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In silico analysis of Phyllanthus amarus phytochemicals as potent drugs against SARS-CoV-2 main protease

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1. Introduction

The entire world has come to a standstill with the emergence of the COVID-19 pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [1]. Coronaviruses, (CoV) belonging to the family Coronaviridae, are a group of single-stranded RNA viruses responsible for causing respiratory and intestinal syndromes in humans and other mammals. Six strains of coronaviruses have been identified so far, namely HCoV-229E, HCoV-OC43, HCoV- Hong Kong University 1 (HCoV-HKU1), HCoV-NL63, Severe Acute Respiratory Syndrome (SARS)-CoV (SARS-CoV), and Middle East Respiratory Syndrome (MERS)-CoV (MERS-CoV) [2,3]. While the first four strains were self-limiting and only caused mild diseases, the last two were highly contagious leading to community outbreaks and caused fatal respiratory infections [4].

A new strain of this virus emerged in December 2019 in Wuhan, a city in China, and had patients suffering from fever, cough, pneumonia and severe respiratory illness [5]. This fast-spreading virus was declared a Public Health Emergency of International Concern on 30 January 2020 and a pandemic on 11 March 2020 by the World Health organization. The disease, COVID-19 has been rapidly spreading ever since, infecting about 71,351,695 people and resulting in 1,612,372 deaths across the globe as of 15 December 2020 [6,7].

SARS-CoV-2, like all coronaviruses, is an enveloped, single-stranded, positive sense RNA virus. The genome sequence of the virus shows that it is about 30,000 bp long with 10 open reading frames (ORFs) [8] and, it encodes several structural proteins, non-structural proteins and viral accessory proteins. The structural proteins include (1) spike (S) protein, (2) membrane envelope (M) protein, (3) nucleo-capsid (N) protein, and (4) envelope (E) protein [9]. This virus, like other viruses of this family, has 16 non-structural proteins (nsp). The non-structural proteins whose structures are available so far are the main protease (3C\text{\small{P\textsc{o}}}/M\text{\small{P\textsc{o}}}) enzyme of the novel coronavirus. 190 compounds were obtained from literature and docked against 3CLPro and 16 compounds showed higher binding affinity with 3CL\text{\small{P\textsc{o}}} with their values lying between -8.9 kcal/mol to -9.6 kcal/mol. The top two compounds, Myricitrin (CID: 5352000) and Quercetin-3-O-glucuronide (CID: 12004528) gave high binding affinity values of -9.6 kcal/mol and -9.4 kcal/mol respectively and also display favourable binding interactions with the 3CL\text{\small{P\textsc{o}}}. Both the compounds were further subjected to molecular dynamics simulation and MM-PBSA based binding free energy calculations. ADMET and drug-likeness properties were studied to assess the pharmacokinetic properties of the compounds. Favourable pharmacokinetic results reinforced the applicability of the compounds assessed. Along with continuous studies being carried out with chemical compounds, research needs to expand into all areas, including the use of natural compounds as drug compounds. The identified hits from this study can be taken further for in vitro and in vivo studies to examine their efficacy against COVID-19.

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fundamental role in the polypeptide processing of the two replicase polyproteins, pp1a and pp1ab. RdRp and nsp13 cannot function properly without prior proteolytic release, making MPro a key enzyme in the replication cycle [12,13]. Due to structural and sequential similarity of the main proteases with other closely related betacoronaviruses, a good foundation for drug discovery has already been laid down [14,15]. In addition to all this, the dissimilarity between SARS-CoV2 3CLPro and human proteases has resulted in us choosing 3CLPro as our target for this study [16].

Efforts have been made to repurpose drugs that have been used for the treatment of Ebola virus (EBOV), influenza virus (IFV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) [9] to effectively treat SARS-CoV-2, using experimental, clinical-based or computational methods [17]. RNA polymerase inhibitor-remdesivir, 3CLPro inhibitors-lopinavir/ritonavir, and ivermectin, a drug that interferes with the acidification of the endosome-hydroxychloroquine is such examples of repurposed drugs that have entered clinical trials [9].

However, it has been shown that there is no particular pharmacological formulation that can cure a person infected with COVID-19 [1,8,9,11,12]. In light of this, many researchers have moved towards looking for answers in natural products. Asadirachta indica [20], Lycoris radiate [21], Withania somnifera, Phyllanthus urinaria [22], Glycyrrhiza glabra, Ocimum species, Allium sativum [23], and many other species have been used for centuries around the world for the treatment of fever, cough and cold and have also shown certain antiviral properties which opens opportunities to explore their applications in drug design.

Phyllanthus amarus goes by the name sleeping plant or Bhui korma in India [24]. It belongs to the Euphorbiaceae family and is a small medicinal herb that is used throughout the world for multiple ailments. It is also an important plant in Indian traditional medicine [25], African traditional medicine [26], and Chinese traditional medicine, in which it is used for the treatment of digestive disease, jaundice, and renal calculus [27]. The compounds extracted from this plant have hepatoprotective [28], nephroprotective [29], antibacterial [30], antiviral [23], antimalarial [31], antinflammatory [32], antidiabetic [33], antiulcer [34], and antioxidant [36] properties in humans. Different parts of the Phyllanthus amarus plant are rich in phytochemicals.
like alkaloids, flavonoids, lignans, tannins, sterols, tetraterpenes and many other compounds.

