run lasting 100 ns was executed. The hydrogen-bonded atoms' bond lengths were limited in every step by the LINear Constraint Solver (LINCS) algorithm [52]. The Particle Mesh Ewald (PME) technique was utilised to calculate electrostatics, with a 1.2 nm cutoff. Using a leap-frog approach, the Newtonian equations of motion were integrated, with a time step of 1 femtosecond for the equilibration stages and 2 femtoseconds for the production steps. Every 0.1 ns of the production run a frame was recorded, summing to 1000 frames per system. A frame was recorded every 0.1 seconds during the production cycle, for a total of 1000 frames per system [53]. The Root mean square deviation (RMSD) for the protein alone, the ligand alone, and the protein-ligand complex, root mean square fluctuation (RMSF) of C α atoms, solvent accessible surface area (SASA), radius of gyration (RoG), number of hydrogen bonds, and separation of center of mass of ligand and protein were all computed.

Binding free energy calculation using MM-GBSA Using the Molecular Mechanics Generalised Born Surface Area (MM-GBSA) method and decomposition analysis to obtain the binding energies of amino acids within 0.5 nm of the ligand using the gmx MMPBSA package, the binding free energy of the two top docked phytochemicals from the initial docking analysis were further determined [54, 55]. The methods used was same as those published in our previous manuscripts [47, 56].

Clustering of molecular dynamic trajectory The trajectories derived from MD simulation of the protein-ligand complexes that were used in TtClust V 4.9.0, an elbow technique clustering analysis programme. Conformers that function as representative structures were selected following the conformation clustering.

Protein-Ligand Interaction profiler (PLIP) [57] was used for the interactive analysis. During the cluster analysis, the amino acid residues were automatically renumbered to begin at number 1 and go all the way up to the last number in the protein sequence, in contrast to the downloaded crystalized structures that had gaps in the numbering.

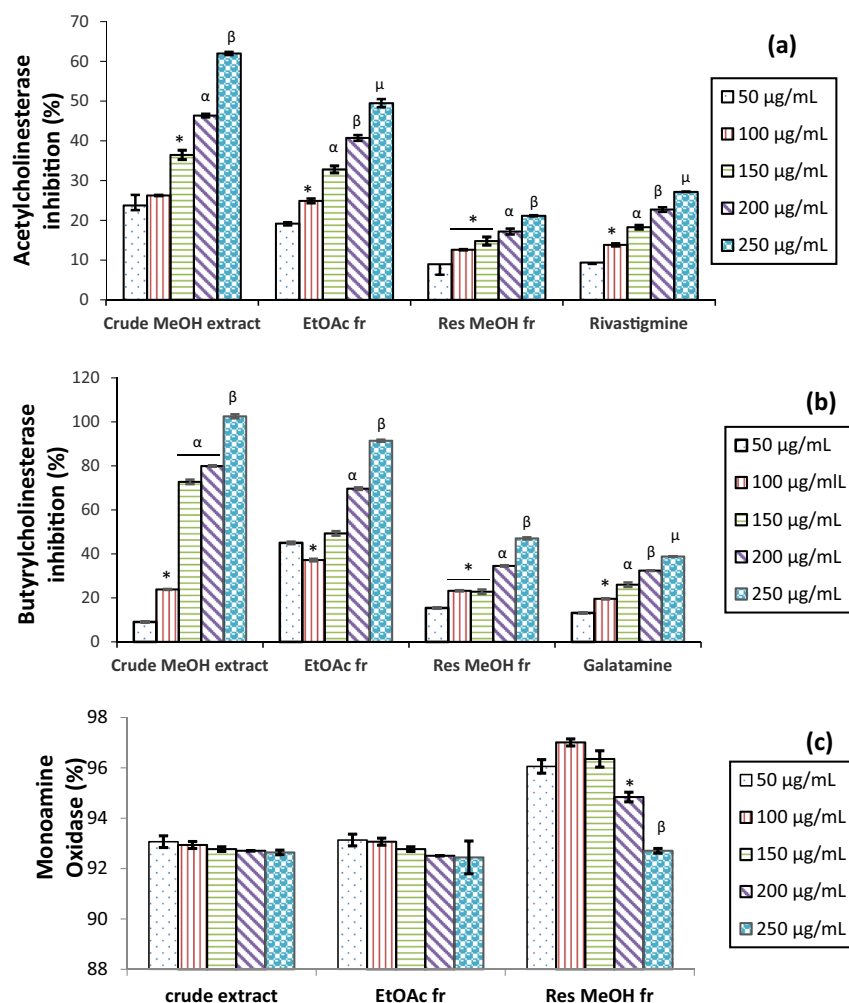
Results

In Vitro Studies

Inhibitory activities of cholinesterase and monoamine oxidase

The results of the inhibitory effect of the MeOH extract, solvent partition fractions, chromatographic fractions and pregnanes on AChE, BChE and monoamine oxidase activities using in vitro inhibition assays showed a concentration-dependent (CD) inhibition of the activities of AChE and BChE by the extracts and solvent fractions (Fig. 1a, b), while the extracts and solvent did not show a CD inhibition of monoamine oxidase activity (Fig. 1c). The evaluation of their IC₅₀ revealed that the crude methanol extract, ethyl acetate solvent fraction, residual methanol fraction and rivastigmine (reference compound) had an IC₅₀ of (AChE = 174.5, 153.0, 144.0 and 148.3 μ g/mL), while the crude methanol extract, ethylacetate solvent fraction, residual methanol fraction and galatamine (reference compound) had an IC₅₀ of (BChE = 135.5, 183.3, 178.3 and 143.5 μ g/mL) respectively. In the case of AChE, it was observed that the two partition fractions (ethyl acetate and residual methanol fractions) had lower IC₅₀ than the crude extract, though the crude extract had a higher percentage inhibition than the fractions, while an opposite trend was observed for BChE. The chromatographic fractions gotten from the fractionation of the ethyl acetate fraction were further analyzed for their inhibitory activities having shown

Fig. 1 **a** Acetylcholinesterase **(b)** butyrylcholinesterase **(c)** monoamine oxidase inhibitory activities of the extract and solvent fractions of *G. latifolium* (Utazi) leaves and reference compounds. Data is expressed as mean $\pm$ SD. MeOH-methanol extract; EtOAc-ethylacetate fraction; Res MeOH-residual fraction after partitioning with n-hexane and ethyl acetate. For each bioactive component bars with different symbol differ significantly ($p < 0.05$)

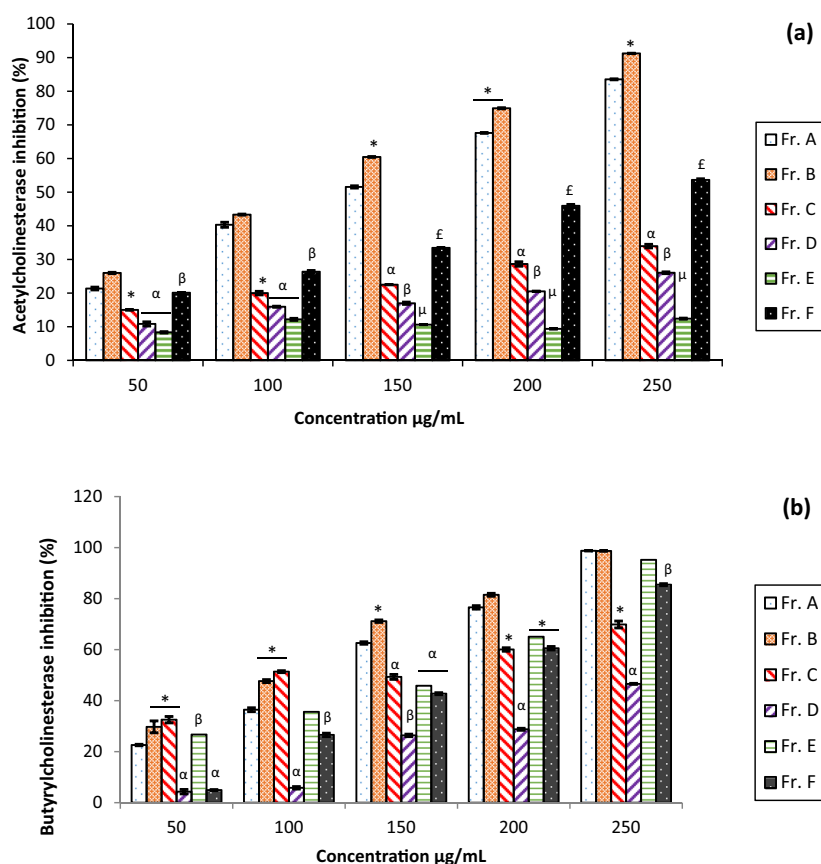


the higher percentage inhibition for both AChE and BChE. The various chromatographic fractions (Fr. A to F) showed a concentration dependent inhibition of AChE and BChE with IC_{50} of (AChE = 140.2, 139.7, 153.0, 149.0, 155.4 and 155.9 μ g/mL) (Fig. 2a) and (BChE = 147.5, 134.9, 145.5, 160.6 179.5 and 146.5 μ g/mL) (Fig. 2b). Fraction B (Fr. B) exhibited the highest percentage inhibition and lowest IC_{50} to both enzymes compare to other fractions. Evaluation of the inhibitory activity of the two pregnane isolates (iloneoside and masectohexol), which were previously isolated from the pregnane-rich fractions in our laboratory, showed that they moderately inhibited the activities of AChE and BChE as shown in Fig. 3. A further evaluation of their IC_{50} revealed that, iloneoside and masectohexol had an IC_{50} of (AChE = 19.28, 184.9 μ M) (BChE = 30.75, 43.4 μ g/mL) respectively. The result suggests a better inhibition of BChE than AChE as both pregnanes demonstrated higher percentage inhibition. Also, iloneoside had a better inhibitory potential than masectohexol to both enzymes as indicated by the higher percentage inhibition for AChE and lower IC_{50} values for both enzymes (Fig. 3).

