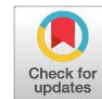


Research Article



In-Silico Identification of Potential NS2B-NS3 Protease Inhibitors against Zika Virus

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Abstract | Zika virus transmitted by mosquito, has emerged as a significant public health threat with the potential to cause global pandemics. This study explores natural phytochemicals as potential inhibitors of Zika virus (ZIKV) infection by targeting the essential NS2B-NS3 protease complex, which plays a crucial role in viral replication. A virtual screening library of 52 natural compounds was prepared, followed by pharmacokinetic (ADME) and toxicity assessments to identify two promising candidates. These selected compounds were further analyzed through molecular docking and extensive 200-nanosecond molecular dynamics simulations to evaluate binding affinity, stability, and interaction with the ZIKV protease structure (PDB ID: 5LC0). Among the tested ligands, PubChem CID: 56649692 showed the most favorable binding affinity at -5.321 kcal/mol, surpassing other compounds, including the control ligand. Molecular dynamics simulation confirmed that CID: 56649692 maintained stability within the acceptable range, as reflected in root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) values. The stability was further supported by the formation of multiple hydrogen bonds and hydrophobic interactions with key residues (LYS1119, ASP1120, and LYS1073) in the protease's active site, indicating strong and stable ligand-protein interactions. CID: 56649692 demonstrates significant potential as a lead compound for ZIKV inhibition based on its high binding affinity, molecular stability, and favorable ADME and toxicity profiles. Targeting the NS2B-NS3 protease complex represents a promising strategy to disrupt ZIKV replication, supporting CID: 56649692 as a viable candidate for further investigation. Future in vitro and in vivo studies are recommended to validate these in silico findings and assess the therapeutic efficacy of CID: 56649692 under physiological conditions.

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Keywords | Zika virus, NS2B-NS3 protease, Molecular docking, In silico screening, Molecular dynamics simulation, Protein inhibitor



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Introduction

Zika virus (ZIKV), an arthropod-borne pathogen from the Flaviviridae family and Flavivirus genus, has emerged as a significant public health threat. Zika virus has the potential to cause global pandemics (Ramharack and Soliman, 2018). Originally identified in Uganda, ZIKV is primarily transmitted by Aedes mosquitoes but has since spread across continents through additional modes of transmission, impacting populations in Africa, Asia, and the Americas. While ZIKV infection in adults often results in mild symptoms—such as fever, rash, and muscle pain—the virus poses severe risks for pregnant women, with the potential to cause congenital malformations, including microcephaly in fetuses (Telehany *et al.*, 2020). Moreover, neurological complications such as Guillain-Barré syndrome, encephalitis, and myelitis have been reported, further underscoring ZIKV's complex pathogenesis and global health impact. Consequently, the World Health Organization (WHO) declared ZIKV a Public Health Emergency of International Concern, emphasizing the need for accelerated research into vaccines, diagnostics, and therapeutic interventions (Sinigaglia *et al.*, 2018).

Research into ZIKV's molecular structure and function has identified it as a single-stranded, positive-sense RNA virus with a 10,794-nucleotide genome, encoding a polyprotein that is processed into structural and nonstructural proteins (Musso and Gubler, 2016). Among these, the NS2B-NS3 protease complex is critical for viral replication and maturation, as it processes the viral polyprotein by cleaving it at specific sites (Tan *et al.*, 2020). The NS2B-NS3 complex, therefore, is an attractive target for antiviral drug development, as inhibiting its activity could disrupt viral replication and halt disease progression. The NS2B protein, anchored to the host cell's endoplasmic reticulum membrane, forms a cofactor necessary for NS3 protease activity, further enhancing its viability as a drug target (Yadav *et al.*, 2021). This protease's essential role in viral replication and its involvement in immune evasion mechanisms make it a primary focus for therapeutic exploration (Valente and Moraes, 2019; Li *et al.*, 2018).

Natural compounds, particularly flavonoids, have shown promising antiviral activities against the NS2B-NS3 protease complex in other flaviviruses. Compounds such as epigallocatechin gallate, curcumin,

and theaflavin-3-gallate inhibit the protease function and have demonstrated potential for ZIKV therapy (Nandi *et al.*, 2018). Targeting the hydrophobic binding pocket and specific allosteric sites of the NS2B-NS3 complex has yielded several leads, with compounds like ZINC00845171 and ZINC08782519 identified as inhibitors in virtual screenings (Fatima *et al.*, 2018; Voss and Nitsche, 2020). Furthermore, natural flavonoids like myricetin and quercetin have been explored for their role in blocking protease function in other related viruses, suggesting a viable pathway for ZIKV inhibition (Mwaliko *et al.*, 2021). Advances in computational methods, including molecular docking and dynamic simulations, have facilitated the rapid screening of large compound libraries, enabling the identification of candidate molecules for further experimental validation (Gorshkov *et al.*, 2019).