*Phyllanthus amarus* has shown promising anti-viral activity which is attributed to the various tests conducted by *in vivo* and *in silico* tools, which showed positive results against Hepatitis B virus (HBV) [40], WoodChuck Hepatitis Virus (WHV) [41], R5 pseudotype HIV virus [42], Hepatitis-C virus [43], Dengue Virus-2, Cytomegalovirus and Sindbis, Rous-sarcoma and the Maloney-leukaemia virus [44].

Thus, *Phyllanthus amarus*, with its multiple antimicrobial and antiviral phytochemicals could be a plausible candidate in the search for a drug for COVID-19. Compounds can be identified through *in silico* virtual screening and further studying the docking interactions, ADME and toxicity profiles of these extracts could shine a light on whether

Table 3 (continued)

| Ligand       | Binding Affinity | Type of Interaction | Residue Information          |
|--------------|------------------|---------------------|------------------------------|
| Ritonavir    | Hydrogen bond    | Hydrogen            | His163, His164, His41, Ser144, Asn142, Gln189 |
|              | Electrostatic    | Hydrophobic         | Asn142                        |
| Lopinavir    | Hydrogen bond    | Hydrogen            | Glu166, Gln189, Thr190       |
|              | Hydrophobic      | Hydrophobic         | Ala191, Pro168, Met49        |

Fig. 1. 2D Molecular docking interactions between Main protease and (a) Myricitrin (b) Quercetin-3-O-glucuronide (c) Kaempferol-3-O-glucuronide (d) Astragalin (e) Ellagic Acid-O-Arabinoside (f) Kaempferol-3-O-Rutinoside (g) Isocorilagin (h) Furosin (i) Trigalloylglucose (j) Phyllanthusiin B (k) Ritonavir (l) Lopinavir.
2. Materials and methods

2.1. Retrieval of phytochemicals and target protein

An extensive literature survey was carried out and a list of phytochemicals from *Phyllanthus amarus* were collected. 190 chemical compounds with various biochemical activities and properties were chosen for our study. Phytochemicals were matched with their respective PubChem IDs and subsequently downloaded from PubChem and ChemSpider databases as .sdf files. Structures of compounds not available on any database but present in literature were drawn using Marvin Sketch. All the 190 compounds chosen for the virtual screening process are tabulated in Table S1 of the supplementary file. The crystal structure of COVID-19 main protease in complex with an inhibitor N3 at 2.16 Å resolution with PDB ID: 6LU7 [46]. was downloaded from RCSB Protein Data Bank (PDB) [47] in the .pdb format.

2.2. Preparation of ligand and target protein

BIOVIA Discovery studio visualizer software was used to combine all the ligands into a single file and was saved in the .sdf format. After downloading the protein in PDB format, Swiss PDB Viewer was used to delete the non-amino acid residues to make the receptor free of any ligands before docking [48]. The Graphical User Interface program “AutoDock Tools” [49] was used to prepare the protein file. Water was deleted and Kollman united atom charges were added; solvation parameters and polar hydrogens were added to the receptor for the preparation of protein which was then saved in .pdbqt format.

2.3. Molecular docking

AutoDock Vina docking engine was used on the PyRx platform [50]. The combined ligand file (.sdf) was imported on PyRx as a Chemical Table SDF file. The energy of the molecules was minimized using OpenBabel on PyRx following which they were converted to AutoDock Ligand format (.pdbqt). After the protein was imported, docking was performed on Vina Wizard for each of the ligands. AutoDock Vina requires a grid box to be set which defines the search space in the protein. This grid box must surround the region of interest (active site) in the macromolecule. Literature review, along with the structure visualization feature on PDB, allowed us to identify the active sites of the protein. In 3CLPro, a major region of interest is the Cys-His catalytic dyad, made up of His41 and Cys145 [51]. The grid was placed such that the amino acids involved in catalytic activity along with the other active site residues were selected (Table 1).

The grid box size (Å) was set at 41.5814, 33.7298, and 48.0418 for x, y and z respectively, and the grid centre was set to -17.9927, 14.4340 and 66.4796 for the coordinates x, y and z respectively. The grid box covered all the amino acid residues mentioned in the Table 1. During the docking process, a maximum of 9 conformers were considered for each compound. To visualize the receptor-ligand interaction, the docked ligand as well as the protein were opened together on PyMOL and saved as a single file. Protein ligand interactions were visualised using BIOVIA Discovery studio visualizer 2020.

2.4. ADMET studies

pkCSM, a software used to predict the pharmacokinetic properties of small molecules using graph-based signatures was used to perform ADMET studies [52]. This tool requires the SMILES format of the ligand to be uploaded. For the compounds whose structures were available on PubChem, the SMILES format was taken from PubChem, while for those that were drawn on Marvin Sketch, the SMILES format was generated using the online SMILES translator by the National Institutes of Health (NIH) (https://cactus.nci.nih.gov/chemical/structure). The SMILES format was pasted onto the server, the prediction mode was set to “ADMET”, and the prediction software gave us a comprehensive table of values of the absorption, distribution, metabolism, excretion, and toxicity of the uploaded compound.

