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Original Article

Exploring the diverse acetylcholinesterase inhibitory potential of girinimbine: insights from in vitro assays, molecular docking, and simulation studies

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Abstract

The search for new treatments for Alzheimer's disease (AD) has led to the exploration of plant-based drugs as potential options. Acetylcholinesterase (AChE) inhibitors are widely used as anti-AD medications. This study aimed to investigate the inhibitory mechanism of girinimbine, a constituent of Murraya koenigii, on AChE. AChE inhibition was assessed by in vitro experiments using the modified Ellman method, as well as in silico molecular docking and molecular dynamic simulation. The results were compared to those of the well-known anti-AChE agents tacrine and propidium iodide. Girinimbine, propidium, and tacrine at concentrations of 3.8X10-5M, 1.1x10-5M, and 6.1x10-7M showed percentages of inhibition percentages of 35.6%, 28.2%, and 76.6%, respectively. The docking and molecular dynamics simulation analyses indicated that girinimbine exhibited a higher binding affinity to AChE compared to propidium and tacrine. This finding was further confirmed by the docking, root mean square deviation (RMSD), root mean square fluctuation (RMSF), and radius of rotation analyses. In conclusion, M. koenigii girinimbine shows promise as an acetylcholinesterase inhibitor for Alzheimer's disease. Further research, including in vivo studies and clinical trials, is needed to explore its potential as a plant-based drug candidate for AD treatment.

Keywords: Girinimbine, Murraya koenigii, Acetylcholinesterase inhibition, Molecular docking, MD simulation.

1. Introduction

Neurodegenerative diseases are known for nerve cell dysfunction and loss of neurons in the central nervous system [1]. One of these ailments is Alzheimer's disease (AD), which affects millions of people. AD causes cognitive loss, short-term memory problems, and the inability to read, talk, and/or think coherently [2]. Current reports on remedial strategies for this harmful ailment are based on the hypothesis of a cholinergic system, particularly on AChE inhibition [3]. Numerous clinical trials have been conducted to find typical and non-toxic drugs to cure AD [4]. The most widely used treatment for AD is tacrine, which has so many side effects that, in most cases, it leads to withdrawal from medication. Many diseases, including AD, share the involvement of oxidative stress in their pathogenesis. Oxidative stress is triggered by free radicals released due to a discrepancy in cellular redox status [5]. This suggests that AD treatment should involve AChE inhibitors and antioxidants that can scavenge excess free radicals and antagonize the consequences of oxidative

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stress [6]. The discovery of plant-based antioxidants is a potential pathway for the development of anti-AD [7].

Murraya koenigii (curry-leaf tree) is native to Asia. Traditionally, the plant is used as a stimulant, stomachic, febrifuge, and analgesic for treating diarrhea, dysentery, and insect bites and to decrease body heat [8]. *M. koenigii* exhibits diverse biological activities and is one of the richest sources of carbazole alkaloids, a class of natural products that possess versatile biological activities such as antioxidant, antimutagenic, and anti-inflammatory activities [8, 9]. Previous studies have reported the antioxidant mechanisms of crude extracts of *M. koenigii* in animal models of AD. The impact of *M. koenigii* leaf total alkaloidal extract on cognitive skills and brain cholinesterase activity in mice was also reported [10]. However, no reports were found on the inhibition of the pure compound girinimbine on AChE and its mechanisms. Therefore, the present study was conducted to examine the inhibitory effect of girinimbine on AChE using a direct colorimetric assay and to understand its mechanism through molecular docking and molecular dynamics simulation. The present study is the first to apply a direct colorimetric test to measure the inhibitory effect of girinimbine on AChE and to use molecular docking and simulation to determine how it works.

2. Materials and Methods

2.1. Isolation of Girinimbine

Girinimbine (Fig. 1) was isolated from the roots of *M. koenigii* according to the method described earlier. The structure of this compound was established by a spectroscopic method and by comparison with previously reported works [11]. The purity of girinimbine determined by HPLC was 97±0.3%, while LC/MS confirmed that the molecular weight of girinimbine ($C_{18}H_{17}NO$) is 263.334 g/mol. All data related to this compound's extraction, spectroscopy, and purity could be obtained from our previous published work [11].

