proceedings

MDPI

Proceedings

In silico study of 5,7-Dimethoxycoumarin and p-Coumaric acid in *Carica papaya* Leaves as Dengue Virus Type 2 Protease Inhibitors †

Samith Rathnayake [1]*, Ayesh Madushanka [2], Asha Wijegunawardana [3], Harthika Mylvaganam [1], Ajith Rathnayake [3], Eranga geethanjana perera [3], Ishara jayamanna [3], Priyantha Chandrasena [3], Ahinsa Ranaweera [1], Prasad Jayasooriya [1] and Chathuranga Bamunuarachchige [3]

[1] Department of Food Technology, Rajarata University of Sri Lanka, Mihinthale
[2] Department of Applied Science, Rajarata University of Sri Lanka, Mihinthale
[2] Department of Bioprocess Technology, Rajarata University of Sri Lanka, Mihinthale
* Correspondence: srathnay@tec.rjt.ac.lk
† Presented at the 1st International Electronic Conference on Biomolecules: Natural and Bio-Inspired Therapeutics for Human Diseases, 1–13 December 2020; Available online: https://iecbm2020.sciforum.net/.

Received: date; Accepted: date; Published: date

Abstract: Dengue virus is a serious public health issue in tropical and subtropical regions. The global incidence of dengue necessitates the potent antiviral medication for the prevention of proliferation of the virus inside the human body. The DEN2 NS2B/NS3 protease presents in the dengue virus, is an attractive drug target due to its essential role in viral replication, survival, and other cellular activities. In traditional medicine, *Carica papaya* leaves have been used for the treatment of dengue fever in Sri Lanka, Pakistan, Malaysia. Therefore, phytochemicals present in *Carica papaya* leaves, have a potential anti-viral activity and can be used as strong drug candidates against the dengue virus. In this investigation, two phytochemical compounds in *Carica papaya* leaves: 5,7-Dimethoxycoumarin and p-Coumaric acid were selected from the literature and then docked against the DEN2 NS2B/NS3 protease. The compounds showed strong interactions with favorable binding energies in the active site of DEN2 NS2B/NS3 protease. To validate the molecular docking results, the docked ligand-protein complexes were subjected to molecular dynamics simulation along with the apo form of the protein for 30 ns. The molecular dynamics simulation analysis comprising of root mean square deviation and fluctuation, the radius of gyration, hydrogen bonding, DSSP, and MM/PBSA revealed the stability of the apo and complex systems. Interactions formed by these compounds with residues Leu149 and Asn152 are found to be essential for the stability of the ligand-protein complex. The findings revealed that these phytochemical compounds depict the promising results against the DEN2 serotype of the dengue virus and the potential to work as therapeutic drugs. Further experimentation on the proposed compounds is necessary to validate the results and can lead to the development of strong inhibitors with improved activity.

Keywords: Dengue Protease Inhibitors; NS2B/NS3 protease; 5,7-Dimethoxycoumarin; p-Coumaric; *Carica papaya* Leaves; molecular docking; molecular dynamics

1. Introduction

Dengue virus (DENV) is a mosquito-borne flavivirus that has causes mild dengue fever to life-threatening complications, dengue hemorrhagic Fever, and dengue shock syndrome, resulting in serious public health problems in tropical and subtropical regions [1,2,3]. The rapid spread of this virus, affecting over a million people has necessitates scientists to search for potent antiviral medication. The Dengue virus genome has consisted of a single-stranded, positive-sense, RNA molecule that encodes three (3) structural and seven (7) non-structural proteins. The three structural proteins as capsid ©, pre-membrane/membrane (prM/M), and envelope (E), and non-structural (NS)

proteins are: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [4]. These non-structural proteins are responsible for viral replication and other cellular functions [5]. There are four types of Dengue virus (DENV-1 to DENV-4). The DENV- 2 is the cosmopolitan genotype of the four serotypes [6]. NS3 is a multifunctional protein with chymotrypsin-like serine protease, that binds to an NS2B cofactor and is involved in cleaving the DENV polyprotein [7-9]. This NS2B-NS3 protease complex plays a vital role in the processes of the viral polyprotein and for virus replication [10]. Thus, it an attractive target for antiviral drug development [11].

