

Article

Through Bioinformatics, Can an Extract from *Diospyros montana* Offer Broad-Spectrum Inhibition Against EV-A71 2C Protein?

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Abstract

Enterovirus A71 (EV-A71), belonging to the *Picornaviridae* family, is the primary causative agent of hand, foot, and mouth disease (HFMD) in children under five years of age, and no specific broad-spectrum antiviral therapy is currently available. Despite having extensive research on the EV-A71 3C protease, another nonstructural protein, the 2C protein, which is a highly conserved protein and a member of the ATPases Associated with diverse cellular Activities (AAA+) helicase family, lacks conclusive details of its mechanism. This study, thereby, performed a comprehensive analysis of the wild-type EV-A71 2C protein by studying the role of Zn²⁺ in structural stability and determining the binding site with two pronounced inhibitors—fluoxetine 12b and dibucaine 6i—as the positive controls via the blind docking method. Then, a virtual screening on a Vietherb database (5071 metabolites from Vietnamese natural sources) yielded 20 top-ranked compounds, which were selected for further molecular dynamics (MD) simulation and cross-variant evaluation. Among these, CID 101282068 extracted from *Diospyros montana* exhibited overall good, predicted binding affinity (docking score of −9.8 kcal/mol and $\Delta G_{\text{bind}} = -33.27$ kcal/mol) and sustained the highest number of persistent hydrogen bonds with critical residues at the oligomerization interface. Further validation against 23 EV-A71 2C variants highlights its hypothetical broad-spectrum characteristics and can be considered a promising lead candidate targeting EV-A71 2C multimers.

Keywords: *Enteroviruses A71*; monomeric 2C protein; dibucaine 6i; fluoxetine 12b; Vietherb database; *Diospyros montana*

Academic Editor: Jack Adam Tuszynski

Received: 11 August 2026

Revised: 23 September 2026

Accepted: 28 September 2026

Published: 30 September 2026

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1. Introduction

Hand, foot, and mouth disease (HFMD), which is brought on by a variety of *Enteroviruses* (EVs) of the family *Picornaviridae*, was first discovered in 1957 in New Zealand [1]. This is known as a common disease occurring in children under five years of age, with major symptoms (e.g., blisters on hands, feet, and tongue; fever; pulmonary edema; and fatality) caused by the two most dominant enterovirus types, namely *Enterovirus A71* (EV-A71) and *Coxsackievirus A16* (CV-A16). Noticeably, various acute neurological problems and a high rate of fatality were associated with the EV-A71 strain compared to the CV-A16

strain [2]. To be specific, epidemiological data from several countries recorded such large outbreaks caused by *EV-A71*, including its first appearance in Hubei province, China (1987). This was then documented as over 3000 deaths and more than 100,000 severe cases reported in the period of 2008–2015 [3–11]. European countries, however, observed less widespread HFMD than in Asia. Meanwhile, Vietnam recorded many disease outbreaks widely in 2008 and 2010 (an average of 10,000 yearly reported cases) after its first occurrence in 2003 [12]. Years later, the number of cases peaked with more than 200,000 hospitalizations and over 200 deaths documented [13,14]. Additionally, southern regions accounted for most of the severe and fatal cases due to their higher average temperature and humidity [14]. According to Vietnam Ministry of Health guidelines, no specific antiviral therapeutics have been approved for the treatment of HFMD in the country. Management therefore relies solely on vigilant clinical supervision, timely recognition of complications, and pharmacological intervention in severe cases, alongside adequate nutritional support to bolster children's overall health and immune resilience. Additionally, the scarcity of current epidemiological data recorded in Vietnam leads to a limited understanding of the fundamental etiology and epidemiology of HFMD. Hence, there is an urgent need to investigate this disease's roots as well as novel antiviral discoveries.

The *EV-A71* genome encodes four structural capsid proteins (VP1–VP4) responsible for host–cell attachment and another seven nonstructural proteins (2A–2C and 3A–3D) supporting viral replication and immune evasion [15]. Notably, the nonstructural 2C protein which aids in generating a negative-sense single-stranded RNA virus used as a template to manufacture more viral genomes, is the most highly conserved protein among picornaviruses, making it an attractive target for broad-spectrum anti-*EV* drug development [16]. Additionally, the protein is catalytically active only in its hexameric state, illustrating its self-oligomerization process in solution [17]. Furthermore, the viral 2C protein functions analogously as an ATPase within the AAA+ family (ATPases Associated with diverse cellular Activities). Its architecture contains canonical Walker A, Walker B, and motif C domains. It consists of ATP-driven molecules that unwind single/double stranded nucleic acids during replication and genome maintenance. These proteins typically assemble into ring-shaped hexameric complexes that use loop insertions projecting into their central pore to engage nucleic acid strands, which are subsequently separated through ATP hydrolysis [18]. However, because the mechanism of the *EV-A71* 2C protein remains poorly understood compared to the 3C protease, heightened efforts are required to develop novel inhibitors. Several small molecules, including (S)-fluoxetine, guanidine HCl, N6-benzyladenosine and some chemically synthesized compounds such as dibucaine 6i derived from dibucaine—a repurposed local anesthetic—showed broad activity on *EVs* ($EC_{50} = 1.238 \mu\text{M}$), pyrazolopyridine analog 7d ($EC_{50} = 0.04\text{--}0.10 \mu\text{M}$), and some fluoxetine-inspired modifications, such as 12b ($EC_{50} = 0.49 \pm 0.26 \mu\text{M}$), 19b ($EC_{50} = 0.89 \pm 0.09 \mu\text{M}$) and 19d ($EC_{50} = 0.27 \pm 0.12 \mu\text{M}$), demonstrated potent inhibition against the *EV-A71* 2C protein [19–22].

Situated within the Indo-Burmese biodiversity hotspot, Vietnam is a tropical nation giving advantages of a rich diversity of herbal plants. In addition, their information, such as metabolites, chemical structures, morphologies, geographical locations, diseases..., has been computerized, standardized, and published online for non-expert users and scientists via a Vietherb database [23]. Thus, the Vietherb library with more than 5000 compounds was used as the ideal source for identifying potential candidates from Vietnamese natural sources via performing virtual screening. Our computational workflow initiated with an assessment of Zn^{2+} and ATP cofactor effects on the wild-type *EV-A71* 2C protein model, following a new binding site detection using a global docking method on fluoxetine 12b and dibucaine 6i targeting the *EV-A71* 2C protein as potential positive controls. Then, all Vietherb ligands were filtered according to Lipinski's Rule of Five (RO5) before

specific docking. Only 20 top-ranked compounds were chosen for further assessment of binding properties within the binding site via molecular dynamics (MD) simulation to characterize binding stability and were screened against 23 *EV-A71* 2C protein variants to assess broad-spectrum potential.

2. Materials and Methods

2.1. *EV-A71* 2C Monomer Structures Preparation

A multimer wild-type strain of *EV-A71* 2C structure was resolved experimentally and retrieved from the Protein Data Bank (PDB ID: 5GRB) including Zn^{2+} ions and ATP cofactors [24]. Three distinct monomeric systems were prepared from chain A using PyMOL (version 3.1.8, Schrodinger Inc., New York, NY, United States): (1) 5GRB-apo, prepared by removing all heteroatoms (HETATM) and water molecules; (2) 5GRB-Zn, retaining the bound Zn^{2+} ion while removing all water molecules and ATP; and (3) 5GRB-Zn&ATP, retaining both Zn^{2+} and ATP while excluding water molecules [25].

