

Halogenated Secondary Metabolites from Higher Plants: Potent Drug Candidates for Chikungunya Using *in silico* Approaches

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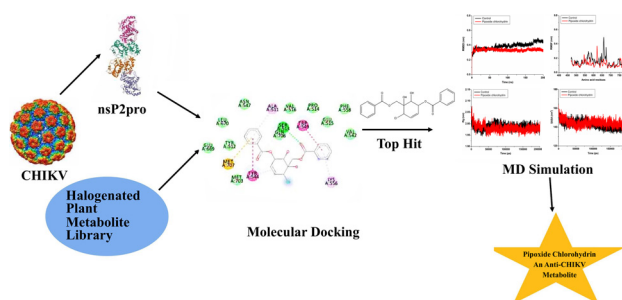
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Abstract

Chikungunya virus (CHIKV) causes a debilitating fever and joint pain, with no specific antiviral treatment available. Halogenated secondary metabolites from plants are a promising new class of drug candidates against chikungunya, with unique properties that make them effective against the virus. Plants produce these compounds to defend themselves against pests and pathogens, and they are effective against a wide range of viruses, including chikungunya. This study investigated the interactions of halogenated secondary metabolites with nsP2pro, a therapeutic target for CHIKV. A library of sixty-six halogenated plant metabolites screened previously for ADME properties was used. Metabolites without violation of Lipinski's rule were docked with nsP2pro using AutoDock Vina. To find the stability of the pipoxide chlorohydrin-nsP2pro complex, the GROMACS suite was used for MD simulation. The binding free energy of the ligand-protein complex was computed using MMPBSA. Molecular docking studies revealed that halogenated metabolites interact with



nsP2pro, suggesting they are possible inhibitors. Pipoxide chlorohydrin showed the greatest affinity to the target. This was further confirmed by the MD simulations, surface accessible area, and MMPBSA studies. Pipoxide chlorohydrin, a halogenated metabolite, was the most potent against nsP2pro in the survey.

Key words: Chikungunya virus, nsP2 cysteine protease, traditional medicine, drug candidates, molecular docking, MD simulation, pipoxide chlorohydrin

Introduction

Chikungunya virus (CHIKV) is a mosquito-borne virus that can cause debilitating joint pain. It is a silent threat, as there is no cure, and the symptoms can last for months or even years. It is transmitted through infected *Aedes* mosquito (Simon et al. 2011). The word “chikungunya” originated from Makonde meaning “which

bends up”, and thus ushers to the distorted appearance of infected persons (de Lima Cavalcanti et al. 2022). CHIKV was first reported in Tanzania in 1954, followed by severe outbreaks in Asia in the 1960s and 1980s. The recent re-emergence of multiple CHIKV strains in Asia and Africa and its established potential to spread to temperate regions cause global concern (Huang et al. 2019). The massive chikungunya outbreak in the

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Congo in 2000 heralded its global emergence in 2004, but the true number of cases is unknown due to limited serological surveillance (Ejaz et al. 2022). Chikungunya virus emerged and spread rapidly from Eastern Africa to Kenya and the Indian Ocean in 2005–2006. On Reunion Island, CHIKV caused a significant outbreak, infecting a large number of people (Imad et al. 2021; Musili 2021). In 2006, CHIKV unfurled progressively toward Asia and spread in most of the regions of India, where it affected a substantial set of populations (Translational Research Consortia (TRC for Chikungunya virus in India 2021). Unexpectedly, in 2010, southeastern France confirmed two autochthonous transmissions of chikungunya fever, and recent outbreaks in the Arabian Peninsula, Southern China, and New Caledonia have shown that this arbovirus can be transmitted widely, even in remote and temperate regions.