2.2. AChE inhibition assay

The anticholinesterase activities of the compounds [girinimbine, tacrine and propidium iodide (Fig. 1)] were evaluated using Ellmann's method with slight modifications, using acetylthiocholine as a substrate and 5, 5'-dithiobis[2-nitrobenzoic acid] (DTNB) to evaluate AChE activity [12]. 110 µL of sodium phosphate buffer (pH 8.0) was added to the 96 wells, followed by 20 µL of sample solution, 50 µL of DTNB (0.126 mM), and 20 µL of AChE enzyme (0.6 U/mL). The mixture was incubated for 50 minutes at 37 ° C. The reaction was then initiated by the addition of 50 µL (0.120 mM) acetylthiocholine iodide, respectively. The hydrolysis of acetylthiocholine was monitored by the formation of a yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine at a wavelength of 412 nm every 30 s for 25 min using a 96-well microplate plate reader (TECAN Infinite M200). The test compounds were dissolved in analytical-grade dimethyl sulfoxide. Tacrine and propidium iodide were used as reference standards. All reactions were performed in triplicate and monitored with a spectrophotometer. The percentage of inhibition of enzyme activity due to the presence of the test compound was obtained from the expression; 100 – (v_i/v_o x *100*), where *vi* is the initial rate calculated in the presence of inhibitors and v_o is the enzyme activity.

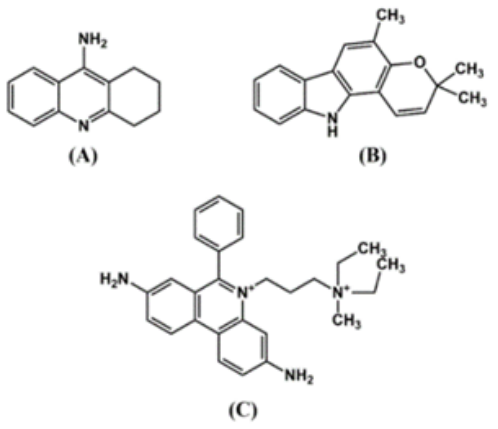

Fig. 1. 2D structures of Tacrine (A), Grinimbine (B), and Propedium (C).

2.3. Molecular docking

The molecular docking studies of the desired compounds have been conducted with AChE utilizing the Molecular Operating Environment (MOE) [13]. Initially, the crystal structure of AChE (PDB ID: 4EY7) [14] was retrieved from the RCSB Protein Data Bank. Before further work, missing residues were modeled using MOE's loop modeler module [15]. After that, the optimization and regularization of the protein target were also carried out by MOE using the AMBER10 force field. The compounds Tacrine, Grinimbine, and Propidium (Fig. 1) were sketched by ChemBioDraw Ultra [16] and then converted to 3D for docking studies applying the MMFF94 force field [17]. Finally, MOE software made the energy minimization and charge application (including naming which charges you use) of all compounds possible. Post-docking analysis, especially protein-ligand interactions, was visualized at the molecular level using the online web-based Protein-Ligand Interaction Profiler (http://plip-tool.biotec.tu-dresden.de/plip-web/plip/index).

2.4. MD simulation

Molecular dynamics simulation (MD) was performed for three complex systems (AChE-Tacrine, AChE-Grinimbine and AChE-Propedium Complex) by GROMACS (V2021) [18], followed by subsequent analysis. The complex systems were obtained from molecular docking studies. For MD simulation, the amber99SB-ILDN force field [19] was selected, which accurately represented several structural and dynamic features of proteins. The ACPYPE software (ACPYPE 2023.10.27) [20] was utilized to parameterize compounds, including tacrine, grinimbine, and propidium. All complexes were solvated in a cubic box of the TIP3P water model and then neutralized with Na and Cl ions before being energy minimized for 5000 steps with the steepest descent approach. After system minimization, a constant number of particles, volume, and temperature (NVT) and a constant number of particles, pressure, and temperature (NPT) were maintained. Furthermore, all MD simulations were executed at 100 ns in the PME algorithm (Particle Mesh Ewald), and the cutoff distance of 10 was used to tackle long-range electrostatic interactions. The shake algorithm was utilized to treat the bonds with hydrogen atoms. A graphical processing unit (GPU) accelerated simulation was performed, and the final MD trajectories were subjected to post-simulation analysis

using VMD. Different stability parameters were accessed for all three systems through the xmgrace tool, such as Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg).