In prior unpublished work conducted by our group, *EV-A71* 2C protein variant sequences were selectively retrieved from the National Center for Biotechnology Information (NCBI) GenBank database using specific filtering criteria to minimize sequence redundancy. The data retrieval and sequence processing steps resulted in a consensus protein sequence representing strains collected in Vietnam and a panel of mutated 2C protein variants as shown in Table 1. Three-dimensional monomeric models containing a single Zn^{2+} ion for each consensus and variant 2C protein were predicted using AlphaFold3 (Google DeepMind and Isomorphic Labs, Cambridge, MA, United States), the latest version of the deep learning-based structure prediction platform developed by DeepMind [25]. Of all the predicted models, only those with the highest-ranking scores of confidence metrics were retained for downstream processing.

Table 1. List of 23 *EV-A71* 2C protein variants and corresponding mutations. Accession numbers were retrieved from the NCBI GenBank database.

No.	Accession	Variants (1-Letter Coded Amino Acid)
1	AHX57810.1	Presented consensus
2	WPH57244.1	P122S
3	BCU73704.1	I126V
4	AHX57702.1	I127V
5	AHX57711.1	G140S
6	AHX57705.1	S153G; Y243F
7	QBP14797.1	D162N
8	AGW52035.1	V171I; N181T; P182R; K209R; Y243F; K268R
9	AHX57658.1	V171I; Y243F; K268R
10	AHX57766.1	V197I
11	AHX57735.1	D198E
12	AHX57651.1	I200V; D257E
13	BCU73715.1	A224S
14	BCU73726.1	S225N
15	QBP14830.1	V232I
16	AHX57641.1	D236N
17	AHX57689.1	Y243F; D257E
18	AJQ21565.1	Y243F
19	QBP14767.1	Y243H
20	AHX57716.1	Y254F
21	QBP14794.1	L258V
22	QBP14820.1	K268R

2.2. Control and Vietherb Natural Compounds Preparation

Reference positive controls (fluoxetine 12b and dibucaine 6i) and 5071 natural metabolites from the Vietherb library were retrieved in SDF format from the PubChem database, while sucrose was obtained from the ZINC2.0 database as a negative control [26,27]. All chemical structures were later processed through geometry minimization using the Merck molecular force field 94 (MMFF94) force field before being converted to PDBQT format using Open Babel (version 2.4.0, United States) [28].

In addition, the drug-likeness of all Vietherb compounds was evaluated using the SwissADME web server [29]. Compounds were filtered based on adherence to Lipinski's Rule of Five (RO5): molecular weight ≤ 500 Da, lipophilicity ($\text{LogP} \leq 5$), hydrogen-bond donors ≤ 5 , and hydrogen-bond acceptors ≤ 10 [30,31]. The number of Lipinski's rule violations was recorded for each compound to assess overall drug-likeness.

2.3. Molecular Docking Simulations

AutoDock Vina (version 1.1.2, The Scripps Research Institute, La Jolla, CA, United States) was used to perform preliminary molecular docking. A docking grid box was generated using the MGLTools package (version 1.5.7, The Scripps Research Institute, United States) that covered the entire 2C protein structure with a grid spacing of 1.0 Å in each direction [32–34]. Several key parameters were set, including optimal exhaustiveness setting, an energy range of 4 kcal/mol, a maximum of 20 binding poses, and 5 repeated screening runs to ensure reproducibility.

Following the high-throughput screening, the shortlisted Vietherb compounds were assessed on different variant proteins using the Autodock4_{Zn} force field with the Zn²⁺ treatment in the protein system because the standard AutoDock Vina scoring function is not specifically parameterized for the coordination preferences of Zn²⁺ in metalloprotein binding sites. Specifically, a pseudo-atom (designated as TZ) was explicitly placed adjacent to the Zn²⁺ to model the ideal tetrahedral coordination geometry. Grid maps, then, were computed using calibrated, non-bonded pairwise potentials specifically optimized for Zn²⁺-coordinating groups (such as N, O, and S) based on empirical metalloprotein structural data. The docking procedure was subsequently performed with these specified parameter sets [35,36]. Then, the system of the first binding mode was used for further MD simulation evaluation.

2.4. Molecular Dynamics Simulation and MM-PBSA Analysis

Hydrogen atoms were added to the ligand-protein systems at pH 7.4 using Open Babel. Partial atomic charges were assigned via the AM1-BCC model using a parameterized program named Antechamber – a part of Ambertools25 package (University of California, United States); atom types were defined using General Amber Force Field 2 (GAFF2), and missing parameters were generated using parmchk2 [28,37–39]. These were then converted into GROMACS format (version 2025.2, KTH Royal Institute of Technology, Sweden/Netherlands) using the ParmEd tool (version 4.3.1, University of California, United States) before preparing force field parameters for both the protein (ff14SB) and ligand via the LEaP module of the Amber25 and Ambertools25 packages [37,38,40–44]. To maintain active-site coordination geometry, the Zn²⁺ ion coordinated by residues Cys270, Cys281, and Cys286 was parameterized using MCPB.py in Ambertools25 [45]. The complex systems were solvated in a TIP3P water box and neutralized with 0.15 M NaCl before minimization to relax the complexes and equilibrated using thermodynamic canonical (NVT) and isothermal-isobaric (NPT) ensembles at 300.0 K and 1 atm for 2 ns and 20 ns, respectively. Production MD simulations were then conducted for 200 ns per complex.

In addition, to calculate the binding free energy ΔG_{bind} , the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) method was used via *g_mmpbsa* with default parameters [46]. Calculations were performed across 500 frames extracted from the system-specific stable trajectory of the final 50 ns.

2.5. Data Analysis and Visualization

The PyMOL and BIOVIA Discovery Studio Visualizer (version 2024, Dassault Systèmes, United States/France) were used to visualize binding mode clusters and non-covalent interactions [25,47]. Basic statistical analyses and data visualizations were executed in Microsoft 365 Excel (version 2508, Microsoft, United States). The trajectory output from MD simulation was processed to calculate Root Mean Square Deviation (RMSD) and hydrogen-bonding profiles. Contact persistence over the 200 ns simulation between ligands and protein was calculated and performed using InterMap (DenMark) [48].

3. Results

3.1. Influence of Zn^{2+} and ATP Cofactors on EV-A71 2C Monomer Stability

The conformational stability of the wild-type EV-A71 2C protein monomer (PDB ID: 5GRB) was evaluated over 200 ns of MD simulation across three monomeric states: the apo, the Zn^{2+} -bound (5GRB_Zn), and the Zn^{2+} -and-ATP-bound (5GRB_Zn_ATP). The backbone RMSD profile was used as the stability metric to understand the subsequent binding site analysis of the EV-A71 2C protein (Figure 1). Specifically, low backbone mobility (0.20–0.25 nm) was exhibited with transient fluctuations between 50 and 170 ns before stabilizing when the Zn^{2+} ion was embedded in the system. Meanwhile, the presence of the ATP cofactor in the 5GRB_Zn_ATP state displayed the lowest and most stable backbone RMSD (<0.20 nm throughout the 200 ns trajectory) among the three models, likely due to additional energetic stabilization provided by ATP within the catalytic site. In contrast, the apo state exhibited substantial flexibility, characterized by a sharp increase in RMSD between 50 and 60 ns before stabilizing near 2.5 Å (0.25 nm) after 90 ns. Therefore, the Zn without ATP state would truly reflect the monomer mechanism of the 2C protein, better representing the unoccupied, ATPase-competent state of the protein for further binding pocket identification and potential candidate screening.