Given the urgency of addressing ZIKV's public health threat and the potential of NS2B-NS3 as a therapeutic target, this study aims to identify natural compound inhibitors that specifically interact with and stabilize the protease complex. Using an *in silico* approach, we screened a library of phytochemicals for binding affinity, pharmacokinetics (ADME), and toxicity profiles. Molecular docking and 200-nanosecond molecular dynamic simulations were employed to characterize ligand-protein interactions and assess binding stability. This study highlights key natural compounds with the potential to inhibit ZIKV replication by targeting NS2B-NS3 protease and provides a foundation for future *in vitro* and *in vivo* investigations into therapeutic applications.

Materials and Methods

Protein Preparation

For computational analysis, preparing a high-quality protein structure is essential. The three-dimensional, non-mutated crystal structure of the Zika virus NS2B-NS3 protease (PDB ID: 5LC0), with a molecular weight of 49.49 kDa and resolution of 2.70 Å, was retrieved from the RCSB Protein Data Bank (<https://www.rcsb.org/>). Chain A was selected as the target for drug design analysis, given its structural similarity to Chain B. Using Discovery Studio Visualizer (Accelrys), unnecessary components, including water molecules, hetero atoms, and Chain B, were removed to streamline the macromolecule for analysis. The prepared protein structure was saved in PDB format for further *in silico* procedures (Madhavi *et al.*, 2013).

Ligand Library Preparation and Similarity Search

The initial ligand library was created by retrieving the 2D structures of reference ligands from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). To identify structurally related compounds, a 60% similarity search was performed using Ambinter (<http://www.ambinter.com/>), yielding 52 natural ligands. Each ligand was separated and saved in SDF format for subsequent computational analysis using Discovery Studio Visualizer (Accelrys).

Prediction of Protein Active Site and Grid Generation

The active site of NS2B-NS3 protease was determined using the Computed Atlas of Surface Topography of Proteins (CASTp) server (<http://sts.bioe.uic.edu/castp/index.html?1bxw>). This platform enables the identification of amino acid residues within the protein's active site that facilitate ligand binding (Opo *et al.*, 2021). A grid for the macromolecule was generated to prepare the protein for the molecular docking process.

ADME Profiling of Ligands

The pharmacokinetics properties of all 52 ligands, including absorption, distribution, metabolism, and excretion (ADME), were evaluated using the Swiss ADME server (<http://www.swissadme.ch/index.php>). ADME profiling included parameters such as molecular weight, H-bond donors, H-bond acceptors, number of rotatable bonds, log P, Lipinski's rule of five compliance, synthetic accessibility, solubility, gastrointestinal absorption, and blood-brain barrier permeability. Based on these criteria, 27 ligands were selected for further analysis (Kshatriya *et al.*, 2019).

Toxicity Profiling of Ligands

To confirm the safety of selected ligands for potential therapeutic application, toxicity assessments were performed using the Pro Tox-II web server (https://tox-new.charite.de/prottox_II/index.php?site=compound_input). Toxicity profiles were evaluated for hepatotoxicity, carcinogenicity, mutagenicity, and cytotoxicity, and only ligands that displayed an inactive status across these parameters were selected. Following these criteria, five natural compounds were chosen for molecular docking analysis (Banerjee *et al.*, 2018).

Molecular Docking

Molecular docking was conducted using Schrödinger software, specifically the GLIDE (Grid-based Ligand Docking with Energetics) module, to determine

the optimal binding mode of each ligand with NS2B-NS3 protease. Proper docking was validated by generating a grid box with coordinates X = 91.37, Y = 50.42, and Z = 143.125, encompassing the active site residues. Docking affinity scores were used to assess the binding interaction and specificity of each ligand for the target protein (Morris and Lim-Wilby, 2008; Pagadala *et al.*, 2017).

Visualization of Protein-Ligand Interactions

Post-docking visualizations of protein-ligand complexes were conducted using Discovery Studio Visualizer (Accelrys) to analyze hydrogen bonds, hydrophobic interactions, and overall binding orientation. Each docked complex was evaluated to ensure optimal ligand positioning within the active site.

