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Polypharmacology of some medicinal plant metabolites against SARS-CoV-2 and host targets: Molecular dynamics evaluation of NSP9 RNA binding protein

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ABSTRACT

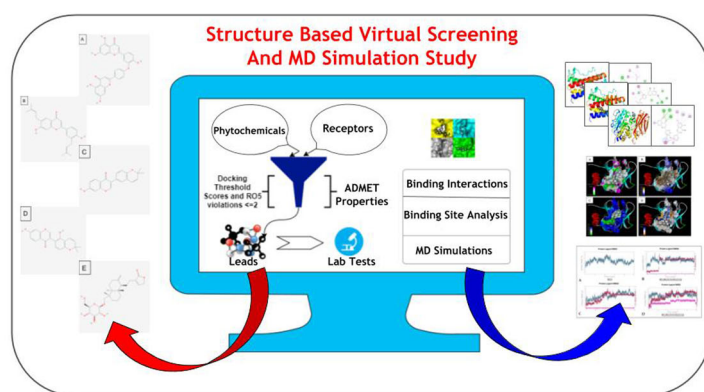
Medicinal plants as rich sources of bioactive compounds are now being explored for drug development against COVID-19. 19 medicinal plants known to exhibit antiviral and anti-inflammatory effects were manually curated, procuring a library of 521 metabolites; this was virtually screened against NSP9, including some other viral and host targets and were evaluated for polypharmacological indications. Leads were identified via rigorous scoring thresholds and ADMET filtering. MM-GBSA calculation was deployed to select NSP9-Lead complexes and the complexes were evaluated for their stability and protein-ligand communication via MD simulation. We identified 5 phytochemical leads for NSP9, 23 for Furin, 18 for ORF3a, and 19 for IL-6. Ochnaflavone and Licoflavone B, obtained from *Lonicera japonica* (Japanese Honeysuckle) and *Glycyrrhiza glabra* (Licorice), respectively, were identified to have the highest potential polypharmacological properties for the aforementioned targets and may act on multiple pathways simultaneously to inhibit viral entry, replication, and disease progression. Additionally, MD simulation supports the robust stability of Ochnaflavone and Licoflavone B against NSP9 at the active sites via hydrophobic interactions, H-bonding, and H-bonding facilitated by water. This study promotes the initiation of further experimental analysis of natural product-based anti-COVID-19 therapeutics.

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Virtual screening; phytochemicals; NSP9; Orf3a; furin; Interleukin-6; molecular dynamics simulation; COVID-19; SARS CoV-2



Abbreviations: ADMET: Absorption Distribution Metabolism Excretion and Toxicity; COVID-19: Corona Viral Disease-2019; IL-6: Interleukin-6; LD50: Lethal Dose 50; MD: Molecular Dynamics; MM/GBSA: Molecular Mechanics/Generalized Born Surface Area; NSP9: Non-Structural-Protein 9; Orf3a: Open Reading Frame 3a; RMSD: Root Mean Square Deviation; RMSF: Root Mean Square Fluctuation; RO5: Rule of Five; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus-2

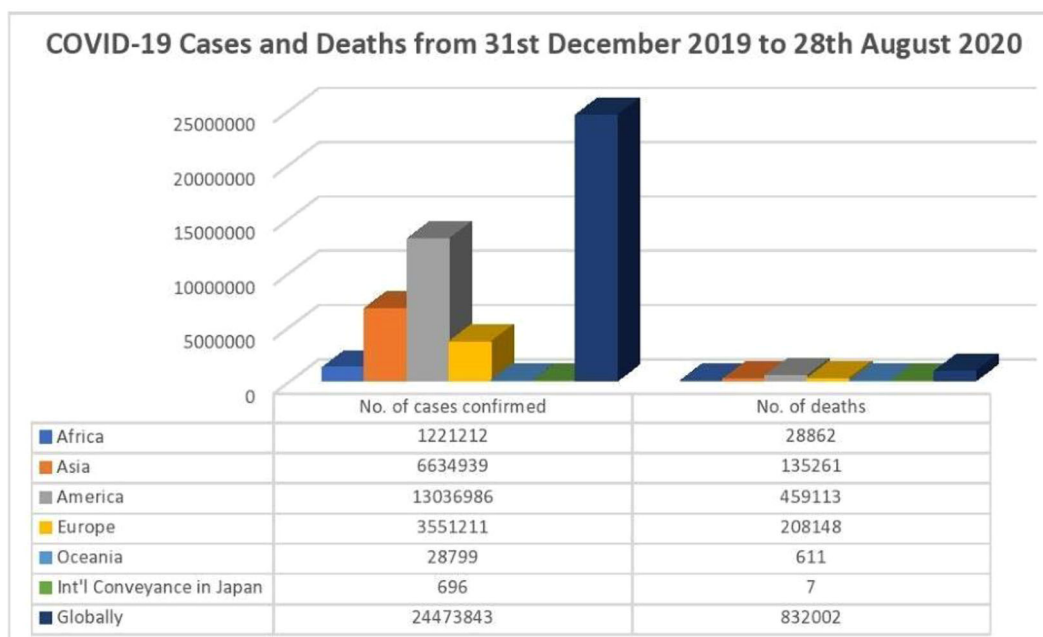


Figure 1. COVID-19 situation report till 28 August 2020. SARS CoV-2 virus disease cases and deaths. Data are based on official information reported by the European Center for Disease Prevention and Control.

Introduction

COVID-19 was first reported in Wuhan, China in December 2019 and declared a global pandemic by the World Health Organization (WHO) in March 2020 (Cucinotta & Vanelli, 2020; Li et al., 2020). The global outlook reported by ECDC as of 28 August 2020 was 24,473,843 confirmed cases and 832,002 deaths among continents (www.ecdc.europa.eu) represented in Figure 1.

The global challenge to health and economy posed by COVID-19 may only be upturned by the development of an effective drug for its management. Highly exploited drug targets for COVID-19 include Receptor Binding Domain (RBD) (Dong et al., 2020), spike protein (S), nucleocapsid (N) (Grunewald et al., 2018; Surjit et al., 2006), non-structural proteins (NSPs) including papain-like protease (Plpro), RNA-dependent RNA polymerase (RdRp) and 3-chymotrypsin-like protease (3Clpro), among others. The non-structural proteins 9 (NSP9) replicase is among the 16 NSPs of SARS-CoV-2 vital for viral replication and transcription (Ziebuhr et al., 2000). Also, unlike other proteins with a single domain, it comprises a unique single folded beta-barrel. It binds with RNA, interacts further with NSP8, and triggers the essential functions (Sutton et al., 2004). Inhibition of NSP9 further inhibits NSP8 and thereby antagonizes viral replication making it one of the attractive targets for drug discovery. Our interest was NSP9 because of its uncharacterized function in the domain of replication. ORF3a is also an interesting viral target for drug discovery. It plays an essential role in induced cell apoptosis and activation of NLRP3 inflammasome (Siu et al., 2019), leading to cytokine storms (Tufan et al., 2020).

Besides the viral targets, there are host proteins that aid viral replication and promote an excessive inflammatory

response. Identification of potential inhibitors of host factors has been less explored via computational studies (Wu et al., 2020; Rahman et al., 1975) when compared with the studies on viral proteins inhibition. Among various host targets (Gil et al., 2020), we were interested in studying the receptors, furin that plays a major role in activating the S-proteins, implicated in the cleavage and activation of viral coat proteins (Cameron et al., 2000), and interleukin-6, a cytokine with a crucial role in virus-induced immunopathological events which causes fatal pneumonia in severe SARS-Cov-2 infections (Channappanavar & Perlman, 2020).

The interaction of drug molecules with multiple targets have been described as polypharmacology. It represents a new lead to rational designing of the next cohort of therapeutic agents with improved efficacy and decreased toxicity. The past decade witnessed a paradigm shift in the drug discovery process as regards solely targeting disease with one drug. Lack of efficacy and high toxicity had been the reasons for the rejection of new drug applications, hence the need for this paradigm change. The rational designs of multi-target ligands have been challenging to achieve. However, their benefits over the use of selective compounds include improved efficacy and less complicated pharmacodynamic and pharmacokinetic characteristics (Proschak, 2014).

Plants are rich sources of bioactive compounds called phytochemicals which play an important role in its growth, and as a defense against cell damage, competitors and other plant predators. These chemicals are reported to be highly effective against numerous pathogens (Hussain et al., 2017). In this context, we present a virtual screening of small-drug like plant metabolites chosen from some selected medicinal plants with antiviral and anti-inflammatory properties, against four targets: NSP9, ORF3a, furin, and IL-6, and the

Table 1. Taxonomy of the plants chosen for the study.

