An *in silico* molecular docking study to evaluate the inhibitory potential of *Cichorium intybus, Saussurea lappa, Trigonella foenum-graecum, Glycin max, Lepidium sativum* and *Nigella sativa* against NS5B of HCV to treat hepatitis C

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Abstract

Hepatitis C virus causes several liver disorders worldwide. Various anti-viral medications treat hepatitis C by targeting HCV's cell cycle. HCV replication and RNA synthesis need NS5B. It's a proven antiviral target. HCV medication discovery targets NS5B protein. This study used in silico methods to explore medicinal plant phytochemicals against HCV NS5B polymerase. Total of 100 phytochemicals derived from *Cichorium intybus, Saussurea lappa, Trigonella foenum-graecum, Glycin max, Lepidium sativum* and *Nigella sativa* were selected and their structures were retrieved from PubChem database. These phytochemicals were passed through filters including Lipinski's rule of 5, toxicity filter, blood brain barrier and TPSA for bioactive profiling. Molecular docking determined phytochemical interaction patterns. Molecular dynamics simulation evaluated docked complex structure stability in receptor binding sites. 6'-O-acetyl daidzin, coumestrol and hederagenin have shown common molecular interaction with Asn291 and Asp318 of HCV NS5B, which are crucial residues to block Hepatitis C virus activity and growth. These phytochemicals also shown low RMSD values in md simulation making them structural stable inside the binding pocket of HCV N5SB. Based on pharmacological properties, structural stability, and target receptor active site, these phytochemicals are suitable herbal treatment of Hepatitis C both in vitro and clinical trials. Hence, 6'-O-acetyl daidzin, coumestrol and hederagenin are found to be most effective phytochemicals due to their comparatively high binding affinity values with NS5B protein of HCV besides their higher binding affinity values than reference drug sofosbuvir. It is recommended to investigate these possible pharmacological hits in vitro and pre-clinically to treat hepatitis C.

Keywords: Molecular modeling, virtual screening, antiviral agents, bioactive compounds, herbal medicine, protein-ligand interaction, viral NS5B protein.

INTRODUCTION

Hepatitis C virus is one of the major cause of hepatic diseases around the world. According to data presented by World Health Organization WHO), HCV has infected around 170 million people worldwide (Chaudhari *et al.*, 2021). The causative contagious agent of HCV was discovered in late 1980 without conventional helping virological aids and methods. It is a RNA virus that causes liver damage and leads towards the carcinoma of hepatocellular cells (Blach *et al.*, 2017). HCV is categorized in the *Flaviviridae* family which also includes dengue and yellow fever viruses. It is an enveloped virus that has E1 and E2 glycoprotein surrounding the nucleocapsid (Aizawa *et al.*, 2016). In the Pacific regions the prevalence of HCV is around 0.3 to 12% and in Pakistan it is increasing at a very high rate, around 10 million people are affected with hepatitis C virus (Sievert *et al.*, 2011). Genotype is the major factor in determining the treatment implication. Around six primary genotypes of HCV have been discovered so far, each with several subtypes (Ashfaq and Idrees, 2014). According to inclusive detailed study in Pakistan, Punjab genotype 3a is the most prevalent genotype with 88.1% (Idrees and Riazuddin, 2008).

Hepatitis is a contagious disease that can cause progressive liver disorders. It can spread from infectious blood to another healthy person by most common way, which is contaminated syringes. In developed countries like Europe major source of HCV 40%) acute type is by unsafe intravenous drugs (Gower *et al.*, 2014). It can be short term or long-term illness depend upon disease condition. More than half of infected person have long term illness, chronic hepatitis which is life threatening act as silent killer and leads towards the liver carcinomas (Kose *et al.*, 2019). There is no FDA approved vaccine for hepatitis C the only way to avoid this disease is through preventive measures. From the discovery of hepatitis virus in 1989, there is concentrated interaction among translational, medicinal and clinical research which is led to incessant progress in diagnostic and treatment strategical tools (Jia *et al.*, 2019).

By the discovery of HCV virus many anti-viral drugs are used for the treatment or eradication of this infectious agent. These drugs target the specific step of cell cycle of HCV in order to restrict the replication, assembly, release and activity of HCV. Many steps in HCV life cycle can be used as target of antiviral therapies (Khan *et al.*, 2013b). Structural and non-structural proteins are the main target of many antiviral therapies. HCV genome encodes six non-structural proteins including NS2, NS3, NS4A, NS4B, NS5A & NS5B (Khan *et al.*, 2013a). NS3 or NS4A protease

are used as target of direct anti-viral drugs, inhibit the intracellular life cycle by effecting polyprotein of viral agent (Ashfaq *et al.*, 2011). The non-structural protein 5B NS5B) protein is required for replication and synthesis of RNA of HCV. It is an authenticated target for antiviral therapy (Mustafa *et al.*, 2020). It is considered to use NS5B protein as target in order to find out the potential drug against HCV. Pakistan is rich in medicinal plants, therefore we focused on phytochemicals derived from different plants including *Cichorium intybus, Saussurea lappa, Trigonella foenum-graecum, Glycin max, Lepidium sativum* and *Nigella sativa*. These phytochemicals can be used as inhibitors as they bind to NS5B polymerase and can stop the replication of HCV.