Molecular Dynamics Simulation

To evaluate the stability of protein-ligand complexes over time, molecular dynamics (MD) simulations were conducted using the Desmond module within Schrödinger, over a 200-nanosecond simulation period. An SPC water system was established with a 10 Å orthorhombic periodic boundary, and a salt concentration of 0.15M was used to achieve system neutrality with added Na⁺ and Cl⁻ ions. The OPLS3e force field was applied for energy minimization, and the Nose-Hoover thermostat was used at a pressure of 1.02326 bar (Ahammad *et al.*, 2021).

Trajectory Analysis

The Desmond module was utilized for trajectory analysis, examining parameters such as root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), protein-ligand interactions, and ligand properties (e.g., radius of gyration). RMSD and RMSF were used to assess the stability and conformational flexibility of the complexes, with RMSD values within the 1-3 Å range considered indicative of stability (Ahammad *et al.*, 2021). The stability of hydrogen bonding and hydrophobic interactions was also analyzed, as these interactions are crucial for effective binding and ligand efficacy (Hassan *et al.*, 2016).

Statistical Analysis

Results from docking scores, MD simulations, and ADME/toxicity profiles were compiled and analyzed to determine the lead compounds. A comprehensive analysis of binding affinity, pharmacokinetics, toxicity, and molecular stability led to the selection of

PubChem CID: 56649692 as a lead compound with high therapeutic potential.

Results

Evaluation of pharmacokinetics properties of ligands Out of 52 natural ligands from the library only two natural compounds (PubChem CID: 56649692 and PubChem CID: 7267140) excluding control ligand (PubChem CID: 127348520) were selected in this research after they fulfilling and maintaining all the parameters of ADME and toxicity profiling. Non mutated and optimized crystal structures of the receptor and ligands are shown in [Figure 1](#).

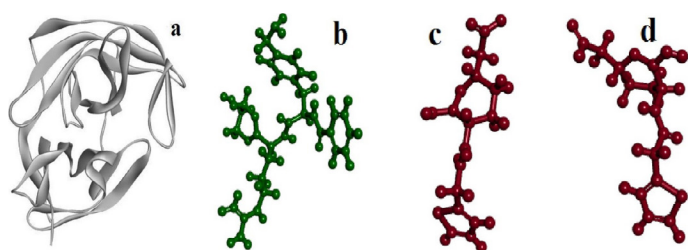


Figure 1: Illustration of optimized protein macromolecule, control ligand and two natural ligands. Legends: (a) Receptor (PDB ID: 5LC0), (b) Control ligand (PubChem CID: 127348520), (c) PubChem CID: 56649692 and (d) PubChem CID: 72671402.

Molecular docking and post molecular docking quantitative analysis and screening

Molecular docking analysis is performed to find out the appropriate and best candidates for research and binding affinity score between protein and ligands determine the finest interaction between them. In

this current research, the control ligand have shown the binding affinity of (-4.808 Kcal/mol) towards the receptor (PDB ID: 5LC0). On the other hand, natural ligand PubChem CID: 56649692 have shown the greatest binding affinity (-5.321 Kcal/mol) with the receptor demonstrating itself as superior to the other selected ligands. Moreover, another ligand (PubChem CID: 72671402) also shown similar binding affinity (-5.314 Kcal/mol) while docked with the receptor.

The control ligand PubChem CID: 127348520-5LC0 macromolecule is complexed with 8 hydrogen bond interactions and 4 hydrophobic interactions. In regards to natural ligand PubChem: 56649692-5LC0 macromolecule is stabilized by 5 hydrogen and 4 hydrophobic interactions. Similarly, other ligand PubChem CID: 72671402-5LC0 complex was stabilized by 3 hydrogen and hydrophobic interactions ([Table 1](#)).

For the control ligands and 5LC0 macromolecule complex, these ASP1086 (2.05 Å), GLN1074 (2.38 Å and 2.46 Å), ILE1165 (2.58 Å and 2.25 Å), LEU1085 (1.74 Å), ASN1152 (3.80 Å) and LYS1073 (2.06 Å) amino residues contain hydrogen bonds while hydrophobic interactions are ASP1086, ASP1071, ILE1123 and LEU79 ([Figure 2](#)).

Profiling with the test ligands, ligand (PubChem CID: 56649692) formed the complex with the receptor 5LC0 via LYS1119 (2.82 Å and 2.88 Å), ASP1120 (1.96 Å and 2.12 Å and LYS1073 (2.59 Å) 5 hydrogen bonds and 4 hydrophobic interactions LYS1119, ILE1123, LEU78 and ALA77 ([Figure 3](#)).

Table 1: A brief description of amino acid interacting with the active site of the receptor 5LC0 with all the ligands while comparing with the control ligand (PubChem CID – 127348520).