CHIKV belongs to the alphavirus genus of *Togaviridae* (Pialoux et al. 2007). A 12 kb enveloped positive-strand RNA virus is a small but mighty pathogen. Its genome is wrapped in a lipid membrane that helps it attach to and enter host cells. Once inside, the virus's RNA genome is translated into proteins that allow it to replicate and spread (Pialoux et al. 2007; Jain et al. 2008). The amino acid sequences of E1 glycoprotein in the envelope unveiled three different CHIKV lineages. The Asian cluster is the most widespread and causes severe disease. The East-Central and South African cluster is less transmissible and causes milder disease. The West African cluster is least transmissible and can cause mild disease (Phadungsombat et al. 2020). However, the origin of CHIKV is believed to be from West Africa. Though the pathophysiology associated with CHIKV remains elusive, various ardent efforts have been made to propose the appropriate mechanism using alphaviruses. Studies using animal models have shown that CHIKV can infect a variety of different cell types, including those found in the skin, muscles, and joints. It provides important insights into how the virus works and how it can be targeted for treatment and prevention (Ganesan et al. 2017). The CHIKV genome contains two ORFs that encode non-structural and structural proteins; including nsP1 to nsP4 and C-E3-E2-6 k-E1 respectively. The nsP1 to nsP4 executed specific enzymatic and non-enzymatic activities. nsP1, a viral protein, has dual functions: it synthesizes caps on viral RNA and anchors the virus to the plasma membrane (Law et al. 2019). The nsP2 protein's N-terminal RNA helicase unwinds RNA strands using ATP, making it essential for viral RNA replication and transcription. Apart from these activities, nsP2 has also performed the cysteine protease activity needed for polyprotein processing. The nsP3 protein's ADP ribosyl-binding and hydrolase activities kick-start viral genome replication, whereas nsP4 directs the RNA-dependent RNA poly-

merase activity (Law et al. 2021). These non-structural proteins form the replication complexes concurrently with host factors, catalyzing the synthesis of a negative-sense (–) antigenome. This event is responsible for synthesizing new genomic and sub-genomic (SG) RNAs, which act as a template for various components of virus assembly such as capsid and glycoproteins (E1, E2, and E3) (Strauss and Strauss 1994). The nsP2pro, among all nsPs, possesses two different enzymatic activities; it is widely characterized as a multifunctional two-domain protein. A close look into the structural details of the nsP2pro has suggested that the central catalytic dyad comprises Cys478 and His548 amino acids. In contrast, the primary substrate-binding residues encompass the other residues such as Asn476, Cys478, Asn547, and Tyr544. Similarly, some additional residues, Met707, Asp711, and Leu670, also exhibit a key role in enzyme-substrate interaction. Trp549, a key residue for substrate binding, is the most preserved in the class of alphaviruses (Narwal et al. 2018). The C-terminal domain (CTD) of nsP2, a key player in viral replication, is a promising drug target for chikungunya therapy.

Since no potent vaccine or drug has been developed against CHIKV, several higher plants with different secondary metabolites owning antiviral, antifungal, and antibacterial effects have gained massive consideration in developing drug and inhibitor molecules. The diversity and molecular complexity of these secondary metabolites still encourage them to be used in designing medicinal drugs and inhibitors against an array of enzymes and membrane receptors. The most prolific origin of marine organo-halogen metabolites is the seaweed “limukohu” (*Asparagopsis taxiformis*), contemplating the availability of chloride and bromide ions in seawater (Gribble 2015). *Scutellaria baicalensis*, a traditional Chinese herb, contains flavonoids with potent antiviral activity against SARS-CoV-2, the virus that causes COVID-19. Likewise, various other herbs, specifically the herbs used in traditional Chinese medicine, possess a variety of compounds such as N-cis-feruloyl tyramine, betulinic acid, moupinamide, cryptotanshinone, quercetin, desmethoxyreserpine, coumaroyltyramine, lignan, and surgical. These compounds can hinder the entrance, multiplication, and interaction of SARS-CoV-2's spike protein with host cells. They are also reported to inhibit PLpro and 3CLpro (Zhang et al. 2020).

Though enormous studies have been conducted using a variety of species from megadiverse areas that divulged significant potential for treating respiratory ailments, the halogenated secondary metabolites have not been widely tested against the Chikungunya virus. Hence, the current study strived to unveil the effect of halogenated metabolites from higher plants on CHIKV and develop them as potent drug candidates against it (Puranik et al. 2019). We have screened the halogen-

ated secondary metabolites using *in silico* analysis. This *in silico* study is the first of its kind, highlighting the importance of *in silico* investigations in this field.

Experimental

Materials and Methods

Halogenated secondary metabolites are sparsely scattered in higher plants, a library of sixty-six such molecules described elsewhere was used for the current investigation (Table SI). The authors previously used Swiss-ADME to assess the ADME profiles of selected compounds and then conducted further investigations (Guex and Peitsch 1997). Lipinski's rule-compliant compounds were docked with nsP2pro (Lipinski et al. 1997).