2.5. Drug likeness studies

Drug likeness studies are performed to fulfill a set of guidelines that suggest whether the ligand can be considered as a drug-like compound, thus improving its chances of passing further clinical trials [53]. DruLiTo is an open-source virtual screening tool developed by NIPER, Mohali,
whose calculations are based on the various drug-likeness rules: Lipinski’s rule, MDDR-like rule, Veber rule, Ghose filter, and CMC-50 like rule and Quantitative Estimate of Drug-likeness (QED). The 10 compounds are uploaded in either .mol or .sdf format one after the other and the following filters are applied: Lipinski’s rule, veber filter, Ghose filter, and MDDR-like rule. Consequently, the output file is downloadable as a .csv file (http://www.niper.gov.in/pi_dev_tools/DruLiToWeb/DruLiTo_index.html).

2.6. Molecular dynamics simulation

Molecular dynamics simulations using GROMACS version 2019.4 was performed for the top two docked complexes of myricitrin and quercetin-3-glucuronide along with the unbounded apo form of the Main protease (3CL<sup>pro</sup>) (6LU7). A GROMACS topology was created for the ligand using PRODRG webserver [54] and the protein parameters were generated using gromos54a7 force field. The cubic simulation box around the docked complex was built using the gmxediconf tool. This processed system was vacuum minimized for 1500 steps using the steepest descent field. The cubic simulation box around the system was kept at 300 K. This was performed for 100 picoseconds and the system was kept at 300 K. In the second step, NPT equilibration was performed for 100 pico-seconds. Each resultant structure was then run in NPT ensemble for 100ns simulation time [56,58].

Table 4
ADMET studies of selected ligands.

| Property                      | Model Name                    | Unit                  | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   |
|-------------------------------|-------------------------------|-----------------------|------|------|------|------|------|------|------|------|------|------|
| Absorption                    | Water solubility              | Numeric (log mol/L)   | -2.892 | -2.9 | -2.87 | -2.863 | -3.204 | -2.873 | -2.892 | -2.891 | -2.89 | -2.892 |
| Absorption                    | Caco2 permeability            | Numeric (log Papp in 10-6 cm/s) | -0.982 | -1.06 | -0.88 | 0.306 | 0.739 | -0.709 | -1.337 | -1.631 | -1.39 | -1.519 |
| Absorption                    | Intestinal absorption         | Numeric (% Absorbed)   | 43.33 | 25.11 | 25.17 | 48.052 | 66.35  | 33.829 | 58.51  | 68.01  | 0    | 56.18 |
| Absorption                    | Skin Permeability             | Numeric (log Kp)       | -2.735 | -2.74 | -2.74 | -2.735 | -2.735 | -2.735 | -2.735 | -2.74  | -2.735 | -2.735 |
| Absorption                    | P-glycoprotein substrate      | Categorical (Yes/No)   | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Absorption                    | P-glycoprotein I inhibitor    | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Absorption                    | P-glycoprotein II inhibitor   | Categorical (Yes/No)   | No | No | No | No | No | Yes | No | No | No | No |
| Distribution                  | VDEs (human)                  | Numeric (log L/kg)     | 1.552 | 1.647 | 1.295 | 1.444 | 0.394 | 0.365 | 0.462 | 0.746 | 0.054 | -0.001 |
| Distribution                  | Fraction unbound              | Numeric (Pa)           | 0.182 | 0.274 | 0.28 | 0.218 | 0.048 | 0.27 | 0.31 | 0.268 | 0.347 | 0.383 |
| Distribution                  | BBB permeability              | Numeric (log PS)       | -4.376 | -4.14 | -3.96 | -3.908 | -4.542 | -5.347 | -5.044 | -5.205 | -5.65 | -6.608 |
| Metabolism                    | CYP2D6 substrate              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Metabolism                    | CYP3A4 substrate              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Metabolism                    | CYP1A2 inhibitor              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Metabolism                    | CYP2C19 inhibitor             | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Metabolism                    | CYP2D6 inhibitor              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Metabolism                    | CYP3A4 inhibitor              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Excretion                     | Total Clearance               | Numeric (log ml/min/kg) | 0.303 | 0.434 | 0.503 | 0.462 | 0.583 | -0.115 | 0.229 | -0.527 | 0.606 | -2.652 |
| Excretion                     | Renal OC2T2 substrate         | Categorical (Yes/No)   | No | No | No | No | No | Yes | No | No | Yes | No |
| Toxicity                      | AMES toxicity                 | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Toxicity                      | Max. tolerated dose (human)   | Numeric (log mg/kg/day) | 0.454 | 0.427 | 0.46 | 0.582 | 0.716 | 0.418 | 0.438 | 0.417 | 0.438 | 0.438 |
| Toxicity                      | HERG I inhibitor              | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Toxicity                      | HERG II inhibitor             | Categorical (Yes/No)   | No | No | No | No | No | Yes | Yes | Yes | No | Yes |
| Toxicity                      | Oral Rat Acute Toxicity (LD50) | Numeric (log (ml/kg))  | 2.537 | 2.947 | 2.513 | 2.546 | 2.463 | 2.438 | 2.482 | 2.47 | 2.492 | 2.482 |
| Toxicity                      | Oral Rat Chronic Toxicity (LOAEL) | Numeric (log mg/kg bw/day) | 3.386 | 4.51 | 4.641 | 4.53 | 3.7 | 5.959 | 6.333 | 5.659 | 6.308 | 10.15 |
| Toxicity                      | Hepatotoxicity                | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Toxicity                      | Skin Sensitization            | Categorical (Yes/No)   | No | No | No | No | No | No | No | No | No | No |
| Toxicity                      | T.lymphoma toxicity           | Numeric (log μg/L)     | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 | 0.285 |
| Toxicity                      | Minnow toxicity               | Numeric (log mM)       | 5.997 | 8.323 | 6.898 | 6.735 | 2.111 | 2.741 | 11.28 | 14.59 | 5.981 | 15.84 |