Medicinal plants have been known and used for millionaires as a rich source of therapeutic agents all over the world. It has been recently reported that the importance of phytochemical compounds against DENV [12,13]. The Carica papaya is a flowering plant, which is native to Mexico and South America and is now cultivated in other tropical countries around the globe. Recent reports have claimed that Carica papaya extracts have to depict several medicinal properties [14-16]. Presently, the use of Carica papaya plant extract has become popular as an unlicensed, also traditional herbal remedy for dengue infection in South East Asian countries like India, Malaysia, Pakistan, and Sri Lanka [17]. The leaves of the Carica papaya contain various types of phytochemicals, specifically phenolic compute [18]. Furthermore, recent studies suggesting that Carica papaya leaves have potential anti-viral activity against DENV.

In this study, two phytochemical compounds: 5,7-Dimethoxycoumarin and p-Coumaric acid from *Carica papaya* leaves have been evaluated for their potential inhibitory activity against DENV NS2BNS3 serine protease, using molecular docking and molecular dynamics methods. Therefore, the knowledge and experience gathered from this study could contribute to the efforts in discovering novel and potent anti-viral agent agonist DENV.

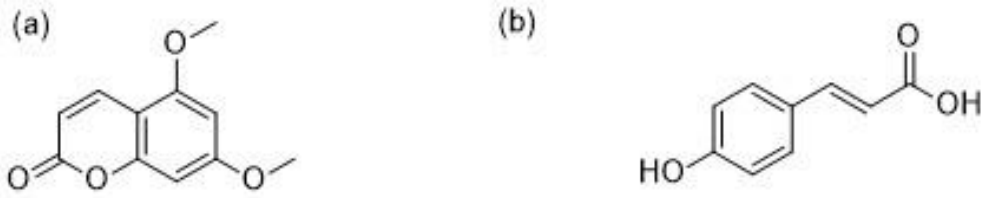

Figure 1. (**a**) 5,7-Dimethoxycoumarin, (**b**) p-Coumaric acid.

2. Materials and Methods

The three-dimensional crystallographic structure of DEN2 NS2B/NS3 serine protease was downloaded from the protein data bank (PDB ID 2FOM, 1.5 Å resolution). 3D structures of the 5,7-Dimethoxycoumarin, p-Coumaric acid compounds (Figure 1), and glycerol as a reference molecule, which was retrieved from the crystallographic structure, were constructed and geometrically optimized using ab initio B3LYP/6-31G(d) density functional theory methodology with the GAMESS software [19]. Molecular docking was performed using DOCK6 software [20,21]. The docking protocol was validated by re-docking the native ligand to the active site of the 2FOM receptor. It was performed using glycerol from the crystallographic structure as a reference. The best conformation was selected regarding the better-superimposed conformation and root mean square fluctuation value recorded. Then, the optimized structure was docked on to 2FOM receptor by DOCK6 software and a flexible docking process was performed using a scoring function 'grid score' for all ligands and then ranked them. Molecular dynamics (MD) simulations were performed with the best poses obtained from docking studies. All the MD simulations were performed by using GROMACS 4.6.5 molecular dynamics software package and the protein topology was generated using the GROMOS54a7 force field [22-24]. The force field parameters for the two (2) ligand compounds were generated using the PRODRG server [25]. The docked complexes were placed in a center in a box of 9×9×9 nm3 and solvated with SPC water [26]. Cl^- ions were added to the system to uphold the electro-neutrality of the systems. The Berendsen's weak coupling algorithm was used to maintained temperature and pressure at 300 K and 1 bar. Electrostatic interactions were modeled by particle mesh Ewald (PME) with a short-range cut off of 1.2 nm while systems were simulated for 50 ns run with 2

fs time step [27]. The same MD protocol was applied for the apo-protein alone to investigate its stability.

The stability of the above systems was studied in terms of root mean square deviations (RMSD) of the backbone of the protein, Root mean square fluctuation (RMSF), and radius of gyration of the protein. Furthermore, the secondary structure profile analysis of trajectories of the protein-ligand complexes and apo-protein was conducted using the Dictionary of Secondary Structure of Proteins (DSSP) analysis [28]. At the end of the simulations of complexes, the non-covalent interactions between ligand and the protein were analyzed by the LigPlot+v.14.5 software The binding free energies of protein-ligand complexes were calculated based on Molecular Mechanics–Poisson Boltzmann Surface Area (MM-PBSA) method using g_mmpbsa GROMACS utility [29,30]. The binding free energy comprises of three energetic terms potential energy in a vacuum, non-polar solvation energy, and polar solvation energy.