Plant Species	Family	No of chosen metabolites	References
<i>Apium graveolens</i> (Celery)	Apiaceae	20	(Zhou et al., 2009)
<i>Astragalus</i>	Fabaceae	25	(Li et al., 2019)
<i>Glycyrrhiza species</i> (Licorice)	Fabaceae	32	(Mamedov & Egamberdieva, 2019)
<i>Oregano</i>	Lamiaceae	21	(Somjen et al., 2004)
<i>Tinospora cordifolia</i>	Menispermaceae	21	(Leyva-López et al., 2017)
			(Sharma et al., 2019)
			(de J. Rostro-Alanis et al., 1975)
<i>Nigella sativa</i> L	Ranunculaceae	50	(Venkatachallam et al., 2010)
<i>Hibiscus sabdariffa</i> L.	Malvaceae	32	(Da-Costa-Rocha et al., 2014)
<i>Lonicera japonica</i>	Caprifoliaceae	50	(Shang et al., 2011)
<i>Scutellaria baicalensis</i>	Lamiaceae	37	(Zhao et al., 2019)
<i>Oplopanax horridus</i>	Araliaceae	39	(Wu et al., 2018)
<i>Phyllanthus urinaria</i>	Phyllanthaceae	93	(Geethangili & Ding, 2018)
<i>Ocimum sanctum</i> L	Lamiaceae	22	(Singh & Chaudhuri, 2018)
<i>Morinda citrifolia</i> (Noni)	Rubiaceae	18	(Abou Assi et al., 2017)
<i>Vigna radiata</i> L	Fabaceae	20	(Silva et al., 2013)
<i>Terminalia chebula</i>	Combretaceae	20	(Riaz et al., 2017)
<i>Andrographis paniculata</i>	Acanthaceae	20	(Hossain et al., 2014)
<i>Phyllanthus amarus</i>	Phyllanthaceae	15	(Gupta & Vaghela, 2019)
<i>Aloysia gratissima</i>	Verbenaceae	19	(Santos et al., 2015)
<i>Psoralea corylifolia</i> L	Fabaceae	15	(Li et al., 2016)
			(Zhang et al., 2016)
Total Molecules		569	
Non-redundant Molecules		521	

analysis of their polypharmacological indications. We also examined the nature and properties of the binding pockets of all the chosen receptors. A Molecular Dynamics (MD) simulation study was executed to validate the findings and investigate the stability of the NSP9 protein-ligand complexes.

Materials and methods

Dataset and ligand preparation

Phytochemicals from 19 different medicinal plants with potential antiviral and anti-inflammatory activities, obtained through literature survey, were compiled and manually curated to get a comprehensive database of 521 non-redundant molecules. The compounds chosen are mainly classified as phenolic acids, alkaloids, flavonoids, terpenoids, coumarins, lignins and glycosides among the other classes of natural products that exhibit excellent antiviral and anti-inflammatory effects. Table 1 enlists all the plants from which the phytochemicals have been chosen for the study, along with the references and with their taxonomic identities.

The 2-D structures of the compounds were retrieved from PubChem Database (Kim et al., 2019), and the database of molecules was prepared by uploading the retrieved structures. As controls for the screening study, standard inhibitors for all the four receptors were downloaded from PubChem. They were minimized by the conjugate gradient algorithm using the *obminimize* function of Open Babel (Boyle et al., 2011) prior to docking.

Evaluation of the binding site

Four receptors, including two from SARS-CoV-2: NSP9 and ORF3a and two from the host: furin and IL-6, were chosen for our study. The protein PDB structures of the receptors with PDB IDs 5JXI (Dahms et al., 2016), 1ALU (Somers et al.,

1997), 6XDC (Kern et al., 2020), and 6W4B (Tan et al., 2020) with resolutions 2.00 Å, 1.9 Å, 2.9 Å and 2.95 Å respectively were downloaded from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RSCB PDB; (<http://www.rcsb.org/>, Berman et al., 2002). The active pockets of the viral targets, NSP9 and ORF3a with PDB IDs 6W4B and 6XDC respectively, were predicted using SiteMap (Schrödinger Release 2018-1: SiteMap, Schrödinger, LLC, New York, USA). Potential binding regions and active residues of the host receptors, IL-6 and furin with PDB IDs 1ALU and 5JXI respectively, have been previously identified, experimentally verified, and resolved in high-resolution crystals. The pockets and residues identified in the active site were further evaluated using ConSurf (Ashkenazy et al., 2016; Celnikier et al., 2013; Ashkenazy et al., 2010) and PrankWeb (Jendele et al., 2019; Krivák & Hoksza, 2018) online servers.

Receptor preparation

Chain A of the polymeric-proteins was chosen for further molecular docking and screening studies. Prior to virtual screening, the protein molecules were cleaned and prepared using AutoDock Tools 1.5.6 (Sanner, 1999; Morris et al., 2009). All the non-polar hydrogens, lone-pairs, water molecules, and non-standard residues were removed. Polar hydrogens were added, followed by the addition and merging of Gasteiger charges. The files were exported and saved as .pdbqt.

The Grid configuration parameters were also generated using AutoDock Tools 1.5.6. For each receptor, the GridBox was set near the active site region, and the size of the box was relaxed, ensuring the inclusion of all essential catalytic, conserved structural motifs and substrate binding amino acid residues inside the box that are vital for the functioning of the receptors to reduce the search space for ligand optimization (Trott & Olson, 2010).

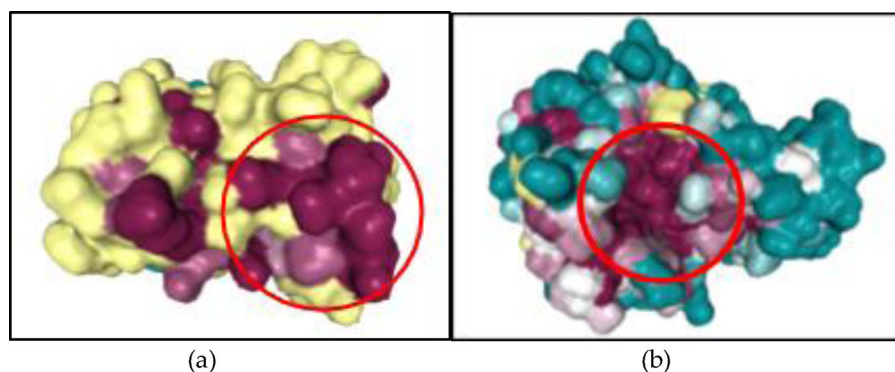


Figure 2. Predicted conserved regions of the active site of target (a) IL-6 [1ALU:A] and (b) Furin [5JXI]. The region marked in shades of purple corresponds to the conserved residues, while shades of cyan correspond to variable amino acid residues. The region in yellow doesn't have sufficient information for conservation analysis. Encircled in red is the active site for the host receptors from reported literature.

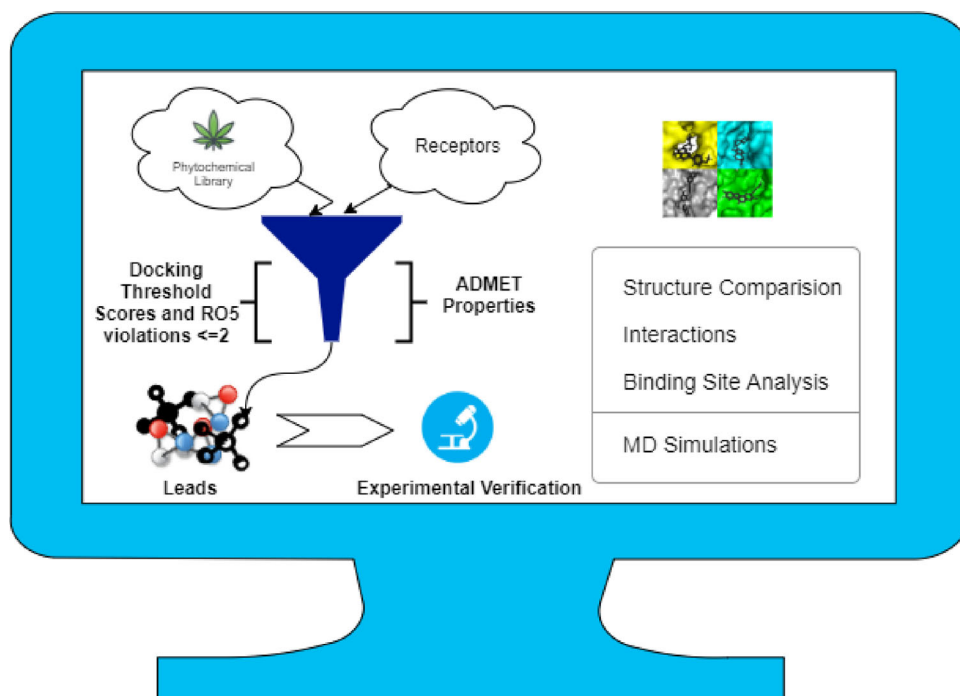


Figure 3. Schematic representation of the workflow.

Virtual screening workflow

Structure-based virtual screening (SBVS) of the prepared phytochemical library was performed using custom workflows of Mcule Drug Discovery Pipeline (<http://Mcule.com>; Kiss et al., 2012). The Mcule server utilized the 3-D structure of the target and fitted the ligands by efficiently using a gradient optimization method in its local optimization procedure. The individual workflow steps added to filter the hits for this study, namely the basic property filter search based on the number of rule-of-five (RO5) violations and the Docking (Vina) workflow step (Trott & Olson, 2010), were sequentially executed (Figure 3).

Very recently, for areas with large unmet medical needs like oncology and virology, extensive studies show the focus and demands of beyond-rule-of-five (BRO5) drug candidates (Zhang & Wilkinson, 2007). To broaden the search of our study for finding anti-COVID phytochemical candidates, we have allowed a maximum balanced violation of two.

The molecules were screened by docking using the Vina docking algorithm (Trott & Olson, 2010). The receptor targets were prepared and fed as an input file. The grid dimensions, as well as the coordinates of the center of the binding site for each receptor molecule, was fed to the workflow step, and other constraints required were set to default. For the inhibitors used as standard controls, molecular docking was manually performed using AutoDock Vina.