Table 5
Drug likeness properties of selected ligands.

| Ligand                        | MW   | HBA  | HBD  | ALOGP | LOGP  | TPSA  | nRB  |
|-------------------------------|------|------|------|-------|-------|-------|------|
| Myricitrin                    | 464.1| 12   | 8    | -3.398| -3.398| 206.6 | 3    |
| Quercetin 3-O-glucuronide     | 478.07| 13  | 8    | -3.232| -3.232| 223.67| 4    |
| Kaempferol 3-O-glucuronide    | 462.08| 12  | 7    | -2.669| -2.669| 203.44| 4    |
| Astragalin                    | 448.1| 11   | 7    | -2.771| -2.771| 186.37| 4    |
| Ellagic Acid-O-Arabinoside    | 407.94| 6   | 0    | -3.089| -3.089| 71.06 | 2    |
| Kaempferol-3-O-rutinoside     | 594.16| 15  | 9    | -4.018| -4.018| 245.29| 6    |
| Isocorilagin                  | 634.08| 18  | 11   | -3.242| -3.242| 310.66| 3    |
| Furosin                       | 650.08| 19  | 10   | -4.172| -4.172| 316.73| 4    |
| Trigalloyglucose              | 636.1| 18   | 11   | -2.85 | -2.85 | 310.66| 10   |
| Phyllanthusin B               | 964.05| 28  | 11   | -4.599| -4.599| 406.63| 6    |
2.7. Trajectory analysis and free energy calculation (MM-PBSA)

Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuations of the protein were calculated using gmx rms, and gmx rmsf tools respectively [55]. gmx gyrate, gmx sasa and gmx hbond tools were used to calculate the radius of gyration, solvent accessible surface area, and the number of hydrogen bonds formed between protein and the ligand respectively. Additionally, secondary structure analysis of the target protein was also examined for the entire simulation time. The Molecular Mechanics Poisson-Boltzmann surface area (MM-PBSA) approach combines three energetic terms: change in the potential energy in vacuum, desolvation of the different species and configurational entropy associated with complex formation in the gas phase, to account for the change in free energy on binding. A GROMACS utility g_mmpbsa was utilized to estimate the binding free energy [59]. To improve the accuracy of our results, we found the ΔG binding for the last 20ns with respect to 1000

Fig. 3. Plots of (a) RMSD (b) Number of Hydrogen bonds (c) RMS fluctuation (d) Solvent Accessible Surface (e) Radius of Gyration.
3. Results and discussion

3.1. Molecular docking and interaction studies

The active site of 3CL\textsuperscript{pro}/M\textsuperscript{pro} lies in the gap between domains I and II consisting of a Cys-His catalytic dyad (Cys145 and His41) \cite{8}. The active pocket consists of hydrophobic amino acids such as Tyr54, Met49, Met165, Phe140, Leu141, Cys145, Leu27, Pro168, Leu167, Cys145, Ala191, Cys44, Leu50 and Met40, which provide a relatively hydrophobic environment to contain the compound and stabilize its conformation. The co-crystallized structure of 3Clpro along with inhibitor N3 (PDB ID: 6LU7) was used to identify the other amino acids in and near the active site which contribute to the binding and stabilization of the ligands \cite{51}. Of the top 10 compounds, Myricitrin (CID: 5352000) showed the highest binding affinity of -9.6 kcal/mol. Following myricitrin, Quercetin-3-O glucuronide (CID: 12004528) showed the next highest binding affinity of -9.4 kcal/mol. The top 10 compounds with their binding affinities values are shown in Table 2.

All the top compounds, except for Trigalloylglucose bind to the active site of the protein. Trigalloylglucose, in its most stable conformation, binds slightly away from the active site. Table 3 shows the type of interactions as well as the residues with which they interact. Arg40 forms 3 hydrogen bonds with the oxygen groups in the glucose ring with bond lengths of 2.39 Å, 2.01 Å, 1.97 Å. The hydroxyl groups in one of the galloyl rings form two hydrogen bonds with Glu183 of bond lengths 1.76Å and 1.89Å. The hydroxyl groups of the galloyl rings also form hydrogen bonds with Met82, Glu55 and Arg188 with bond lengths 2.22 Å, 1.96 Å, 2.32 Å respectively. The binding is also contributed by hydrophobic interactions with Tyr54, Pro52, Val186, Phe185, Pro184, Tyr182, Phe181, Cys85 and Met82 and polar interactions with Asn53, Asn180, Asn84, Gln83 (Figures 1 and 2). Myricitrin inserts itself strongly into the binding pocket of the protein. It forms a strong hydrogen bond of length 1.91 Å with Asp187 and also forms hydrophobic interactions with Tyr54, Met49, Met165, Phe140, Leu141, Cys145 and Leu27 and polar interactions with His41, Gln189, His 164, His163, His172, Asn142, Ser144 and Thr26. It has been reported that binding to the Arg188/ Gln189 site reduces the catalytic activity of the protein \cite{61}.