Ligand and protein preparation. OpenBabel was used to generate 3D structures of selected metabolites from canonical SMILES inputs. Open Babel was used to add explicit hydrogen atoms and generate a PDB structure (O'Boyle et al. 2011). Open Babel tools in PyRx were used for energy minimization and optimization of the molecules, and the resulting structures were saved in PDBQT format (Dallakyan and Olson 2015). The RCSB database's 3D coordinates for nsP2pro (PDB ID: 4ZTB) were retrieved (<https://doi.org/10.2210/pdb4ZTB/pdb>). Eliminating insignificant molecules like water was performed using UCSF Chimera for further investigation (Pettersen et al. 2004). Protein optimization was carried out employing Discovery Studio (Dassault Systèmes, USA), and the resulting structures were stored in PDB format. The MGL tool was chosen to add polar hydrogen and Kollman charges to the molecule and the results were stored in PDBQT file format.

Molecular docking of ligands. AutoDock Vina was used to dock halide compounds (Trott and Olson 2010). The grid was centered at (20.223, 1.321, and 16.419) in the X, Y, and Z directions, and had dimensions of 16 Å × 16 Å × 16 Å. Exhaustiveness was set to 16. The default values were used for the other parameters. The pipoxide chlorohydrin molecule with the lowest binding score was further analyzed to investigate its docking interactions and stability of the pipoxide chlorohydrin-nsP2pro complex using MD Simulation (University of Groningen, Netherlands). The interactions between the two molecules in the complex were visualized in two dimensions using Discovery Studio and PyMol (Schrödinger and DeLano 2020).

Molecular dynamics simulation. MD simulations were used to investigate the dynamic parameters of the halide phyto molecule-nsP2pro complex using the GROMOS 43a1 (University of Groningen, Netherlands) force field and the GROMACS 5.1.4 package (Van Der Spoel et al. 2005). PRODRG was used to create ligand

topology files (Schüttelkopf and Van Aalten 2004). A cubic box was used to dissolve protein complexes and ions were added to neutralize the system. The system's energy was minimized using the steepest descent method, with a stopping criterion of 1,000 kJ/mol/nm to avoid steric collisions. PME was used to calculate long-range electrostatic interactions, with a 9 nm cutoff radius for both van der Waals and Coulombic interactions. There were two stages to the equilibration process. In the first phase of the simulation, the solvent and ions were allowed to move freely for 100 picoseconds, while the protein and protein-ligand complexes were restrained. This was done in the NVT ensemble, which keeps the system at constant volume, number of particles, and temperature. In the second phase of the simulation, the restraint weight on the protein and protein-ligand complexes was gradually decreased for 100 picoseconds. This was done in the NPT ensemble, which keeps the system at constant pressure, number of particles, and temperature. The Linear Constraint Solver (LINCS) algorithm was used to enforce the constraints that all hydrogen bonds in the system must be maintained (Hess et al. 1997; Hess 2008). The temperature and pressure of the system were controlled using Parrinello-Rahman pressure coupling and Berendsen's temperature coupling, respectively (Berendsen et al. 1984). The LeapFrog integrator was used to integrate the equations of motion for the production simulation. The step size was 2 fs, which is a common value for production simulations. Periodic boundary conditions were used to ensure that the system did not interact with itself at the edges of the simulation box. The simulation was run for a total of 200 ns, which is a long enough time to observe the system's behavior over a significant timescale. The available GROMACS were used to examine the MD simulation trajectory.

MMPBSA free energy calculation. The Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method was used to calculate the free energy difference between the bound and unbound states of the protein-ligand complex. This included the binding energy, which is the energy released when the ligand binds to the protein, and the per-residue energy contributions, which are the energy changes that occur at each residue in the protein when the ligand binds. In MMPBSA, the polar solvation energy was calculated using the Poisson-Boltzmann equation, which is a method for calculating the electrostatic interactions between a molecule and a solvent. The non-polar solvation energy was calculated using a linear relationship to the solvent-accessible surface area. This investigation used the GROMACS `g_mmpbsa` module to assess several elements of complexes' binding free energy (Kumari et al. 2014). The last 10 ns of the simulation were used to identify any trends or changes in the system.