Post-docking visualization and binding site analysis

The structures of the top conformation of 30 docked Phyto-ligands complexed with the receptor macromolecules having the lowest binding affinity values (highest vina docking scores) were retrieved from Mcule, and the poses were visualized using EduPyMol version 2.3.4. The top screened molecules were further analyzed for the receptor-ligand

interactions (with the binding site residues), the type of bonds, the bond distances, using the various functionalities available in Discovery Studio Visualiser (BIOVIA, Dassault Systèmes, 2020). Among multiple interactions, we mainly looked for the occurrences of hydrogen bonding, van der Waal, π - π interaction, π -sigma, π -sulfur, and the other existing non-covalent interactions. The characteristics of the binding site pocket were also of interest and hence were analyzed for the nature of aromaticity, H-bond donor/acceptor distribution, hydrophobicity, and solvent accessibility (SAS) using DS Visualiser.

Molecular dynamics simulation

For the challenges with respect to the computational expenses, we chose NSP9 for further evaluation mainly because we were interested to understand the dynamics of the binding site residues of a viral protein that has not been appropriately characterized concerning its biological function. Molecular dynamics simulations were performed using Desmond tool (Bowers et al., 2006; Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY; Maestro-Desmond Interoperability Tools, 2018). The ligand-receptor complex was placed at the center of a periodic orthorhombic box, and the addition of TIP3P water molecules performed solvation. Sodium and chloride ions were added to neutralize the system at a concentration of 0.15 M to mimic physiological conditions. For long-range electrostatics, the smooth particle mesh Ewald (PME) estimation was used, while for nonbonded interactions at a cut-off of 9 Å MSHAKE (Lambrakos et al., 1989) algorithm was utilized. The Default Desmond protocol was followed for system relaxation. After energy minimization, the system was gradually heated to a temperature of 300 K. It was maintained by Nose-Hoover thermostat for a constant number of particles, volume, and temperature simulation of 500 ps with 2 fs time step, followed by equilibration of pressure to 1 bar and was maintained via Martyna-Tuckerman-Klein barostat, during constant number of particles, pressure, and temperature (NPT) of 500 ps with 2 fs time step. MD Simulation was performed for each of the three protein-ligand complexes and the apo-state protein for 100 ns with 2 fs time step. Computation of the trajectories was executed using multiple time-step RESPA integrators. We analyzed the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) of the main backbone of carbon atoms, RMSD of the ligands and the protein-ligand contacts were also assessed. Simulation results analysis was performed using the Desmond SID in maestro.

ADMET properties

The resultant library generated after the virtual screening and the benchmark docking scores for all the four receptors were subjected to *in silico* predictions of pharmacokinetics and ADMET properties using the admetSAR 2.0 webserver (Yang et al., 2019). The molecules were specifically checked for human intestinal absorption, blood-brain barrier

permeance, phosphoglycoprotein substrate binding, cytochrome inhibition (CYP2D6), carcinogenicity, and AMES mutagenicity to give us the final leads for the receptors.

Rodent acute toxicity studies

In silico prediction of acute toxicity in rodent models for the leads of NSP9 and with possible polypharmacological prospects was performed using the GUSAR web server (Lagunin et al., 2011). Using GUSAR, compounds can be evaluated based on the Quantitative Neighborhoods of Atoms Descriptors and Prediction of Activity Spectra for substances algorithm. The result thus obtained is correlated using the SYMYX MDL toxicity database. Further, it is classified using the chemical classification manual of the Organisation for Economic Co-operation and Development (OECD) (Salman et al., 2020).

Results and discussions

Binding pocket identification

For the proteins, especially NSP9 and ORF3a, which are dimeric and tetrameric with similar chain sequences, chain A was chosen for the study for simplicity. For SARS CoV-2 receptors, NSP9 and ORF3a, the binding pockets were predicted using SiteMap (Schrodinger). Residues in the active site pockets of NSP9 mainly included M13, Y33, G38, G39, R40, F41, V42, L43, F57, P58, K59, S60, I66, Y67, T68, E69, I92, K93, G94, L95 and N96, among others. The sequence region 94-97 is found to be rich in polar and hydrophobic residues (Sutton et al., 2004). The active site residues in ORF3a include mainly K75, F79, N82, Q116, S117, N119, F120, R122, I123, L127 and L139, among others. The second or third transmembrane region is properly responsible for Golgi localization. The residues K75, F79 and N82, lie in the second transmembrane helix domain, while residues Q116, S117, N119, F120, R122 and I123 in the third transmembrane helix region (UniProtKB - P0DTC3; <https://www.uniprot.org/>, The Uniprot Consortium, 2019). For the furin binding region, residues D153, H194, A252, S253, W254, P256, E257, N295, D301, E331, and S368 were obtained. Interleukin-6 contained four helices linked with loops (Somers et al., 1997). The C-terminal region (175-181) was found to be the receptor-binding domain with ARG179 as the key residue (Fontaine et al., 1993). A and D helices played vital roles in signal transduction and receptor binding.

The degree to which amino acid residues will be conserved is highly dependent on the structural as well as functional significance. The ConSurf server used in this study estimated the same based on the phylogenetic relations between homologous sequences (Ashkenazy et al., 2016; Celniker et al., 2013; Ashkenazy et al., 2010), while PrankWeb uses the Jensen-Divergence method for calculating the conservation scores (Jendele et al., 2019; Krivák & Hoksza, 2018). Figure 2 shows the surface distribution of conserved areas at the host receptors evaluated using ConSurf. The data received from PrankWeb verifies the same (Figure S1 in SI).

Table 2. Details of inhibitor chosen for each receptor and their docking scores upon binding with their respective receptors. The docking score threshold was set up for the receptors taking into consideration the highest score of the standard molecule and the range of docking scores of the top 30 retrieved molecules for all the receptors.

Receptor	Inhibitor	Docking Score (kcal/mol)	Set Cut-off (kcal/mol)	References
NSP9	Verdinexor	−7.7	≤ -7.8	Gordon et al., 2020
	Dabrafenib	−6.8		
ORF3a	Emodin	−6.9	≤ -7.1	Schwarz et al., 2011; Shah, 2020
	Tranilast	−7.0		
IL-6	Madindoline A	−6.9	–	Hayashi et al., 2002; Hong et al., 2015
	LMT28	−5.4		
Furin	Phenylacetyl arg-val-arg 4-amidino benzylamide	−7.8	–	Becker et al., 2010

In silico virtual screening

Phytochemicals have been reported to inhibit SARS-CoV-2 via a number of mechanisms (Ghildiyal et al., 2020; Gurung et al., 2020). Most phytochemicals like alkaloids, flavonoids, terpenoids, coumarins, and lignins inhibit the virus in the host due to their antioxidant activities, scavenging abilities, inhibition of DNA and RNA synthesis, or the blocking of viral reproduction (Naithani et al., 2010). Harnessing this potential of plant-based bioactives to speed up the much-desired development of COVID-19 therapeutics is strengthened by advances in computer-aided drug discovery (CADD) that deploys several computational technologies to the drug design process (Ebhoimen et al., 2020). This computational study identified promising phytochemicals from some of the reported antiviral and anti-inflammatory medicinal plants screened against SARS Cov-2 Nonstructural protein 9 (NSP9) RNA binding protein, and further scrutinizing the probable binding interactions it can have with other targets involved in COVID-19. The other targets included the open reading frame protein 3a (ORF3a) which has been well established to induce cellular apoptosis (Ren et al., 2020), furin convertase that activates the spike glycoprotein of SARS Cov-2 and interleukin-6, one among the many critical inflammatory cytokines that can significantly contribute to fever, lymphopenia, coagulation, lung injury, and multi-organ failure (MOF) (Abbasifard & Khorramdelazad, 2020). The custom workflow options of Molecule make the virtual screening process easier for execution, and the cloud computing server facilitates faster processing for quick screening. To identify the potent phytochemicals, virtual hits from the in house generated library of 521 phytochemicals, from the first filter of the workflow, were checked for the total number of RO5 violations; a total of 452 molecules were found to have either two or fewer violations. According to the Lipinski Rule of Five (RO5), if a compound has a molecular weight (Mw) > 500 Da, calculated M log $p > 4.15$, hydrogen-bond donors > 5, hydrogen-bond acceptors > 10, it is unlikely to be further pursued as a potential oral drug, because it would perhaps lack properties that are necessary for its absorption, distribution, metabolism, and excretion (Lipinski et al., 1997). Traditionally, a prominent number of drugs that target viral receptors deviate from Lipinski's RO5 (Doak & Kihlberg, 2017) and have now opened immense potentials for compounds beyond RO5 drug space. Hence, to increase the chemical discovery space, we kept the maximum number of RO5 violations to two for further narrowing down the library of molecules to the most relevant molecules for the receptors of our choice.

These drug-like compounds were further taken for the next workflow step of Vina docking. The Vina docking algorithm (Trott & Olson, 2010) is the most frequently used tool for docking because of its high precision and robustness. The returned result for each of the receptors after the screening was set to the top 30 molecules with the best docking scores. Docking scores of the resultant phytochemicals ranged from a value of −7.1 to −9.1 kcal/mol for NSP9 (6W4B), −7.0 to −8.5 kcal/mol for ORF3a protein (6XDC), −6.9 to −8.1 kcal/mol for Interleukin-6 (1ALU) cytokine and −8.4 to −9.6 kcal/mol for the furin convertase (5JXI). Based on the binding energies of the virtual hits and the control standards, the cutoff values of docking scores were selected for all the four receptors for the identification of potential inhibitors. Vina docking was manually performed for known inhibitors of all the four target proteins. Table 2 enlists the docking scores for the inhibitors used against each of the receptors.