Macromolecule Ligands (PDB ID)	Binding Affinity (Kcal/mol)	Amino acid that are involved in interaction	
		Hydrogen bond Interaction	Hydrophobic bond interaction
5LC0 PubChem CID: 127348520 (Control Ligand)	-4.808	ASP1086 (2.05 Å), GLN1074 (2.38 Å & 2.46 Å), ILE1165 (2.58 Å & 2.25 Å), LEU1085 (1.74 Å), ASN1152 (3.80 Å), LYS1073 (2.06 Å)	ASP1086, ASP1071, ILE1123, LEU79,
5LC0 PubChem CID: 56649692 (Ambinter ID: Amb33718069)	-5.321	LYS1119 (2.82 Å & 2.88 Å), ASP1120 (1.96 Å & 2.12 Å, LYS1073 (2.59 Å)	LYS1119, ILE1123, LEU78, ALA77
5LC0 PubChem CID: 72671402 (Ambinter ID: Amb34891301)	-5.314	GLN1074 (2.09 Å & 2.97 Å), TRP1089 (3.31 Å)	ILE1123, LEU78, ASP1120

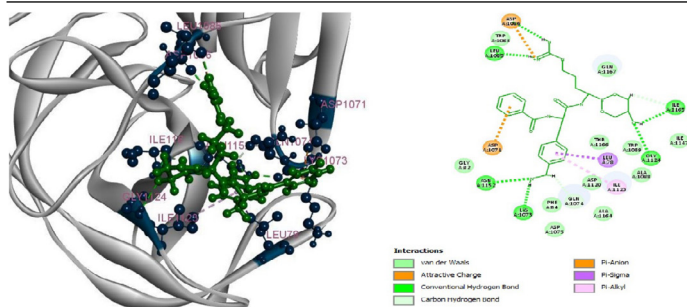


Figure 2: Represent the interaction between the protein 5LC0 and Control ligand (PubChem CID: 127348520). In the left side, the interaction is shown in 3D and in right side in 2D.

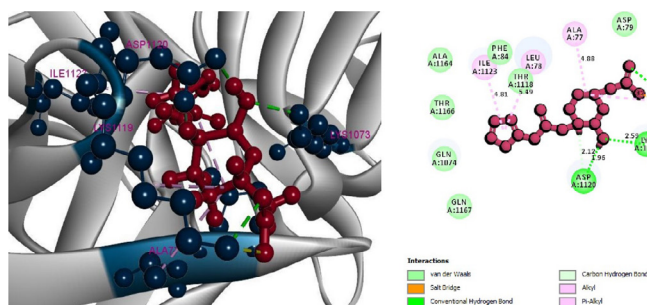


Figure 3: Represent the interaction between the protein 5LC0 and PubChem CID: 56649692. In the left side, the interaction is shown in 3D and in right side in 2D.

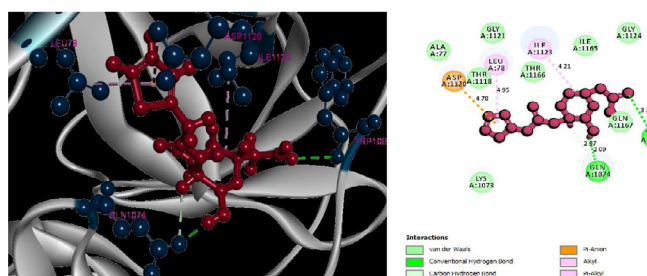


Figure 4: Represent the interaction between the protein 5LC0 and PubChem CID: 72671402. In the left side, the interaction is shown in 3D and in right side in 2D.

Similarly, three 3 hydrogen bonds GLN1074 (2.09 Å and 2.97 Å) and TRP1089 (3.31 Å) and 3 hydrophobic interactions ILE1123, LEU78 and ASP1120, stabilized the complex of receptor 5LC0 and Ligand (PubChem CID: 72671402) (Figure 4).

Molecular dynamic simulation (MDS)

Stability and integrity of the complexed structure of protein-ligand can be ensured by molecular dynamic simulation study in an earmarked and virtual platform. Our study was performed in 200ns MDS to examine the conformational change, stability and steady nature of our desired protein-ligand complexes. Simulation analysis was carried out with 5LC0 protein and two specific ligands compared to a control

ligand to measure their binding ability and stability. The outcome was interpreted with parameters; such as RMSD, RMSF, Protein interactions and ligand properties (radius of gyration).