In vitro antioxidant activities

The chromatographic fractions with the highest AChE and BChE inhibitory activities (fraction B), along with its two pregnane isolates, were evaluated for ferric ion-reducing power, hydroxyl radical (OH), and superoxide (O_2^-) scavenging activities. The OH-scavenging activity of fraction B, iloneoside, and masectohexol is shown in Fig. 4a, while the IC_{50} is shown below the graph. The three test samples exhibited concentration-dependent hydroxyl radical scavenging activity, with fraction B having the highest OH. scavenging activity. Iloneoside exhibited higher radical-scavenging potential than masectohexol ($P < 0.05$). The O_2^- scavenging activities of fraction B, iloneoside and masectohexol are presented in Fig. 7, while the IC_{50} is shown below the graph. The three test samples exhibited concentration-dependent O_2^- scavenging activity, with fraction B having the highest activity. Iloneoside did not show a significant difference in radical scavenging activity when compared to masectohexol ($P < 0.05$) (Fig. 4b). The ferric ion-reducing activity of fraction B, iloneoside, and

Fig. 2 a Acetylcholinesterase (AChE) **(b)** Butyrylcholinesterase (BChE) inhibitory effects of chromatographic fractions of *G. latifolium* (Utazi) leaves. Fractions (Fr. A to Fr. F) are chromatographic fractions of the EtOAc fraction of *G. latifolium* (Utazi) leaves. Data is expressed as (mean $\pm$ SD). For each concentration, fractions with different symbol differ significantly ($p < 0.05$)



marsectohexol is presented in Fig. 4c, while the IC_{50} is shown below the figure. Fraction B, iloneoside, and marsectohexol showed dose-dependent ferric ion-reducing activity. The ferric ion-reducing activity of iloneoside was significantly different from that of marsectohexol ($P < 0.05$). Figure 4a Hydroxyl radical scavenging (b) *Superoxide radical scavenging* (c) Ferric ion reducing activities of Fr. B: fraction B, iloneoside and marsectohexol. Lines with different number of asterisks differ significantly ($p < 0.05$) (means $\pm$ SD).

In Silico Analysis

Validation of molecular docking protocol

An essential stage in estimating the docking protocol's accuracy is validation. The first step was validating the docking procedure that would be utilised for the molecular docking analysis of the pregnane compounds to the proteins. The native ligand was aligned with the docked poses of the reference compounds (co-crystallized ligands) having the lowest binding energy. The protein's extracted conformer of co-crystallized ligands was overlaid with the chosen docked conformers from our docking study. For donepezil, propidium, and aminoquinoline compound, respectively, a very low root mean square deviation

(RMSD) of 0.5761, 0.6005, and 0.000 $\text{\AA}$ was observed (Fig. S1). With the help of BIOVIA Discovery Studio Visualizer 20.1.0 (San Diego, CA, USA 2020), the RMSD was determined.

Molecular docking study

Table 1 displays the findings of the docking process for the pregnanes compounds and reference compounds against hAChE, hBChE, and hBACE-1. The results showed that the binding energies of donepezil and propidium to hAChE and hBChE were (−12.3 and −11.2 Kcal/mol) and (−10.2 and −9.8 Kcal/mol), respectively, whereas the binding energy of aminoquinoline pH was −9.8 Kcal/mol. The best docked pregnane, iloneoside, bound to hAChE, hBChE, and hBACE-1 with binding energies of −9.8, −9.9, and −9.4 Kcal/mol, respectively. Although compared to donepezil, the binding energy of iloneoside was lower, iloneoside had comparable binding energy with propidium and aminoquinoline compounds (Table 1).

Amino acid interactions of references compounds and pregnanes with the active site of target enzymes

For interactive analysis, iloneoside and reference chemicals were used. The different binding interactions in the

Fig. 3 Acetylcholinesterase and butyrylcholinesterase inhibitory activities of iloneoside and marsectohexol isolated from *G. latifolium* (Utazi) leaves. Lines with different number of asterisks differ significantly ($p < 0.05$) Data is expressed as mean $\pm$ SD

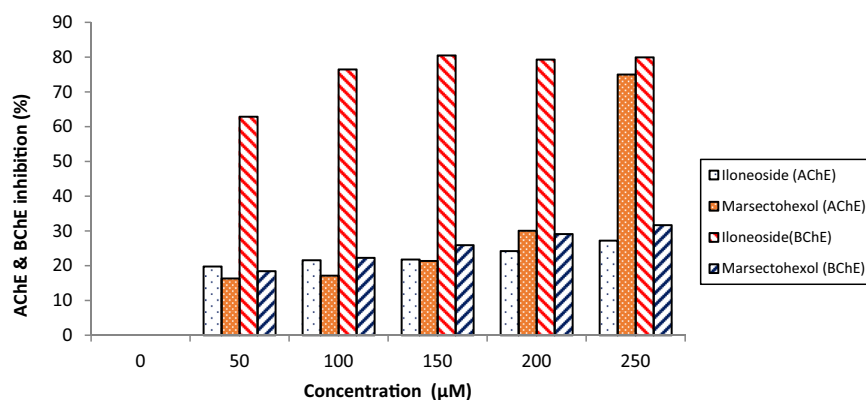
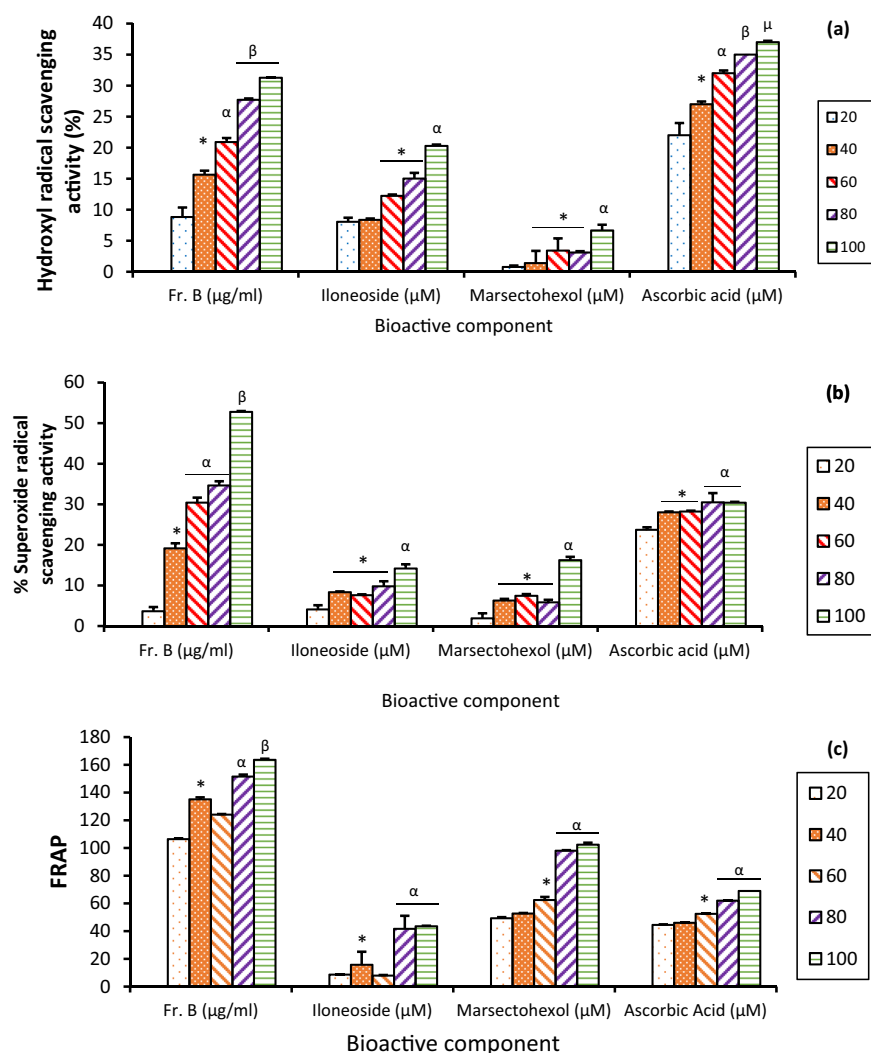


Fig. 4 Antioxidant properties (a) Hydroxyl radical scavenging activity (b) Superoxide radical scavenging activity (c) Ferric ion reducing activity of Fr. B: fraction B, iloneoside, marsectohexol and ascorbic acid. For each bioactive component bars with different symbol differ significantly ($p < 0.05$) (means $\pm$ SEM). OH: Hydroxyl radical; O₂[•]: % Superoxide radical and FRAP: ferric reducing antioxidant power



proteins' active grooves are displayed in Table 2. Donepezil was shown to be docked into the active site gorge based on interactions between its several functional groups (Fig. 5M).