2.5. Statistical analysis

Data was entered and managed using SPSS (Version 26). Analysis of Variance (ANOVA) was used to analyze the difference between the groups.

3. Results

The anticholinesterase activities were evaluated by Ellmann's method with slight modifications, using acetylthiocholine as substrate and DTNB to evaluate AChE activity. Girinimbine, propidium, and tacrine at concentrations of $3.8X10^{-5}$M, $1.1x10^{-5}$M and 6.1×10^{-7}M showed percentages of inhibition of 35.6, 28.2, and 76.6, respectively (Table 1).

Molecular docking studies were used to explore the binding mode and mechanism of three compounds complexed with the target protein and to graphically portray receptor-ligand interactions. MOE was used for molecular docking studies to investigate how desired medications attach to the active site residues of the AChE protein Tyr72, Trp86, Trp286, Phe297, Tyr337, Phe338, Tyr341, and His447. The docked compounds (Fig. 1) were an active AChE site. The binding score determined the best-docked compound postures. Tacrine, grinimbine, and propidium have docking scores of -5.65, -6.31, and -8.42 kcal/mol, respectively, indicating strong binding affinities to the target AChE. The docking research found Trp86, Thr337, Phe338, and Tyr341 residues in the protein binding pocket, which is crucial. On the basis of binding interaction research, all drugs have hydrophobic groups and touch pocket residues hydrophobically. The pi-pi benzene and pyridine moieties of Tacrine stack with Tyr341 and Tyr337, and its hydrophobic interactions with Tyr86, Tyr124, Phe297, Tyr337, and Tyr338 are shown in Fig. 2A. In Fig. 2B, Grinimbine has two hydrophobic contacts with Trp86, Trp286, Tyr337, and Phe338, and four hydrophobic interactions and one pi-pi with Tyr341. The nitrogen atom of Asp74 and Grininbine's pyrrole ring formed a single 2.9 hydrogen bond. Propidium interacts hydrophobically with Asp74, Trp286, and His447, Trp86, and Tyr341. In Fig. 2C, the electrostatic groups in amine sustain hydrogen bonds with Tyr72, Asp74, Tyr133, Glu202 and Ser203.

To check the comparative stability of three compounds in complex with AChE, we investigated the deviation average protein Cα backbone root mean square deviation (RMSD) of the C backbone protein deviation as shown in Fig. 3. From the plot (Fig. 3), it is clear that the reference AChE-tacrine complex (black) has an average root mean square deviation value of ~0.2 nm. On the contrary, the AChE-Grinimibine complex (red) has a slightly lower mean root mean square deviation of ~0.17 nm. The AChE-Propidium complex (green) has a more significant average root mean square value of ~0.25 nm among all systems. This indicates a smaller difference between the stabilities of the backbone of proteins, although, according to numerical values, the Grinimbine complex is stable among the systems. Grinimbine is the compound that holds and stabilizes the system well.

To assess the flexibility of protein residues, the mean square fluctuations (RMSFs) were determined (Fig. 4). The fluctuation of amino acid residues can be explained using the RMSF values obtained from the 100-ns MD simulation for the three complex systems to analyze the conformational changes generated by each inhibitor. The low average RMSF value indicated that individual amino acid residues were stable in the protein's dynamic state during the MD simulation. Fig. 4 indicates that all the complexes have similar fluctuations in amino acid residues.