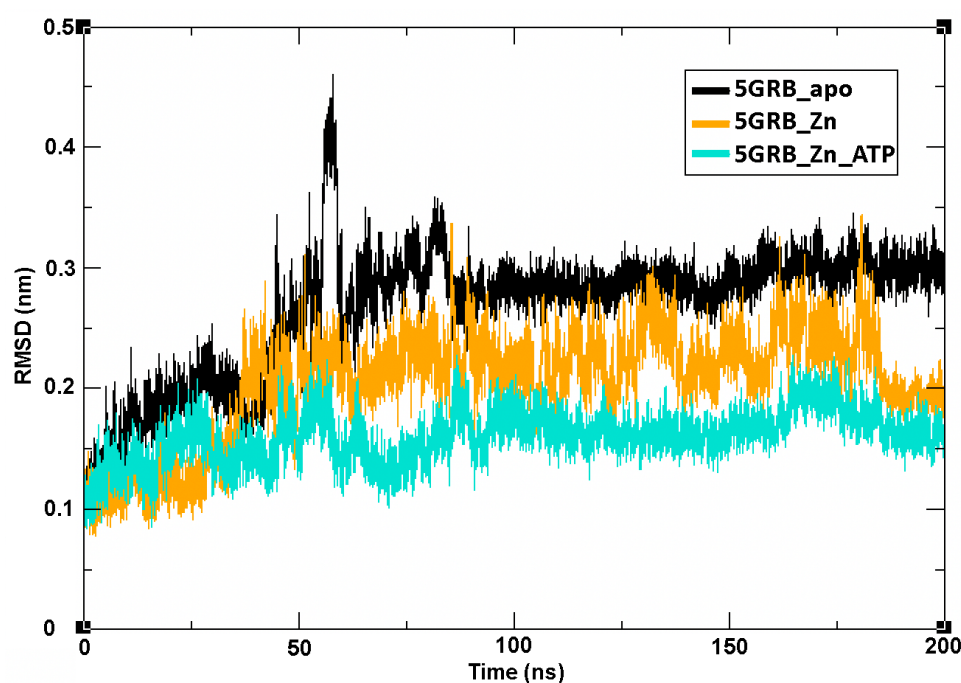


Figure 1. Conformational stability of EV-A71 2C monomer states across 200 ns MD trajectories. Backbone Root Mean Square Deviation (RMSD) profiles of the apo, Zn²⁺-bound (5GRB_Zn), and Zn²⁺-and-ATP-bound (5GRB_Zn_ATP) states of the wild-type monomer (PDB 5GRB) evaluated over 200 ns of molecular dynamics simulation.

3.2. Two Mechanisms for Potential Inhibitors Against the 2C Protein: Inhibiting Oligomerization or Targeting the ATPase Site

The 5GRB_Zn model was then blind docked to access the binding pocket using either selected positive controls (fluoxetine 12b and dibucaine 6i) or a negative control—sucrose. Although multiple potential interaction sites were sampled, the deep pocket located at the C-terminus, designated as Cluster 1 and intrinsically associated with 2C self-oligomerization, emerged as the primary, thermodynamically favored binding site for the positive controls. A secondary region adjacent to the Zn²⁺-binding domain (the ATP-binding site), called Cluster 2, showed less favorable binding for both control compounds in the monomeric state (Supplementary Figure S1).

Notably, residues 270–286 were previously identified as the zinc-binding region, linked to the ATPase domain (Cluster 1) through antiparallel β -sheet interactions ($\beta 5$ and $\beta 6$). Several key residues are involved in Cluster 1 including Ile141, Arg144, Ala145, Asp148, Asn277, Phe278, Lys279, and Arg280, with Arg144 shown to mediate inter-subunit interactions between adjacent 2C monomers [17]. Prior work by Li and colleagues reported that N6-benzyladenosine bound within the deep pocket situated between the N-terminal membrane-binding and ATPase domains of the 2C protein, mediated by similar key residues including Arg144, Asp148, Leu269, Cys270, Ser271, Asn273, Phe278, Lys279, Arg280, Cys281, and Ser282 [22]. Our docking simulations consistently show key hydrogen bonds between fluoxetine 12b (docking score -7.3 kcal/mol) and Arg144 and Asp148 of the 5GRB_Zn model as well as other hydrophobic interactions with Ala145 and Phe278. In comparison, dibucaine 6i exhibited a weaker, less favorable docking score (-6.9 kcal/mol) and formed only one hydrogen bond with Lys279 while more van der Waals interactions were formed with Ile141 and Phe278. This reduced affinity is potentially attributable to a comparatively rigid and planar structure, with fewer suitably oriented polar groups (Figure 2). Because no experimentally co-crystallized ligand is present in PDB 5GRB, re-docking could not be performed; virtual screening was thus focused directly on Cluster 1.