The aim of this study was to screen the phytochemicals from different medicinal plants having pharmacological properties against HCV NS5B polymerase using in silico approaches.

MATERIALS AND METHODS

This study was designed to dock the 100 phytochemicals derived from *Cichorium intybus, Saussurea lappa, Trigonella foenum-graecum, Glycin max, Lepidium sativum* and *Nigella sativa* against NS5B polymerase of HCV. Molecular docking was performed via MOE v2015) software. The interactions between selected phytochemicals and NS5B protein was visualized by using PyMOL program (Seeliger and de Groot, 2010).

Retrieval of phytochemicals:

The Dr. Duke's phytochemical database https://phytochem.nal.usda.gov/) (Imran *et al.*, 2022) and previous literature were searched to find a total of 100 phytochemicals. Majority of these phytochemicals have anti-viral properties. PubChem database https://pubchem.ncbi.nlm.nih. gov/) was used to retrieved the 3D chemical structures of selected phytochemicals. PyMOL program is used to translate ligand SDF files, obtained from PubChem database, into PDB files. The sofosbuvir, a known anti-hepatitis drug, was used as a reference ligand in order to compare the docking results of selected phytochemicals (Keating and Vaidya, 2014).

Screening of Phytochemicals:

All the selected phytochemicals were passed through various filters to find out their bioactive profile. The phytochemicals were passed through four filters including Lipinski's rule of 5, toxicity filter, blood brain barrier and TPSA.

Drug likeness of Phytochemicals:

The Lipinski rule of five was measured using SwissADME software. It is used to determine whether a phytochemical with certain pharmacological characteristics may be employed as a human orally active medication (Nawaz *et al.*, 2020, 2022b; Hassan *et al.*, 2021). This law determines the ADME qualities of any chemical. Phytochemicals that broke more than one rule were dismissed. This filter aids primary progress in pre-clinical studies while simultaneously reducing the risk of clinical failure (Benet *et al.*, 2016; Attique *et al.*, 2019).

The blood brain barrier, which is highly selective and functional, prevents non-essential chemicals from entering the brain. The BBB shields the brain from the detrimental side effects of drugs and external molecules (Gupta *et al.*, 2019). Phytochemicals having a TPSA value of less than $70A^2$ are not able to cross the blood-brain barrier. The TPSA value for each chemical was determined using SwissADME software. The study excluded active compounds that penetrate the blood-brain barrier.

Toxic doses are usually represented as LD50 values and are given in mg/kg body weight. The LD50 is the median lethal dosage, or the level at which 50% of test subjects die following exposure to a drug. Based on the LD50 value in mg/kg, the global harmonized system GHS) of chemical categorization and labelling has recognized six toxicity classes. Protox-II was used to screen the phytochemicals, phytochemicals with LD50 value $\leq$ 300mg/kg are unable to pass through the filter (Mansouri *et al.*, 2021).

Retrieval of receptor protein:

The three-dimensional structure of NS5B was retrieved from Protein Data Bank (Berman *et al.*, 2000; Attique *et al.*, 2020) using PDB ID: 3QGH. In order to optimize the protein, water molecules were removed using MOE. Based on the data obtained from PDB, specific site was chosen for further in-silico analysis. That specific site was placed in the center of the grid box to conduct the docking. The structure obtained after these preparations was used as the receptor protein for docking analysis.

Molecular Docking and MD simulation:

Molecular Operating Environment v2015 (Vilar *et al.*, 2008; Alghamdi *et al.*, 2021; Mustafa *et al.*, 2022; Nawaz *et al.*, 2022a) program was used to bind the selected phytochemicals with the receptor protein. Molecular docking was carried out on 28 phytochemicals out of 100 that has passed all the filters. The human HCV NS5B polymerase PDB ID: 3QGH) was docked using the Molecular operating environment (Harris *et al.*, 2005) version 2015 (Attique *et al.*, 2019; Nawaz *et al.*, 2020; Alghamdi *et al.*, 2021; Hassan *et al.*, 2021). and Schrodinger (Alogheli *et al.*, 2017) to visualise possible inhibitors' binding conformation inside the protein binding area. We chose structure-based drug design approach because both ligand and the receptor structure availability. The receptor was kept being static in docking while the drug candidate was thought kept flexible. The estimated centroid point's coordinates are X: 29.12, Y: 8.23, and Z: -6.32, with 10Å radius for the area around the binding pocket. This point selection technique was used to select the protein binding site. A maximum of 200 pose run were allowed for each drug candidate. The slow docking method was chosen because of its high degree of accuracy. The generated confirmations were ranked using the fitness function in the s-score, which served as the fitness function. The GBVI/WSA dG scoring system, which is based on the generalised Born solvation model, was also used to alter the docking results GBVI). GBVI/WSA dG, a force field-based scoring function (Keating and Vaidya, 2014) utilized for binding potential study.