RMSD analysis of the protein and ligands

Initially, our protein 5LC0 (light green color) was inside the acceptable range of RMSD value and well aligned with the reference value. Here, the RMSD value was measured for the ligand's CID: 137348520 (control ligand), CID: 56649692 and CID: 72671402 which were complexed with the 5LC0 protein, displayed in the Figure 5 and Table 2. All three ligands consist of control ligands were in the standard range (1-3Å) of RMSD value. At the starting of 0-2ns the simulation, both ligands and control ligand with protein showed a linearity. However, the moderate fluctuation starts for all the ligands after the 2ns simulation. CID: 72671402 (yellow color); this ligand showed a sharp increased fluctuation at 86ns simulation. On the other hand, same kind of fluctuation was observed for CID: 56649692 (blue color) at 155ns simulation. Though control ligand (dark green color) showed a minimum fluctuation from 100ns to 185ns simulation. Nevertheless, the both desired ligands (blue and yellow color) showed a great alignment with the protein from the 185ns to 200ns, where CID: 56649692 (blue color) ligand's result was more significant.

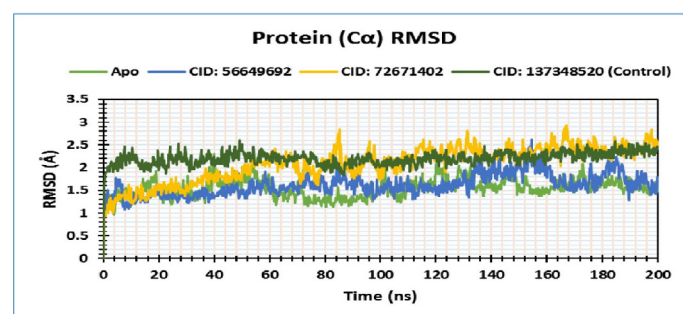


Figure 5: Represent the RMSD value from the complexed structure of protein-ligands from molecular dynamic simulation at 200ns run. Receptor (PDB ID: 5LC0, Control ligand (PubChem CID: 127348520), PubChem CID: 56649692 and PubChem CID: 72671402.

RMSF analysis of the protein and ligands

From the Figure 6, we have found the main peaks between the 30 to 60 residue indexes. The compound CID: 72671402 (ash color) have shown the highest peak and fluctuation of 6.8 Å. Another fluctuation of the same compound was appeared at 3.1Å. The compound CID: 56649692 (orange color) demonstrated two peaks between the 30 to 40 residue indexes.

Table 2: *Physicochemical properties part 1 (Formula, Molecular weight, No. Heavy atoms, No. Arom. Heavy atoms, no. rotatable bond, no. H-bond acceptors, No. H-bond donors) of control ligand and other two desired ligands.*

Ligands PubChem (CID)	Physicochemical properties						
	Formula	MW (g/mol)	No. Heavy atoms	No. Arom. Heavy atoms	No. rotatable bond	No. H-bond acceptors	No. H-bond donors
137348520 (Control ligand)	C ₂₅ H ₃₅ BN ₆ O ₅	510.4	37	12	14	7	6
56649692	C ₁₂ H ₁₆ BN ₅ O ₅	297.14	20	5	6	5	3
72671402	C ₁₂ H ₁₆ BN ₅ O ₅	297.14	20	5	6	5	3

Table 3: *Physicochemical properties part 2 (Lipophilicity, Water solubility, Pharmacokinetics, Drug likeness, Medicinal chemistry) of control ligand and other two desired ligands.*

Ligands (PubChem CID)	Lipophilicity	Water solubility	Pharmacokinetics	Drug like-ness	Medicinal chem-istry	Lipophilicity
	Consensus Log P o/w	Log S(ESOL)	GI absorption	BBB permeant	Lipinski rule violation	Synthetic accessible
137348520 (Control ligand)	.30	-2.19	Low	No	Yes; 0 violation	4.85
56649692	.11	-1.75	High	No	Yes; 0 violation	3.62
72671402	.11	-1.75	High	No	Yes; 0 violation	3.62

Both the peak showed a fluctuation of 2.9Å. However, our control ligand displayed comparatively low fluctuation; the highest fluctuation of the control ligand (yellow color) was observed at 2.4Å as well as control ligand did not sustain an average minimum distance for further residues. Since, both the ligands CID: 72671402 (ash color) and CID: 56649692 (orange color) showed a great fluctuation, it indicates both are in proper and appropriate complexed from with the 5LC0 protein.