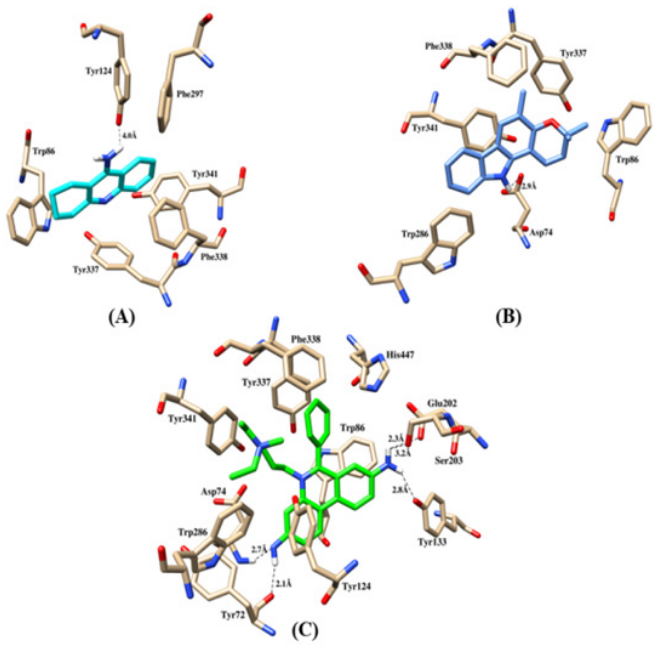

Fig. 2. Selected best-fitting docking poses of Tacrine (A), Grinibine (B), and Propodium (C) in the active site of AChE.

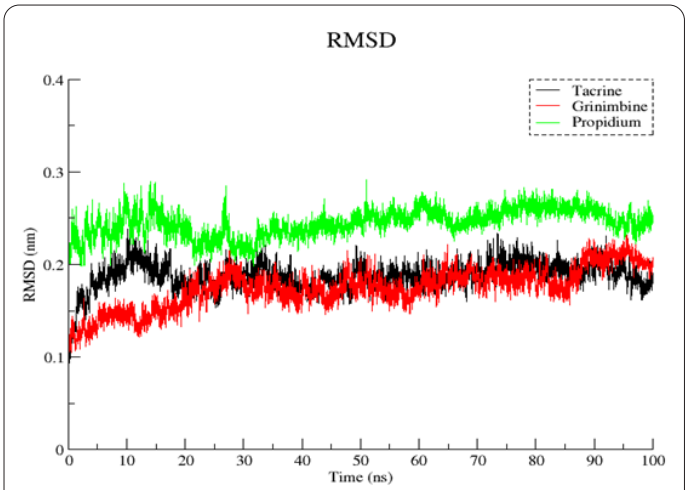

Fig. 3. RMSD for the backbone of complex systems, tacrine complex (black), grinimbine complex (red), and propidium complex (green); all complexes are with target protein AChE.

Table 1. *In vitro* effects of girinimbine, propidium, and tacrine on the AChE enzyme.

Activity	Girinimbine	Propidium	Tacrine
% of Inhibition (Final Concentration)	($3.8X10^{-5}$M) 35.6±1.3	($1.1x10^{-5}$M) 28.2±2.3	($6.1x10^{-7}$M) 76.6±8.4

However, some regions of amino acids 75-110, 250-300 and 335–380 have significant fluctuations, as we observed that the Tacrine (reference) and Propidium complexes (black and green, respectively) have slightly higher fluctuations than the AChE–Grinimbine complex.

The Rg values were used to assess the folding and compactness of proteins in the presence of three compounds. Fig. 5 shows the Rg plot of α-carbon atoms vs. simulation time. The Rg values can indicate the stability of the system during the 100-ns MD simulation time. The tacrine complex (black) Rg value was consistent at about 2.33 nm and served as a reference. The grinimbine complex (red) has the same value of 2.33 nm as the reference compound. The propidium complex has the highest value of around 2.39 nm among the three systems. These results demonstrated that the binding of grinimbine compacted and rigidified the protein structure.

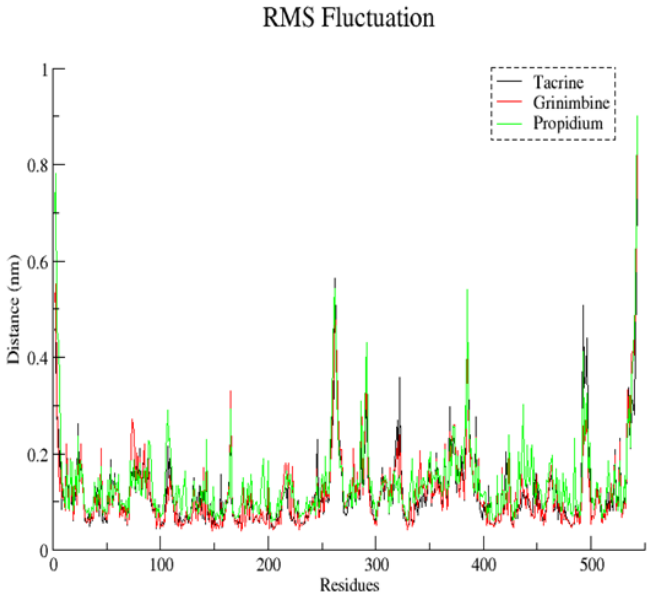

Fig. 4. RMSF for the amino acid residues of complex systems, tacrine complex (black), grenimbine complex (red), and propidium complex (green).