Results and Discussion

Molecular docking. The binding affinities of thirty-six drug-like halogenated metabolites ranged between -6.1 to -1.3 kcal/mol (Table SII). Pipoxide chlorohydrin (-6.1 kcal/mol) with the lowest binding score was selected to analyze its interaction with nsP2pro. This rarely occurring and hardly the scientists worked upon was reported by a few workers from *Piper hookeri* and *Piper attenuatum* (Jensen et al. 1993; Parmar et al. 1997). The best pose of pipoxide chlorohydrin and nsP2pro complex was observed for non-covalent interactions. The 2D structure of the selected complex showed a conventional hydrogen bond with amino acid residue Ser513. Van der Waals and π -sulphur interactions were observed with amino acid residues Gln706 and Met707, respectively. Two π - π stacking and π - π , T-shaped bond with Tyr544 and Trp549 also the π - π alkyl interactions were seen with Lys556, Tyr544, and Ala511 residues were observed (Fig. 1). The interactions put fingers towards sufficient stability as well as the flexibility of the ligand-protein complex. Mishra et al. (2016) used a computational approach to study the target (nsP2pro) of their antiviral compounds MBZM-N-IBT and MIBT. Similar studies by Jain et al. (2017) reported binding energies of -6.26 kcal/mol, -6.11 kcal/mol, and -6.01 kcal/mol for catechin-5-O-gallate, rosmarinic acid, and arjungenin, respectively against CHIKV structural proteins. For nsP2 compounds, stevioside, bacopaside II, and jujubogenin isomer of bacopasaponin shows inhibitory effects.

Molecular dynamics simulation. In docked complex pipoxide chlorohydrin-nsP2pro interactions were further analyzed and validated through MD simulation studies. A ligand's interaction with a protein may cause

a various conformational disturbances. These analyses aimed to investigate the structural alterations induced by ligands in proteins and evaluate the stability of the protein-ligand complexes.

Root mean square deviation. From its beginning, the root mean square deviation (RMSD) of backbone C α atoms in the protein backbone was calculated using MD simulation trajectories. The RMSD of nsP2pro C α atoms from the beginning structure fluctuates between 0.33465 – 0.38566 nm and hits the maxima in 20 ns. Little fluctuations in RMSD indicate that nsP2pro showed stable conformation during free and in the complex state. Whereas the mean RMSD of pipoxide chlorohydrin was observed 0.6465 nm and nsP2pro-pipoxide chlorohydrin complex exhibited an average RMSD of 0.33977 nm. Since the RMSD for every system was less than 0.4 nm, it can be said that the nsP2pro initial structure hasn't changed substantially throughout the simulation. Moreover, the protein backbone does not significantly change in structure upon interaction with pipoxide chlorohydrin (Fig. 2).

Root mean square fluctuation. The root mean square fluctuation (RMSF) per residue was calculated to understand the flexibility and dynamics in the complex. Most enzyme residues' RMSF swings between 0.122 and 0.04224 nm in intensity. The overlapped residue fluctuation profile suggested significant alteration in the position of amino acid residues due to the binding of pipoxide chlorohydrins to nsP2pro (Fig. 3). Hence, the dynamic attributes are not affected by the interaction.

Radius of gyration. Protein compactness is measured by the radius of gyration (Rg), and protein stable conformation is correlated with low Rg values. As binding to a ligand may cause a protein to unfold, Rg vari-

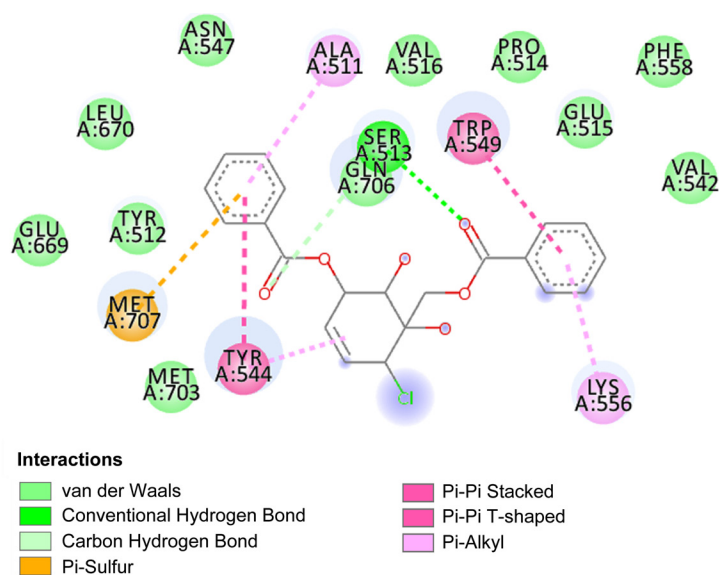


Fig. 1. 2D interaction diagram of pipoxide chlorohydrin-nsP2pro complex.