3. Results

3.1. Molecular Docking

The validation results of the re-docking of the reference molecule showed the RMSD value of 1.02 Å. Since an RMSD value is <2.0 Å for the best-scored conformation, it is a successful prediction [31]. A view of the 5,7-Dimethoxycoumarin docked into protein is given in Figure 2(a). The docked structure of the p-Coumaric acid-protein is given in Figure 2(b). The recorded best grid score for the 5,7-Dimethoxycoumarin-protein complex was -26.35 kcal mol^{-1}. For p-Coumaric acid-protein was -27.08 kcal mol^{-1} and it was bound to the same binding site as 5,7-Dimethoxycoumarin in protein.

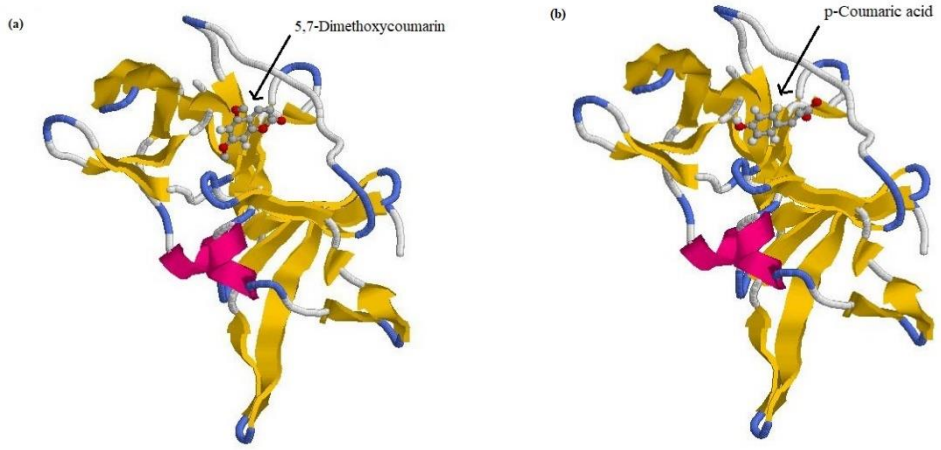

Figure 2. Docked complexes of (**a**) 5,7-Dimethoxycoumarin and (**b**) p-Coumaric acid.

3.2. Molecular Dynamics Analysis

Figure 3 represents the RMSD of the backbone of the ligand-protein complexes and apo-protein. The RMSD parameter indicates reasonable stability of proteins after binding with the ligands, and also in the apo-protein.

The compactness of the ligand-protein systems presented in Figures 4 by calculates the radius of gyration (Rg) of the protein. The same parameter was calculated for apo-protein and is presented in the panel (c) of Figure 4. All the Rg values appear to be spread between 1.45 nm to 1.60 nm as can be seen in the figures. All the proteins exhibit reasonable stability by having a plateau variation of Rg in the last 10-20 ns of the MD trajectories. Therefore, it can be emphasized that the binding of ligands would not affect the structural stability of the protein.

Conformational differences of individual amino acids in protein were compared by calculating the Root Mean Square Fluctuations (RMSF) of amino acids and are given in Figures 5. Close

examination reveals some minor differences in fluctuations of the amino acids of proteins in ligand-protein complexes and that of in apo-protein. However, it can be seen that the overall structural integrity of proteins was preserved strengthening the conclusions drawn from the previous calculations.

The DSSP analyzes were carried out for the configurations taken at every 100 ps of the 50 ns MD trajectories using the do_dssp utility of GROMACS software package. The time evaluation of the secondary structure of the ligand-protein complexes and apo-protein are presented in Figures 6. The results revealed that the secondary structure of all the systems including the apoprotein remains almost the same throughout simulations, indicating no significant secondary structure changes.

The LigPlot analysis of the protein structures indicates that the ligands form strong hydrogen bonds with DEN2 NS2B/NS3 serine protease, specifically residue Leu149 and Asn152 (Figure 7,8). The number of hydrogen bonds was studied using the g_dist tool in the GROMACS program. Throughout the simulation time, the distance between the centers of mass of the two groups of atoms which were involved in hydrogen bond formation was maintained nearly at a constant value confirming the continuance, stability, and effectiveness of the hydrogenbonding.