The docking scores of NSP9 against Verdinexor and Dabrafenib as inhibitors (Gordon et al., 2020) were found to be −7.7 and −6.8 kcal/mol, respectively. Therefore, particularly, based on Autodock Vina docking scores (ΔG) of the known inhibitors for NSP9, the cut off value was set as ≤ -7.8 kcal/mol. Similarly, upon docking ORF3a, with two of its inhibitors Emodin and Tranilast (Schwarz et al., 2011; Shah, 2020), the docking scores found were −6.9 and −7.0 kcal/mol, respectively. Hence a strict benchmark of binding affinity for the identification of potential Phyto ligands for ORF3a was set as ≤ -7.1 kcal/mol. However, the docking scores of IL-6 and Furin with its inhibitor were far less than the maximum docking scores of the top 30 resultant molecules retrieved after screening. Hence, for Furin and IL-6, all the molecules were chosen for further analysis. While for the SARS CoV2 viral targets, cut-off values ≤ -7.8 kcal/mol for NSP9 RNA binding protein and ≤ -7.1 kcal/mol for ORF protein 3a obtained formed the basis for considering 6 and 23 compounds, respectively for further analysis. The top 6 ligands for NSP9 were further assessed for their MM-GBSA energy scores prior to the Molecular Dynamic simulation study. Table S2 (SI) contains the docking scores for all molecules that passed the docking thresholds for each of the receptors, along with the number of RO5 violations.

Of interest was how the top six phytochemicals, namely Ochnaflavone, Hispaglabridin B, Corylin, Glabrone, Licoflavone B, and Neoandrographolide, that passed the minimum cut off threshold for binding affinity scores for NSP9, performed for the three other target receptors that were chosen. Among these six potential phytochemicals, we found

Table 3. Potential plant metabolite inhibitor for NSP 9 and their respective plant sources, geographical locations and compound classes.

S/N	COMPOUND NAME	BOTANICAL NAME	FAMILY	Compound Class	Geographical Location
1	Ochnaflavone	<i>Lonicera japonica</i>	Caprifoliaceae	Biflavonoid	China, Japan, Korea, East Asian.
2	Hispaglabridin B	<i>Glycyrrhiza glabra</i>	Fabaceae	Flavonoids	Spain, Italy, Turkey, Central Asia.
3	Corylin	<i>Psoraleae corylifolia</i>	Fabaceae	Flavonoids	Western part of China
4	Glabrone	<i>Glycyrrhiza glabra</i>	Fabaceae	Flavonoids	Spain, Italy, Turkey, Central Asia.
5	Licoflavone B	<i>Glycyrrhiza glabra</i>	Fabaceae	Flavonoids	Spain, Italy, Turkey, Central Asia.
6	Neoandrographolide	<i>Andrographis paniculata</i>	Acanthaceae	Diterpenoids	Pakistan, India,

out that Ochnaflavone, Hispaglabridin B, and Licoflavone B passed the docking threshold for all other receptors. Also, Corylin was found to have passed the docking threshold for Furin and ORF3a, while Glabrone passed the docking threshold for Furin besides NSP9. Neoandrographolide was unique for the Nonstructural protein 9 and was not in the top 30 screened libraries for any of the other three receptors. A few other top NSP9 docked molecules were scrutinized, although they didn't pass the set threshold based on the standard drug chosen, but seemingly have good polypharmacological properties. Hispaglabridin A, that had a binding affinity score of -7.7 kcal/mol for NSP9, is possibly a potent inhibitor for the other three receptors since it passed the threshold for ORF3a, Furin, and IL-6 with docking scores -7.3 , -9.2 , and -7.1 kcal/mol, respectively. Psoralidin, which had a binding affinity value of -7.6 kcal/mol for NSP9, was found to have a docking score of -8.1 kcal/mol against IL-6 and -9.0 kcal/mol against Furin. Licoflavone A, Licoflavonone, Kanzonol U, Hydnocarbin, and Glabrol are among the others that have promising affinity values for multiple targets.

Pharmacokinetics and ADMET screening

Pharmacokinetic properties of drugs usually refer to the properties defining the movement of the drug into, through, and out of the body. Pharmacokinetics can simply be defined as the variation in drug concentration with time due to several processes involved in the absorption, distribution, metabolism, and excretion (ADME). Adverse drug reactions in the body mainly depend on patient-related factors (like renal function, genetic makeup, sex, age), and on the physicochemical properties of the drug, so is pharmacokinetics (Alomar, 2014). Pharmacokinetic properties of the compounds need to be identified before performing *in vitro* and *in vivo* studies. Additionally, the behavioral action of compounds inside host organisms needs to be ascertained in terms of its rate of absorption and excretion, metabolism, and distribution. Prediction of the drug ADME and toxicity (ADMET) properties at the early stages of the drug discovery pipeline not only helps in removing the compounds that have poor ADMET properties, but it also helps in reducing research and development costs. The physicochemical properties of the molecules were obtained from the Mucle server, and admetSAR was used to get the ADMET properties for the drugs that met the threshold docking score. Table S3 (SI) displays the results for all the computed molecules. With the exception of Ononin and Calycosin 7-o-glucoside, all the potential ligand hits possessed human gastrointestinal absorption, while not many of them possessed blood-brain permeance capability.

As the final step to filter potential inhibitors for the targets chosen, among many properties, there was extensive focus on the phosphoglycoprotein (Pgp) substrate binding, cytochrome p450 (CYP2D6) inhibition, carcinogenicity, and AMES mutagenicity to filter out the potential inhibitors for the receptors of our study. In general, cytochrome P4502D6 plays a fundamental role in drug metabolism, involved in the metabolism of a variety of liver substrates (Lynch & Price, 2007). Major cases of drug-drug interactions occur due to its inhibition by any medication. Pgp is an ATP dependent efflux transporter glycoprotein, which reduces the efficacy of drugs that are Pgp substrates (Finch & Pillans, 2014). Interestingly, except for Hispaglabridin B, all the top virtual hits of NSP9 are noncarcinogenic, non-AMES mutagenic, non-inhibitor of CYP2D6 and are not Pgp substrate either, and hence were chosen as the final leads for NSP9. Table 3 depicts the respective plant sources, geographical locations, and compound classes for the 5 final leads of NSP9, and their 2D structures (retrieved from PubChem) are demonstrated in Figure 4. Upon further filtering, the hits for ORF3a, Furin, and IL-6 targets, 18, 23, and 19 final potential lead compounds were found for each of the receptors, respectively. As a side note, we must not also forget that development in drug discovery research has been successful in manipulating some of the functionalities to modify ADMET properties for efficient lead optimization. Small tweakings in the molecular structures can result in remarkable property manipulations.

RAT acute toxicity assessment

Acute toxicity is an adverse effect that occurs after single or multiple exposures to a substance, usually within 24 h. Thus the knowledge of the rodent acute toxicity of lead compounds is essential in drug design. The prediction of the chosen ligand's acute toxicity was performed using a free web server, GUSAR (Lagunin et al., 2011), which considers various routes of drug administration (subcutaneous, oral, inhalation, intravenous and intraperitoneal). LD50 values correspond to acute toxicity dose that leads to 50% mortality in 24 h after the administration of the substance. Acute toxicity determined by inhalation or oral administration is pertinent for assessing overall toxicological risk, whereas that for intravenous is important for drug development (Polish et al., 2019). Rat acute toxicity evaluation was carried out for the five leads of NSP9 (including Hispaglabridin B), which presumably possess polypharmacological properties. Despite Hispaglabridin B failing the ADMET tests, it's predicted LD50 was analyzed hence, its inclusion here too. The results of the rodent acute toxicity presented in Table 4 shows that the chosen ligands - Licoflavone B, Ochnaflavone, Corylin, and Glabrone can be considered to