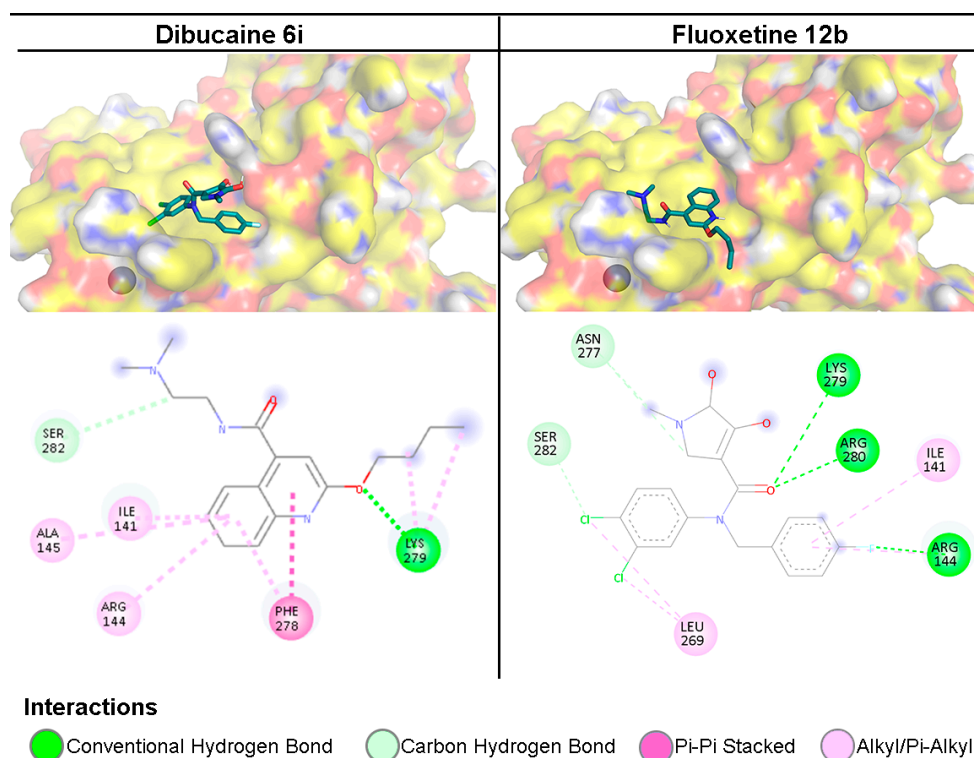


Figure 2. Predicted binding modes and non-covalent interactions of positive controls within Cluster 1. Representative docked poses of dibucaine 6i and fluoxetine 12b bound to the oligomerization pocket (Cluster 1) of the monomer 5GRB_Zn model (Zn²⁺ ion is depicted as a gray sphere). Key active-site residues and types of non-covalent interaction networks are shown in stated colors. The interactions were visualized using BIOVIA Discovery Studio Visualizer (version 2024).

3.3. Virtual Screening of the Vietherb Database

To identify potential compounds from natural sources targeting the EV-A71 2C protein, a virtual screening of the Vietherb library (3502 compounds after being filtered with Lipinski's RO5) was carried out using AutoDock Vina against the 5GRB_Zn monomer model. Predicted binding scores spanned a wide energetic range. Withametelin N (CID 16757187) extracted from *Datura metel* exhibited the most favorable docking score (−11.0 kcal/mol), while the methanethiol (CID 878) compound extracted from *Raphanus sativus* was bound unfavorably at the oligomerization interface (−1.1 kcal/mol). As shown in Figure 3, a docking-score distribution histogram illustrates the most compounds clustering between −8.0 and −5.0 kcal/mol, peaking around −7.0 to −6.0 kcal/mol. Although Lipinski filtration reduced the initial library size by ~31%, the overall distribution profile remained nearly unchanged. The top 20 candidate compounds achieved docking scores ranging from −11.0 to −9.8 kcal/mol, outperforming both positive control groups. These prioritized compounds were subsequently re-docked using the Autodock4_{Zn} scoring function to ensure that tetrahedral Zn²⁺ coordination geometry did not alter predicted binding modes prior to MD simulations.

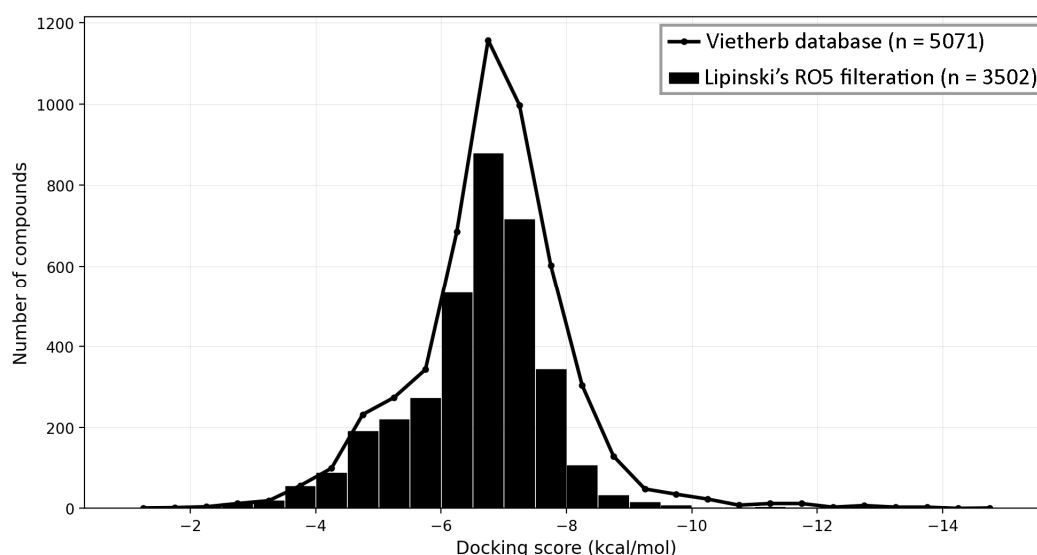


Figure 3. Docking score distribution of the Vietherb natural compound library. Frequency distribution histogram of top-ranked docking scores for Vietherb natural metabolites screened against the monomer 5GRB_Zn model before and after filtration using Lipinski's Rule of Five.

3.4. Molecular Dynamics Simulation and MM-PBSA Binding Free Energy Evaluation

The thermodynamic stability of top-ranked compounds and reference controls was determined through a 200 ns MD simulation on the 5GRB_Zn complex. The resulting trajectories were quantified using two complementary RMSD profiles: the ligand heavy-atom RMSD fitted to the protein backbone to monitor active-site retention, indicating ligand rearrangement within the binding pocket, and the protein backbone RMSD reflecting global protein conformational stability (Figures 4 and S2).

Overall, 2-(1,5-dihydroxy-3-methyl-8-oxo-6,7-dihydro-5H-naphthalen-2-yl)-5-hydroxy-7-methylnaphthalene-1,4-dione (CID 101282068) and (-)-Porrigenin B (CID 177402) compounds demonstrated the most stable MD profiles. CID 101282068 witnessed both low backbone (0.10–0.25 nm) and ligand (0.15–0.25 nm) RMSD values throughout the 200 ns trajectory, indicating a tight accommodation within the binding pocket and yielding the most favorable MM-PBSA binding free energy ($\Delta G_{\text{bind}} = -33.27$ kcal/mol) (Table 2). Interaction fingerprinting revealed that CID 101282068 formed the most diversified polar network, establishing persistent hydrogen bonds with Asp148, Ala267, Lys279, Arg280, and Ser282 (Figure 5). For the CID 177402 ($\Delta G_{\text{bind}} = -32.72$ kcal/mol), an early pose reorganization was revealed by its ligand RMSD plot during the first 40 ns, following a stable plateau at around 0.15 nm till the end of the simulation. This stability was driven by two hydrogen bonds with Arg280 and Ser282 maintained at ~100% occupancy, alongside stable hydrophobic contacts with Ile141. This coincided with a consistently low backbone RMSD (~0.10–0.20 nm), suggesting the overall fold remained largely undisturbed despite the ligand's positional shift. In contrast, (-)-Neoporrigenin A (CID 177405) maintained a comparatively modest backbone RMSD (0.15–0.30 nm), reflecting reasonable overall protein stability. Likewise, such a high rearrangement of this ligand conformation was observed at several intervals, especially around 155–160 ns (spiking to ~0.85 nm) and a less favorable MM-PBSA energy ($\Delta G_{\text{bind}} = -29.25$ kcal/mol), forming only a single hydrogen bond with Lys279. A similar condition was observed on Gangetinin (CID 101316460), where its ligand RMSD fluctuated persistently between 0.2 and 0.8 nm across the entire trajectory and lacked hydrogen bonding, engaging purely via hydrophobic interactions within the pocket.