Visual analysis of docking findings based on interaction between molecules, the best complexes 6'-o-Acetyl-daidzin-, Coumestrol-, Hederagenin-HCV NS5B polymerase) were selected for the Molecular Dynamics MD) simulation investigation. To run the MD simulation, Schrodinger's Desmond Module was used (Bowers *et al.*, 2006). A solvent solution drenched in water was used for the forecasting. We are looking into the possibility of using the TIP3P water model to fix the issue. This orthorhombic simulation was built with a periodic boundary box that was at least 10Å away from the protein's surface. For system neutralisation, the right number of counter-ions were introduced. By introducing 0.15 M NaCl into the simulation box, a consistent isosmotic state was established. Prior to the production run of the simulation, an equilibration phase was performed as per the specifications. The conditions used in the MD simulation were 300 K and 1.013 atm of pressure. In all, 1000 snapshots of the simulated trajectory were saved over the course of 100 ns.

In order to investigate the MD-simulation path, the technique known as the Simulation Interaction Diagram was utilised.

RESULTS

Screening of phytochemicals:

The phytochemicals selected from Dr. Duke's phytochemical database and previous literature were derived from different medicinal plants Table 1). PubChem database was used to obtain the chemical structures of phytochemicals. All the 100 phytochemicals were passed through filters to determine their bioactive profile. First, the phytochemicals were screened for Lipinski's rule of 5, phytochemicals with no or one rule violation were further screened for toxicity filter. Out of 92 phytochemicals that passed Lipinski's rule of 5, 11 phytochemicals have LD50 value ≤ 300mg/kg and are unable to pass through the toxicity filter while the rest of phytochemicals were screened for BBB and TPSA filter. After BBB and TPSA screening, 28 phytochemicals were selected for further in silico analysis. The screening results of top three phytochemicals are given in table 1.

Table 1. Top screened phytochemicals from numerous plants with their physicochemical properties

PC	MF	PN	PP	MW D)	HBA	HBD	MR	Mop	LD50 mg.kg)	TPSA
6'-O-ACETYL-DAIDZIN	$C_{23}H_{22}O_{10}$	*Glycin max*	Seed	458.41	10	4	113.83	-0.71	3750	155.89
COUMESTROL	$C_{15}H_8O_5$	*Glycin max*	Leaf	268.22	5	2	73.81	1.76	2991	83.81
HEDERAGENIN	$C_{30}H_{48}O_4$	*Nigella sativa*	Seed	472.7	4	3	137.82	4.97	2000	77.76

PC: Phytochemicals
MF: Molecular formula
PN: Plant name
PP: Plant part
MW: Molecular weight

HBA: Hydrogen bond acceptor
HBD: Hydrogen bond donor
MR: Molecular refractivity

Molecular Docking Analysis:

Docking was used to find out the binding energies of these phytochemicals with NS5B protein. The grid box for proteins was created by adjusting the location of a specific site. All the phytochemicals were docked with the catalytic site of HCV NS5B polymerase. The phytochemicals that showed good binding affinities with NS5B polymerase as well as the docking results of reference drug sofosbuvir are given in table 3. The results of docking were interpreted by using PyMOL. Binding conformations and hydrogen bonding between the ligand and protein were also observed. The interactions between the ligand and Protein were visualized by using PyMOL.

In molecular modelling, molecular docking is the s-score for determining which proteins and ligands have tendency to interact with one another. It permits the investigation of fundamental biological procedures by following the actions of chemical compounds at the binding site of a target protein. Binding energy, docked confirmation and binding affinities inside the binding pocket were used to rank docking poses. Tabular data displaying the top docking rankings for drug candidates that have been successfully docked is provided in Table 2. 6'-O-ACETYL-DAIDZIN CID: 156155), a chemical molecule, has been demonstrated to have a strong binding affinity -10.8 kcal/mol) with HCV NS5B polymerase.