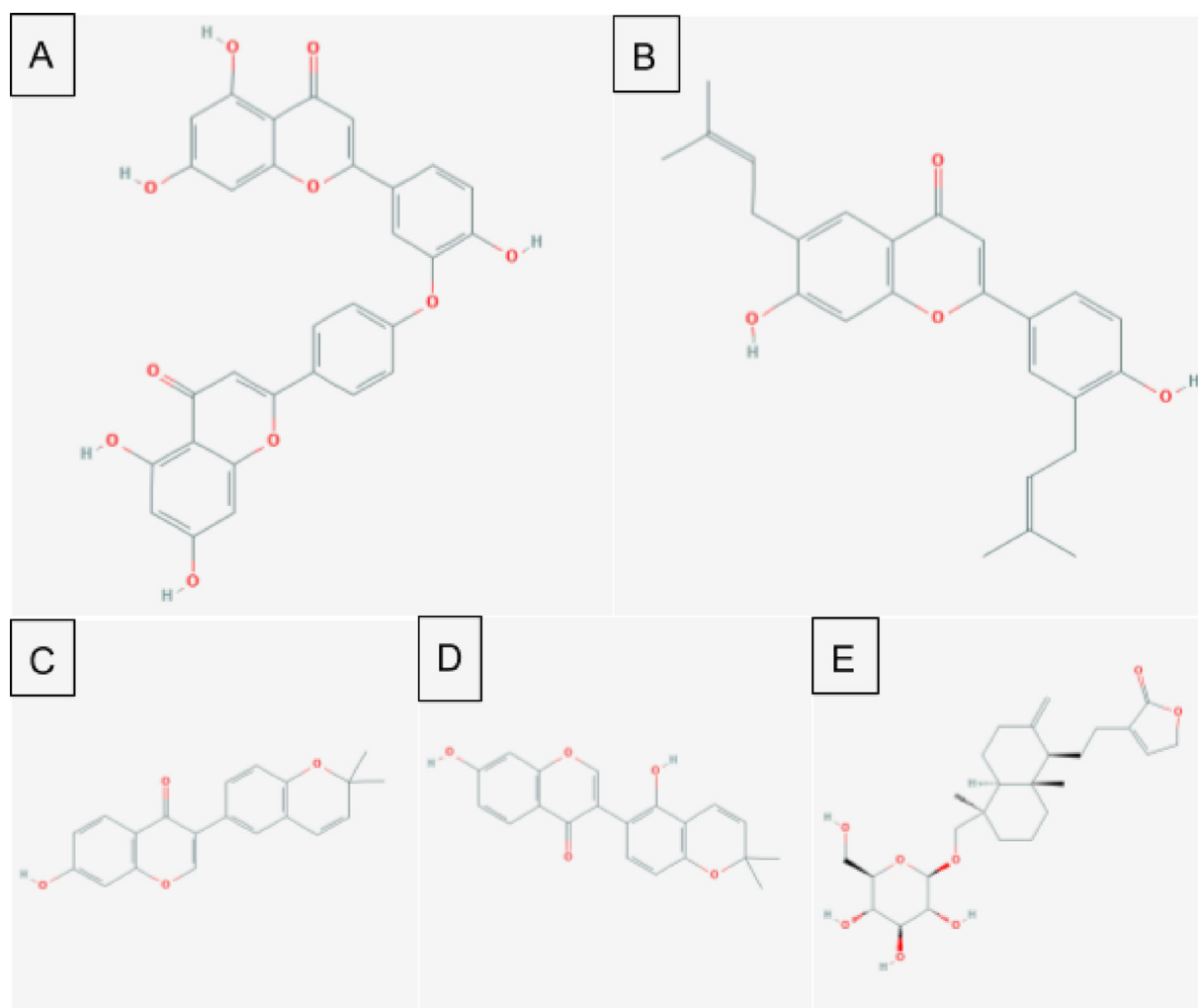


Figure 4. Lead compounds for NSP9 (A) Ochnaflavone, (B) Licoflavone B, (C) Corylin, (D) Glabrone, (E) Neo-andrographolide.

Table 4. In silico prediction of acute toxicity in rodent models and chemical classification of selected phyto ligands with promising ADMET properties.

Compound	Rat IP*		Rat IV*		Rat Oral*		Rat SC*	
	LD50 (mg/kg)	LD50 Classification	LD50 (mg/kg)	LD50 Classification	LD50 (mg/kg)	LD50 Classification	LD50 (mg/kg)	LD50 Classification
Hispaglabridin B	432,800 in AD	Class 4 in AD	61,130 in AD	Class 4 in AD	114,500 in AD	Class 3 in AD	347,800 in AD	Class 4 in AD
Licoflavone B	914,200 in AD	Class 5 in AD	276,100 in AD	Class 4 in AD	906,500 in AD	Class 4 in AD	1866,000 in AD	Class 5 in AD
Ochnaflavone	2065,000 in AD	Non Toxic in AD	459,000 in AD	Class 5 in AD	2218,000 in AD	Class 5 in AD	7027,000 in AD	Non Toxic in AD
Corylin	976,800 in AD	Class 5 in AD	155,600 in AD	Class 4 in AD	663,300 in AD	Class 4 in AD	1479,000 in AD	Class 5 in AD
Glabrone	913,900 in AD	Class 5 in AD	188,800 in AD	Class 4 in AD	512,200 in AD	Class 4 in AD	1580,000 in AD	Class 5 in AD

*IP - Intraperitoneal route of administration, IV - Intravenous route of administration, Oral - Oral route of administration, SC - Subcutaneous route of administration, in AD - the compound falls in applicability domain of models, out of AD - the compound is out of applicability domain of models.

be low toxicity drugs (class 4 and 5). Ochnaflavone was non-toxic in intraperitoneal and subcutaneous routes of administration. However, Hispaglabridin B was observed to be of class 3 toxicity in oral administration. This also shows evidence for further experimental preclinical studies for the molecules as lead compounds for the treatment of COVID-19.

Binding interactions with NSP9

We identified various non-covalent interactions of the ligands with the amino acid residues of the binding pocket of NSP9, namely H-bonding, van der Waals, pi-pi T shaped, pi-pi

stacked, pi-sulfur, pi-sigma, alkyl, pi-alkyl, carbon-hydrogen, and pi-donor hydrogen bonds. Table S4 (SI) lists all the existing interactions between ligands and NSP9 active site residues for the ligand-receptor poses. The best-ranked lead molecule, Ochnaflavone, interacted with the binding site of the target with a binding affinity of -9.1 kcal/mol. This interaction is strengthened by two hydrogen bonds with GLY94 and ARG40 with bond lengths 2.29 Å and 2.11 Å respectively, Vander Waals interaction with the residues THR68, ILE92, GLY 39, GLY38, PHE57, LYS59, MET13, LYS93, LEU95, SER60 of the binding pocket, a pi-pi interaction with PHE41 and alkyl, and pi-alkyl interactions with VAL42, ILE66, and ARG40. Among the other lead molecules that establish hydrogen bonding

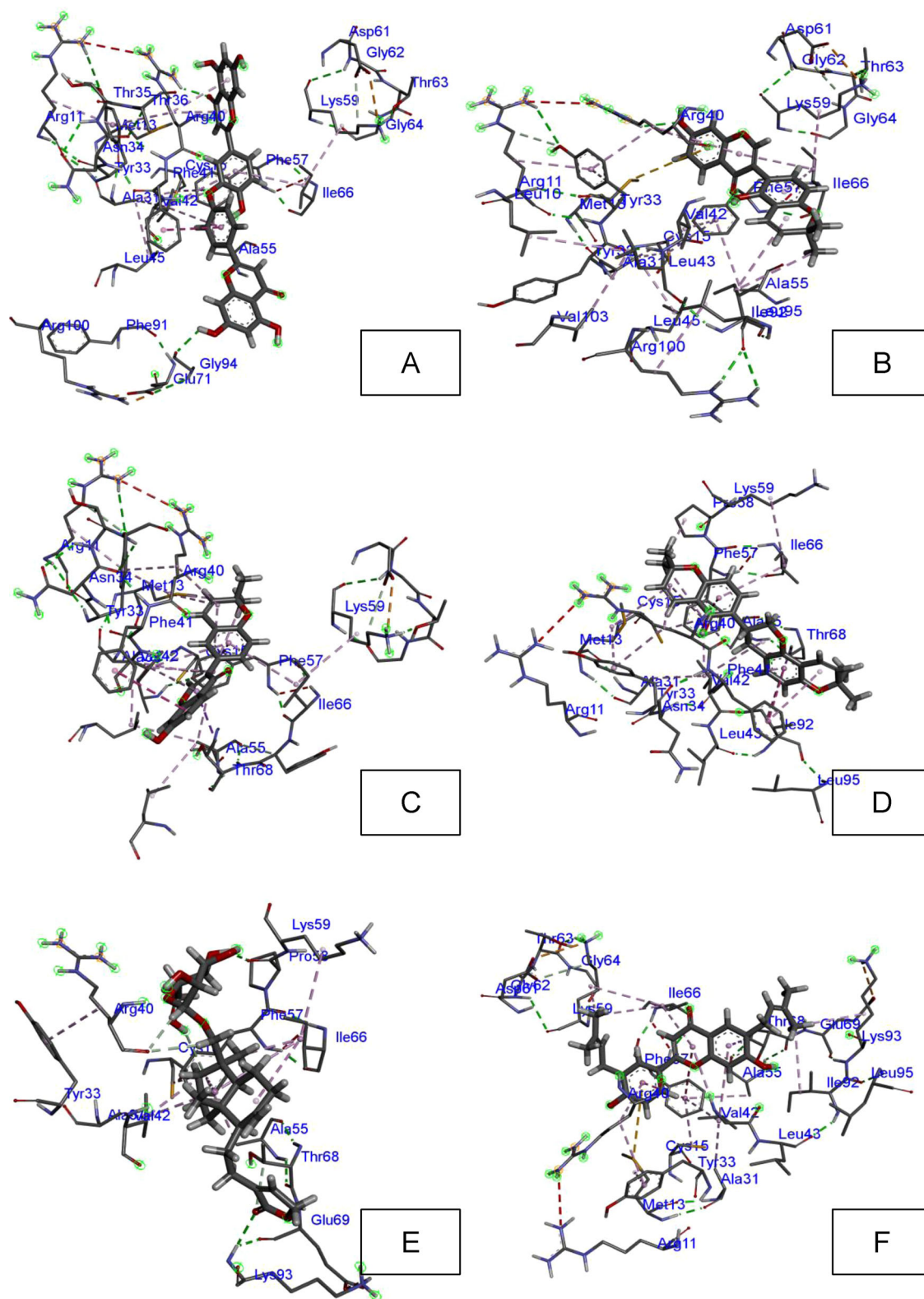


Figure 5. Protein-ligand interactions of the top-scoring phytochemical inhibitors with NSP9. NSP9 residues interacting with the ligand atoms via hydrogen bonds, π -interaction with alkyl or sulfur, Van der Waals interaction, hydrophobic bonds are labeled with the corresponding three-letter residue code along with their position in the protein sequence. The hydrogen bonds, π -interaction, pi-sulfur, alkyl interactions, and Van der Waals are displayed using green, magenta, yellow, pink, and cyan dotted lines, respectively. Images show NSP9 complexed with (A) Ochnaflavone, (B) Hispaglabridin B, (C) Corylin, (D) Glabrone, (E) Neoandrographolide, (F) Licoflavone B.