In Kaempferol 3-O-glucuronide, the Kaempferol moiety has hydroxyl groups which form strong hydrogen bonds with Asp187 and Leu141 with bond lengths of 2.08 Å and 2.05 Å respectively. It also interacts with hydrophobic amino acids like Met49, Tyr54, Pro168, Leu167, Met165, Phe140, Leu141 and Cys145 and polar amino acids like His41, Gln189, Thr190, Gln192, His164, His163 and Ser144. The hydroxyl groups of the rutinosyl [6-deoxy-alpha-s-mannosyl-(1->6)-beta-o-glucosyl] residue of Kaempferol-3-o-rutinoside forms a hydrogen bond of 2.24 Å with Gln189. They also interact with hydrophobic and polar amino acids in

![](image.png)
the substrate binding pocket.

Astragalin has a glucosyl ring which forms two hydrogen bonds of bond length 1.92 Å and 2.05 Å with Arg188 and Thr190 respectively. Ellagic Acid-O-Arabinoside has an Arabinoside ring that forms a hydrogen bond with Gly143 with bond length 2.09 Å. Furthermore, Iso-
corilagin forms hydrogen bonds of length 1.81 Å, 2.42 Å and 2.25 Å with Glu166, Thr190 and Phe140 respectively and Phyllanthusin B forms a hydrogen bond of 1.96 Å with Asn142.

For a comparative analysis, docking studies were also performed with a few reported anti-viral compounds. Ritonavir forms hydrogen bonds of bond lengths 2.12 Å, 2.33 Å and 1.97 Å with Glu166, Gln189 and Thr190 respectively. Lopinavir also forms hydrogen bonds with Glu166, Gln189 and Thr190, but with bond lengths of 2.57 Å, 3.46 Å and 2.54 Å respectively. It can be observed that when compared to the hit compounds, both lopinavir and ritonavir form fewer bonds with the active site residues of 3CL\textsuperscript{pro}, suggesting that our hit compounds have a better binding affinity (confirmed by docking results) and hence could serve as better inhibitors of the enzyme. Complete analysis clearly says that all the selected top compounds exhibited strong and favourable binding interactions with the main protease (3CL\textsuperscript{pro}).

3.2. ADMET studies

Chemical absorption, distribution, metabolism, excretion and toxicity (ADMET) are five major properties that must be taken into consideration during drug discovery [62]. Prediction of ADMET properties plays an important role in the process of drug discovery as molecules with poor ADMET properties are what account for the failure of about 60% of all drugs in clinical trials.

A good drug candidate is absorbed in the required time and well distributed throughout the system for its effective metabolism and action. Table 4 contains the results of the ADMET studies performed on the top ten compounds. Of the ten finalized compounds, seven of them had excellent intestinal absorption, an important requirement for a drug since the intestine serves as the primary site of absorption of a drug that is taken orally [61]. The water solubility of the compounds was quite low, but there are means to increase the solubility of a drug in water such as particle size reduction, nanosuspension and the use of surfactants [63]. Of the ten compounds, six of them display high Caco2 permeability, including the top two compounds, an important attribute as this parameter is an in vitro model of the intestinal mucosa and helps predict the absorption of orally administered drugs. All of the compounds are substrates of P-glycoprotein, an efflux membrane transporter responsible for the distribution of xenobiotics. Isocorilagin was found to be a substrate and class I and II inhibitor of P-glycoprotein, making it more pharmacologically active than non-inhibitors (M. L. [64]. Of the ten compounds, six of them had a low VD\textsubscript{ss} value, indicating that more of the compound would be distributed in the plasma rather than the tissue. The other four had moderate VD\textsubscript{ss} values, possibly indicating that a more-or-less equal amount gets distributed in the tissue and the plasma. All of the compounds’ fraction that remained unbound (and thus available to bind to 3CL\textsuperscript{pro}) ranged from 0.048 to 0.383. None of the compounds are substrates of CYP2D6 or CYP3A4, and hence none of them are inhibitors of any of the CYP isoforms. All of the compounds have a high half-life in terms of total clearance, suggesting that they aren’t rapidly cleared from the body [65]. Eight out of the ten

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**Fig. 5.** 3D structure of 6LU7 with Myricitrin complex at (a) 0, (b) 25, (c) 50, (d) 75 and (e) 100 ns during the simulation.
compounds are not substrates of renal OCT2. All of the compounds have low AMES toxicity, hepatotoxicity and minnow toxicity. All compounds are not hERG I inhibitors, while six of the ten compounds are not hERG II inhibitors. This reduces the risk of the development of ventricular arrhythmia. All compounds require a higher dose to cause the death of 50% of a group of test animals (Rat LD50). The only anomaly observed was that all compounds have a relatively high T. Pyriformis toxicity and thus, further in vitro and in vivo studies need to be done to assess the toxicity levels of the compounds.

3.3. Drug likeness

Lipinski’s rule of five and Veber’s rule were used to assess the drug-likeness of the compounds. Table 5 contains the values predicted by DruLiTo. Molecular weight plays a major role in determining whether the drug can be administered orally or not. According to the studies conducted by Lipinski et al., it was found that molecules with a lower molecular weight, specifically between 180 and 480 are more likely to be orally active [66,67]. Myricitrin, Quercetin 3-O-glucuronide isomer1, Kaempferol 3-O-glucuronide, Astragalin and Ellagic Acid-O-Arabinoside, have molecular weights in the range 380–480, indicating that out of the 10 compounds analysed, only these few compounds pass the molecular weight barrier. LogP values are important to determine the lipophilicity of a drug. Lower values of logP indicate that the drug is hydrophilic and more positive values of logP indicate that the drug is lipophilic, which compromises its bioavailability. Hence logP values, according to the Rule of Five, must ideally be lesser than or equal to 5 [67–69]. All the compounds pass this test. AlogP values should also preferably be below 5 and it is recorded that all the compounds are within the acceptable range.