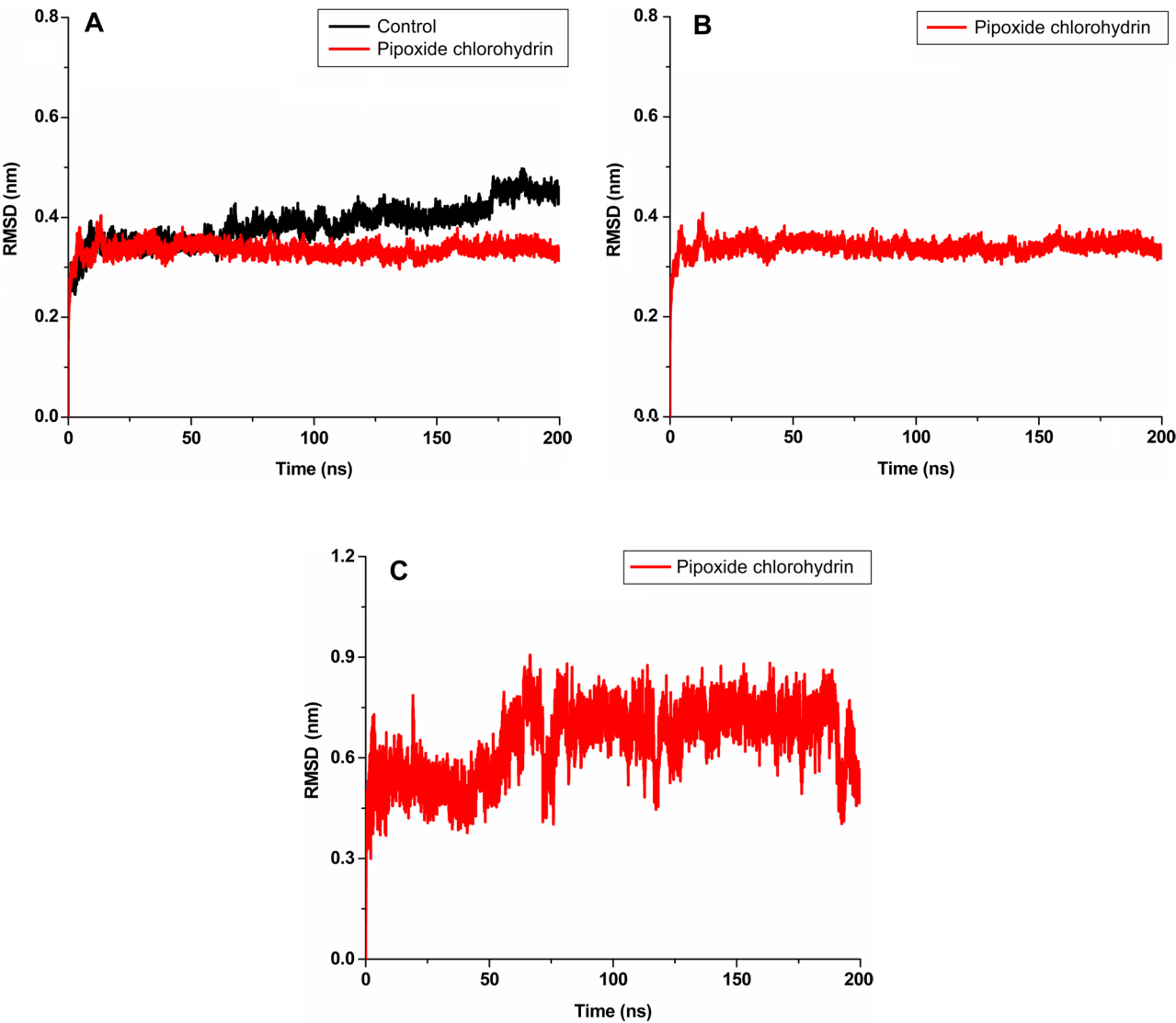


Fig. 2. The root mean square deviation (RMSD) profile A) ns2Pro backbone, B) pipoxide chlorohydrin-nsP2pro complex, C) pipoxide chlorohydrin during the simulation length.