The co-crystallized ligand and the revalidated docked donepezil were orientated in the gorge in comparable ways. Donepezil's 1-benzyl unit interacts with Trp86 and His447

via aromatic Pi-Pi stacking. Two pi-Alkyl interactions between the piperidine ring and hAChE's Tyr337 and Tyr341 were created. Between the 5-methoxy unit of inden-1-one, two pi-sigma bonds with Trp286 and Phe338 were observed respectively. These bonds mostly involve charge transfer and may aid in the intercalation of the

Table 1 Binding energies of pregnane compounds and reference compounds to target enzymes

	Binding energy (Kcal/mol)					
	hAChE		hBChE		hBACE-1	
S1	Donepezil (E = 306.68)	−12.3	Donepezil (E = 306.68)	−10.2		
S2	Propidium (E = 668.97)	-	Propidium (E = 668.97)	−9.8	Aminoquinoline pH (E = 291.99)	−9.4
Preg	Iloneoside (E = 1647.67)	−9.8	Iloneoside (E = 1647.67)	−9.9	Iloneoside (E = 1647.67)	−9.4
Preg	Marsectohexol (E = 1048.30)	−6.8	Marsectohexol (E = 1048.3)	−6.6	Marsectohexol (E = 1048.3)	−6.8

S reference compound, *Preg* Pregnane; after minimising, the energy (E) in KJ/mol is represented by the values in brackets

inhibitor in the binding site. The carbonyl oxygen of the indenone ring and Phe295 bonded to create the lone hydrogen bond (Fig. 5a). Iloneoside was also docked into the active site gorge of hAChE, interacting in a different pattern with the active site residues. The glycosyl units of iloneoside were oriented into the gorge, interacting with the “swinging gate” residue (Tyr337), while the 17 β -marsdenin aglycon was docked to the entrance of the gorge. A hydrogen bond was observed between the 2-hydroxyl unit of the allopyranosyl sugar and Asp74. The C6 of the allopyranosyl and oleandropyranosyl sugar units formed several alkyl contacts with Phe338, His447, leu289, Tyr124, Tyr72, Tyr341, and Trp286. The A ring of the aglycon made 2 Pi-alkyl contacts with Val294 and Trp286, while the C21-carbonyl oxygen and the 3-O-methyl oxygen of the oleandropyranosyl sugar made 2 hydrogen bonds with Gly34 and Tyr124 of hAChE (Fig. 5b).

Propidium was inserted deeply into the active site gorge of hBChE, aligning with the native ligand in the same fashion, while the acyl-binding pocket's groove accommodated the phenanthridinium ring (Fig. 6M). The amino group of the first ring of phenanthridine formed a H-bond with Ser198, while the ring also formed a Pi-Pi T-shaped and a Pi-donor hydrogen connection to Trp231.

Leu286 and Phe329 interacted via Pi-sigma and Pi-Pi T-shaped hydrophobic interaction with the first and second rings. Tyr332, a P-Site residue, and the 6-phenyl moiety connected to the phenanthridinium ring additionally formed a Pi-Pi T-shaped contact. One of the ethyl groups connected to the propidium's azanium moiety showed two Pi-sigma interactions, and the azane's nitrogen atom established a Pi-cation contact with Trp82 and an attractive charge with Glu197, respectively (Fig. 6a). Iloneoside was docked at the gorge entrance with the 12-O-tigloyl and the carbonyl moiety of the 17 β -marsdenin aglycon directed into the groove of the acyl-binding pocket. The 11 and 12-O-tigloyl units made several alkyl contacts with Trp82 of the A-site, Ala328, Try440, His432, Leu, 286, Tyr332 of the P-site, and Val288. A hydrogen bond was formed between Trp82 and the C21-carbonyl oxygen of 17 β -marsdenin. The 14-hydroxyl on the aglycon, the 2-hydroxyl of allopyranosyl,

and the 3-O-methyl of the oleandropyranosyl sugar moiety formed H-bonds with Thr120, Glu276 and Asn289, respectively (Fig. 6b).

The co-crystallized molecules and the docked aminoquinoline (3-(2-Amino-6-(3-methylpyridin-2-yl) quinolin-3-yl)-N-(3,3-dimethylbutyl)-2-methylpropanamide) (compound 1) showed the same pose and pattern. The protein's S1' and S2' sub-pockets were occupied by the reference compound, while the S3 and S3 subpocket (sp) remained accessible. Compound ('s two amino groups contributed two hydrogen atoms to Asp228 whereas Asp32 received two hydrogen atoms from both amino groups and the quinoline ring. Gly34 received a hydrogen atom from the amino group of the N-methylpropanamide group.

Tyr71 and the quinoline ring's benzyl ring formed a Pi-Pi T-shaped stacking, while Phe108 and Tyr71 and 3-methylpyridin-2-yl made two Pi-alkyl interactions, respectively (Fig. 7a). The BACE-1 enzyme's Val69 and Tyr71 were in multiple alkyl interactions with the 3,3-dimethylbutyl moiety. Iloneoside was in the substrate binding pocket in different docking modes. Iloneoside assumed an orientation that was stretched beyond the binding area of the reference inhibitor; iloneoside stretched into the S3' and S3 subpockets of the binding pocket. The carbonyl group of the 2-tigoyl moiety attached to C11 and C12 of the steride moiety of iloneoside made two hydrogen bonds with Thr231 and Tyr71 of the flap region of BACE-1. Tyr198 made 3 hydrogen bonds with the glycosyl moieties, while the B ring of iloneoside made 2 pi-alkyl contacts with Phe108 and Ile118 (Fig. 7b).

Molecular dynamics simulation of lead complexes

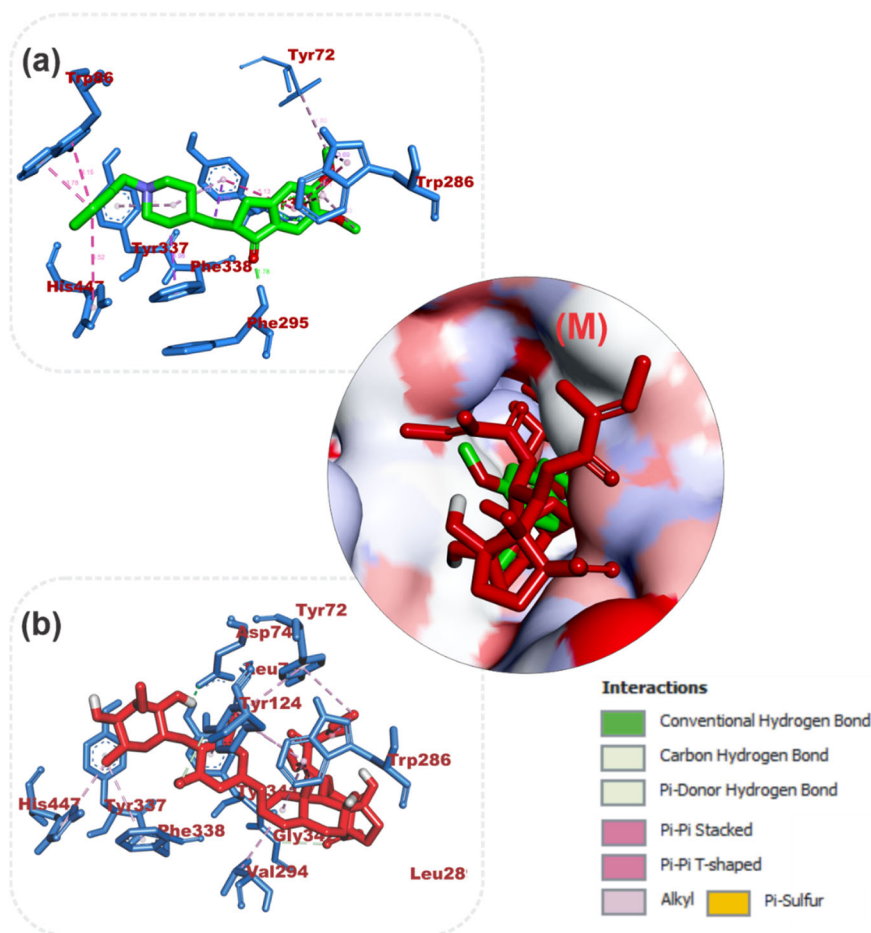
The structural integrity and stability of the complexed systems after the binding of the lead compounds and reference inhibitors were compared to the apo proteins through the analysis of the parameters (number of H-bonds, RoG, SASA, RMSF, and RMSD) that were computed from the trajectories obtained from the MD simulation using the VMD Tk console scripts. For each of the graphs plotted, the parameters were calculated as a function of the time frame.