with pocket residues include Glabrone (with ARG40: bond length 2.42 Å) and Neo-andrographolide (with LYS93 AND PRO58: bond lengths 2.60 Å and 2.31 Å, respectively). Figure

5 shows the NSP9-lead compounds binding interactions (including Hispaglabridin B). Corylin and Licoflavone B develop a pi-sigma interaction with THR68, while Neo-

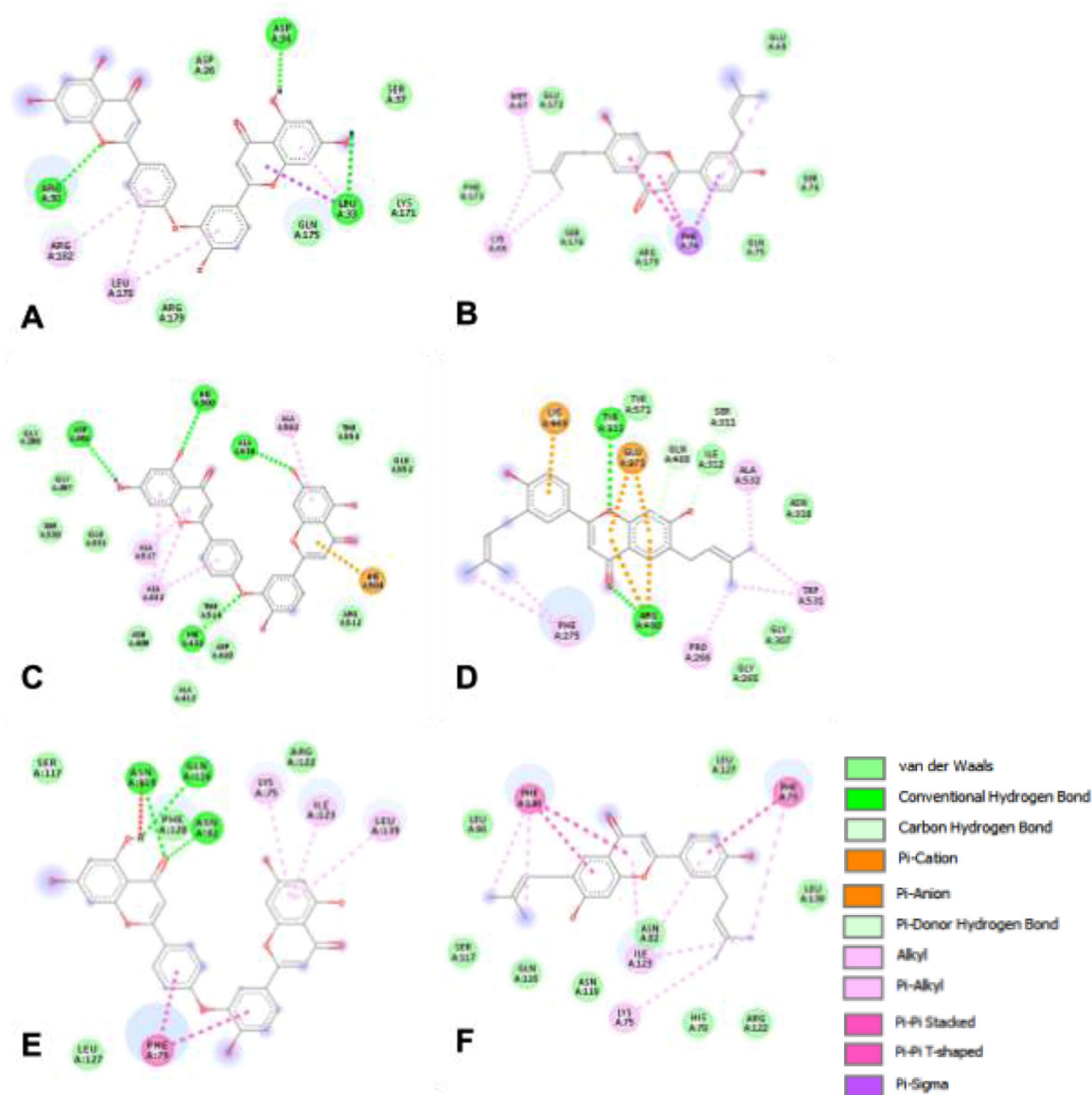


Figure 6. 2D representation of the interactions between ligands and the receptors ORF3a, Furin and IL-6; (A) Ochnaflavone and IL-6 (B) Licoflavone B and IL-6 (C) Ochnaflavone and Furin (D) Licoflavone B and Furin (E) Ochnaflavone and ORF3a (F) Licoflavone B and ORF3a.

andrographolide interacts with the same via pi-donor hydrogen bond. The interaction of Neo-andrographolide is also interesting because there is a possible interaction with PHE57 and ARG40 via carbon-hydrogen bonds. Glabrone and Licoflavone B are observed to interact by a pi-sulfur bond with MET13. Alkyl interactions with ILE66 and VAL42 are prominent in all the ligands. Among all the pocket residues, it seems LYS93, MET13, ARG40, VAL42, PHE57, ILE66, and THR68 play a vital role in strengthening the bonds between the ligands and the receptor.

Interactions of the polypharmacological leads with ORF3a, furin and IL-6

We evaluated the binding interactions of the target receptors with the two compounds, Ochnaflavone, and Licoflavone B,

that took our interest due to their potential multi-target inhibition properties, promising ADMET properties, and RAT acute toxicity results (Lagunin et al., 2011). Figure 6 shows the 2D representation of all the different kinds of interactions that occur between the ligands and IL6, furin, and ORF3a. We found that Ochnaflavone established prominent hydrogen bonds with the residues present in the binding pockets for all the three targets besides NSP9. At the same time, Licoflavone B was seen to form hydrogen bonding interactions just with the residues of Furin. Apart from hydrogen bonds, the major contributions of the interaction of Ochnaflavone come from pi-alkyl, pi-pi T shaped and stacked, and Van der Waals interaction with nearby residues. Licoflavone B was seen to establish pi-sigma, pi-anion/cation, alkyl, and pi-alkyl, pi-pi T shaped and stacked, and Van der