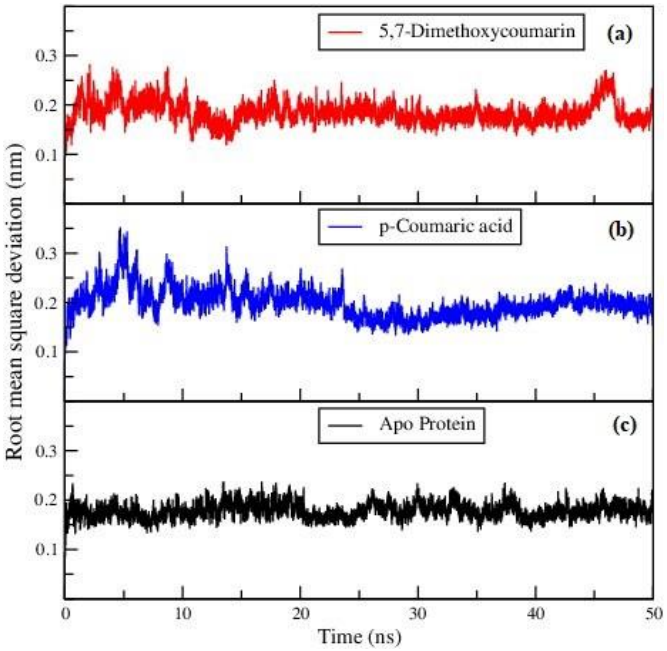

Figure 3. Backbone RMSD of (**a**) 5,7-Dimethoxycoumarin-Protein, (**b**) p-Coumaric acid-Protein complexes and (**c**) Apo Protein.

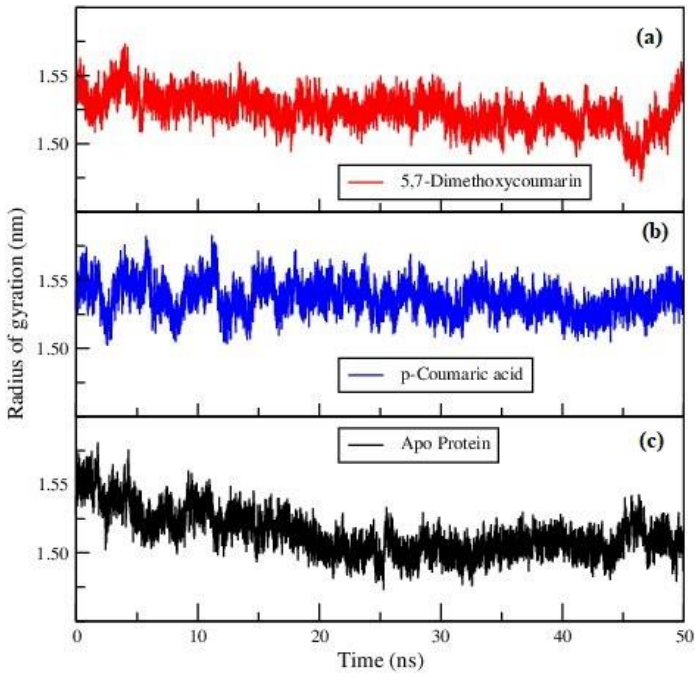

Figure 4. Radius of gyration of (**a**) 5,7-Dimethoxycoumarin-Protein, (**b**) p-Coumaric acid-Protein complexes and (**c**) Apo Protein.