Table 2. Binding affinities of the top docked drugs against the human HCV NS5B polymerase

Sr.no	Phytochemical	Pub Chem ID	Binding Affinity kcal/mol)	Hydrogen bonding residues	Pi-pi interacting residues
1.	6'-O-ACETYL-DAIDZIN	156155	-10.8	Asn291, Asp225, Asp318, Asn411, Tyr448	Tyr448
2.	HEDERAGENIN	73299	-9.5	Asn291, Asp318, Glu446, Tyr561	
3.	COUMESTROL	5281707	-8.7	Asn291, Asp318, Glu446	
4.	SOFOSBUVIR	45375808	-8.5	TYR 176, TYR 191, TYR 452	

As shown in figure 1A, 6'-O-ACETYL-DAIDZIN, HEDERAGENIN and COUMESTROL have shown common hydrogen bonding with Asn291 and Asp318 residues of HCV NS5B polymerase reflecting the importance of these two residues toward crucial role in drug binding. Whereas, 6'-O-ACETYL-DAIDZIN has shown hydrogen bonding with Asp225, Asn411, Tyr 448, HEDERAGENIN with Glu446, Tyr 561 and COUMESTROL with Glu446 respectively. In addition to hydrogen bonding Tyr448 of HCV NS5B polymerase sharing pi-pi interaction with 6'-O-ACETYL-DAIDZIN.

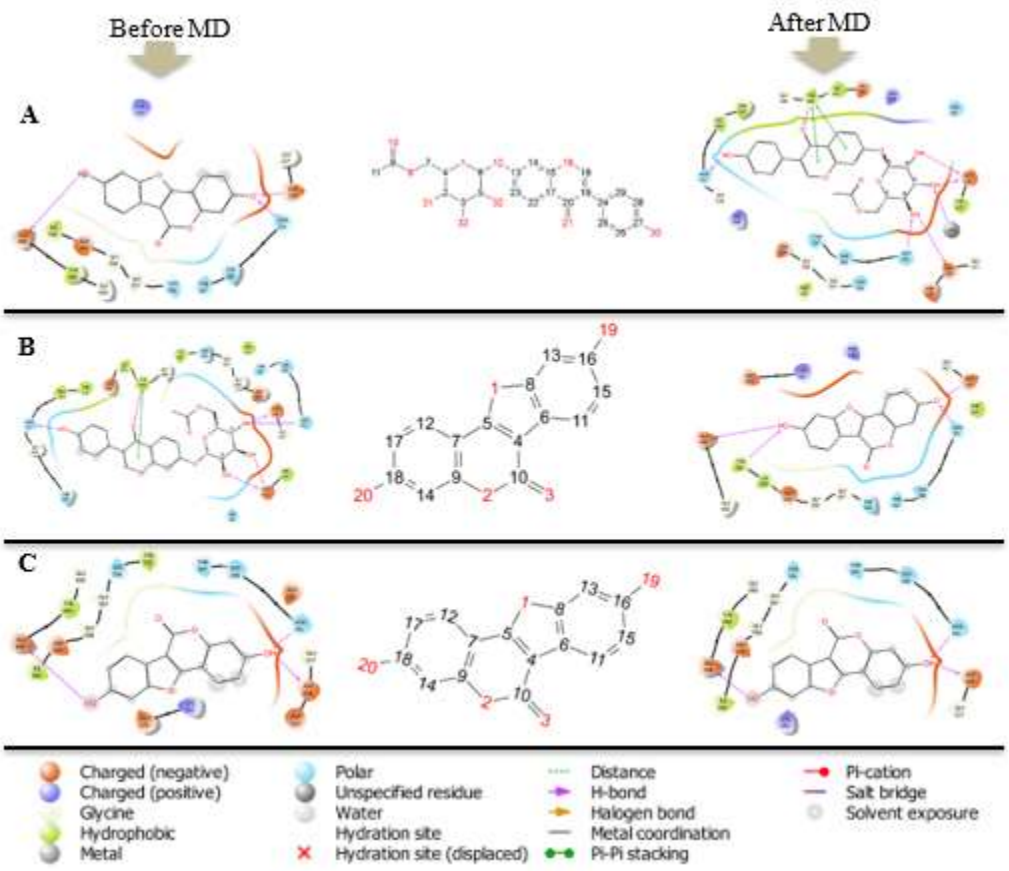

Figure 1. Drug candidates' molecular interactions before and after md simulation: Where **A)** 6'-O-ACETYL-DAIDZIN, **B)** HEDERAGENIN and **C)** COUMESTROL correspondingly have shown binding potential with key residues of HCV NS5B polymerase. Used drug candidates interacting elements are shown in red numbering in middle, structural representation of compounds).