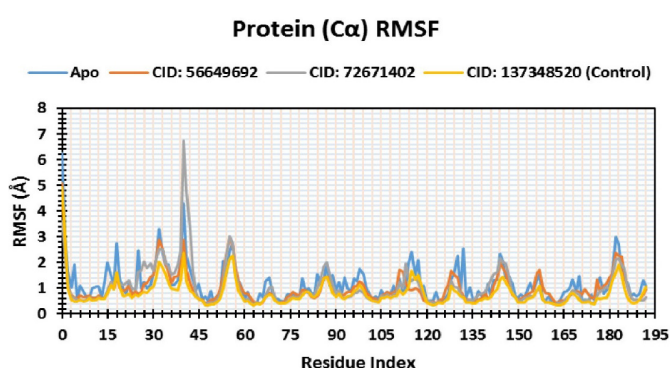


Figure 6: *Represent the RMSD value from the complexed structure of protein–ligands from molecular dynamic simulation at 200ns run. Receptor (PDB ID: 5LC0, Control ligand (PubChem CID: 127348520), PubChem CID: 56649692 and PubChem CID: 72671402.*

Interaction of protein with ligands

The complexed form with the control ligand and desired ligands as well as their intermolecular interactions were examined through Simulation Interac-

tion Diagram (SID) while maintaining the 200ns simulation run. The intermolecular interactions between the protein 5LC0 and the selected ligands [CID: 137348520 (control ligand), CID: 56649692 and CID: 72671402] were illustrated based on hydrophobic interactions, hydrogen bond interactions, ionic interactions and water bridges; shown in Figure 7. For the ligand (CID: 56649692), there were two interactions showing Interaction Fraction Value (IFV) more than 1 at TRP1089 and ASP1122 consisting hydrogen, hydrophobic, ionic interactions and water bridges interactions which means they maintained those specific interactions more than 100 percent of simulation time. Additionally, this ligand had another IFV nearly 80 percent at ARG73. On the contrary, the ligand (CID: 72671402) showed only one IFV at LYS1072 more than 1 and another IFV at 58 with amino acid GLU80. In case of the control ligand (CID: 137348520) have shown 4 IFV in between 80-97 percent at ASP83, LYS1073, ASP1120 and GLN1167 respectively. The highest hydrogen bonds were observed at control ligand. However, out of two desired ligands, CID: 56649692 demonstrated more interactions with the protein molecule via hydrogen bonds (Table 3). Since, hydrogen bond interaction is a significant interaction in stabilizing the ligand–protein complex, impacting drug specific and accelerating of metabolism and absorption (Hassan et al., 2016). As a result, ligand (CID: 56649692) could be a more potential inhibitor of Zika virus influx in the human body.

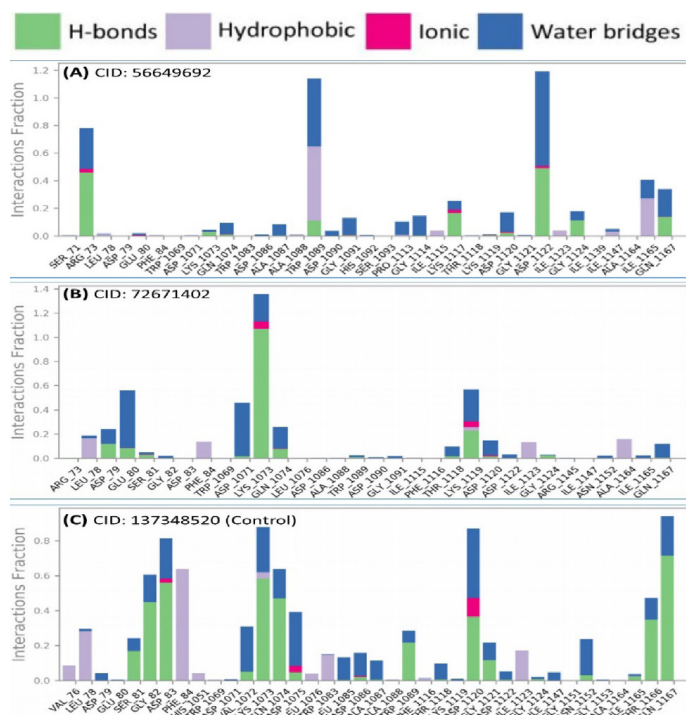


Figure 7: Represent the protein-ligand interaction via hydrogen bonds, hydrophobic interactions, ionic interaction and water bridges from molecular dynamic simulation at 200ns run. Receptor (PDB ID: 5LC0, Control ligand (PubChem CID: 127348520), PubChem CID: 56649692 and PubChem CID: 72671402.

Radius of gyration

Radius of gyration of a body about the axis of rotation is defined as the radial distance to a point which would have a moment of inertia the same as the body's actual distribution of mass, if the total mass of the body were concentrated there.