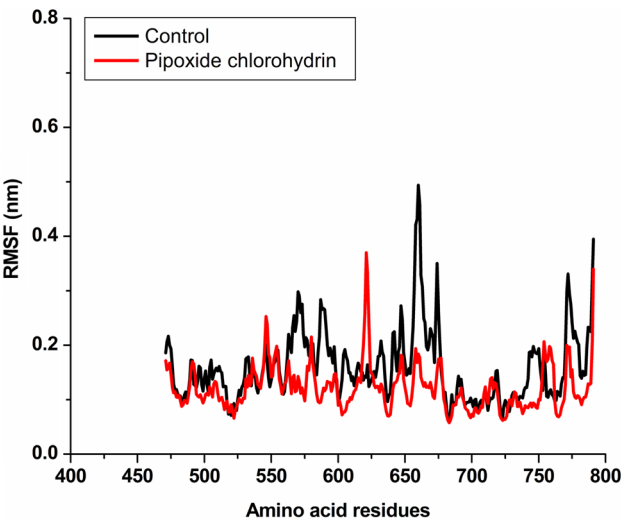


Fig. 3. Comparison of the root mean square fluctuation (RMSF) profiles of control and pipoxide chlorohydrin-nsP2pro complex.

ation was calculated in each scenario throughout the simulation. Similar values of $R_g 1.98671 \pm 0.01127$ and 1.98117 ± 0.01406 nm were observed for free and bound states of nsP2pro respectively. R_g values indicate that the binding of pipoxide chlorohydrin does not affect the compactness and integrity of nsP2pro. R_g was centered on 1.98 nm for unbound nsP2pro (Fig. 4).

Solvent-accessible surface area. The globular structure of proteins is stabilized by hydrophobic interactions among non-polar residues, which protect them within hydrophobic cores, shielding them from the surrounding aqueous environment. Solvent-accessible surface area (SASA) of the simulated complex indicates change in protein surface area exposed to the solvent. Expansion and compression of protein surface area are indicated by the higher and lower SASA values, respectively. SASA calculations enable the determination of solvation-free energy associated with every atom in the

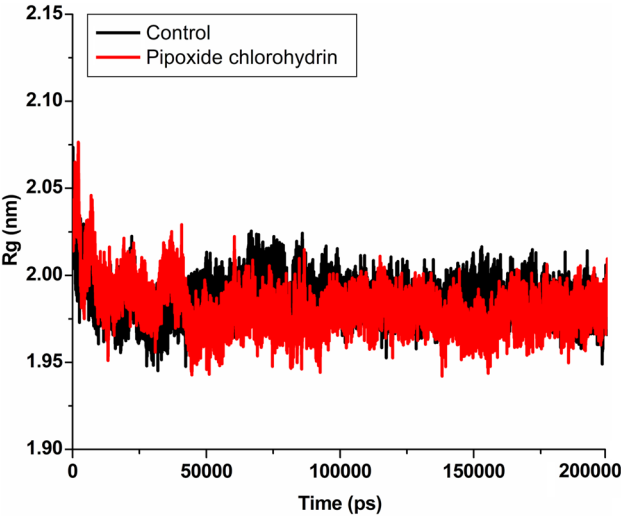


Fig. 4. Radius of gyration comparison between control and pipoxide chlorohydrin-nsP2pro complex.