Docking alone cannot provide information about the binding mechanism, stability, or dynamics of prospective ligands. Desmond module of Schrödinger's equation allowed us to do MD simulation for several nanosecond time frames depending on the stability point of the docked complexes with human HCV NS5B polymerase. Used docking protocol validated from md simulation results as molecular interaction pattern of crucial residues before and after of all used drug candidates Figure 1 & S4) remains same.

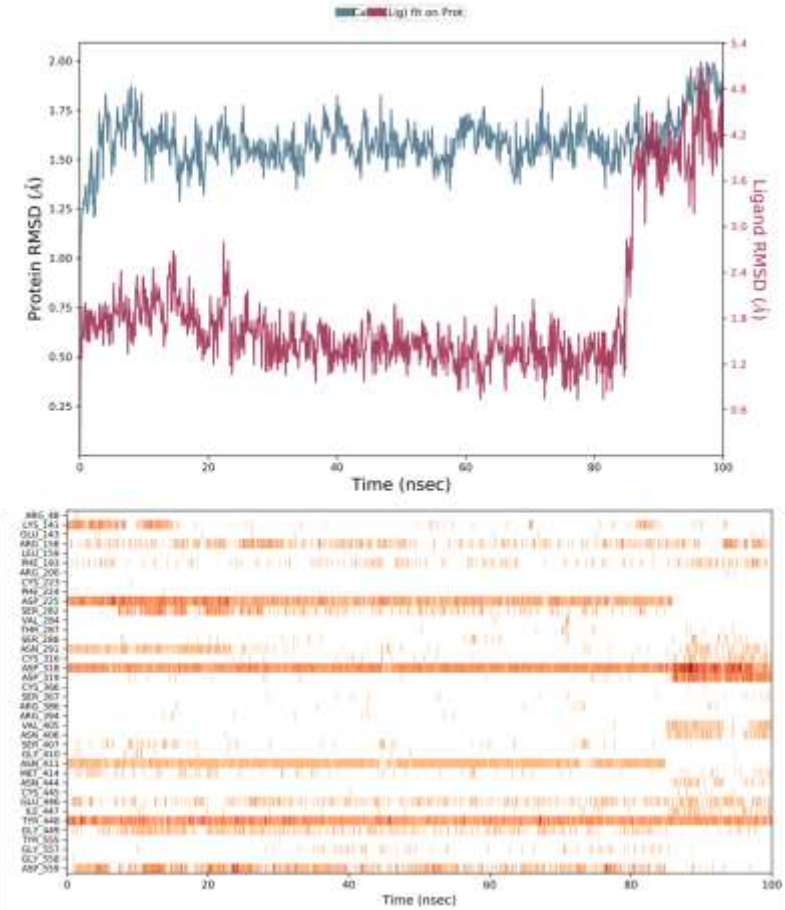

Figure 2. HCV NS5B polymerase receptor) and 6'-O-ACETYL-DAIDZIN ligand) interaction complex, **A)** RMSD plot for C-alpha atoms Å) sites from the starting structure vs simulation time ns). **B)** Hydrogen bonding timeline plot validates key residues bonding concistency throuought the simulation nominating this complex as structuraly stable

6'-O-ACETYL-DAIDZIN has shown structural stability with HCV NS5B polymerase figure 2A) as we can see the complex RMSD value is lower than 4Å. Consistent hydrogen bonding of interacting residues Asp225, Asn291, Asp318, Asn411, Tyr448 and Asp559 figure 2B) also

validates this complex 6'-O-ACETYL-DAIDZIN- HCV NS5B polymerase structural stability. RMSD trajectory gap from 0ns to 84ns is due to fitness of 6'-O-ACETYL-DAIDZIN within the binding pocket of receptor due to higher number of rotatable bonds in 6'-O-ACETYL-DAIDZIN figure S1). As we can see from figure S1, 6'-O-ACETYL-DAIDZIN has 10 rotatable bonds, resulting its fitness deviation by different angels figure S1A) but hydrogen bonding with crucial residues remains same before and after md figure S1B).

HEDERAGENIN has shown structural stability with HCV NS5B polymerase as we can see the complex RMSD value is lower than 4Å figure 3A). HEDERAGENIN just fluctuated during md time frame from 25ns to 65ns to change its confirmation, breaking hydrogen bonding with Asp318 but after 65ns it again gains its previous confirmation and retaining bonds with key residues figure 1B, S2 & S4). But RMSD value and hydrogen bond histogram confirms HEDERAGENIN- HCV NS5B polymerase complex structural stability throughout simulation.