Table 2 Molecular interactions of the amino acid residues of *human* acetylcholinesterase and *human* butyrylcholinesterase with reference inhibitors and lead pregnane compound from *G. latifolium*

Compounds	Protein	H-bonds Bond distance (Å)		Hydrophobic and other Interaction	
		Numbers of bonds	Interacted residues	Numbers of bonds	Interacted residues
Donepezil		1	Phe295 (2.88)	2	Tyr337 Phe338 Trp286 Tyr341 Trp286 Trp86 His447
Iloneoside	<i>hAChE</i>	3	Asp74 (2.57) Gly342 (3.21) Try124 (3.46)	2	His447 Phe338 Leu289 (2) Trp337 Trp286 (4) Tyr341 (2) Val294 (2) Tyr72
Propidium		2	Ser ¹⁹⁸ (2.81) Trp ²³¹ (3.03)	7	Trp82(2) Glu197 Trp82 Trp231 Leu286 Phe329(2) Tyr332
Iloneoside	<i>hBChE</i>	5	Glu276 (2.14) Asn289 (2.16) Thr268 (2.88) Trp82	10	Ala328 Try440 His432(2) Leu286 Tyr332 Val28 Ala277
PH	BACE-1	5	Gly34 Asp32(2) Asp228(2)	8	Phe108 Val69(2) Tyr71(5)
Iloneoside		6	Gly34 Tyr71 Thr231 Tyr198(3)		Tyr71(2) Ile118 Phe108

human Acetylcholinesterase: *hAChE*; *human* butyrylcholinesterase: *hBChE* and *human* β -secretase: *hBACE-1*

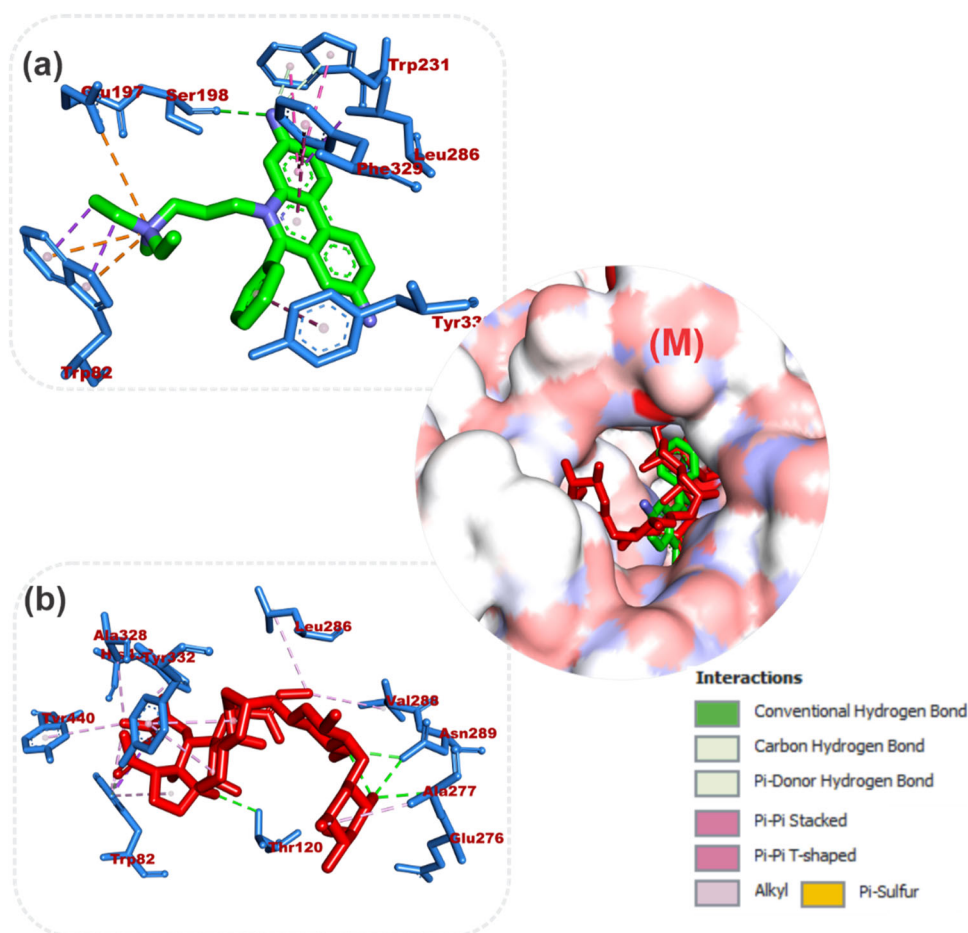
Fig. 5 Amino acid interactions of iloneoside and reference inhibitor in the active site groove of *human* Acetylcholinesterase. Stick representations of the ligands are shown by colours (a) Green: propidium (b) Red: iloneoside (M) surface representation



The plots obtained from the RMSD parameters reveal the extent of the deviation of the individual frames of the complexed structure compared to the unbound structure; hence, they are used to evaluate how stable the systems are in the dynamic environment. Figure 8 shows the RMSD plots of the three enzyme systems. The complexed systems

and the unbound system achieved equilibration around 10 ns, after which minimal fluctuations were recorded throughout the progression. All the systems for *hAChE* revealed the same pattern of RMSD, with mean RMSD values of 3.9031, 4.2362, and 4.03161 Å for the apo-enzyme, donepezil, and iloneoside complexes, respectively.

Fig. 6 Amino acid interactions of iloneoside and reference inhibitor in the active site sites of human butyrylcholinesterase. Stick representations of the ligands are shown by colours (a) Green: propidium (b) Red: iloneoside (M) surface representation



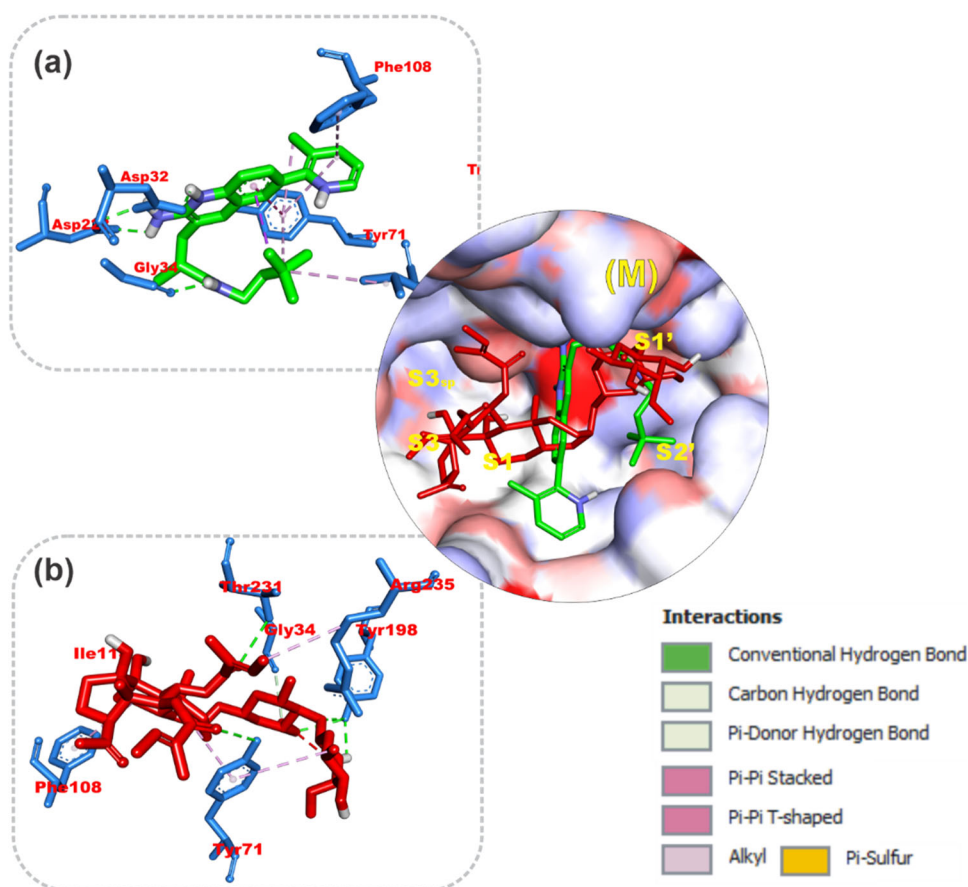
The unbound enzyme, propidium, and iloneoside complexes mean RMSD values for the hBChE systems were 3.9582, 4.111, and 3.7906 Å, respectively, whereas the BACE-1 systems had 4.2591, 3.8524, and 3.8819 Å for the unbound enzyme, compd 1, and iloneoside complexes systems (Fig. 8).

The RMSF plots reveal the flexibility of distinct areas of the protein structure. Usually, there are high spikes occurring at both the C and N terminals of the proteins, which correspond to the terminal motions. The average RMSF values for the hAChE systems are 2.0213 Å for the apo enzyme, 2.1693 and 1.8282 Å for enzymes complexed with donepezil and iloneoside, respectively. All the hAChE systems experience mild fluctuations. The hBChE systems had average RMSF values of 1.9993 for the apo enzyme, 1.4947 and 1.67555, respectively, for propidium and iloneoside complexes. The apo system experiences high fluctuations around the amino acids Val445 and Pro447 that are involved in a turn, but the binding of the compounds significantly reduces the fluctuation in that region. The average RMSF values of the BACE-1 systems were 1.7285 and 1.787 Å for the BACE-1 enzyme complexed with Iloneoside and Compd 1, and 1.932 Å for the apo enzyme (Fig. 9).

The unbound enzyme experienced the highest fluctuations around Ser58-Tyr68 that are involved in a loop, and the binding of the compounds significantly reduced the fluctuations around the amino acid residues. More compacted complexes may be seen by looking at the complex system's RMSF values, which are lower than those of the unbound system.

The RoG measures the compactness of the systems. A minimally fluctuating RoG plot is an indication of a stably folded protein structure. Figure 10 presents the RoG plots of the bound and unbound systems. Throughout the simulation period, the hBChE and BACE-1 systems displayed minimal variation, whereas the graphs for the hAChE systems revealed a constant progression. The average RoG values for the three enzyme systems were within a narrow range. The average RoG values for the unbound enzyme, donepezil, and iloneoside complexed systems are 23.206, 23.203, and 23.280 Å, respectively, as derived from the plots of the hAChE systems. The values for the apo enzyme for the hBChE are 23.0294, whereas the values for the enzyme complexed with propidium and iloneoside are 23.1665 and 23.037 Å, respectively. The average RoG values of the BACE-1 enzyme systems for the apo, compd

Fig. 7 Amino acid interactions of iloneoside and reference inhibitor in the active site of BACE-1. Stick representations of the Ligands are shown by colours (a) Green: Compound 1 (reference inhibitor) (b) Red: iloneoside (M) surface representation



1, and iloneoside complexed with BACE-1 were 21.9142, 20.8247, and 22.1471 Å, respectively (Fig. 10).