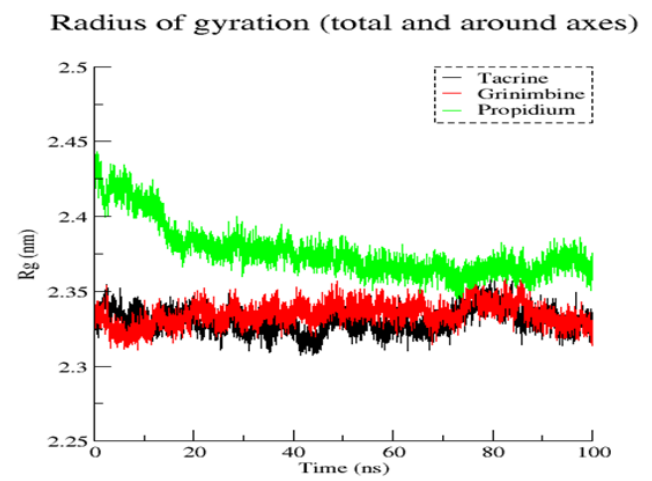

Fig. 5. Radius of gyration (Rg) of complex systems, Tacrine complex (black), grenimbine complex (red) and propidium complex (green).

4. Discussion

The current study was designed to investigate AChE inhibitory effects and molecular docking simulation of girinimbine. Natural products are characterized by unorthodox and often unanticipated chemical structures that offer novel approaches to clinically useful medications. The myriad of structurally diverse compounds found in nature make them an important resource for drug discovery. However, in addition to their amazing diversities, natural products remain largely unexplored, making them a rich source of drugs. A growing number of natural products and natural-product-derived drugs have been developed in the industrialized world. To date, most of the FDA-approved AD drugs are natural products or natural product-derived compounds. For example, of a total of 877 new chemical drugs produced between 1981 and 2002, approximately 61% were natural product-based compounds. Moreover, approximately 60% of antitumor and anti-infective drugs are of natural origin [21].

As AChE inhibitors are an effective remedial strategy in AD, efforts are being made to find new compounds with anti-AChE activity [22]. The fact that phytochemicals are known to be a potential source of new inhibitors has led to the discovery of an important number of compounds and crude plant extracts with the capacity to inhibit the enzyme AChE. According to cholinergic theory, it increases the levels of the neurotransmitter acetylcholine in the brain, thus enhancing cholinergic functions in patients with Alzheimer's disease and alleviating symptoms of this neurological disorder [23, 24]. The current study was designed to investigate AChE inhibition and molecular modeling of girinimbine from *Murraya koenigii* (L.) Spreng. The anticholinesterase activity of girinimbine, propidium, and tacrine was evaluated using Ellmann's method using acetylthiocholine as a substrate.

Girinimbine showed percentages of inhibition of 35.6 (Table 1). Kumar et al., 2010, investigated some Indian plants for their inhibitory potential for AChE through bioassay-guided isolation. They managed to isolate a carbazole alkaloid, mahanimbine [3, 5-dimethyl-3-(4-methyl pent-3-enyl)-11H-pyrano [5, 6-a] carbazole], from the petroleum ether extract of the leaves of *M. koenigii* [25]. *M. koenigii* extract has previously been reported to inhibit brain cholinesterase activity and prevent oxidative stress in mice [10, 26]. However, studies that use a direct test to examine the AChE inhibitory activity of girinimbine have not yet been published. The present study is the first to apply a direct colorimetric test to measure girinimbine's inhibitory effect on AChE and molecular docking to figure out how it works.