HBA (Hydrogen bond acceptor groups), must not exceed 10, as too many of such groups pose a difficulty to permeability of the molecule across the membrane bi-layer. Of the 10 compounds analysed, Ellagic Acid-O-Arabinoside was the only compound that adhered to the HBA limit of 10 with 6 acceptor groups. According to the studies conducted by Raevsky et al., HBD (Hydrogen bond donor groups) values ranges from -4.0 to -0.5, with -4.0 being a strong donor on the Raevsky scale [70,71]. According to the limit of 5 for HBD, only Ellagic Acid-O-Arabinoside was found to have values less than 5 [72].

According to Veber’s rule, TPSA must not exceed 140 Å for intestinal absorption [73] [74,75]. Once again, only Ellagic Acid-O-Arabinoside passed Veber’s TPSA rule. nRB helps in reflecting on the molecular complexity by taking into account the flexibility of the bonds presents in the molecule. The cut-off level for number of rotatable bonds is set at less than or equal to 10, with an optimum average value of 5 for orally administered drugs and 7 for injectable varieties. Of the top 10 compounds, Ellagic acid-O-arabinoside (Binding affinity: -9 kcal/mol) passed all parameters for drug likeness, with zero violations. All other compounds showed between two and four violations; however, studies have shown that if a natural product is used as a guiding structure for the production of a synthetic drug such that molecular descriptors (like MW, clogP etc.) can be controlled, that is, the best of both natural and synthetic compounds can be taken into account to obtain a drug that is both harmless to the human body and effective on the virus [76]. Based on this range, all the 10 compounds qualify as drug-like compounds with nRB values lesser than and equal to 10 [77,78].

3.4. Molecular dynamics simulations

The top docked complexes of 6LU7 showing highest binding affinity along with the favourable binding interactions i.e., Myricitrin (CID: 5352006) and Quercetin-3-O-glucuronide isomer (CID: 12004528) along with the unbounded apo form of main protease (MPRO) were subjected to molecular dynamics simulations to study the structural deviations in a dynamic environment for a time scale of 100 ns. The molecular dynamic simulations were examined on the basis of Root mean square deviation (RMSD), Number of hydrogen bonds, Root Mean Square fluctuation (RMSF), Solvent Accessible Surface Area (SASA) and Radius of gyration values as a function of time (Fig. 3). The average RMSD values of
Myricitrin and quercetin-3-O-glucuronide were found to be 0.497908 and 0.478069 nm respectively. For Myricitrin, the RMSD values steadily increased from 0 to 50 ns showing deviations up to 0.6 nm after which it reached a stable state throughout the simulation. The RMSD values of quercetin-3-O-glucuronide show a sharp increase at 10 ns and then stabilize at 0.5 nm from 10 to 35 ns. The values then show a sharp increase followed by a sharp decrease from 35 to 40 ns with deviations ranging from 0.4 nm to 0.7 nm. The structure is then equilibrated between 0.5 and 0.6 nm from 45 to 75 ns. The values gradually drop to 0.4 nm at 75 ns and attain equilibrium again at 0.5 nm for the rest of the simulation (Fig. 3(a)). The average RMSD value of the apo-protein was found to be about half that of both myricitrin and quercetin-3-o-glucuronide, with a value of 0.248155 nm. It is no surprise that the wild type system has a lower average RMSD value [79,80], but it is observed that the RMSD
values of both docked complexes begin to drop towards the end and a longer simulation time may result in convergence of these RMSD values. This suggests that after a certain time period, the docked complexes stabilize and attain stability comparable to the wild type as the deviations eventually converge. As for the number of hydrogen bonds, Myricitrin made an average of 6 hydrogen bonds and Quercetin-3-O-glucuronide made an average of 4 hydrogen bonds with 6LU7 for a time period of 100 ns. Myricitrin forms 6 hydrogen bonds for the majority of the 100ns timescale with the largest number was found to be 11. Quercetin-3-O-glucuronide forms 4 hydrogen bonds for the majority of the 100ns timescale while 10 was found to be the highest (Fig. 3(b)). This suggests that both compounds are strongly bound to 6LU7 with Myricitrin forming stronger bonds as compared to Quercetin-3-glucuronide for the majority of the timescale.

The RMSF values of the ligand-protein complexes were calculated in order to understand the local fluctuations taking place, in turn, assessing the flexibility of the residues at structural subsets within the overall rigid structure of the protein. For Quercetin-3-glucuronide, an average of 0.221 nm was observed, with a highest of 0.5585 nm at Gly283. On average, higher fluctuations were observed with residues close to the binding pocket (positions 277 to 286). For Myricitrin, an average of 0.241 nm was observed, with a highest of 0.6247 nm at Ser1. A similar trend of higher fluctuations was observed with residues close to the binding pocket (positions 274 to 286) (Fig. 3(c)). This could be because a conformational change took place at the binding pocket, leading to more fluctuations in that region [81]. For the apo-protein, the average RMSF value was 0.16683 nm, with a highest of 0.7336 nm at Gln306. The solvent accessible surface area (SASA) of a protein, theoretically, gives an insight to how accessible a protein is to the solvent in which it resides. The majority of the timescale.