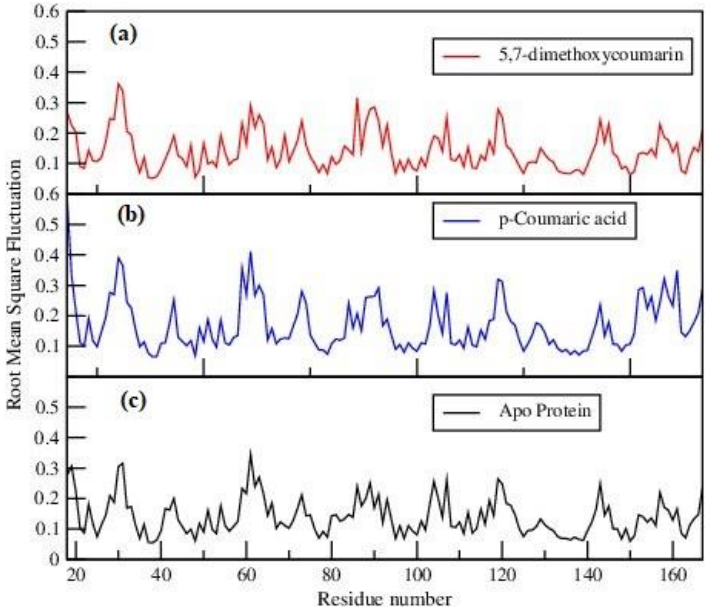

Figure 5. Root mean square fluctuation of (**a**) 5,7-Dimethoxycoumarin-Protein, (**b**) p-Coumaric acid-Protein complexes and (**c**) Apo Protein.

3.3. MM-PBSA Calculations

The MM-PBSA analyses were conducted for the two (2) complexes to investigate their protein-ligand interactions. The potential energy in the vacuum (van der Waals and electrostatic energy), polar solvation energy, nonpolar solvation energy (under the solvent-accessible surface area model), and the MM-PBSA binding free energy obtained from g_mmpbsa module in GROMACS molecular dynamics software package are listed in Table 1 below. The resultant binding free energies are large negative values indicating two ligands are spontaneously binding to the protein.

Table 1. Binding energy component and binding free energy for ligands from MM-PBSA (all energy terms are in kJ/mol).

System	Van der Waals energy	Electrostatic energy	Polar solvent enrgy	Non polar solvent energy	Binding free enrgy
5,7-Dimethoxycoumarin	-85 ±11	-10 ±1	54 ±7	-7.7 ±0.8	-47 ±3
p-Coumaric acid	-74 ±14	-208 ±58	155 ±62	-10 ±1	-138 ±31