The SASA plots are often used to assess how much of the proteins' outside surface is accessible to solvents. By comparing the unfolded protein structure to its original structure, SASA and RoG plots both show how much of the structure has unfolded. The graphs for the enzyme systems obtained from the SASA study are displayed in Fig. S5. Throughout the course of the simulation, a consistent progression was noticed following the three enzyme systems' early equilibration.

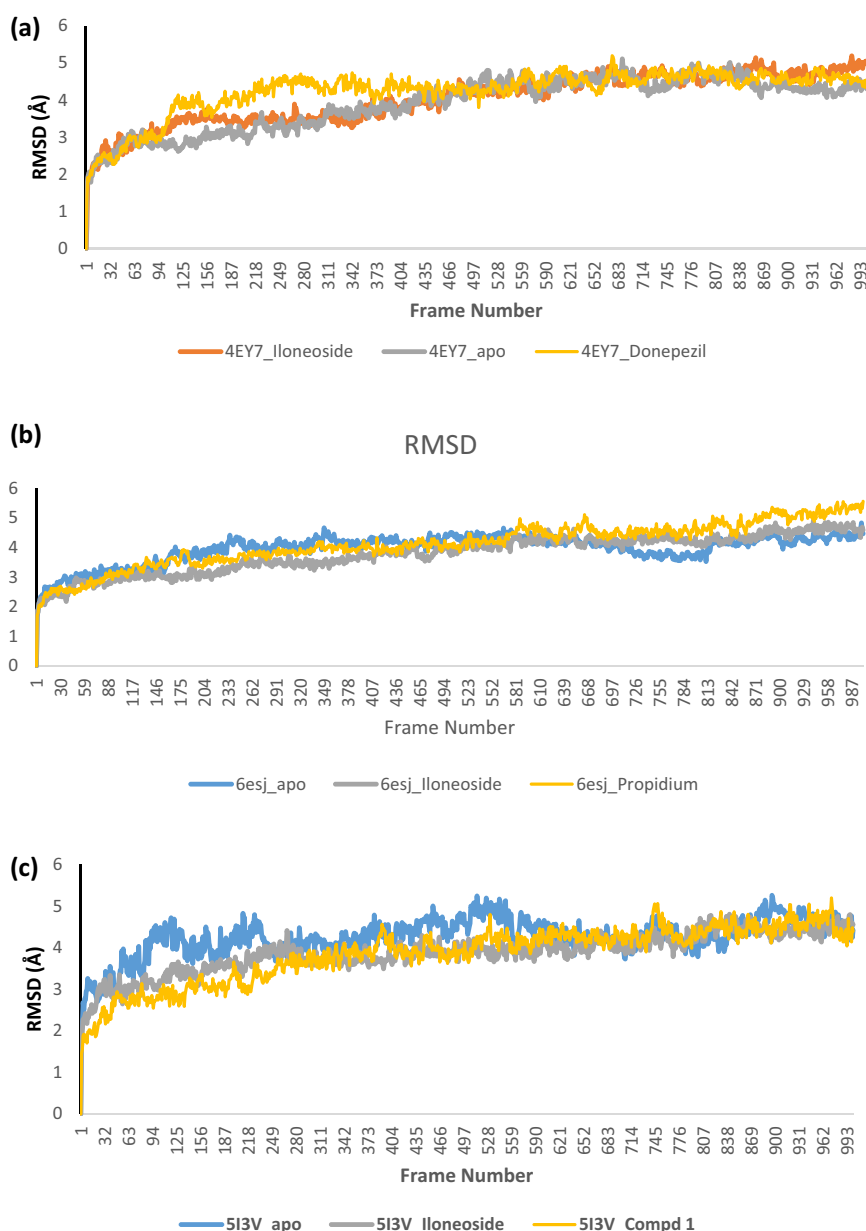
The apo-enzyme, donepezil, and iloneoside complexed with hAChE had mean SASA values of 23615.56, 24332.62 and 25539.85 Å², respectively, for the hAChE systems. The unbound enzyme, propidium, and iloneoside complexed with hBChE presented mean SASA values of 24252.01, 232731.04, and 23998.98 Å², for the hBChE enzyme system. In contrast, the BACE-1 systems experienced reduce average SASA values of 18470.53, 17750.3, and 18425.85 Å², for the apo enzyme, compd 1, and iloneoside complexed with hBChE, respectively (Fig. 11). Based on the plots of the unbound enzyme, donepezil, and iloneoside complexes, the average number of H-bonds in the hAChE system is 71.702, 70.55544, and 67.65, respectively. The unbound enzyme, propidium, and iloneoside complexes in

the hBChE enzyme system had mean numbers of hydrogen bonds of 71.1673, 68.7642, and 68.8171. whereas the average number of hydrogen bonds in the apo-BACE-1, compd-1, and iloneoside complex systems was 42.33, 47.322, and 46.623. For the hBChE and BACE-1 systems, there was a rise in the mean number of H-bonds in the complex system as compared to the apo protein, but a minor decrease in the mean number of H-bonds was seen in the complexes as compared to the ap-enzymes in the hAChE systems. The ligand-induced increase in intramolecular hydrogen bonds in hBChE and BACE-1 may be the cause of the system's observed decrease in fluctuation from RMSF plots, indicating a more stable complex (Fig. 12).

Molecular mechanics generalized born surface area (MMGBSA) analysis

Using the MMGBSA approach, the free energy of binding of iloneoside for enzymes the protein targets was calculated. Iloneoside's binding free energy was found to be −50.17, −22.48, and −39.3 Kcal/mol for the hAChE, hBChE, and BACE-1 systems, respectively. Table S2 presents the study of several components of the total binding free energy. Figure 13 shows the breakdown of the total binding free energy based on contributing amino acids and the system's energy

Fig. 8 The Backbone-RMSD plots of molecular dynamics (MD) simulation of ligands complexed to (a) *human* acetylcholinesterase (b) *human* butyrylcholinesterase (c) *human* β -secretase (BACE1)



changes across the simulation time. The analysis of various components of the total binding free energy is presented in Table S2.

Cluster analysis of trajectories of MD simulation of the enzyme-ligand complexes

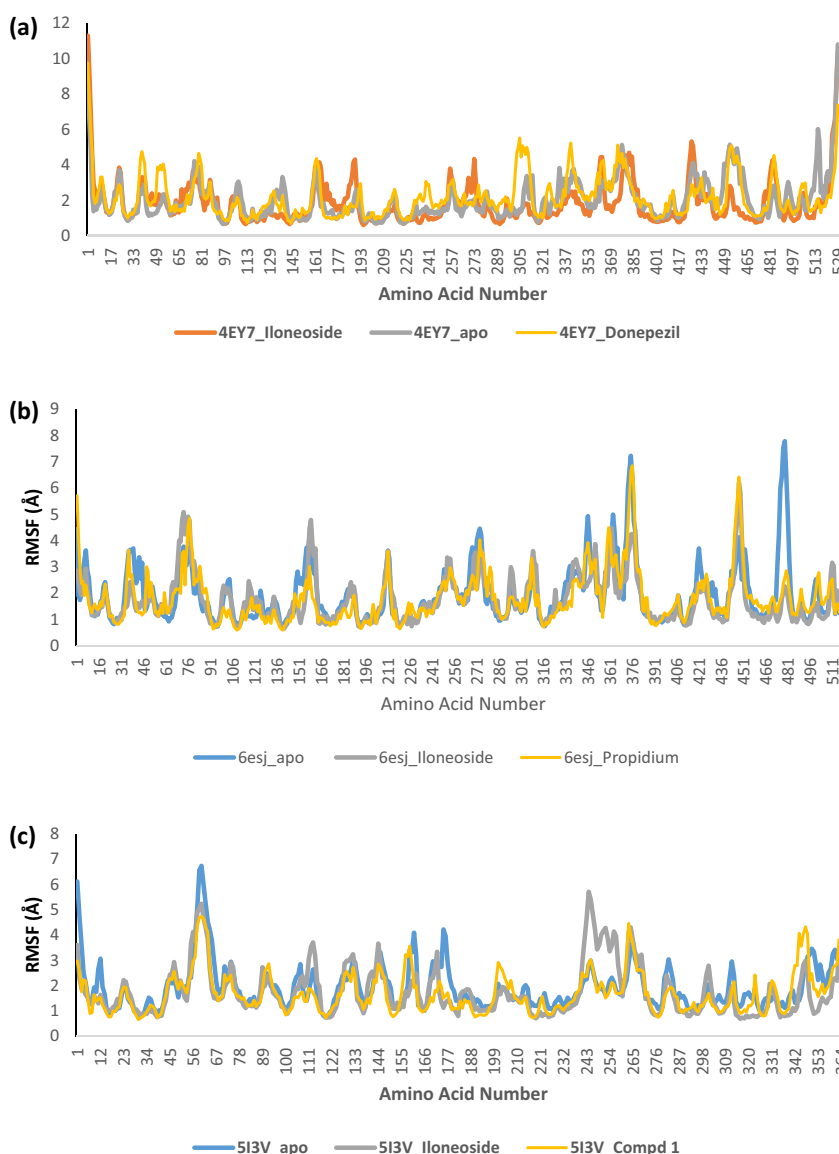
In order to study the interactions of the pregnane compounds and reference compounds with the enzymes in a dynamic environment, the trajectories of the MD simulations were clustered based on their conformational patterns during the 100 ns simulation. From each of the clusters, representative conformers were selected for further analysis (Tables S3–S5). Figure 13 shows the interactions of the

ligands in the first and last conformations of the enzymes. The tables and Figs. further show that the interactions of the ligands with important active site residues that are involved in catalysis were conserved all through the course of the simulations.