3.5. Cross-Docking Validation Across Protein Structural Variants

The robustness of top-ranked compounds was also assessed by additional docking against a panel of 23 *EV-A71* 2C protein models (including the consensus model AHX57810.1 and 22 variant models) (Figure 6). Across all tested variants, all five lead candidates consistently showed more favorable docking affinity (approximately -10.5 to -9.0 kcal/mol) to the multimerization site and substantially outperformed dibucaine 6i (-7.5 to -6.5 kcal/mol). Meanwhile, fluoxetine 12b and CID 101316460 experienced broadly similar docking patterns with all the 2C protein models. This suggested a remarkably favorable binding of identified leads relative to the controls irrespective of structural variants used. In particular, CID 177402 and CID 101282068 compounds displayed the most consistently strong and stable docking scores across the full panel of protein variants (-10.6 to -9.0 kcal/mol), while CID 182649 and CID 177405 displayed comparatively greater variability across specific variants, with noticeable weakening against QBP14767.1. These results demonstrate that CID 101282068 binds robustly to the 2C oligomerization site regardless of sequence variations across *EV-A71* strains. The reliability and generalization of the predicted binding affinities were supported by the docking scores of all five shortlisted compounds across this structurally diverse protein panel relative to the control.

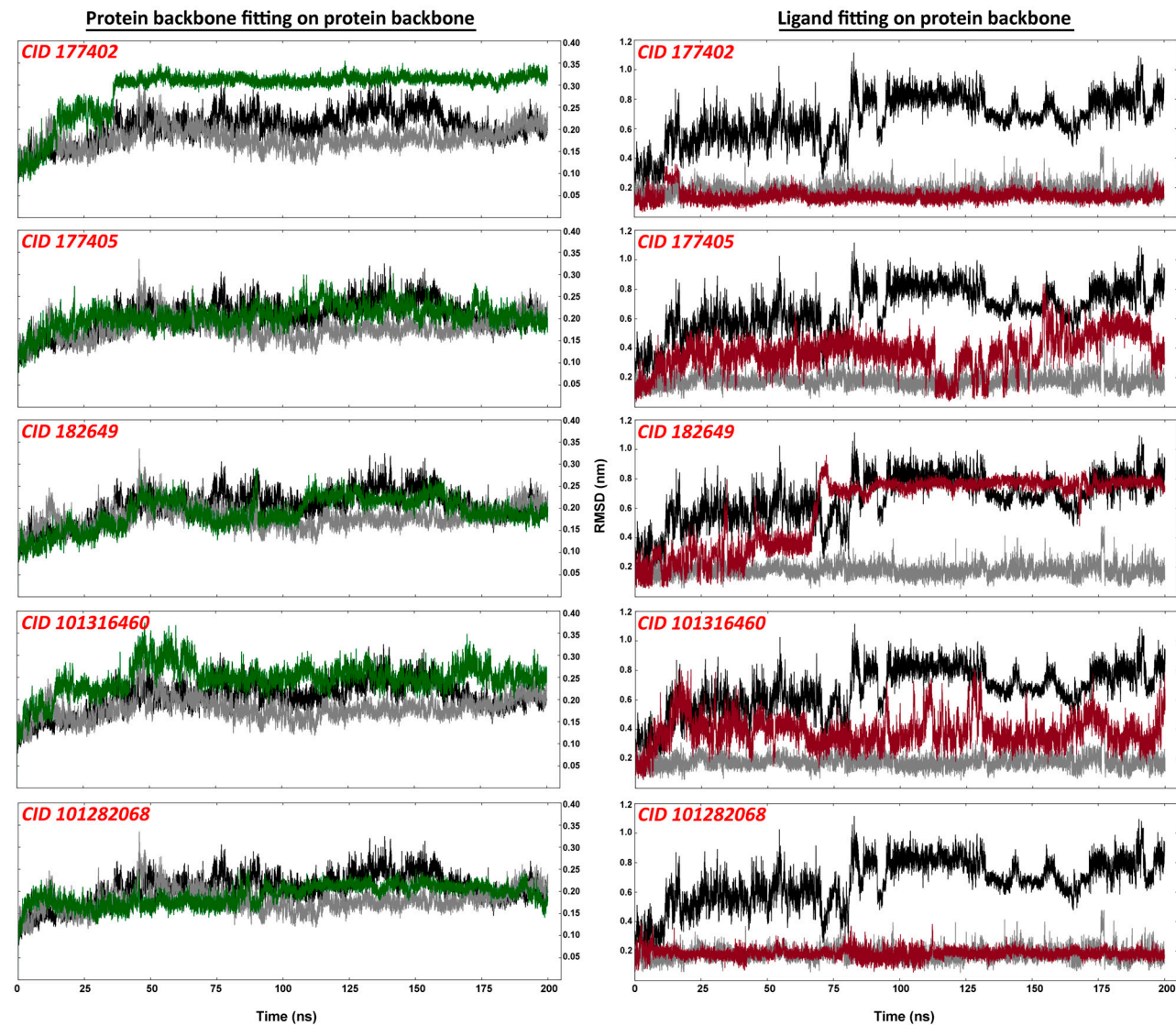


Figure 4. Structural stability profiles of the top 5 lead candidates and positive controls during 200 ns MD simulations. Molecular dynamics trajectory analysis displaying protein backbone RMSD (green traces) and ligand heavy-atom RMSD fitted to the protein backbone (velvet traces) for the top five shortlisted Vietherb compounds and reference controls (dibucaine 6i and fluoxetine 12b as black and gray traces, respectively) bound to the 5GRB_Zn model.

Table 2. Computational parameters of the top 5 shortlisted Vietherb natural compounds against the monomer 5GRB_Zn protein model. Docking scores were collected from AutoDock Vina, ΔG_{bind} was collected from MM/PBSA, and interactions were evaluated within the C-terminal oligomerization interface of the monomer 5GRB_Zn protein model.

Compound Name (IUPAC Name)	Pubchem ID (CID)	Plant Source	Docking Score (kcal/mol)	ΔG_{bind} (kcal/mol)	Interacted Residues
Fluoxetine 12b			−7.3	−31.50	Leu137, Gly140, Ile141, Arg144, Asp148, Phe278, Arg280, Ser282, Val285
Dibucaine 6i			−6.9	−22.26	Leu137, Gly140, Ile141, Arg144, Ala145, Asn277, Phe278, Lys279, Arg280
(-)-Porrigenin B	177402	<i>Allium porrum</i>	−10.3	−32.72	Leu137, Gly140, Ile141, Arg144, Val154, Ser156, Ala267, Phe278, Arg280, Ser282, Leu284, Val285
(-)-Neoporrigenin A	177405	<i>Allium porrum</i>	−10.3	−29.25	Ile141, Arg144, Ala145, Asp148, Lys149, Ala267, Phe278, Lys279, Arg280
(1R,2S,4S,5'S,7S,8R,9S,12S,13R,15R,16R,18S,19R)-5',7,9,13-tetramethylspiro[5-oxapentacyclo [10.8.0.02,9.04,8.013,18]icosane-6,2'-oxane]-15,16,19-triol	182649	<i>Allium porrum</i>	−10.2	−31.94	Leu137, Gly140, Ile141, Arg144, Ser156, Ala267, Phe278, Lys279, Arg280, Ser282, Leu284, Val285
Gangetinin	101316460	<i>Desmodium gangeticum</i>	−9.8	−28.24	Leu137, Ile141, Arg144, Asn277, Phe278, Lys279, Arg280, Val285
2-(1,5-dihydroxy-3-methyl-8-oxo-6,7-dihydro-5H-naphthalen-2-yl)-5-hydroxy-7-methylnaphthalene-1,4-dione	101282068	<i>Diospyros montana</i>	−9.8	−33.27	Ile141, Arg144, Ala145, Asp148, Ala267, Leu269, Phe278, Lys279, Arg280, Ser282, Val285