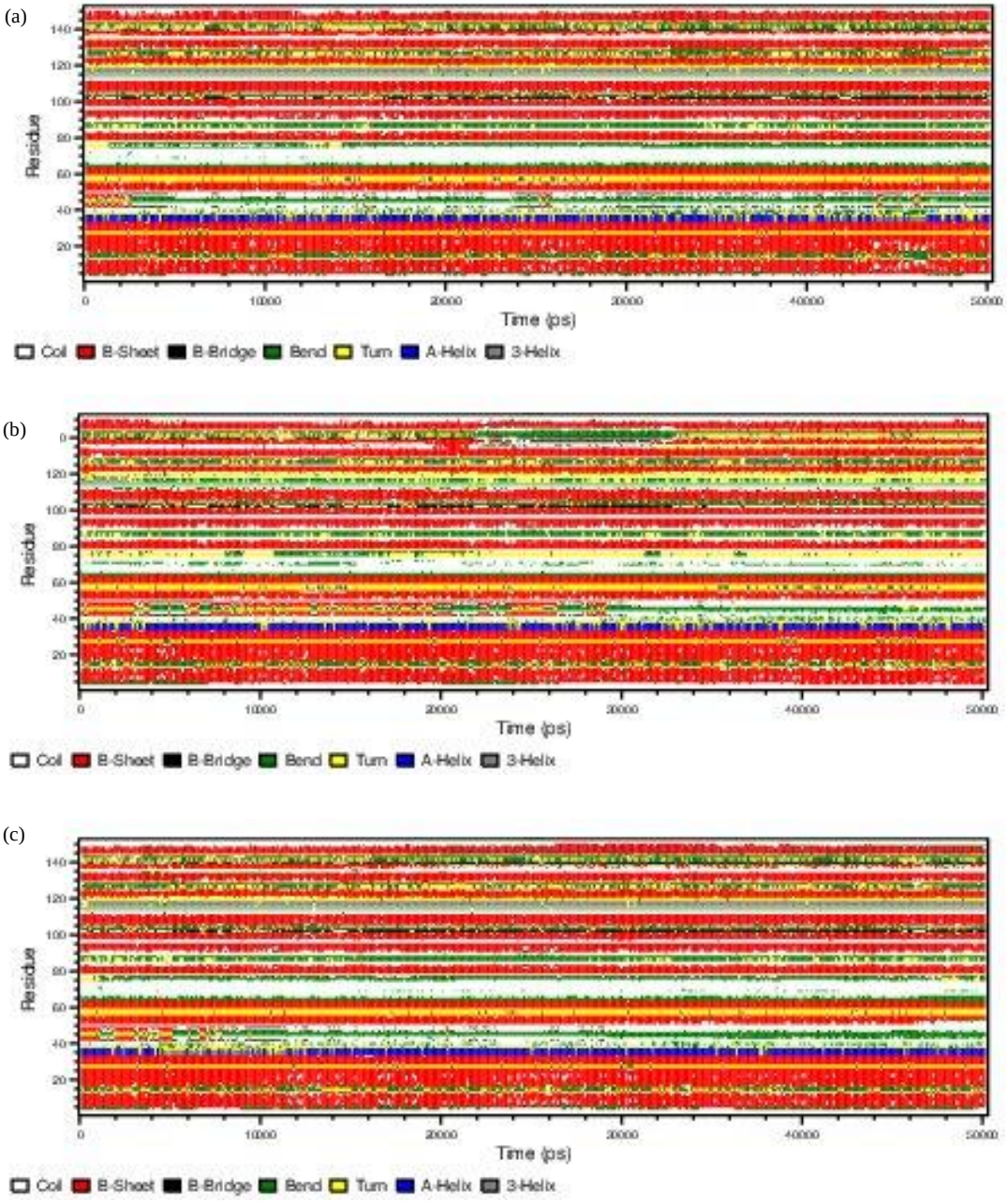

Figure 6. The evolution of secondary structure changes of (**a**) 5,7-Dimethoxycoumarin-Protein, (**b**) p-Coumaric acid-Protein complexes and (**c**) Apo Protein.

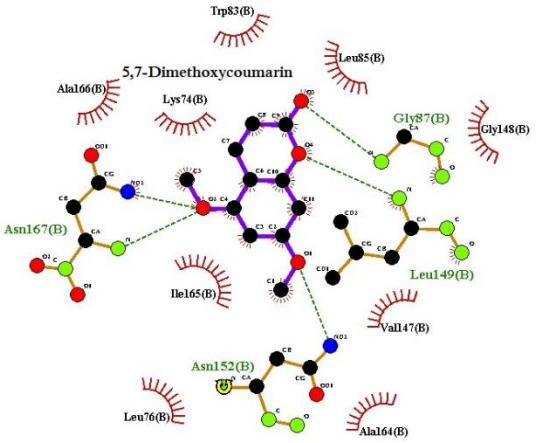

Figure 7. Hydrogen bond interactions of 5,7-Dimethoxycoumarin-Protein complex from LigPlot v.145.

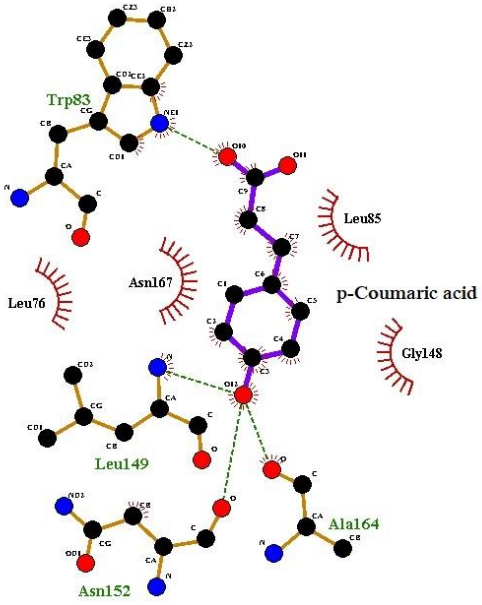

Figure 8. Hydrogen bond interactions of p-coumaric acid-Protein complex from LigPlot v.145.

4. Conclusion

The molecular docking and molecular dynamics results indicate that the binding affinity of 5,7-Dimethoxycoumarin, is less than that of p-Coumaric. However, overall results indicated that the ligands bind to the binding site of DEN2 NS2B/NS3 receptor via hydrophobic and hydrogen bonding interactions. Specifically, the MD study provides an important contribution to understand the stability of the DEN2 ligand-NS2B/NS3 serine protease complex system in aqueous solution. Thus it could be suggested that the binding of 5,7-Dimethoxycoumarin and p-Coumaric acid molecules have potential inhibitory activity against NS2B-NS3 serine protease. Moreover, it is a indication of *Carica papaya* might employ its antiviral activity by blocking the viral assembly mechanism of DENV2 virus. Therefore, this study provides a good platform to further investigations in terms of the pharmaceutical potential and biological functions of 5,7-Dimethoxycoumarin and p-Coumaric acid when it binds to DEN2 NS2B/NS3 serine protease at the molecular level and in vivo as well.