Discussion

The usage of *G. latifolium* in ethnobotanical medicine to treat a variety of neurological conditions, together with experimental data from several leaf extracts' documented ethnopharmacological activity [18, 19] informed the need to further investigate the neurotherapeutic constituents of the

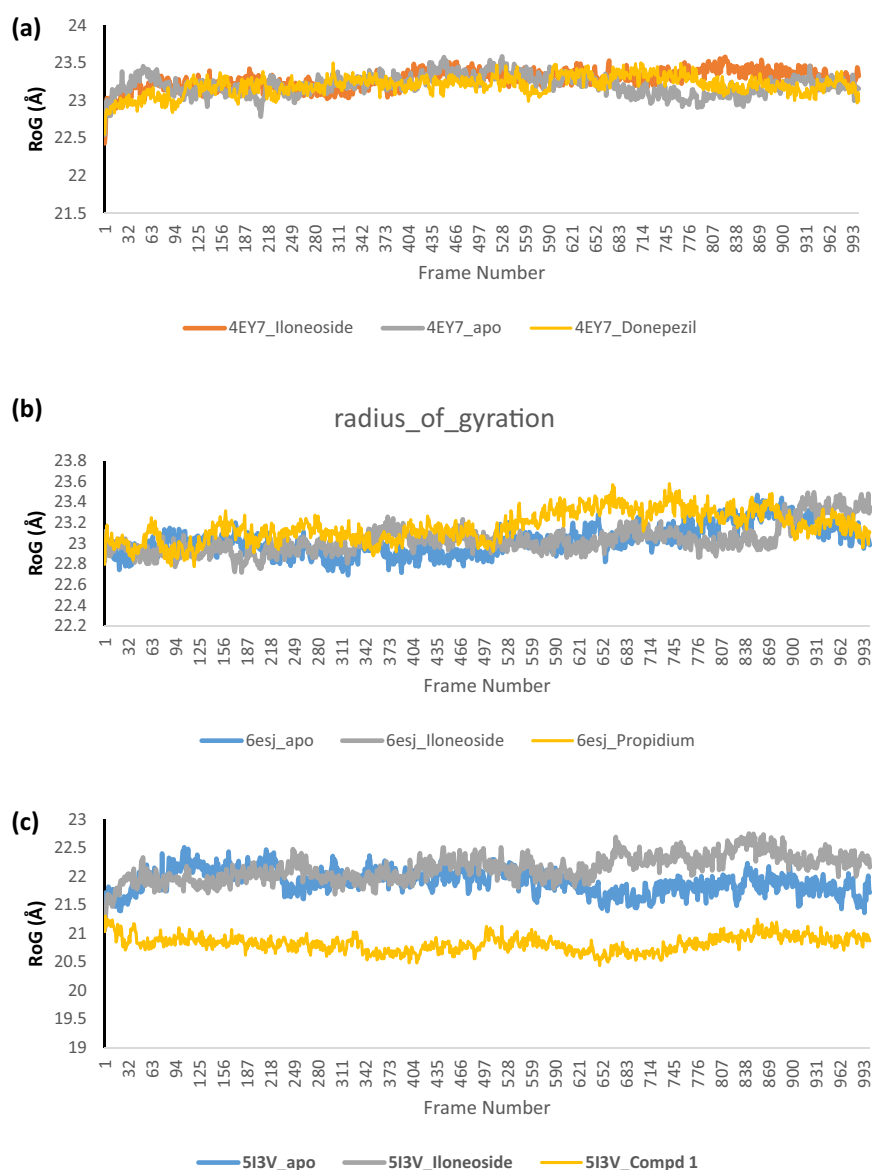
Fig. 9 Per residue RMSF plots of molecular dynamics simulation of various ligands complexed to (a) human acetylcholinesterase (b) human butyrylcholinesterase (c) human β -secretase



leaf. The present investigation revealed that the methanol extract and solvent fractions of *G. latifolium* exhibited concentration-dependent inhibitory activities of AChE and BChE. These findings were consistent with earlier research conducted on the same leaf extracts [18, 19] and other leafy vegetables [58, 59]. Compared to other extracts from plants, *G. latifolium* leaf extract presented lower IC_{50} value to a combination of fruits from *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* ($180 \pm 14.6 \mu\text{g/ml}$) [60], *Nelumbo nucifera* rhizome ($185.55 \pm 21.24 \mu\text{g/ml}$) [61], *Nardostachys jatamansi* rhizome ($130.11 \pm 12 \mu\text{g/ml}$) [61] and a higher lower IC_{50} value when compared to *Punica granatum* ($77 \pm 6.2 \mu\text{g/ml}$) [62] and *Rauwolfia serpentina* ($22 \pm 4.9 \mu\text{g/ml}$) [63]. In a recent study by [64], base of the IC_{50} of the AChE and BChE inhibitory activities of medicinal plants they were categorized as low potency

($200 < IC_{50} < 1000 \mu\text{g/ml}$); moderate potency ($20 < IC_{50} < 200 \mu\text{g/ml}$) and high potency ($IC_{50} < 20 \mu\text{g/ml}$) [65], based on this categorization, *G. latifolium* leaf presented moderate cholinesterase inhibitory activities. The chromatographic fractions obtained from the ethyl acetate fraction of the methanol extract showed different levels of AChE and BChE inhibitory activities. The best chromatographic fraction (Fr. B) was selected based on a higher percentage inhibition and a lower IC_{50} . Two pregnanes that have been previously isolated from this fraction further exhibited AChE and BChE inhibitory activities, with iloneoside having the best activities against both enzymes. The structural elucidation of these pregnanes by Gyebe et al. [29] showed that though both pregnanes consist of the 17β -marsdenin skeleton ring, iloneoside is a di-glycosylated and ditigloylated pregnane. The difference in the

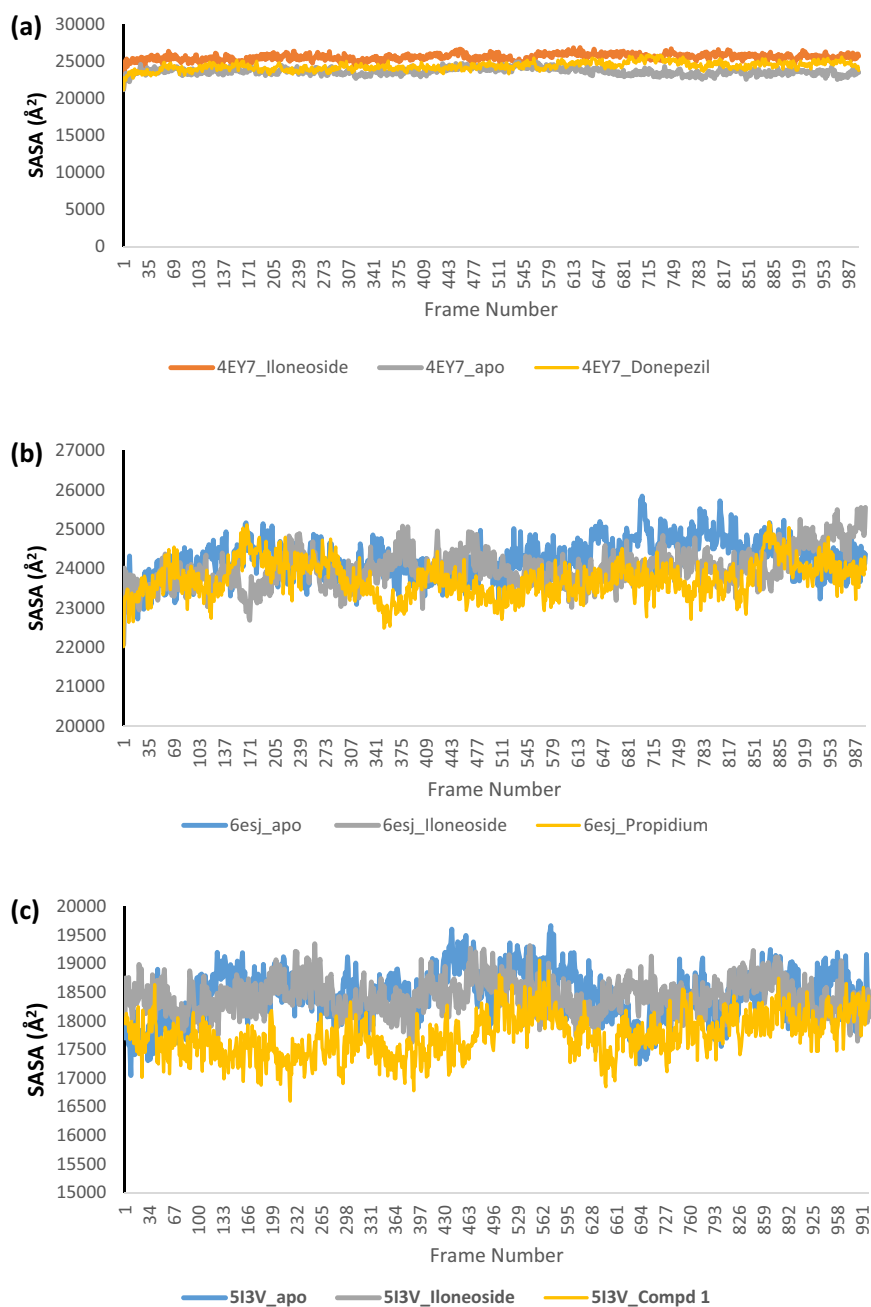
Fig. 10 The Radius of gyration plots of molecular dynamics simulation of various ligands complexed to (a) *human* acetylcholinesterase (b) *human* butyrylcholinesterase (c) *human* β -secretase