Mathematically the radius of gyration is the root mean square distance of the object's parts from either its center of mass or a given axis, depending on the relevant application. It is actually the perpendicular distance from point mass to the axis of rotation. One can represent a trajectory of a moving point as a body. Then radius of gyration can be used to characterize the typical distance travelled by this point. For the compound of CID: 56649692 and CID: 72671402 has been showed the radius of gyration where these two lead compounds has been rotated towards the amino acid residue during 200 ns simulation time in the range of 4.4 Å subjected to control ligand shown in Figure 8 and 9.

Discussion

This study identified natural ligands with potential

inhibitory effects on the Zika virus (ZIKV) NS2B-NS3 protease using an insilico approach, which included ADME and toxicity screening, molecular docking, and molecular dynamics simulation. Among the 52 compounds initially screened, PubChem CID: 56649692 emerged as the most promising candidate based on its binding affinity, stability, and pharmacokinetic profile.

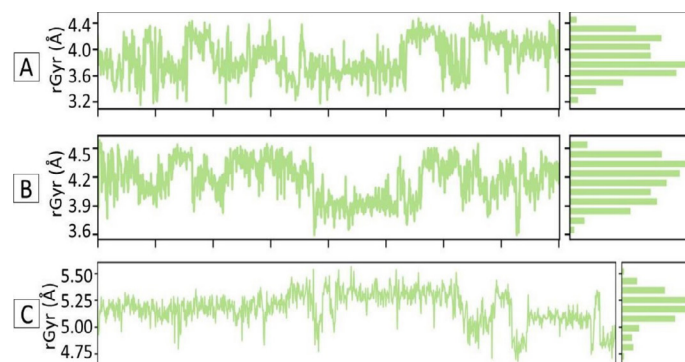


Figure 8: Represent ligand properties (Radius of gyration) of ligands. Here, A is CID: 56649692, B is CID: 72671402 and C is CID: 137348520 (Control ligand).

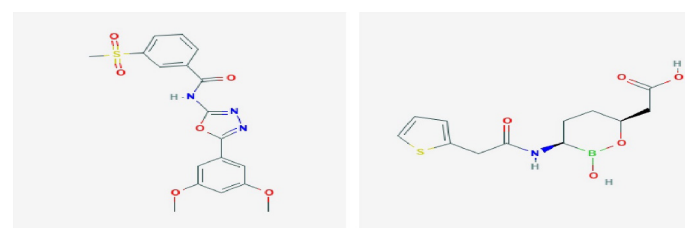


Figure 9: 2D structures of (PubChem CID: 72671402 and PubChem CID: 56649692).

The NS2B-NS3 protease is a critical component in ZIKV replication, facilitating polyprotein processing essential for viral maturation (Yadav *et al.*, 2021). By targeting this protease, compounds that inhibit its activity can effectively impede viral proliferation. Recent studies have shown that natural compounds, particularly flavonoids, exhibit inhibitory action on proteases in flaviviruses, validating the relevance of our focus on natural ligands (Nandi *et al.*, 2018; Fatima *et al.*, 2018). This study's approach aligns with such research, identifying lead compounds that could provide a foundation for ZIKV treatment strategies.

Evaluation of Lead Compound CID: 56649692

CID: 56649692 displayed the highest binding affinity among tested compounds with a docking score of -5.321 kcal/mol, which was notably superior to the control ligand (-4.808 kcal/mol). This suggests that CID: 56649692 has a stronger interaction potential

Table 4: Toxicity profile (*Ames toxicity, oral rat acute toxicity (LD50), Oral rat chronic toxicity (LOAEL), Hepatotoxicity, Carcinogenicity, Cytotoxicity*) of control and selected ligands.

Toxicity profile of ligands						
Ligands (PubChem CID)	AMES toxicity	Oral rat acute toxicity (LD50)	Oral rat chronic toxicity (LOAEL)	Hepatotoxicity	Carcinogenicity	Cytotoxicity
137348520 (Control ligand)		2.573	4.435	Yes	No	No
56649692	No	2.749	1.988	No	No	No
72671402	No	2.749	1.988	No	No	No

with the ZIKV NS2B-NS3 protease. The molecular docking results demonstrated significant hydrogen bonding and hydrophobic interactions, primarily involving key residues LYS1119, ASP1120, and LYS1073. These interactions are essential for stabilizing the ligand-protein complex, a finding consistent with studies highlighting the role of hydrogen bonds and hydrophobic interactions in enhancing binding specificity and stability (Bhargava *et al.*, 2019).