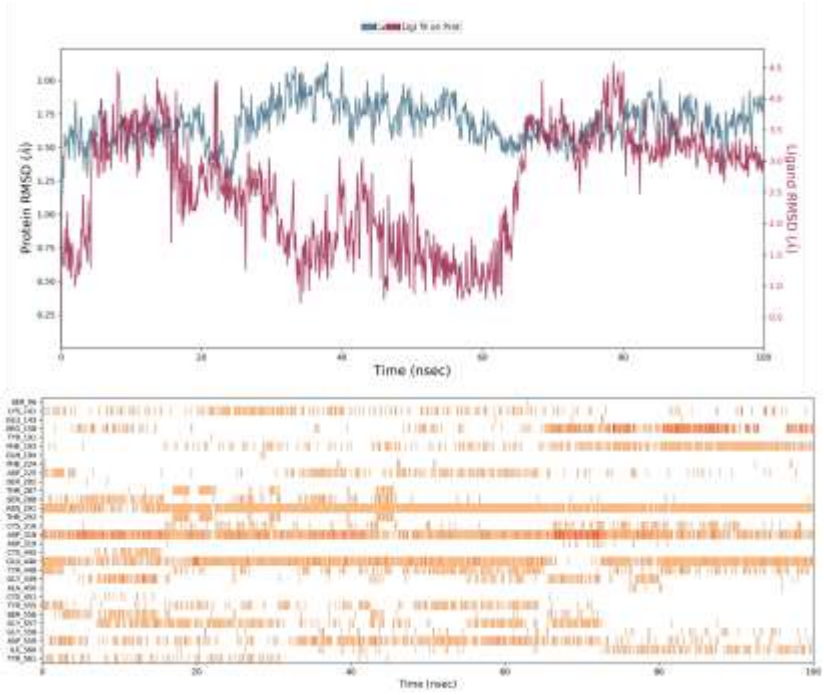

Figure 3. HCV NS5B polymerase receptor) and HEDERAGENIN ligand) interaction complex, **A)** RMSD plot for C-alpha atoms Å) sites from the starting structure vs simulation time ns). **B)** Hydrogen bonding timeline plot validates key residues bonding concistency throuought the simulation nominating this complex as structuraly stable

Third ranked drug candidate "COUMESTROL" in complex with HCV NS5B polymerase has also shown structural stability due to lower RMSD figure 4A) and hydrogen bond figure 4B) consistency throughout simulation timeframe. However, this complex RMSD sharp jump from 0.75Å to 1.5Å from 42ns to end of md is due to COUMESTROL spatial rotation within the binding pocket of receptor and breaking h-bond with Glu446 due to distance). But after 42ns this drug complex remains in equilibrium form and hydrogen bonding with key residues Asn291 and Asp318 remains consistent figure 4B, S3 & S4).