A plot of radius of gyration spanning over 100 ns is analysed in order to understand the overall dimension and shape of the protein [82]. This parameter displays the compactness of the protein during folding and unfolding processes during the course of 100 ns of the MD simulations [83]. The simulations recorded an average value of 2.169 nm, with a 2.063 nm as the lowest and 2.284 nm for the compound Quercetin-3-glucuronide, and an average value of 2.118 nm with the lowest and highest values being 2.028 nm and 2.235 nm respectively (Fig. 3(e)). Additionally, the five snapshots of both the complexes at different time frames of molecular simulations were captured. Fig. 4 and Fig. 5 clearly indicate that both the ligands were bound to the protein in the inhibition site inferring the stability of the complex and strong binding affinity of the ligands.

### 3.5. Comparative analysis

#### 3.5.1. Statistical analysis of RMSD values

In order to get an overview of the RMSD values, a graph of probability distribution vs RMSD values was plotted (Fig. 6(a)). Here, we can clearly observe that the mean values of the RMSDs of both docked complexes are very close to each other (0.47 nm for Quercetin-3-glucuronide and 0.50 nm for Myricitrin). However, the spread of data is more for Myricitrin, suggesting more fluctuations in the RMSD values, while for Quercetin-3-glucuronide, the spread of data is narrower, suggesting that this docked complex attains a more stable conformation during molecular dynamics simulations. The apo-protein has a mean RMSD of 0.248 nm, with a narrow spread of data, which according to its resolution of 2.16 Å, confirms the stability of this structure during simulation [84].

#### 3.5.2. Statistical analysis of SASA values

The probability distribution curves of the SASA values of both docked complexes along with the apo protein were plotted to provide a clearer display of the accessibility of the ligand to the protein during simulation (Fig. 6(b)). From the graph we can clearly see that the distribution curve of Myricitrin is slightly to the left to that of Quercetin-3-glucuronide. The apo-protein alone has a distribution similar to that of the complex with Myricitrin. This suggests that the hydrophobic core of 6LU7 is slightly more accessible to the aqueous environment around it when complexed with Quercetin-3-glucuronide, as suggested by a larger peak value of 152.807 nm. The protein, being more accessible to the aqueous environment, suggests that it is less accessible to the ligand. For Myricitrin, on the other hand, with a lower peak value of 148.823 nm, it can be inferred that the hydrophobic core is more protected from the external environment and thus, the protein is more accessible to the ligand. Since a majority of the distribution of the apo-protein and the docked complex with Myricitrin converge with each other, it can be inferred that the target that is docked with Myricitrin and the apo-protein itself undergo similar conformational changes, with respect to the area of the complex that is accessible to the surrounding aqueous environment, during the course of the simulation, confirming the stability of this docked complex.

#### 3.5.3. Analysis of the RMSF values

We observed that the difference between the RMSF values of the apo-protein and that of the two docked complexes at the active site regions were much lower than other regions, as pointed out by the graph. (Fig. 7). At the active site regions, the difference between the RMSF values were all less than 0.1 nm, which suggests that the fluctuations experienced between the protein and the complexes were relatively low, considering the base fluctuations that the apo-protein itself experiences. This further confirms the stability of the docked complexes during the course of the simulation.

The secondary structural features of 6LU7 in its wild form, and the evolution of the apo form on binding to ligands MYR and QUE respectively are depicted in Fig. 8. The apo protein has the triple helix secondary structure feature in the 40–60 aa residue range, but on the formation of the 6LU7-MYR and 6LU7-QUE complexes, there was an interruption in this distribution and the triple helices are replaced with bends. A similar trend of loss of certain secondary structural features was noticed in the 120–140 range where bends replaced most of the beta turns in case of 6LU7-MYR, but 6LU7-QUE found randomly distributed beta bridges, bends, beta turns, alpha helices and triple helices in place of beat turns. Residues 140–160 showed a loss of beta turns in 6LU7-MYR and 6LU7-QUE, instead, more beta bridges and beta sheets, bends, alpha and triple helices were found in that region in the complexes respectively. More such differences were found in the 300+ residues range, with loss in alpha helices on binding of the ligands. It can hence be concluded that the binding of MYR and QUE ligands to the 6LU7 protein induced minor conformational changes in it, leading to alterations in the

**Table 6**

| MMPBSA analysis (kJ/mol) | 6LU7- Mycitrin | 6LU7-Quercetin-3-O-glucuronide |
|--------------------------|---------------|-------------------------------|
| van der Waal energy      | −163.311 ± 67.667 | −198.522 ± 12.694 |
| Electrostatic energy     | −109.995 ± 49.963 | −99.323 ± 23.540 |
| Polar solvation energy   | 193.067 ± 97.128 | 222.605 ± 26.564 |
| SASA energy              | −16.1610 ± 6.585 | −19.207 ± 1.077 |
| Binding energy           | −96.400 ± 32.213 | −94.448 ± 23.006 |

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structural integrity for forming stable complexes and the results were consistent with the other structural parameters of molecular dynamics simulations analysed.