Acknowledgments: This work was supporated by the Faculty of Technology, Rajarata University Sri Lanka.

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

References

1. Heinz: FX.; Stiasny K. Flaviviruses and flavivirus vaccines. *Vaccine*. 2012, *30(29),* 4301-4306. doi:https://doi.org/10.1016/j.vaccine.2011.09.114.
2. Hasan, S.; Jamdar, SF.; Alalowi, M.; Al Ageel Al Beaiji, SM. Dengue virus: A global human threat: Review of literature. *J Int Soc Prev Community Dent*, 2016, 6(1), 1-6. doi:10.4103/2231-0762.175416.
3. Narvaez, F.; Gutierrez, G.; Pérez, MA. et al. Evaluation of the Traditional and Revised WHO Classifications of Dengue Disease Severity. *PLoS Negl Trop Dis*, 2011, *5(11),* e1397. doi:https://doi.org/10.1371/journal.pntd.0001397.
4. Harapan, H.; Michie, A.; Sasmono, R.T.; Imrie A. Dengue: A Minireview. *Viruses*, 2020, 12, 829. doi: https://doi.org/10.3390/v12080829
5. Nobori, H.; Toba, S.; Yoshida, R. et al. Identification of Compound-B, a novel anti-dengue virus agent targeting the non-structural protein 4A. *Antiviral Res*. 2018, *155,* 60-66. doi:https://doi.org/10.1016/j.antiviral.2018.05.003.
6. Ernst, T.; McCarthy, S.; Chidlow, G. et al. Emergence of a New Lineage of Dengue Virus Type 2 Identified in Travelers Entering Western Australia from Indonesia, 2010-2012. *PLoS Negl Trop Dis*, 2015, *9(1),* e0003442. doi:https://doi.org/10.1371/journal.pntd.0003442.
7. Matusan, AE.; Pryor, MJ.; Davidson, AD.; Wright, PJ.; Mutagenesis of the Dengue Virus Type 2 NS3 Protein within and outside Helicase Motifs: Effects on Enzyme Activity and Virus Replication. *J Virol*, 2001, *75(20),* 9633 LP-9643. doi:10.1128/JVI.75.20.9633-9643.2001.
8. Gebhard, LG.; Kaufman, SB.; Gamarnik, A V. Novel ATP-Independent RNA Annealing Activity of the Dengue Virus NS3 Helicase. *PLoS One*, 2012, *7(4),* e36244. https://doi.org/10.1371/journal.pone.0036244.
9. Wengler, G.; Wengler. G. The carboxy-terminal part of the NS 3 protein of the West Nile Flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology*, 1991, *184(2),* 707-715. doi:https://doi.org/10.1016/0042-6822(91)90440-M.
10. Assenberg R, Mastrangelo E, Walter TS, et al. Crystal Structure of a Novel Conformational State of the Flavivirus NS3 Protein: Implications for Polyprotein Processing and Viral Replication. *J Virol*. 2009, *83(24)*. 12895 LP-12906. doi:10.1128/JVI.00942-09.
11. Tomlinson SM, Watowich SJ. Anthracene-based inhibitors of dengue virus NS2B–NS3 protease. *Antiviral Res*, 2011, *89(2),* 127-135. doi:https://doi.org/10.1016/j.antiviral.2010.12.006.
12. Zandi K, Teoh B-T, Sam S-S, Wong P-F, Mustafa MR, AbuBakar S. Novel antiviral activity of baicalein against dengue virus. *BMC Complement Altern Med*. 2012;12(1):214. doi:10.1186/1472-6882-12-214.
13. Chattopadhyay, D.; Sarkar, MC.; Chatterjee, T. et al. Recent advancements for the evaluation of anti-viral activities of natural products. *N Biotechnol*, 2009, *25(5),* 347-368. doi:https://doi.org/10.1016/j.nbt.2009.03.007.
14. Ranasinghe, P.; Ranasinghe. P.; Abeysekera, WPKM. et al. In vitro erythrocyte membrane stabilization properties of *Carica papaya L*. leaf extracts. *Pharmacognosy Res*, 2012, *4(4),* 196-202. doi:10.4103/0974-8490.102261.
15. Baskaran, C.; bai, VR.; Velu, S.; Kumaran, K. The efficacy of *Carica papaya* leaf extract on some bacterial and a fungal strain by well diffusion method. *Asian Pacific J Trop Dis*. 2012, *2,* S658-S662. doi:https://doi.org/10.1016/S2222-1808(12)60239-4.
16. Vij, T.; Prashar, Y.; A review on medicinal properties of *Carica papaya* Linn. *Asian Pacific J Trop Dis*. 2015,

5(1), 1-6. doi:https://doi.org/10.1016/S2222-1808(14)60617-4.