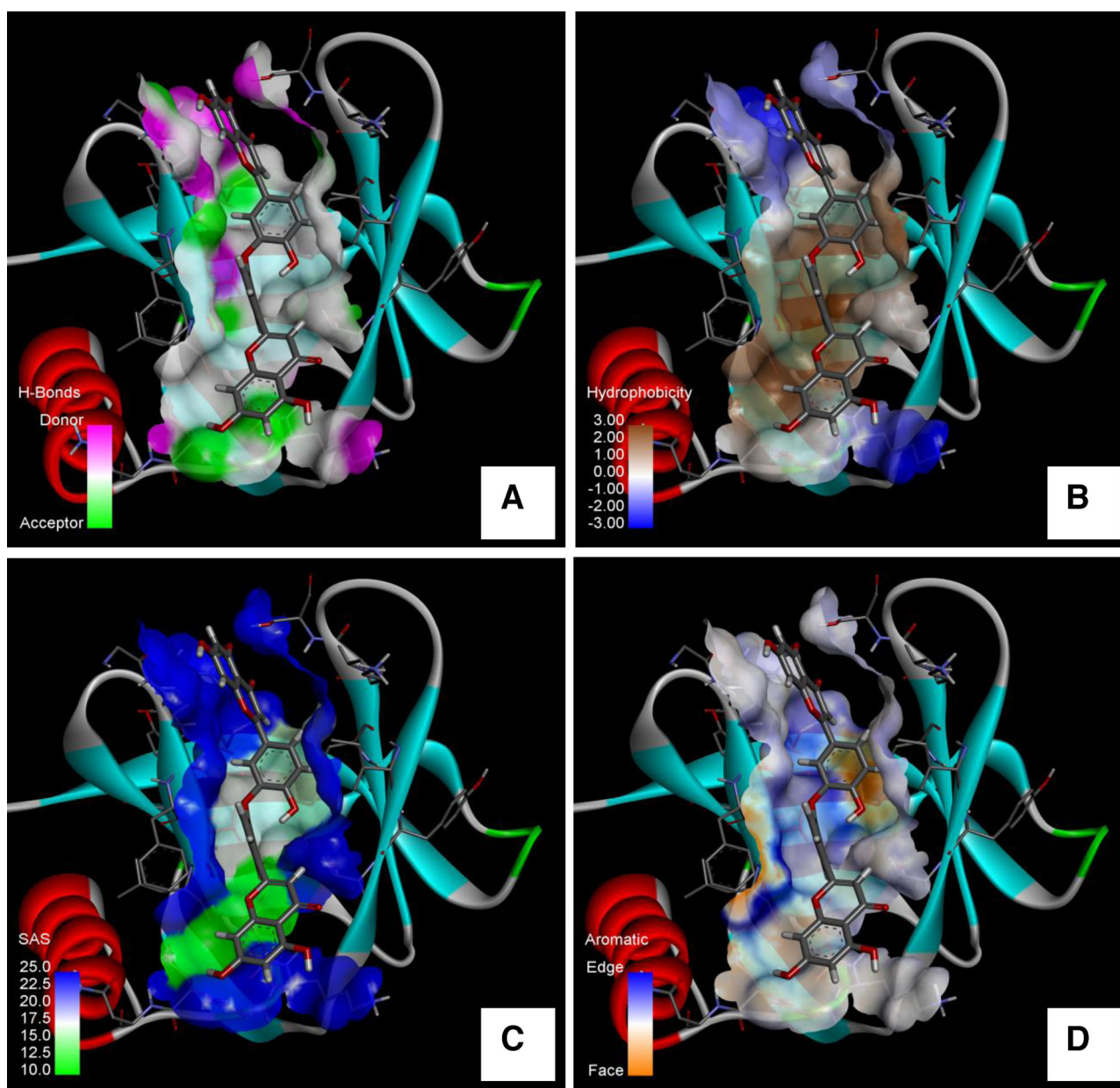


Figure 7. Representation of the characteristics including (A) distribution of H-bond donor/acceptor sites (B) Hydrophobicity (C) Solvent Accessibility and (D) Aromaticity across the surface of the binding site of NSP9 RNA replicase.

Table 5. MM-GBSA scores for the three inhibitors and the standard drug used as a control.

Complex	MMGBSA dG Bind
Hispaglabridin B	-42.88
Licoflavone B	-42.76
Ochnaflavone	-41.43
Verdinexor	-27.64

Waals interaction with the surrounding residue. Ochnaflavone interact via hydrogen bonding with ASP301 and via Van der Waals interaction with GLU331, two key residues of the active site. Licoflavone B interacted with SER176 and ARG179 (one of the most critical active residues of IL-6) via Van der Waals interaction. Ochnaflavone established pi-alkyl interactions with LEU178 and ARG182, besides having Van der Waals interaction with ARG179. Both Ochnaflavone

and Licoflavone B are observed to develop strong bonding interactions (as is shown in Figure 6) with the amino acid residues of the second and third transmembrane helix domain that plays an important role in the Golgi localization.

Binding site analysis

Besides analyzing the binding interactions of the ligands with the amino acid residues in the pocket site, the physico-chemical nature of the binding site was examined, and the spread in the aromaticity, solvent accessibility, hydrophobicity, and distribution of H-bond donor/acceptor sites across the surface of the binding site were evaluated. Figure 7 gives the representation of the distribution of the mentioned properties. It was observed that for ORF3a, which becomes membrane protein in its biochemical pathway, the binding site is

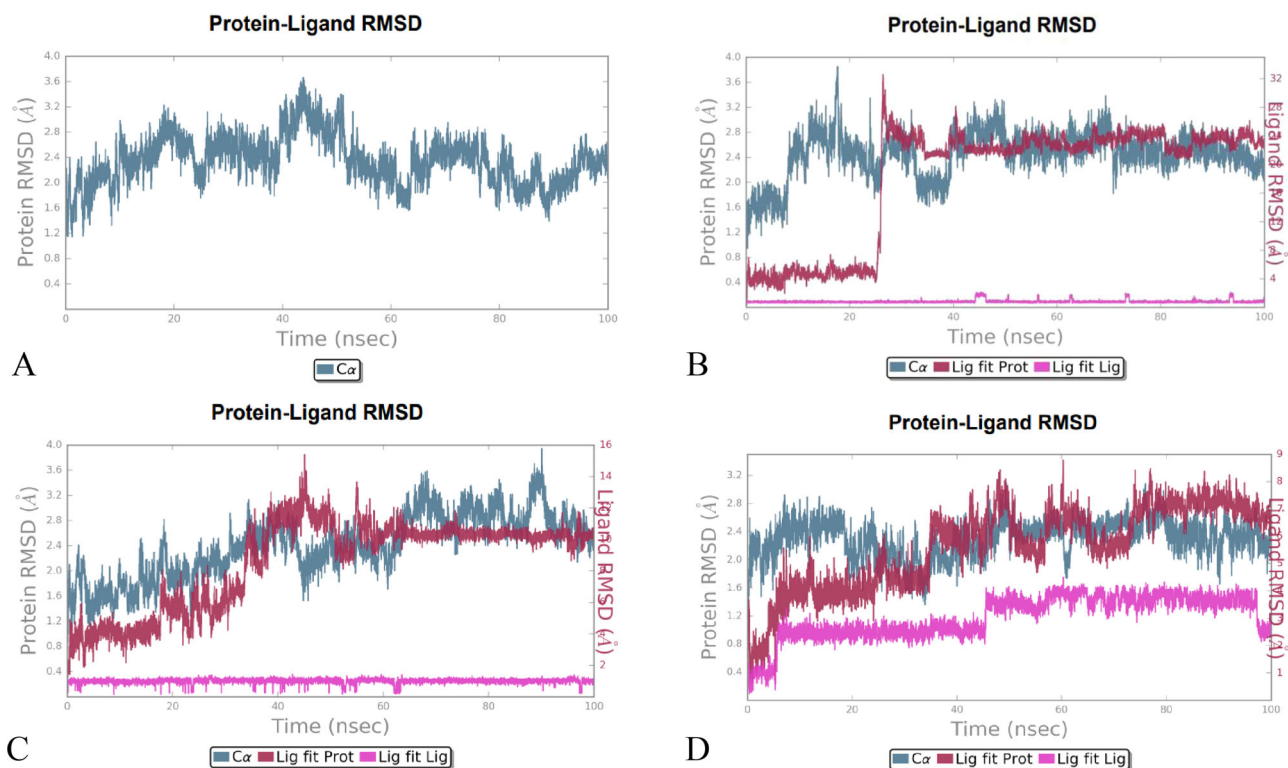


Figure 8. (A) RMSD of the apo-state of NSP9 (B) RMSD of NSP9 and Hispaglabridin B (C) RMSD of NSP9 and Licoflavone B (D) RMSD of NSP9 and Ochnaflavone. ['Lig fit Prot' shows the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference, and then the RMSD of the ligand and heavy atoms is measured. 'Lig fit Lig' shows the RMSD of a ligand that is aligned and measured just on its reference conformation.].

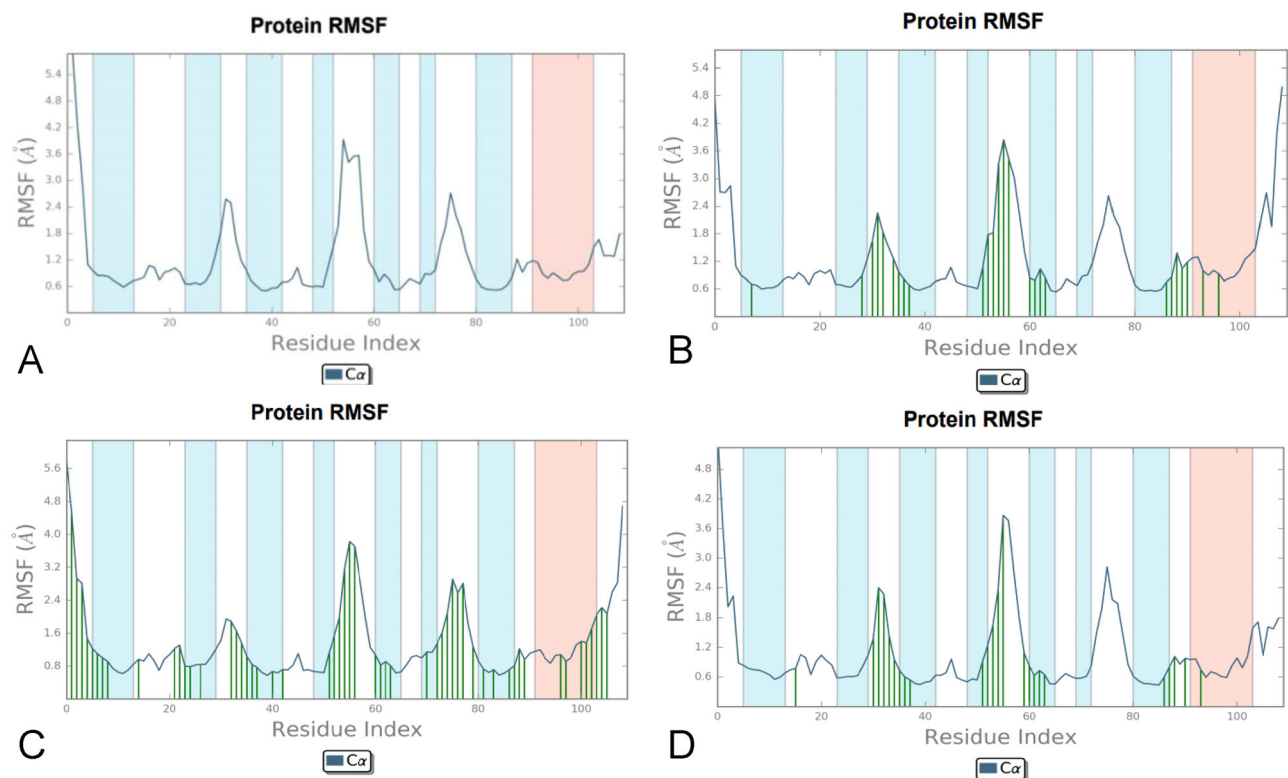


Figure 9. RMSF value of $C\alpha$ of (A) Apo-state NSP9, (B) NSP9-Licoflavone B, (C) NSP9-Hispaglabridin B, and (D) NSP9-Ochnaflavone (from left to right) [Alpha-helical and beta-strand regions are highlighted in red and blue backgrounds, respectively. Protein residues that interact with the ligand are marked with green-colored vertical bars].