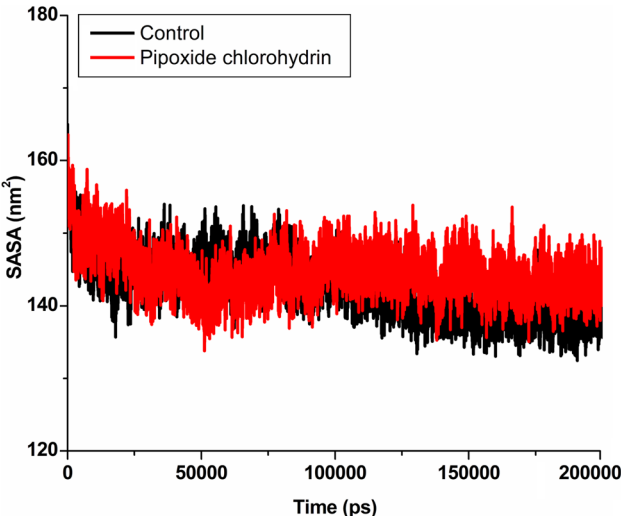


Fig. 5. Solvent accessible surface area profile of control and pipoxide chlorohydrin-nsP2pro complex.

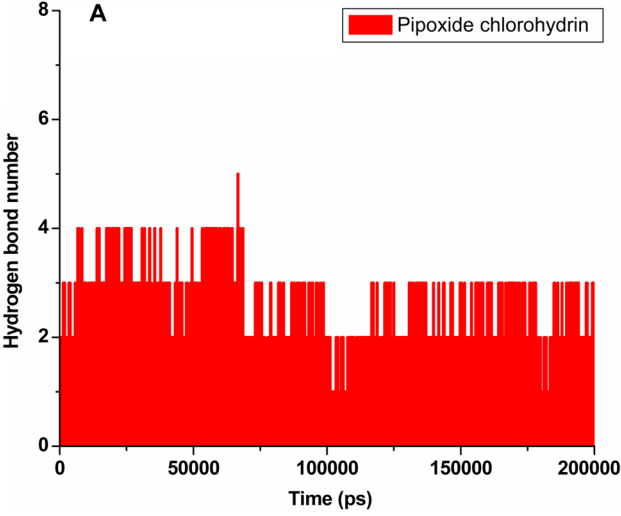


Table I

Average MD simulation parameters.

Parameter	Control	Pipoxide chlorohydrin
Backbone RMSD	0.38566 ± 0.04087	0.33465 ± 0.01483
Ligand RMSD		0.6465 ± 0.10423
Complex RMSD		0.33977 ± 0.01469
RMSF	0.15901 ± 0.06542	0.122 ± 0.04224
Radius of gyration	1.98671 ± 0.01127	1.98117 ± 0.01406
SASA	142.80578 ± 3.86427	144.61529 ± 3.2153

system. Unbound nsP2pro was found to have a peak SASA profile at around 142.806 nm², which shifts to around 144.615 nm² in the presence of pipoxide chlorohydrin (Fig. 5). Since the SASA scores are not as skewed as in control, the binding of pipoxide chlorohydrin exerts little impact on protein folding. The Table I summarizes the mean values for the molecular dynamic simulation parameters.

Hydrogen bond analysis. To determine the hydrogen bond formation between pipoxide chlorohydrin and nsP2pro, the GROMACS module h_bond was employed. A maximum number of hydrogen bonds developed between 0.25 and 0.35 nm and were spread throughout the 100 ns run. The highest distribution of hydrogen bonds was seen at a distance of 0.30 to 0.35 nm between the hydrogen bond source and acceptor, according to the hydrogen bond distribution graph (Fig. 6). Similar studies by Jadav et al. (2017) were conducted on the N-terminal active site concluded that five arylalkylidene derivatives of 1,3-thiazolidin-4-on show nsP2pro inhibitory potential using MD simulations. Another investigation used a high-throughput screening process to find a natural compound called ID1452-2 that can target the chikungunya nsP2pro. However,

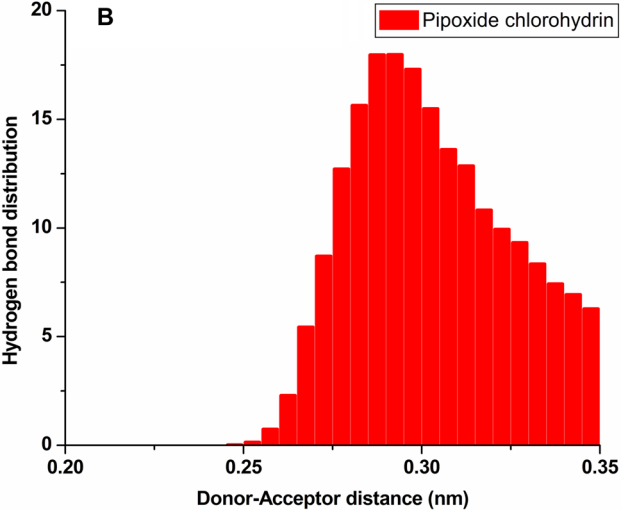


Fig. 6. Number and distribution of hydrogen bonds between pipoxide chlorohydrin and nsPro2pro; A) hydrogen bond number, B) hydrogen bond distribution.