17. Rajapakse, S.; de Silva, NL.; Weeratunga, P, Rodrigo, C.; Sigera, C.; Fernando, SD. *Carica papaya* extract in dengue: a systematic review and meta-analysis. *BMC Complement Altern Med*. 2019, *19(1)*, 265. doi:10.1186/s12906-019-2678-2.
18. Canini, A.; Alesiani, D.; D'Arcangelo, G.; Tagliatesta, P. Gas chromatography–mass spectrometry analysis of phenolic compounds from *Carica papaya L.* leaf. *J Food Compos Anal*. 2007, *20(7),* 584-590. doi:https://doi.org/10.1016/j.jfca.2007.03.009.
19. Schmidt, MW.; Baldridge, KK.; Boatz, JA. et al. General atomic and molecular electronic structure system. *J Comput Chem*. 1993, *14(11),* 1347-1363. doi:10.1002/jcc.540141112.
20. Ewing, TJA.; Makino, S.; Skillman, AG.; Kuntz, ID. DOCK 4.0: Search strategies for automated molecular docking of flexible molecule databases. *J Comput Aided Mol Des*. 2001, *15(5),* 411-428. doi:10.1023/A:1011115820450.
21. Allen, WJ.; Balius, TE.; Mukherjee, S.; et al. DOCK 6: Impact of new features and current docking performance. *J Comput Chem*. 2015, *36,* 1132-1156. doi: 10.1002/jcc.23905.
22. Spoel, DVANDER.; Lindahl, E.; Hess, B.; Groenhof, G. GROMACS: Fast, Flexible, and Free. *J Comput Chem*. 2005, *26(16),* 1701–1718. doi:10.1002/jcc.20291.
23. Hess, B.; Kutzner, C.; van der Spoel, D.; Lindahl, E. GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J Chem Theory Comput*. 2008, *4(3),* 435-447. doi:10.1021/ct700301q.
24. Schmid, N.; Eichenberger, AP.; Choutko, A.; et al. Definition and testing of the GROMOS force-field versions 54A7 and 54B7. *Eur Biophys J*. 2011, *40,* 843-856. doi:10.1007/s00249-011-0700-9.
25. Schüttelkopf, AW.; van Aalten, DMF. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr Sect D*. 2004, *60(8),* 1355-1363. doi:10.1107/S0907444904011679.
26. Berendsen, HJC.; Postma, JPM.; Gunsterenand WF va.; Hermans, J. Interaction Models for Water in Relation to Protein Hydration. In: In: Pullman B. (Eds) Intermolecular Forces. The Jerusalem Symposia on Quantum Chemistry and Biochemistry: Springer, Dordrecht. doi:https://doi.org/10.1007/978-94-015-7658-1_21.
27. Essmann, U.; Perera, L.; Berkowitz, ML.; Darden, T.; Lee, H.; Pedersen, LG.; A smooth particle mesh Ewald method. *J Chem Phys*. 1995, *103(19),* 8577-8593. doi:10.1063/1.470117.
28. Kabsch, W.; Sander, C.; Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers.* 1983, *22(12)*, 2577-2637. doi:10.1002/bip.360221211.
29. Kumari, R.; Kumar, R.; Lynn, A. G-mmpbsa--A GROMACS tool for high-throughput MM-PBSA calculations. *J Chem Inf Model*. 2014, *54(7),* 1951-1962. doi:10.1021/ci500020m.
30. Genheden, S.; Ryde, U. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. *Expert Opin Drug Discov*. 2015, *10(5),* 449-461. doi:10.1517/17460441.2015.1032936.
31. Kontoyianni, M.; McClellan, LM.; Sokol, GS. Evaluation of Docking Performance: Comparative Data on Docking Algorithms. *J Med Chem*. 2004, *47(3),* 558-565. doi:https://doi.org/10.1021/jm0302997.