functionalized 17 β -marsdenin ring may have been responsible for the difference in activities of the pregnanes as demonstrated earlier [29, 66]. The anti-cholinesterase activities of pregnanes and pregnane glycosides isolated from other plants have been previously reported. Cynatrosides A, B, and C from *Cynanchum atratum* inhibited the activities of AChE [67]. The rate of AChE inhibition was dependent on the type of skeletal frame (aglycone) of the pregnane glycosides and the type and arrangement of the moieties [67]. In another study conducted later, the activity of AChE was inhibited in a reversible, non-competitive, and concentration-dependent manner by cynatroside B with an IC_{50} value of 3.6 μ M. Also, scopolamine-induced memory impairments in the mice were significantly ameliorated by 1.0 mg/kg body weight i.p. of cynatroside B [68]. The pregnane (iloneoside) and other phytochemicals identified

in this study may enhance the cholinergic transmission of acetylcholine by limiting the degradation of acetylcholine [69]. Furthermore, the pregnane rich fraction from which 2 pregnanes were isolated from and pregnanes had stronger inhibitory activities against BChE than AChE, this is desirable because in late-stage of AD in humans, the predominating ChE is the BChE [58]. Additionally, the inhibition of BChE activity has been reported to decelerate the formation of neurotoxic plaques, which are implicated in the development of AD [70]. As demonstrated in our present study, previous studies have also reported better inhibitory activity for the BChE enzyme than the AChE enzyme by different plant extracts [71]. The buildup of iron in the brain and the resultant oxidative stress, due to its inherent ability to cross the blood-brain barrier, is a key etiological factor that is associated with the development and

Fig. 11 The Surface Accessible Surface Area plots of molecular dynamics simulation of ligands complexed to (a) *human* acetylcholinesterase (b) *human* butyrylcholinesterase (c) *human* β -secretase

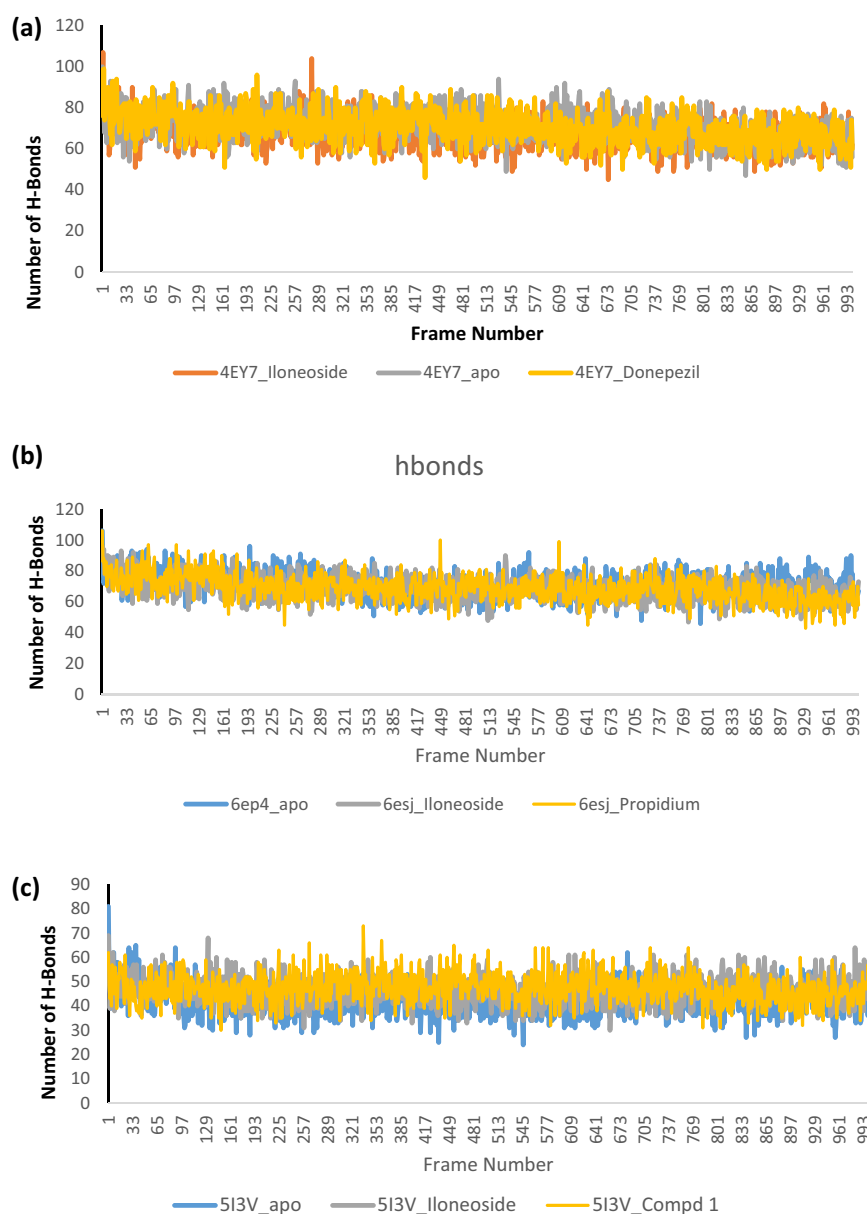


progression of NDDs [72]. Consequently, iron chelating is another strategy used for the prevention and management of NDDs. The results from this study showed that the chromatographic fractions B and the pregnanes compounds had good hydroxyl radical scavenging, ferric ion reducing activity, and superoxide radical scavenging in a dose-dependent manner. The scavenging activities of these compounds may be attributed to the various diverse functional groups in their structural moiety, including hydroxyl groups and oxygen atoms, which may serve as electron donors to oxidised intermediates, hence performing the function of antioxidants [73]. This property has been

reported to have protective effects against many infectious and NDDs such as AD and PD [74]. The extracts and fractions of *G. latifolium* leaves and the isolated compounds presented moderate antioxidant activities based on the comparison between their IC₅₀ and the reference compound (ascorbic acid) [75].

The advances in protein crystallographic techniques and the development of several virtual-screening software programmes with the inherent ability to model protein-ligand interactions have enhanced the various stages of drug design and development [76]. In order to understand the interactions at the molecular level, molecular docking

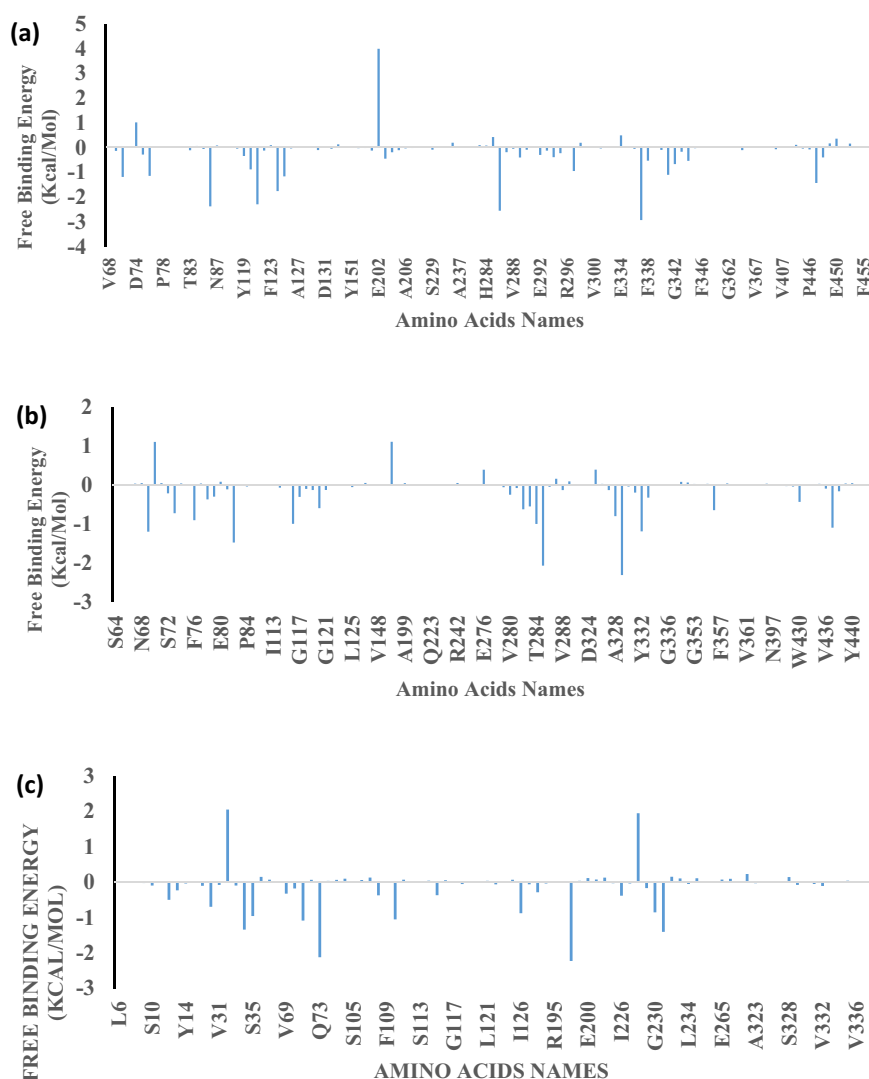
Fig. 12 The changes in the total number of H-bond throughout the trajectory of the MD simulation of the ligands complexed to (a) *human* acetylcholinesterase (b) *human* butyrylcholinesterase (c) *human* β -secretase



techniques were employed in silico. Iloneoside and marsectohexol from *G. latifolium* leaves were docked to the catalytic regions of AChE and BChE enzymes. The respective co-crystallized ligands and known inhibitors of AChE and BChE have provided insight into their catalytic mechanisms, in addition to the important residues of the enzymes that are implicated in the inhibition of their catalysis [77–79]. The results from the docking of iloneoside and marsectohexol were compared with those of reference inhibitors of the enzymes. The molecular docking results corroborated the in vitro inhibition activities, with iloneoside having a better binding affinity to both enzymes than marsectohexol. Iloneoside was found to be accommodated in the long, narrow, hydrophobic gorges of BChE and AChE [80] in comparable binding mode as donepezil

similar to the more intricate binding mechanism of donepezil in comparison to other inhibitors, these phytochemicals engaged in interactions with catalytic residues that span the active site gorge of AChE [81]. They interacted with the Glu334, His447, and Ser203 of the catalytic triad [82] and other aromatic residues that project into the catalytic gorge of AChE. Iloneoside exhibited significant π - π stacking with aromatic amino acids, particularly with P-site residues located near the rim of the gorge, which provide both inhibitory and allosteric binding sites [79]. Tyr334, Phe331, and Trp86 are the residues in the anionic choline-binding site where the interactions between the iloneoside and residues were extended. Trp86 and Try337 are two of these aromatic residues that have been shown to be crucial for the inhibition of the hAChE enzyme [77, 83]. The