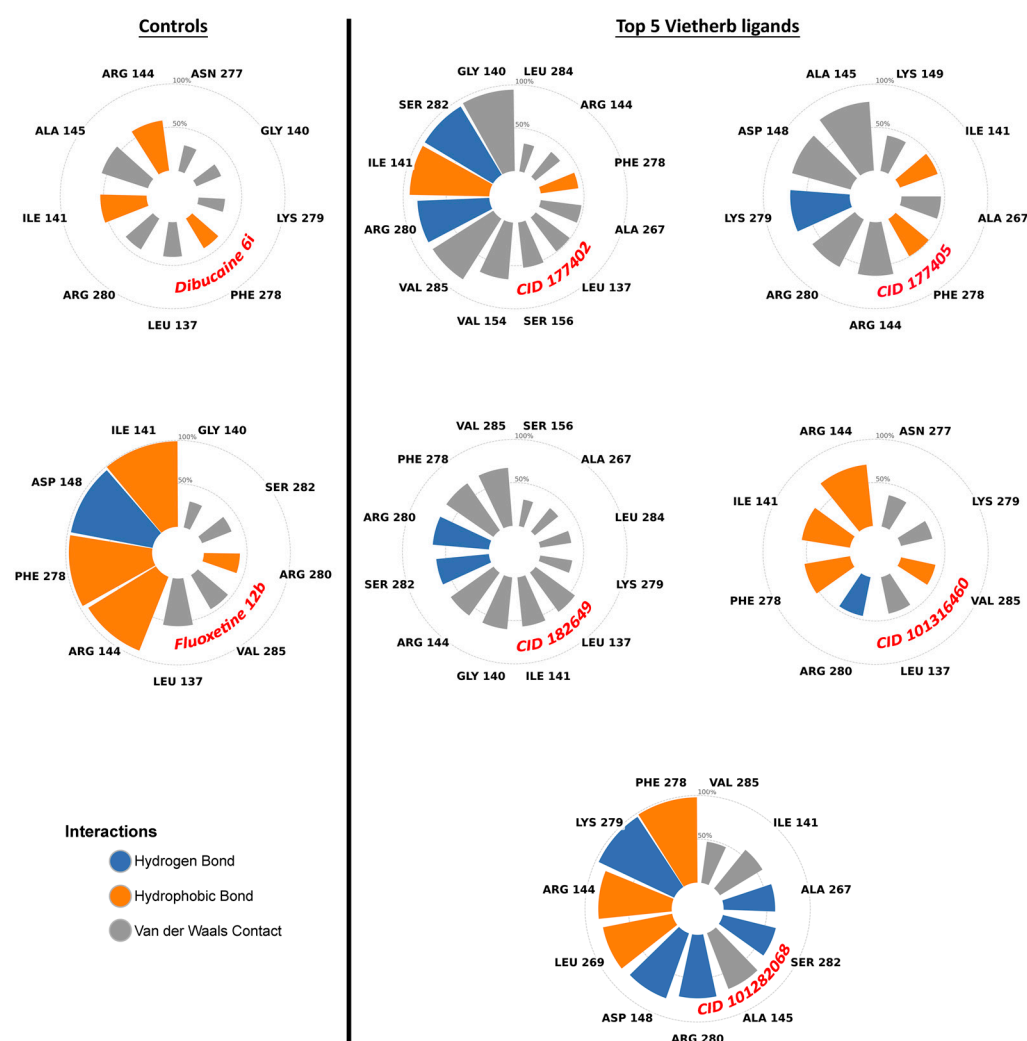


Figure 5. Per-residue protein-ligand interaction fingerprints and contact occupancies of the top 5 candidates. Dominant non-covalent binding residues displayed as wedges (colored by the residue's dominant interaction type) with their contact occupancy recorded as a percentage (rings representing 50% and 100% trajectory persistence thresholds).

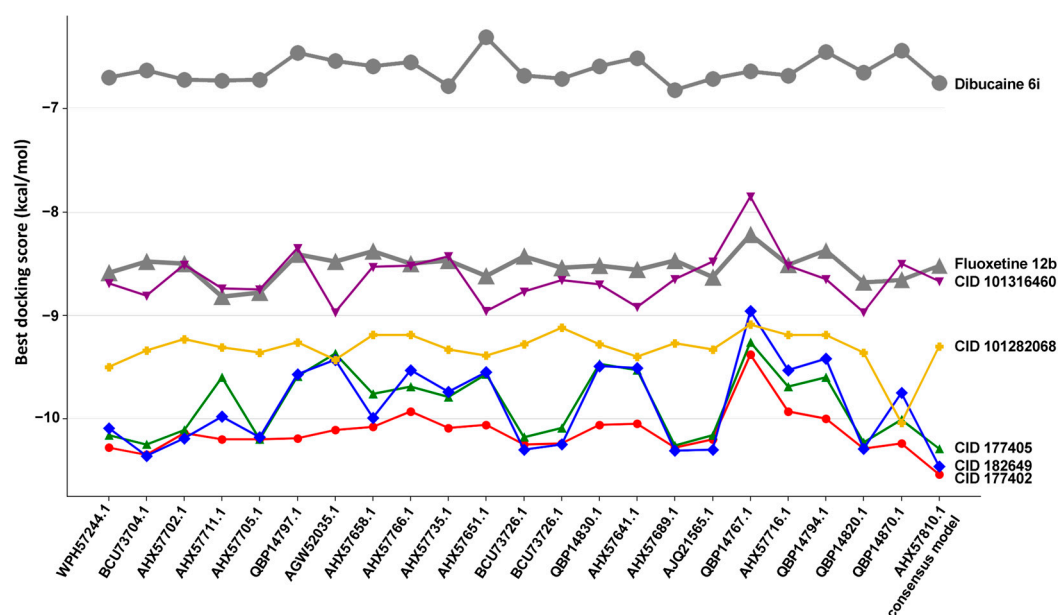


Figure 6. Cross-docking evaluation of the top 5 Vietherb ligands across 23 EV-A71 2C variant models. The bold gray lines present the docking values of positive controls (fluoxetine 12b and dibucaine 6i) on protein models, including the consensus sequence (AHX57810.1).

4. Discussion

Hand, foot, and mouth disease caused by *Enterovirus A71* (EV-A71) remains a major public health concern, especially in the Asia-Pacific region, where recurring outbreaks are frequently accompanied by severe neurological complications and associated mortality. Notably, this ongoing public-health burden was further illustrated in Vietnam, with over 200,000 infected cases and 207 deaths recorded at the end of 2011 and early 2012 [49,50]. Despite this disease burden, no licensed antiviral therapy specifically targeting EV-A71 is currently available. In addition, compared to a well-known non-structural 3C protease protein, EV-A71 2C, a less-researched protein, represents a particularly promising therapeutic target due to its ATPase activity, oligomerization, involvement in RNA replication, and especially being highly conserved across different strains [17,22]. This study focuses on identifying potential compounds from Vietnamese natural sources as EV-A71 2C antivirals using structure-based virtual screening and molecular-dynamics analyses.