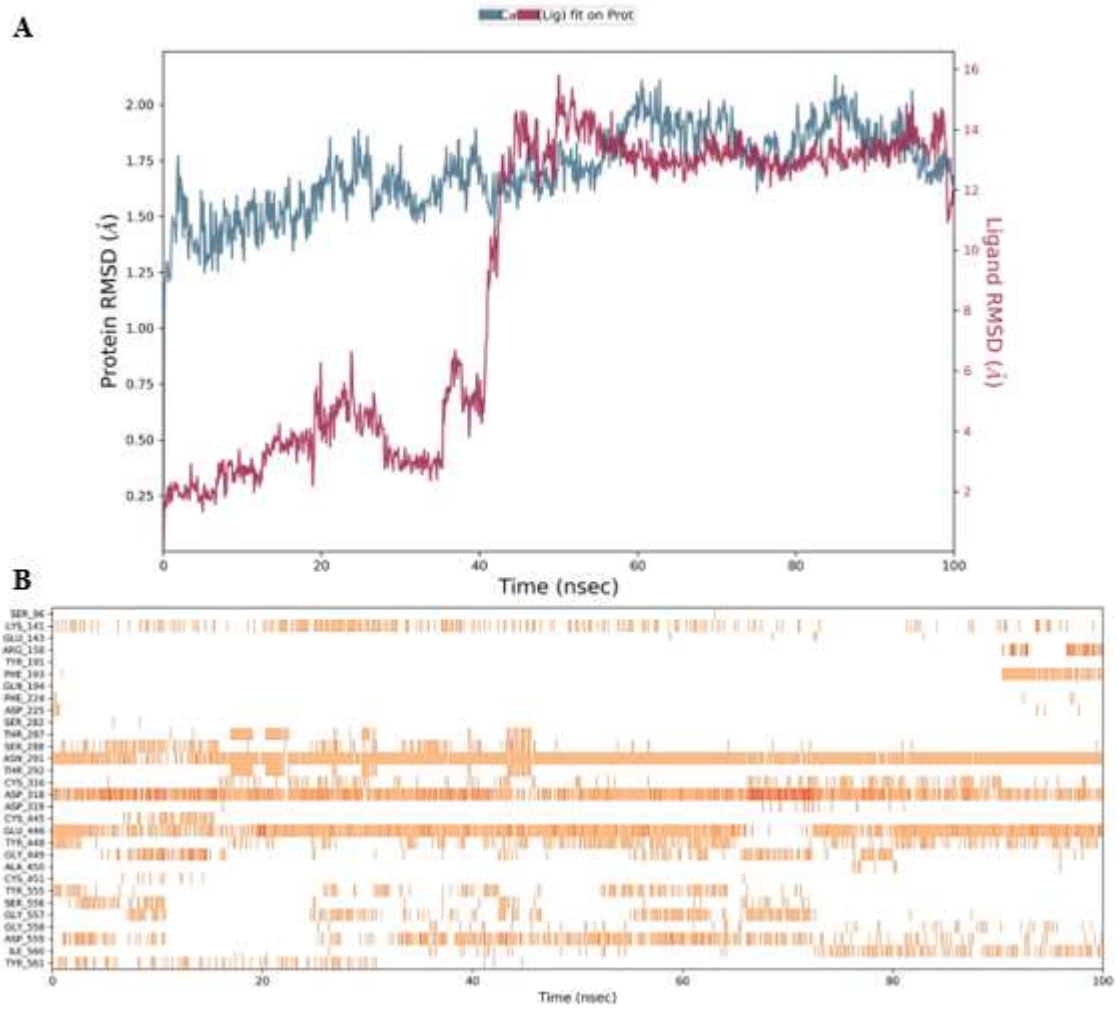

Figure 4. HCV NS5B polymerase receptor) and COUMESTROL ligand) interaction complex, **A)** RMSD plot for C-alpha atoms Å) sites from the starting structure vs simulation time ns). **B)** Hydrogen bonding timeline plot validates key residues bonding concistency throuought the simulation nominating this complex as structuraly stable

DISCUSSION

HCV is a hepatocyte infection that affects the liver and eventually leads to cirrhosis. One hundred and seventy million patients are estimated to be present worldwide, according to approximate estimates. Many of these individuals almost 85%) were unable to recover from the infection on their own, resulting in chronic illness (Spaan *et al.*, 2016). Previous studies showed that HCV genome is responsible for the disease as well as treatment response. HCV genome encodes for the single large polyprotein, proteolysis of this protein leads to the development of structural and non-structural proteins. These structural proteins are involved in the formation of infectious virus particles (Paul *et al.*, 2014). Thus, these structural proteins can be used as an attractive target for the therapeutic interventions of HCV. NS5B polymerase is one of these structural proteins of HCV virus that comprises of 590 amino acids. One of the attractive property of NS5B is its simple structure as this makes it easier to understand its interactions with different phytochemicals(Barreca *et al.*, 2014).

Computational approaches are effective tools for predicting the potential of these ligands binding before they are produced and estimated in the lab. Docking and other approaches are mostly used to uncover the binding patterns of small molecules against their targets in the context of medication discovery and development (Wei *et al.*, 2016). Lately, different phytochemicals have been used in targeted therapies for the treatment of various diseases. Due to this scientists started working on phytochemicals to search for more effective and tolerated agents. In a previous study, it was observed that the phytochemical thiazolidinone and some of its derivatives showed a good anti-HCV activity (Yan *et al.*, 2007). Thus, this study was designed to find the alternative treatment and management options for HCV infection.

This study was based on the molecular docking of phytochemicals derived from some medicinal plants including *Cichorium intybus, Saussurea lappa, Trigonella foenum-graecum, Glycin max, Lepidium sativum* and *Nigella sativa* against NS5B polymerase. The total of 100 phytochemicals derived from these plants were selected and passed through various filters including Lipinski's rule of 5, toxicity filter, blood brain barrier and TPSA in order to find out their drug like properties. After the screening of selected phytochemicals, 28 phytochemicals fulfilled all the requirements and passed through these filters. These phytochemicals were then docked with three-dimensional structure of NS5B protein retrieved from Protein Data Bank using PDB ID: 3QGH. As a result of

docking the binding energies for phytochemicals are obtained and compared. And the results of docking were further analyzed based on number of interactions by using pymol.