Table II
The Molecular Mechanics Poisson-Boltzmann
Surface Area (MMPBSA) computed energy components.

Energy components	Values (kJ/mol)
van der Waals energy	−238.144 ± 15.134
Electrostatic energy	−17.868 ± 5.685
Polar solvation energy	65.878 ± 10.659
SASA energy	−17.349 ± 1.177
Binding energy	−207.483 ± 15.414

more research is needed to determine how effective and safe ID1452-2 is in humans (Lucas-Hourani et al. 2013). Molecular docking and MD simulation studies in this investigation prove that chlorohydrin pipoxide is a potential inhibitor to the C-terminal domain of nsP2pro. A great fraction of research is still devoted to synthetic antivirals or non-halogenated compounds. Halogenated natural metabolites need more exploration to find a potent anti-CHIKV drug.

Binding free energy (ΔG_{bind}) calculation. To determine the ΔG_{bind} , which is an indication of the binding ability of ligands, MM/PBSA MMPBSA analysis was carried out (Table II). All compounds bind to the active site of nsP2pro to create a stable complex, according to the results of MMPBSA. MMPBSA results revealed that the negative values of ΔG_{bind} (−134.142 kJ/mol) for complex pipoxide chlorohydrin-nsP2pro showed a high binding affinity. The energy components of van der Waals interactions (ΔE_{vdw}), electrostatic energy (E_{EEL}), and SASA energy with negative values contributed to lower ΔG_{bind} and favored binding affinity. The positive values of the polar solvation energy (ΔG_{psolv}) component were crucial for the resultant ΔG_{bind} . Table II contains the ligands’ MMPBSA results.

The hunt is on for effective drugs against CHIKV. This study explored pipoxide chlorohydrin, a natural metabolite, as a potential inhibitor of the nsP2pro protein, which is crucial for CHIKV replication (Sharma et al. 2016). Several antiviral drugs approved previously belong to the protease inhibitors category, like protease inhibitors for HIV-1 and hepatitis C virus, are potent antiviral drugs (Lamarre et al. 2003; Hsu et al. 2006). The active site dyad Cys478-His548, Asn547, and Trp549 play a significant role in polyprotein processing during CHIKV replication (Narwal et al. 2018). We targeted nsP2pro that chlorohydrin pipoxide established multiple non-covalent interactions with different residues Ser513, Gln706, Met707, Tyr544, and Trp549 in the C- a terminal binding pocket of nsP2pro suggesting it’s potential to be a lead molecule. Trp549 is a conserved residue among alphaviruses; the non-covalent interaction with it will adversely influence its functions. Singh et al. (2018) carried the peptidomimetic studies *in silico* along with in vitro validation of results

revealed that nsP2pro inhibition is crucial for drug development against CHIKV. MD simulation results validated the molecular docking findings RMSD, RMSE, Rg, SASA, and hydrogen bond analysis justify the formation of stable complex throughout the simulation length. Negative values of binding free energy calculated using MMPBSA pinpoints the stability of nsP2pro complex with pipoxide chlorohydrin. Kumar et al. (2019) performed extensive molecular docking and MD simulation experiments to repurpose previously FDA-approved drugs against the same target and found two drugs, ribostamycin sulfate and E-64, to be efficient inhibitors like ours. Kasabe et al. (2023) identified nine drugs with anti-CHIKV activity, including temsirolimus as the most potent. Similarly, pipoxide chlorohydrin showed the potential to inhibit nsP2pro.

Conclusion

The results of this study suggest that pipoxide chlorohydrin is a promising drug candidate for the treatment of Chikungunya virus infection. It is important to note that further in vitro and in vivo studies are needed to confirm the efficacy, safety, and toxicity of pipoxide chlorohydrin as a potential antiviral drug.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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