Structurally, the EV-A71 2C protein consists of a central AAA+ ATPase core that contains Walker A/B motifs, motif C, an inter-subunit R-finger, an N-terminal amphipathic helix anchoring to membranes, and a C-terminal helix. This C-terminal domain plays a critical role in forming a functional replication complex in picornaviral 2C proteins. This was mentioned in Guan et al.'s (2017) research, revealing self-oligomerization characteristics of the C-terminal region; consequently, disruption of this domain would impair the interaction between adjacent 2C monomers, resulting in a non-functional protein [17]. Thus, a candidate drug capable of blocking protein polymerization could represent a viable therapeutic strategy [17]. Likewise, another functionally critical region was the ATPase domain that served as the anchor for ATP binding responsible for activating the protein's enzymatic function [16]. In addition, the cysteine-rich Zn²⁺-finger also plays a main role in EV-A71 2C protein supporting ATPase core stabilization via hydrophobic interactions [51]. Although the C-terminal Zn-finger domain is essential for 2C oligomerization and function, structural investigation of wildtype EV-A71 2C remains limited, with 5GRB being the only experimentally resolved wild-type model. In 2019, this was first used as a structural template for a *Coxsackievirus B3* (CV-B3) 2C homology model in identifying the inhibitory effect of (S)-fluoxetine and (R)-fluoxetine against an allosteric site on the CV-B3 2C protein [16]. Last year, EV-A71 2C (PDB ID: 5GRB) and CV-B3 2C (PDB ID: 6S3A) were observed upon ligand binding, resulting in choosing CV-B3 2C as a reference for the EV-D68 2C homology model to study the binding and resistance mechanisms of pyrazolopyridine compounds Jun571/Jun6504 [52]. However, the effect of Zn²⁺ ions on this 2C protein has not yet been discussed in previous studies as well as no documentation of using 5GRB to study the inhibitor mechanism against EV-A71 2C has been recorded. Our molecular dynamics simulations demonstrate that Zn²⁺ contributes to the structural stability of the 2C monomer and substantially reduces backbone mobility (RMSD 0.20–0.25 nm) across the ATPase domain independently of ATP. This observation is consistent with previous computational studies in other Zn²⁺-containing proteins that showed that nearby residue fluctuations were reduced by metal coordination and the local secondary structures were also preserved. These studies further showed that the stabilizing effect of Zn²⁺ was not restricted to residues directly involved in metal coordination, but could extend to other regions of the protein through changes in the surrounding interaction network [53,54]. In the present system, similar stability was witnessed with only Zn²⁺ and Zn²⁺-ATP further suggesting the main contribution of Zn²⁺ itself to maintain the 2C ATPase domain conformation, whereas slightly greater stability of the Zn²⁺-ATP system may arise from an

additional stabilizing contribution from the ATP cofactor within the active site [55,56]. Additionally, Yeager and his colleagues proposed a two-step mechanism showing that the catalytic geometry was not fully locked by the α - and β -phosphates of ATP. Thus, a truly hydrolysis-competent state was only achieved when RNA was also engaged by the 2C oligomer [57]. Our MD results from the 5GRB_Zn&ATP complex system were consistent with previous studies indicating that *EV-A71* 2C preferentially assembles with Zn and ATP molecules. This might refer to the requirement of forming a process from monomer to hexamer oligomers, together with ATP for functional RNA-binding activity [58].

Given that Zn^{2+} coordination was essential for maintaining a stable and representative conformation of an ATPase core, the 5GRB_Zn system was subsequently selected as the structural basis for identifying new cryptic binding sites. This was done using a straightforward approach—blind docking—that searched globally for the entire protein surface, and AutoDock Vina is a good example of this conventional sampling-based group. Its scoring function has been improved from AutoDock4.0 that leverages gradient-based optimization of ligand conformations together with multithreaded processing, thereby, remarkably reducing computational time while enhancing the accuracy of predicted binding poses. Nevertheless, the expanded conformational search space inherent to global docking heightens the risk of insufficient sampling under the program's stochastic search algorithm, meaning that result reliability is contingent upon an adequately rigorous search protocol. To address this, the exhaustiveness parameter was optimized which strongly affected docking outcomes across different protein-ligand systems, by assessing the convergence of both binding pose and docking score across successively increasing exhaustiveness values, as indicated in our previous finding [59]. At exhaustiveness values < 128 , the control group produced more clusters, indicating non-specific binding. However, the higher the exhaustiveness value, the smaller the clusters obtained, and the more favorable docking scores remained. These results agree with our earlier study on *EV-A71* 3C protease, where an exhaustiveness value of 256 gave the fewest clusters and the most consistent docking scores [59]. In addition, more computational resources were required for exhaustiveness ≥ 512 , despite no remarkable change in the docking results.

Structural analysis of the predicted binding pockets revealed that Cluster 1 involved in the self-oligomerization interface and located at the C-terminus, consisted of important residues such as Ile141, Asp148, Lys149, Ala267, Leu269, Phe278, Lys279, Arg280, Ser282, Leu284, and Val285 which were consistent with a previous study [17]. Meanwhile, Cluster 2 (Gly132, Gly134, Lys135, Ser136, Asp177, Arg260, Leu261, and Ala263) being situated adjacent to the zinc-binding domain, showed less favorable and weaker contacts for both fluoxetine 12b and dibucaine 6i controls. These findings suggest that Cluster 1 reflects a more druggable and energetically favorable target site than the ATP-adjacent Cluster 2, likely having more hydrophobic/hydrogen bonding interactions and a deeper cavity. Because 2C self-oligomerization is indispensable for viral helicase activity, targeting Cluster 1 provides a potent therapeutic strategy to sterically block subunit assembly and abolish viral replication.