Fig. 13 MM-GBSA plot of binding free energy contribution per residue human acetylcholinesterase (*hAChE*) -phytochemical complexes **(a)** *hAChE*-Iloneoside **(b)** *hBChE*-Iloneoside **(c)** BACE1-Iloneoside



primary distinction between the AChE and BChE enzymes has been identified as arising from their interactions with the P-site; this may have had a role in the different levels of binding affinities exhibited by the pregnanes and reference inhibitors [82]. It has been demonstrated that the variations in the number of aromatic residues located in the gorge—only four of the aromatic residues in the gorge of *hAChE* are present in the gorge of *hBChE*—are the cause of the differences in the specificity of ligand binding between BChE and AChE.

In accordance to the AChE enzyme's catalytic function, the P-site residues are crucial for the substrate's orientation and downward slide as it travels to the A-site, where it correctly aligns with a particular catalytic residue (serine). The acyl-binding residues (Trp236, Phe295, and Phe297) firmly enclose the acetyl portion of the substrate when it is properly positioned for catalysis [84]. Iloneoside was oriented in the gorge and interacted with residues of the A-sites Trp82, Trp231 and Phe329 (Trp86, Trp236

and Phe338 in *hAChE*) and P-sites Tyr332 (Tyr341 in *hAChE*) in a binding pattern identical to that of the propidium.

Tyr332 and Asp70, which are located near the flip of the *hBChE* active site gorge, interact with positively charged substrates like butyrylcholine to guide them down the catalytic gorge.

[85]. Moreover, the catalytic activities of *hBChE* have been linked to residues Trp82 and Asp70 of the cysteine Ω -loop, which is known to create a direct linkage between the choline-binding pocket in the P-site and A-site. Strong interactions between these catalytic residues and compounds such as propidium will prevent the enzymes from catalysing substrate catalysis. Iloneoside showed comparable or greater affinity for the catalytic site residues of human beta-site APP cleaving enzyme 1 (BACE1) than the reference co-crystallized inhibitor, in addition to its strong binding tendencies to the ChE enzymes. Preventing the enzyme's substrate-binding cleft from being accessed by the

aspartate-catalytic dyad (Asp32/Asp228), which is positioned in the middle of the cleft and has a β -hairpin loop covering it [86] has been shown to be the most effective way to reduce its catalytic activity [87]. Iloneoside was securely positioned within the enzyme's binding cleft. It exhibited substantial interaction with many sub-sites (S1, S3, and S3') and the flip (67–75) residues of the binding pocket, but not with the aspartic residues of the catalytic dyad. It has been found that interaction with the residues located on the flip region alters the conformation of the binding cleft entrance by preventing the substrate from entering the cleft [88, 89]. In addition, the orientation, specificity, and stability of the ligand at the binding site are influenced by the many hydrogen bonds and alkyl interactions that iloneoside forms with the enzymes [90, 91]. By use of MD simulated analysis, the structural conformation of the enzyme bound to the pregnane compound (iloneoside) was compared with that of the unbound enzyme and enzymes bound to the reference compounds. Among the computed thermodynamic parameters, the stability of protein structures was investigated from RMSD plots by observing the deviation from conformation of the initial structure [92]. The RMSD plots and the computed mean RMSD values revealed that the binding of iloneoside to the catalytic region of the enzymes did not alter the structural integrity of the enzymes. The close RMSD mean values of the pregnane-bound enzyme complexes with the unbound enzymes further indicate a compacted iloneoside enzyme with reference to the initial structure conformation [92]. The flexibility of individual amino acids of the enzyme was investigated from the RMSF analysis. Residues with higher fluctuations will have higher RMSF values [93]. In the pregnane bound complexes, limited fluctuations in RMSF were observed around the residues that are involved in interaction, previous studies have reported an increase in RMSF around the interacting ligands groups [93]. The reduction in RMSF mean values in the complexes compared to the unbound enzymes further indicates more compacted complexes [93]. The compactness of the iloneoside bound complexes was investigated from the RoG [94], while the extent of solvent accessibility is computed from the SASA plots.

Proteins with increased SASA and RoG value indicates an unfolding that deviates from its original structural conformation. The bound complexes presented a steady RoG and SASA progression that was preserved throughout the period of simulation. The property further indicates compacted complexes with firm intermolecular bonds and a well folded protein structure [94]. The increase in the mean number of H-bonds in the iloneoside-bound hBChE and BACE-1 enzyme complexes may have resulted from the additional H-bonds formed between the enzymes and the ligands. This further demonstrates that the enzymes'

structural integrity, particularly their intermolecular hydrogen bonds, remained unaltered [92].

It has been demonstrated that simulation-based quantitative analysis of the energy of ligand-receptor interaction in a dynamic setting yields significantly more precise and reliable binding affinity estimations [95]. As a result, the binding free energy calculations offer detailed information on the lead compounds' binding mechanisms from the outset of drug design and development [96]. The post dynamics findings validated the static docking calculations, as demonstrated by the binding free energy computation based on the MMGBSA techniques and the breakdown analysis of the total free energy to contributing amino acids. In the simulated dynamic environment, the interactions with residues from active sites were maintained.

The MD simulation trajectories' cluster analysis and the interaction analysis of the clusters' representative conformers demonstrate that the hydrophobic and H-bond interactions seen in the static docking environment were preserved throughout the simulation at various time points. This further implies that the binding interactions found in this study may be maintained in a more dynamic setting, which may make them suitable for use in further experiments.

H-bonds, stacking, and other hydrophobic interactions that are produced between the iloneoside in both enzymes may generally be attributed to one of the following functions: the prevention of amyloid fibrillogenesis via competitive suppression of the enzymes' catalytic activity or by inhibiting the A β identification zone at the P-site [97, 98]. It's possible that iloneoside, which showed dual strong binding inclinations to both cholinesterases, had a role in the leaf extract's significant in vitro cholinesterase inhibitory activity, which our work validated and was first published.

Conclusion

This study employed both in vitro and in silico methods to investigate extracts, fractions, and pregnanes from *G. latifolium* against protein targets associated with the pathology and progression of neurological disorders. The methanol extract, partition fractions, and six chromatographic fractions inhibited the activities of AChE and BChE in a concentration-dependent manner. Fr. B exhibited the highest inhibitory potential against these enzymes. The pregnane compounds that were previously isolated from Fr. B also inhibited the activities of both enzymes. Both Fr. B and its pregnane isolates (iloneoside and marsectohexol) showed inhibitory activity against both cholinergic enzymes and antioxidant potential in a concentration-dependent manner. Between the pregnane compounds, iloneoside had higher binding affinities of -9.8 , -9.9 , and -9.4 Kcal for hAChE, hBChE, and hBACE-1, respectively, which were

comparable with the reference inhibitors of the protein. The antioxidant and anti-cholinesterase properties of the well-known ethnomedicinal and documented neuroprotective properties of *G. latifolium* leaf may be partially attributed to the isolated pregnanes, particularly iloneoside, either alone or in concert with other pregnanes. Iloneoside is recommended for more research since it may serve as lead compounds for the development of therapeutics against neurodegenerative disorders.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s12013-024-01467-7>.

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Author contributions G. A.G. conceptualized the study, G. A.G., O.M.O, I.M.I wrote the original draft performed formal analysis, data curation, and software, J. C. E. methodology, S. O. A., G.O. A. reviewed & edited the manuscript, J.O.A., O.E.O. and M.K,Initial project funding and supervision.

Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

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Abbreviations

GLME	<i>G. latifolium</i> methanol extract
NDDs	Neurodegenerative disorders
AD	Alzheimer's disease
PD	Parkinson's disease
MAO	Mono amine oxidase
BACE1	β -secretase enzyme
APP	amyloid precursor protein
AChE	acetylcholinesterase
BChE	butyrylcholinesterase
hAChE	Human acetylcholinesterase
hBChE	Human Butyrylcholinesterase
hBACE1	Human β -Secretase Enzyme 1
NBT	Nitro blue tetrazolium (NBT)
PDBID	Protein Data Bank ID
SDF	Structure Data Format
UFF	Universal Force Field
MM-GBSA	Molecular Mechanics Generalized Born Surface Area
MDs	Molecular dynamics simulation

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