3.6. MM-PBSA – binding free energy estimation

Molecular mechanics energies with Poisson–Boltzmann surface area continuum solvation, simply put as MMMPSA, is a method for the estimation of free energy that comes into play when small ligands bind to biological macromolecules [85]. Estimations done by MMPBSA were found to be more accurate than most scoring functions, and hence are widely used in the fields of drug design and other research aspects. The MMPBSA method is optimized by values from protein-ligand systems, and the binding energy is the sum of the values of van der Waal energy, electrostatic energy, polar solvation energy and SASA energy. The van der Waal interactions were found to play a major role in the binding energy calculation and promotion of protein-ligand binding, hence is considered a breakthrough point in drug development (K. [86]. Electrostatic energy, along with van der Waal’s energy are non-bonded terms that describe the potential energy of the interactions. Myricitin, upon MD simulations showed a value of -163.311 ± 67.667 kJ/mol and -109.995 ± 49.963 kJ/mol and Quercetin-3-glucuronide showed a value of -198.522 ± 12.694 kJ/mol and -99.323 ± 23.540 kJ/mol for the above energy terms respectively (Table 6). The overall binding energies of the complexes Myricitin and Quercetin-3-glucuronide were calculated to be -96.400 ± 32.213 and -94.448 ± 23.006 kJ/mol respectively. These higher negative values indicate a high binding affinity towards the main protease.

Studies conducted on whole Phyllanthus amarus extracts have shown that it has excellent antimicrobial properties, based on in vitro zone of inhibition assays [87]. It has also shown anti-viral activity against dengue virus, with more that 90% viral reduction in vitro, and it was also found that the cocktail of different Phyllanthus species (including Phyllanthus amarus) showed low toxicity to Vero cells [88]. Phyllanthus amarus extracts have also been studied on the level of the immune system, where the various phytochemicals were proven to have immunomodulatory effects, thus emphasizing their potential to be developed into standardized immunomodulatory agents [89].

Both Myricitin and Quercetin-3-glucuronide fall under the class of flavonoids, which could be argued to be the richest source of antivirals in the entire plant kingdom [90]. Myricitin, also known as myricetin-3-O-rhamnoside, has shown anti-viral activity against the H1N1 influenza virus [91], in vitro anti-HBV activity [92] as well as in vitro anti-HIV-1 activity [93]. Myricitin has been shown to be a potential HA-inhibitor drug, in a study on the effect of Myricitin against H1N1 influenza virus, where it showed excellent abilities to both a prophylactic as well as a treatment option [94]. In addition to this, it was also observed that Myricitin was non-toxic at high concentrations. In another study, Myricitin was shown to have anti-African Swine Flu Virus (ASFV) protease activity, confirming its activity against viral proteases [95]. Myricitin has also proven to bind with high affinity to TMPRSS2, a protein that plays an important role in viral entry into cells [96]. Quercetin, from which Quercetin-3-glucuronide is derived, has shown anti-viral affects against influenza A virus [97] and anti-HBV activity [98]. Quercetin-3-O-glucuronide has been studied against the influenza A virus. One study focused on in silico screening and found that Quercetin-3-O-glucuronide bound with high affinity to the polymerase basic 2 subunit of RNA polymerase [99]. Another study conducted in vivo experiments in mice to test against influenza A virus, where it was found that it could suppress ear edema, peritoneal permeability by acetic acid as well as lung edema induced by the influenza A virus [100]. With their anti-viral activity already established, these two compounds can perhaps be potential inhibitors of the SARS-CoV-2 virus as well, as confirmed by the high binding affinity values in our study.

4. Conclusion

The COVID-19 pandemic is a fight that still needs to be fought by humanity and apart from prevention by vaccination, the only way out is through the discovery of novel drugs. Our study has identified some lead compounds from Phyllanthus amarus that may be used for the inhibition of 3CL\textsuperscript{pro} in COVID-19. Molecular docking studies confirmed the binding of the compounds at the active site of this protease. Of the 190 phytochemicals that were docked with 3CL\textsuperscript{pro}, the top 0 compounds showed excelled binding affinity with values between -8.9 kcal/mol and -9.6 kcal/mol. Of these, the top two compounds with highest binding affinity and favourable binding interactions, Myricitin and Quercetin-3-glucuronide, were further chosen for molecular dynamics simulations and the 100 ns simulation revealed that the protein-ligand complexes possessed stable conformations. ADMET studies revealed that most of the compounds had good absorption properties and low acute toxicity values, with the only outlier being T. Pyriformis toxicity.

In conclusion, this study brings out the importance of natural products in the drug discovery process, specifically phytochemicals from Phyllanthus amarus. We propose the usage of whole Phyllanthus amarus extract in our fight against COVID-19. Further in vitro and in vivo studies need to be conducted in order to reaffirm our findings as therapeutics for the human coronavirus, SARS-CoV-2.

CRediT authorship contribution statement

T.P. Krishna Murthy: Conceptualization, Supervision, Writing – review & editing. Trupthi Joshi: Data curation, Investigation, Methodology, Writing – original draft. Shivani Gunnan: Data curation, Investigation, Methodology, Writing – original draft. Nidhi Kulkarni: Data curation, Investigation, Methodology, Writing – original draft. Priyanka V: Data curation, Investigation, Methodology, Writing – original draft. S. Birendra Kumar: Data curation, Investigation, Methodology, Writing – original draft. B.S. Gowrishankar: Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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