Further supporting these findings, molecular dynamics simulation over 200 nanoseconds revealed that CID: 56649692 maintained stability within an acceptable RMSD range, with minimal fluctuation after 185 ns. This stability indicates that CID: 56649692 is less likely to dissociate from the protease, suggesting a reliable binding conformation conducive to inhibiting ZIKV replication. The RMSF analysis highlighted that fluctuation in CID: 56649692 were localized, with limited impact on the ligand's overall stability. In comparison, CID: 72671402, while showing high binding affinity, exhibited greater RMSD fluctuation, indicating less stable binding interactions with NS2B-NS3.

Comparative Analysis with Control Ligand and Implications

The control ligand, though stable, showed lower binding affinity and fewer sustained interactions than CID: 56649692. Notably, CID: 56649692 had higher Interaction Fraction Values (IFVs) with residues TRP1089 and ASP1122, maintaining these interactions for over 100% of the simulation time. These sustained interactions suggest that CID: 56649692 may offer higher efficacy as an inhibitor, as stable protein-ligand interactions are essential for prolonging drug action and enhancing inhibitory effectiveness (Voss and Nitsche, 2020). The higher interaction frequency also aligns with findings from molecular dynamics studies, indicating that strong IFVs contribute to ligand efficacy

in inhibiting viral proteases (Mwaliko *et al.*, 2021).

ADME and Toxicity Profiles

The pharmacokinetics and toxicity analysis reinforced CID: 56649692's viability as a lead compound. With favorable ADME properties, including high gastrointestinal absorption and compliance with Lipinski's rule, CID: 56649692 exhibited an ideal pharmacokinetic profile for drug development (Table 4). Moreover, the absence of hepatotoxicity, carcinogenicity, and mutagenicity in toxicity screening further supports the compound's safety for potential therapeutic use. This aligns with prior studies highlighting the importance of non-toxic natural compounds as antiviral agents, particularly against protease targets (Banerjee *et al.*, 2018; Kshatriya *et al.*, 2019).

Study Limitations and Future Directions

While these *in silico* findings indicate that CID: 56649692 holds promise as a ZIKV NS2B-NS3 protease inhibitor, further validation is required. *In vitro* assays will be essential to confirm the binding efficacy observed *in silico*, followed by *in vivo* studies to assess bioavailability, pharmacodynamics, and potential side effects. Additionally, structural optimization of CID: 56649692 could enhance its binding affinity and selectivity, addressing any off-target interactions that may arise in biological systems. Future studies could also explore other ZIKV proteins involved in replication and immune evasion, providing a broader target scope for comprehensive antiviral therapies.

This study has identified PubChem CID: 56649692 as a promising lead compound for ZIKV inhibition, based on strong binding affinity, stable protein-ligand interactions, favorable pharmacokinetics, and a robust safety profile. Targeting the NS2B-NS3 protease with CID: 56649692 offers a novel approach to ZIKV therapy, aligning with ongoing efforts to develop effective antiviral agents. Further experimental research will be

necessary to validate these findings and advance CID: 56649692 as a candidate for clinical investigation in the treatment of Zika virus infection.

Conclusions and Recommendations

This study identified PubChem CID: 56649692 as a potential inhibitor of the Zika virus NS2B-NS3 protease through a comprehensive in silico approach, including ADME and toxicity profiling, molecular docking, and molecular dynamics simulation. CID: 56649692 demonstrated a strong binding affinity to the protease, high stability in simulation analyses, and favorable pharmacokinetic properties without observable toxicity risks, distinguishing it as a promising lead compound. Targeting the NS2B-NS3 protease complex effectively inhibits viral replication, making CID: 56649692 a viable candidate for further development in antiviral therapies against ZIKV.

Future work should include in vitro and in vivo studies to validate these computational findings and assess the bioavailability, efficacy, and safety profile of CID: 56649692 under physiological conditions. Additionally, structural optimizations could enhance its potency and selectivity. This study underscores the utility of computational methods in accelerating drug discovery, particularly for emerging global health threats such as Zika virus, and provides a foundational step towards developing an effective therapeutic solution for ZIKV infections.

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Novelty Statement

This study explores natural phytochemicals as novel inhibitors of the Zika virus (ZIKV) NS2B-NS3 protease complex, a key target for viral replication. Unlike prior research on synthetic compounds, it employs advanced computational techniques to identify PubChem CID: 56649692 as a highly stable and potent inhibitor. These findings provide a foundation for future in vitro and in vivo studies, advancing the development of natural anti-ZIKV therapeutics.

Authors Contributions

NJL, MZSM, HH and TM conceptualized, designed the study and wrote the manuscript. ATB, FA and AS edited, and reviewed the manuscript. KAS and PB developed the manuscript, analyzed the data, wrote, and revised the final manuscript. All authors contributed to the review of the manuscript and approved the final manuscript.

Conflicts of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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