Following the binding site identification, structure-based virtual screening against monomeric 5GRB_Zn was performed on the Vietherb natural compound library which had been filtered using Lipinski's RO5. The highest-ranked hit—Withametelin N (CID 16757187) extracted from *Datura metel*—obtained a docking score of -11.0 kcal/mol while five top-ranked compounds achieved docking scores of -10.3 to -9.8 kcal/mol. Nevertheless, the docking score is not a suitable estimation of actual binding affinity due to its action on rigid bodies while either the protein or ligand is dynamic in a biological environment. Thus, MD simulation was subsequently performed to assess how well ligands stayed intact within the binding site as well as binding free energy ΔG_{bind} by MM/PBSA. Both controls performed differently within the pocket which favored fluoxetine 12b over

dibucaine 6i, despite a marginal difference in docking score. In addition, fluoxetine 12b maintained relatively stable interactions with Asp148 and Arg144 via H-bonds, compared to the dominant hydrophobic contacts formed between dibucaine 6i and 5GRB_Zn. Regarding the top five candidates, compound CID 101282068 from *Diospyros montana*, an aromatic binaphthalene-like quinone, presents a greater density of accessible carbonyl and hydroxyl groups and is less rigid than compound (-)-Porrigenin B (CID 177402 from *Allium porrum*). It achieved a comparable stable RMSD profile and a greater number of hydrogen bonds with main residues in that oligomerization interface, despite exhibiting a slightly less favorable docking score and binding free energy than compound (-)-Porrigenin B. Moreover, its inhibitory effect against a broad spectrum of 23 variants of the EV-A71 2C protein showed strong and stable binding compared to two positive controls, overall resulting in the most potential candidate among those five. Meanwhile, the chemical structures of CID 177402 and CID 177405 compounds both extracted from the *Allium porrum* plant were mainly similar in their core skeleton, except for the presence of a hydroxyl group rather than a C-2 ketone in (-)-Porrigenin B, which introduced greater conformational flexibility and a desolvation penalty, thereby destabilizing the ligand-receptor complex. This was, additionally, emphasized through a lower number of H-bonds and a weaker binding free energy in Neoporrigenin A. In addition, (-)-Porrigenin B carrying an unusually oxygen-rich spirostane scaffold and extracted from *Allium porrum*, has been reported to exhibit antibacterial activity against bacterial Mur ligases involved in peptidoglycan biosynthesis [60]. It consists of two secondary hydroxyl groups with the spiroketal's two ring oxygens leading to simultaneous H-bond donor/acceptor sites rather than a single 3-OH typical of most sapogenins. Likewise, a hydroxylated analog of (-)-Porrigenin B is the ((1R,2S,4S,5'S,7S,8R,9S,12S,13R,15R,16R,18S,19R)-5',7,9,13-tetramethylspiro[5-oxapentacyclo[10.8.0.0.02,9.04,8.013,18]icosane-6,2'-oxane]-15,16,19-triol) compound (also extracted from *Allium porrum*) having the replacement of the rigid, sp²-hybridized C-2 carbonyl with an additional sp³ hydroxyl group. This, thereby, introduced greater rotational freedom and a less geometrically defined hydrogen-bonding interaction, leading to less favorable binding to the multimerization site, although it had the second-most favorable docking score and binding free energy. The weakest compound in this top-ranked list is CID 101316460 (Gangetinin) extracted from *Desmodium gangeticum*, which has been evaluated in an in silico study of human ACE2 (COVID-19), displaying a comparatively docking score of -9.8 kcal/mol with no hydrogen bonding identified with the monomeric 5GRB_Zn model. Although (-)-Porrigenin B and Gangetinin have been investigated for their antibacterial/antiviral activity, the rest have not yet been validated or reported for antiviral activity. Thus, further in vitro assays for their inhibitory effect against the EV-A71 2C protein should be considered to validate the computational results.

5. Conclusions

Currently, it has been reported that no therapeutics exist specifically targeting EV-A71-infected disease, leaving clinical management reliant solely on supportive care and symptomatic treatment. Little understanding of the 2C protein includes its function as an ATPase in RNA replication that highly depends on C-terminus-mediated oligomerization. Moreover, a major challenge remains in the absence of a complete crystallographic structure for the active oligomeric complex, which has hindered a comprehensive understanding of its structural features. Thus, this study aims to overcome these existing knowledge gaps by performing a comprehensive in silico evaluation of the wild-type EV-A71 2C monomer. This started with understanding how Zn²⁺ ions affected the overall structural stability of the ATPase core, followed by the primary binding landscape determination with a global blind docking using two established control inhibitors. As a result, working on the monomer structure, the oligomerized region (Cluster 1) was the primary,

thermodynamically favored binding site. Subsequent virtual screening of 5071 natural metabolites from the Vietherb database identified 20 top-ranked candidates that were also prioritized. Among these, the 2-(1,5-dihydroxy-3-methyl-8-oxo-6,7-dihydro-5H-naphthalen-2-yl)-5-hydroxy-7-methylnaphthalene-1,4-dione (CID 101282068) compound, which is abundant in *Diospyros montana*, displayed more stable thermodynamic stability profiles during 200 ns MD simulations as well as maintaining the highest number of persistent hydrogen bonding with major oligomerization interface residues, and was validated through its favorable binding free energy ΔG_{bind} and the docking score across EV-A71 2C variants. However, a notable limitation of initial large-scale screening is the scoring function of AutoDock Vina, which does not explicitly account for zinc-ligand coordination chemistry, potentially underestimating the contribution of the structural Zn^{2+} ion. Then, the 20 top-ranked candidates were subsequently subjected to zinc treatment with the Autodock4_{Zn} force field when performing docking on multiple variant 2C proteins. Nevertheless, future studies employing zinc-aware scoring functions throughout the entire screening pipeline would further improve the accuracy of predicted docking scores. Additionally, while initial rigid docking poses provided a baseline for candidate selection, dynamic contact persistence was rigorously verified through 200 ns explicit-solvent MD simulations. Although these in silico findings successfully narrow down potential lead candidates and reduce the time and cost, these results do not validate the true binding affinity and binding site; experimental validation remains essential. Therefore, the inhibitory effect of potential compounds targeting the EV-A71 2C protein—particularly CID 101282068—should be confirmed through in vitro biophysical assays to determine exact binding affinity K_D and kinetics, in vivo enzymatic ATPase/helicase inhibition and cell-based antiviral IC_{50} and EC_{50} assays, and followed by pharmacokinetic and immunological evaluation to assess drug exposure, safety, and host responses. Finally, evaluating against a broader panel of human *Enteroviruses* beyond the selected EV-A71 will validate the therapeutic potential and possible broad-spectrum activity of the identified compounds.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biophysica6050096/s1>, Figure S1. (a) Clustering of models generated from the exhaustiveness optimization between positive controls (dibucaine 6i and fluoxetine 12b) and the 5GRB_Zn with different exhaustiveness values, illustrated by ChimeraX version 1.12 [61]. (b) The correlation between exhaustiveness values and docking scores of each of the 20 binding poses of the positive (fluoxetine 12b and dibucaine 6i) and negative (sucrose) controls on the 2C protein. The correlation was calculated and plotted in RStudio, following a logarithmic trendline with R correlation coefficients of 0.224, 0.392 and 0.226, respectively (p -value < 0.001); Figure S2. (a) RMSD profiles of the 20 top-ranked systems were evaluated to assess how ligands were sustained within the binding pocket. (b) The assessment of protein structure deviation from its origin after ligand binding was exhibited via RMSD backbone plots; Data S1: the raw data and 3D coordinate files from AutoDock4_{Zn} against all 23 EV-A71 2C variant-inhibitor complexes.

Author Contributions: Conceptualization, Investigation, Methodology and Writing: V.T.T.L. and P.-C.D.; Data Curation and Validation: V.H.P.V., T.T.V. and T.M.L.; Visualization: V.T.T.L. and T.M.L.; Supervision: P.-C.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Vietnam National University Ho Chi Minh City (VNU-HCM) under grant number C2024-28-28. We also acknowledge International University, VNU-HCM for supporting this study.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: During the preparation of this study, the author(s) used Claude Opus (version 5.5, Anthropic PBC., United State) for the purposes of graph generation and styling. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

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