The molecular docking studies investigated the binding of three compounds (tacrine, grenimbine, and propidium) to the target protein AChE. The compounds showed strong binding affinities with AChE, as indicated by their docking scores. Specific residues at the active site of the protein, such as Tyr72, Trp86, Trp286, Phe297, Tyr337, Phe338, Tyr341, and His447, were found to be crucial for binding interactions. The compounds exhibited various types of interactions, including hydrophobic contacts, pi-pi stacking, and hydrogen bonding, with these key residues. Hydrophobic interactions occur between nonpolar or weakly polar regions of molecules [27], and in this case, Grinimbine's hydrophobic groups are likely to interact with the hydrophobic regions of the mentioned residues in the protein. These interactions contribute to the overall stability of the protein-ligand complex [28]. Pi-pi stacking refers to the attractive interaction between aromatic rings [29]. In this case, Grinimbine's aromatic structure, pos-

sibly a carbazole ring, stacks or aligns itself with the aromatic ring of Tyr341 in the protein [30]. This interaction is stabilized by attractive forces between the electron-rich regions of the aromatic rings. Hydrogen bonding occurs when a hydrogen atom is shared between a hydrogen bond donor (in this case, Grinimbine's nitrogen pyrrole ring) and a hydrogen bond acceptor (Asp74) [31]. The distance between the donor and acceptor atoms, mentioned as 2.9, indicates the proximity and strength of the hydrogen bond. This hydrogen bond contributes to the specific binding between Grinimbine and the AChE protein [32]. These findings provide valuable insight into the binding modes and mechanisms of these compounds, which can guide further optimization efforts or the development of new compounds targeting AChE.

Molecular dynamics simulations are computational techniques used to study the behavior and interactions of atoms and molecules over time. They simulate the movement and interactions of particles on the basis of classical physics principles. These simulations provide information on the dynamics, structure, and thermodynamics of biological systems at the atomic level. In the context of protein-ligand interactions, molecular dynamics simulations help to understand binding processes, analyze complex stability, and investigate dynamic behavior. They are valuable tools in drug discovery, protein engineering, and the study of biological processes at the molecular level [33]. The stability of three compounds (tacrine, grenimbine, and propidium) in complex with AChE was compared using the average protein Cα backbone root mean square deviation of the C protein backbone (RMSD) plot. The plot showed that the AChE-Grinimbine complex had a slightly lower average RMSD value (~0.17 nm) compared to the AChE-Tacrine complex (~0.2 nm). However, the AChE-Propidium complex had a higher average RMSD value (~0.25 nm), indicating relatively less stability. These findings suggest that Grinimbine contributes to a stable complex with AChE.

RMSF analysis of protein residues revealed insights into flexibility and conformational changes [34] induced by Tacrine, Grinimbine, and Propidium during 100-ns MD simulations. The low average RMSF values indicate the overall stability of individual amino acid residues in their dynamic state [35]. However, certain regions (amino acids 75-110, 250-300, and 335-380) displayed significant fluctuations, suggesting localized flexibility within the protein structure. Tacrine and propidium complexes exhibited slightly higher fluctuations compared to AChE-Grinimbine, indicating that these inhibitors induce more pronounced conformational changes in these regions. These findings imply that while protein stability is maintained, specific regions undergo flexibility and conformational changes upon inhibitor binding. Further analysis and experimental validation are needed to understand the functional implications of these observations in inhibiting AChE.

5. Conclusions

In conclusion, this study highlights Girinimbine, a plant-derived carbazole alkaloid, as a promising candidate for Alzheimer's disease treatment. Girinimbine effectively inhibits acetylcholinesterase (AChE) and exhibits comparable binding affinity in relation to tacrine and propidium. Further experimental and clinical investigations are warranted to fully explore Girinimbine's therapeutic potential in AChE-related disorders, such as Alzheimer's disease. This research contributes to the development of novel and effective treatments for these conditions.

List of abbreviation

AChE: Acetylcholinesterase; AD: Alzheimer Disease; DTNB: , 5'-dithiobis[2-nitrobenzoic acid]; FDA: Food and Drug Administration; GPU: Graphical processing unit; LC/MS: Liquid chromatography–mass spectrometry; MD: Molecular dynamics; MOE: Molecular Operating Environment; NPT: Number of particles, pressure, and temperature; NVT: Number of particles, volume, and temperature; Particle Mesh Ewald; PME; Rg: Radius of Gyration; RMSD: Root mean square deviation; RMSF: Roots mean square fluctuation.

Ethics approval and consent to participate

There is no form of human subject involved in this manuscript; therefore, ethics approval is not required.

Consent for publication

Not applicable.

Availability of data and material

Data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; participated in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be responsible for all aspects of the work.

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