Our results showed that are the most effective phytochemicals against HCV infection are 6'-O-acetyl daidzin, coumestrol and hederagenin. 6'-O-Acetyl daidzin is a glycosyloxyisoflavone that is daidzein. It is derived from medicinal plant named *Glycin max* soybean) and can easily be extracted from the seeds of the plant. It has antioxidant properties (Correa *et al.,* 2010). It showed -10.8 kcal/mol binding affinity value with NS5B protein. It showed strong interactions with Asn291, Asp225, Asp318, Asn411 and Tyr448 of NS5B protein. Second phytochemical is coumestrol that is a polyphenol and can be extracted from the leaves of *Glycin max* plant. It has antioxidant and anti-cancer properties (Montero *et al.*, 2019). It showed -8.7 kcal/mol binding affinity value with NS5B protein. It showed strong interactions with Asn291, Asp318 and Glu446. Third phytochemical is hederagenin that is pentacyclic triterpenoid, extracted from many plants including *Nigella sativa.* It has anti-tumor, anti-inflammatory, anti-depressant and anti-viral activity (Zeng *et al.*, 2018). It showed -9.5 kcal/mol binding affinity value with NS5B protein. It showed strong interactions with Asn291, Asp318, Glu446 and Tyr561. The binding conformations and interactions of these phytochemicals with the NS5B protein has been shown in figure 1. The binding affinity values of all three phytochemicals are compared with the binding affinity value of reference drug Sofosbuvir) that is -8.5 kcal/mol. These three phytochemicals have also shown good fitness figure 5) within the spatial binding pocket of HCV NS5B reflecting best binding potential.

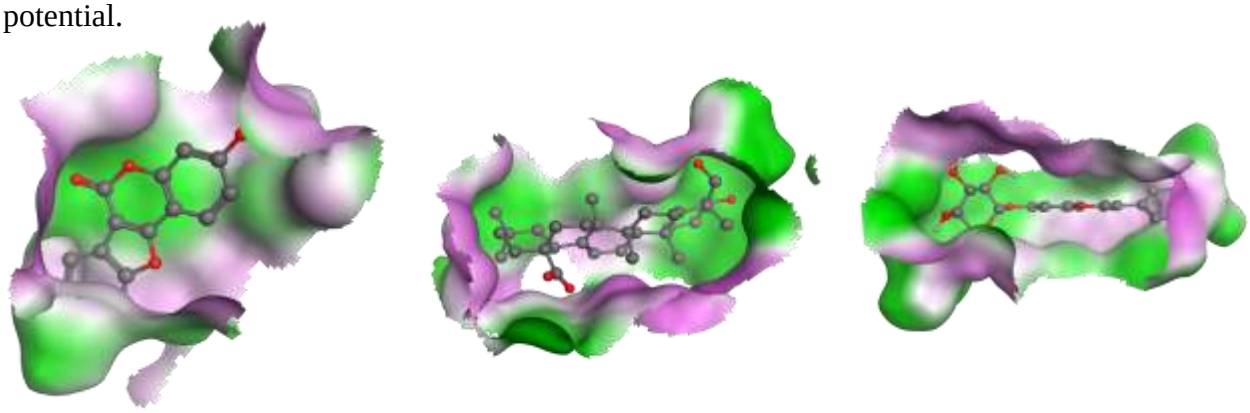

Figure 5. Complementarity fitness of **A)** 6'-O-ACETYL-DAIDZIN, **B)** HEDERAGENIN and **C)** COUMESTROL within the binding pocket region of HCV N5SB.

Drug candidate's structural stability within the targeting receptor sites is mandatory to propose a drug before it's clinical trials Our proposed three drug candidate's 6'-O-ACETYL-DAIDZIN, HEDERAGENIN, COUMESTROL) md simulation reflects their structural stability figure 2, 3 and 4). The binding pattern and dynamics of these drug candidates with respect to time under defined conditions is graphically explained in supplementary data. The binding affinity values of all three phytochemicals are greater than the value of reference drug. These phytochemicals have potential to be used as drugs for the treatment of Hepatitis C in future. These phytochemicals require further in vitro analysis and pre-clinical trials to be used in the treatment of Hepatitis C in future.

Conclusion

To conclude, from the comparative analysis of in silico results 6'-O-acetyl daidzin, coumestrol and hederagenin are found to be most effective phytochemicals, as they showed comparatively high binding affinity values with NS5B protein of HCV as well as their binding affinity values are higher compared to the binding affinity value of the reference drug sofosbuvir. These phytochemical's structural stability, significant binding potential and good drug likeness proved them potential drug agents for the therapeutic treatment of Hepatitis C by inhibiting replication and synthesis of RNA in HCV. In future, we are planning to perform in vitro analysis and pre-clinical trials of these proposed drug hits in order to be used in the treatment of Hepatitis C in future.

***Conflicts of Interest*:** All authors have disclosed no conflicts of interest.

Authors Contribution Statement: Ayesha Munir performed basic write up, docking analysis, results interpretation, discussion formulation**.** Dr Shah Jahan validated the results and assisted in simulation. Dr Faiza Saleem also verified and validated the outcomes. Hafiz Muhammad Zeeshan Raza proofread the write-up, referencing, and managed submission process.

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