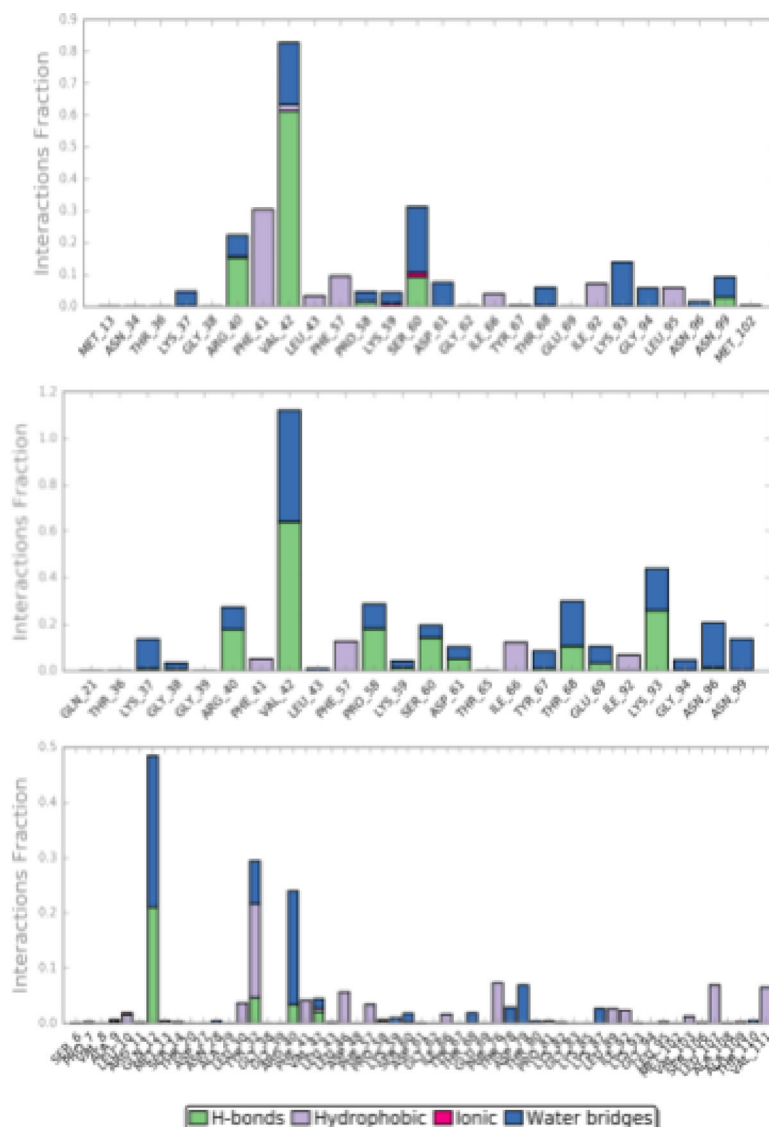


Figure 10. Protein-ligand contacts and interactions with the amino acid residues at the binding site of NSP9 in presence of ligands Licoflavone B, Ochnaflavone and Hispaglabridin B (top to bottom).

highly solvent accessible and is dominated by hydrophobic regions, as is for the case of NSP9. IL-6 binding region does not possess aromaticity. However, furin and interleukin-6 were both dominated by hydrophilic regions (Figure S5 in SI). H-bond donor/acceptor sites were approximately equally distributed throughout the binding region for all the four receptors.

MM-GBSA binding energy of top inhibitors

The molecular mechanics energies combined with generalized Born Surface Area (MM-GBSA) is one of the attractive approaches that is widely and successfully used to improve the docking scores and results of virtual screening, apart from reproducing experimental outcomes. Post-scoring compounds utilizing MM-GBSA have been shown to have a better correlation to their observed binding affinity when compared to docking (Tripathi et al., 2013; Greenidge et al., 2013).

Docking scores are not always accurate, and the MM-GBSA method gives a better approach to estimate the free

binding energies of the protein-ligand complexes with a notably higher degree of accuracy.

The molecular docking scores that we obtained for Ochnaflavone, Hispaglabridin B, Licoflavone B, and Verdineox against NSP9 were -9.1 kcal/mol, -8.5 kcal/mol, -8.1 kcal/mol, and -7.7 kcal/mol, respectively. We, however, observed differences in the binding energy evaluated using the MM-GBSA approach. Hispaglabridin B returned the highest MM-GBSA score of -42.88 kcal/mol, followed by Licoflavone B with -42.76 kcal/mol; both of them were higher than that of Ochnaflavone (-41.43 kcal/mol) as presented in Table 5. Compared to the Vina docking scores, the top three inhibitors have been predicted to have better binding energy values when compared with the reference molecule (Verdineox), suggesting even stronger binding.

Molecular dynamics simulation study

In order to assess the protein-ligand complex stability for the top three inhibitors identified for NSP9, MD Simulations of

100 ns each were performed for Licoflavone B, Ochnaflavone, and Hispaglabridin. A 100 ns MD run for the free protein (apo-state) was also carried out. For the investigation of the stability, the root mean square deviation (RMSD) of the α atoms of the protein and the ligand bound to the protein, and the root mean square fluctuations (RMSF) of the α atoms of the protein was evaluated. Also, of interest was understanding the dynamics of the protein-ligand contacts with time.

The RMSD values for NSP9 free state were within 1.20 Å to 3.6 Å, while the average RMSD asymmetric carbon was 2.22 Å. The RMSD of the α atoms of NSP9 is pretty stable for Ochnaflavone and Hispaglabridin B after 40 ns, while for Licoflavone B, the stability is observed after 60 ns simulation run time. In the case of Licoflavone B (Figure 8C), a very stable RMSD of the ligand after 60 ns was also observed. Hispaglabridin B (Figure 8B) showed a stark increase in its RMSD at around 25 ns and remains stable throughout the rest of the duration. This is perhaps due to a conformational change. On the other hand, Ochnaflavone showed a largely stable RMSD after 50 ns. Figure 9 shows the RMSF values of the apo-NSP9 and protein-ligand complexes. RMSF was employed to follow local changes along the NSP9 amino acid residues for the 100 ns simulation time. It was observed that the alpha helices and beta strands of the apo structure and docked systems oscillated within 0.8 Å to 1.6 Å. The loop regions of all the simulated systems showed large fluctuations up to 4.8 Å, except for the apo-state that showed change over 5.4 Å. Remarkably, there were no noteworthy instabilities in the loop regions when compared across the complexes. Understandably, the large fluctuations of the loops observed were due to their intrinsic flexible nature. Our results showed that the lead molecules complexed with NSP9 were found to be stable in the binding site with negligible structural movements and lesser conformational changes to the overall protein structure, hence presenting them as potential inhibitors against NSP9.

Upon analysis of the protein-ligand contacts, it was found that both Licoflavone B and Ochnaflavone have consistent H-bonding as well as H-bonding mediate by water interaction with VAL42 throughout the entire duration of 100 ns of the simulation run. Licoflavone B was found to be in a prominent hydrophobic interaction with PHE41, while Ochnaflavone developed stable interactions with ASN96 and ASN99 via H-bonding mediated by water at 60% of the simulation run time. PRO58 and LYS93 are among the other important residues of contact for Ochnaflavone. Hispaglabridin B shows a variable interaction profile with the residues. It communicated with ARG40 via hydrogen bonding and water bridges at 26% at a time. Consistent interaction with GLN12 was maintained at 52% through H-bonding together with water bridge contacts, and more robust interactions with TYR32 via H-bonding, water bridge, and hydrophobic contacts. Also, compared to Ochnaflavone and Licoflavone B, Figures 9 and 10 Hispaglabridin B shows a much greater number of residues contact with NSP9 throughout the duration; however, most of the contacts were less than 10% the simulation time.

Conclusion

COVID-19 disease has remained a menace to the world, and the case to fatality percentage has remained on the rise. In order to tackle this menace, there is a need to develop therapeutic intervention rapidly. Here, extensive screening of our manually curated library via docking thresholds and stringent ADMET filters identified 5 leads for NSP9, and 23, 18, and 19 leads for Furin, ORF3a, and interleukin-6, respectively. Among the top five leads of NSP9, Ochnaflavone, a biflavonoid extract from *Lonicera japonica*, and Licoflavone B, a flavonoid extract from *Glycyrrhiza glabra* showed promising polypharmacological prospects, as identified by their docking scores, ADMET properties, and binding interactions with the receptor site residues. Atomistic simulations of 100 ns validated the stability of the NSP9-Ligand complex. The current study comes up with convincing evidence about the antiviral and anti-inflammatory indications of these phytochemicals with great potential to inhibit simultaneous viral entry, replication, and disease progression, further opening up the quest for pre-clinical experimental studies.

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