ORIGINAL ARTICLE

Procyanidin B2 of *Cassia fistula* a Potent Inhibitor of COVID19 Protease: A Molecular Dynamic Simulation Analysis

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Abstract

Based on molecular dynamic simulation studies and "Siddha" medicinal practice, this study proposes that, *Cassia fistula* fruit pulp consists of procyanidin B2, which is a potential protease inhibitor that can be used for the treatment of COVID19 infection. Virtual drug screening method and MD simulation were used to analyze 430 phytochemicals of 20 ethnobotanical medicinal plants suggested by Siddha medicine practitioner as antiviral sources and treats symptoms related to COVID19. MD simulation strongly suggested that procyanidin B2 exhibits strong protease inhibition potential, better than the co-crystallized reference ligand O6K. Scientific evidence states that *C. fistula* exhibits significant antiviral activity against human viral infections in addition to other medicinal activities. The fruit pulp of *C. fistula* is a readily market available product that can significantly aid in recovering the COVID19 infected patients from complications. It is proposed to the research centers and clinics that are currently treating the COVID19 patients to make an attempt to use the *C. fistula* fruit pulp as a hot water extract to improve the patient's response and prevent further mortalities due to the same.

Key words: *Cassia fistula*, COVID19 protease, GROMACS, molecular dynamic simulation, protein data bank 6Y2G, procyanidin B2

INTRODUCTION

A dire situation where all the major countries of the world are suffering from the outbreak of novel COVID19, with limited treatment options, global medical fraternities are making numerous attempts to deal with this crisis situation.[1] Hydroxychloroquine and azithromycin combinations are one possible treatment in the current scenario. Lopinavir and ritonavir combinations are also being considered as treatment options to control the disease.[2] This study is an attempt to identify a potential natural product source as a treatment alternative for COVID19. The molecular dynamic simulation results of this study conclude that the procyanidin B2 molecule present in the fruit pulp of *Cassia fistula* can be used as an antiviral drug (protease inhibitor) to prevent the multiplication/replication of COVID19 in humans.

C. fistula is a well-known medicinal plant in India that is also found in other Asian countries such as China, Hong Kong, Philippines, Malaysia, Indonesia, and Thailand. It is commonly known as "Golden Showers" or "Indian Laburnum" in English. It has various other names in different localities of India, such as "Amaltas" in Hindi, "Konrai" in Tamil, "Vishukonnai" in Malayalam, "Sonali" in Bengali, "Kakkemara" in Kannada, and "Chaturangula" in Sanskrit.[3] It is a state flower of Kerala and national flower of Thailand. *C. fistula* is a deciduous tree of 20~35 feet height, with characteristic yellow pendulous raceme flowers, which contributes to the name "golden showers," and the fruits are cylindrical pods that are about 30~45cm long.

C. fistula is an ethnobotanical plant, with well-known use in "Siddha" and "Ayurveda" medicines for its anti-microbial, anti-diabetic, wound healing, antipyretic, cardiac health, respiratory disorders, anti-parasitic activities, etc.,[4] that were reported repeatedly in local practices and traditional

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medicines. Aiding to these properties of *C. fistula*, it is commercially available in the market as a ready consumable product that is already popular as a food supplement and treatment for asthma, respiratory infections, diabetes, skin diseases, etc. The commercially available product has been reviewed by the public for its positive effect on diabetes, skin diseases, constipation, general health, etc.

C. fistula bark has been reported by a research institute in Tokyo, Japan, that exhibits significant antiviral activity against human viruses such as cytomegalovirus, poliovirus, and measles virus. It has been proven to reduce the multiplication of the virus particle. The bark of this plant has been patented in the U.S.A by the same researchers, for its broad-spectrum antiviral property against human viruses.[5] A review done on the biological activity of *C. fistula* also reported that the ethanolic extract of pod and stem bark was active against Ranikhet disease virus and vaccinia virus, ethanol extract of fruit was also reported to be active against foot and mouth disease virus.[6] A study from Sharma *et al*., (2010) from GLA University, Mathura, India, showed that the fruit pulp and leaf extracts of this plant have significant antiviral activity against infectious bovine rhinotracheitis (IBR) virus that infects cattle.[7] This IBR virus that infects cattle shares a reasonable similarity with symptoms of COVID19, since they both infect the respiratory tract of the host. These evidences strongly suggest that the tree *C. fistula* has significant antiviral application and has the capability to prevent multiplication of the virus within the host.

C. fistula has been reported by many scientific publications for its anti-diabetic activity on mouse and rat models,[8] antimalarial, laxative, antipyretic, skin disease, respiratory disorders, and anti-microbial activities against human pathogens. The scientific *in-vitro* and *in-vivo* studies strongly support the facts observed form "Siddha" and "Ayurveda" practices for its medicinal impact.

In the current study, *C. fistula* has been proposed to be a possible protease inhibitor for COVID19, suggesting its application for the treatment of infected patients. The phytochemical secondary metabolite of *C. fistula* – procyanidin B2 was identified to be the possible protease inhibitor, similar to that of other commercial protease inhibitor drugs such as Atazanavirchloro, Prezista, Lexiva, Prixivan, and Kaletra used in treatment for HIV.[9] Procyanidin B2 is reported to be present in the highest concentration in the fruit pulp of *C. fistula*[3,10] and hence, this study proposes to use the fruit pulp powder for application in COVID19 treatment.

The suggested mode of administration by Siddha practitioners is that the fruit pulp is to be mixed with hot water until it is evenly distributed, followed by filtration and concentration under mild temperature that results in a pasty crude essence of the pulp. This crude essence extract can be administered up to 5 g a day for patients for a positive response. Alternatively, 5 g of the fruit pulp can be stirred well in 100 ml of hot water, followed by filtration, and can be administered as kashaya to the patients.

MATERIALS AND METHODS

Identification and retrieval of ligand

Ethnobotanical data regarding medicinal plants that are used in the treatment of symptoms related to COVID19 were discussed with certified "Siddha practitioners" to identify potential natural product sources for investigation. The phytochemical secondary metabolite composition of each identified medicinal plants was gathered from various literature sources and databases (https://www.ncbi.nlm.nih.gov/pubmed/). A total of 430 phytochemical secondary metabolites were identified from the chosen 20 medicinal plants. The chemical structures of these 430 phytochemicals were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and were stored in.sdf file format.

Drug target

The protease enzyme of COVID19 was chosen to the protein target in this study, due to the well-known properties of protease inhibitors as antiviral agents. The crystal structure of the main protease enzyme of COVID19 was retrieved from protein data bank (PDB website: www.rcsb.org). The protein structure with PDB ID: 6Y2G was retrieved to be used in this study. The protein was cleaned to remove all the non-amino acid residues, i.e. co-crystallized ligands, ions, and water molecules. The retrieved structure was homodimer in nature and hence, the homologous dimer part was also removed to perform a docking with the clean single monomer chain of the protein.

Virtual drug screening of phytochemical ligands

PyRx virtual screening tool was used to perform virtual drug screening analysis, where the inbuilt Open Babel and AutoDock Vina were utilized for this study.[11] The 430 phytochemical ligands retrieved from the PubChem database were subjected to energy minimization using the Open Babel tool integrated within PyRx virtual screening software. Energy minimized molecules were converted into AutoDock ligands, i.e., conversion into ".pdbqt" file format. Further, the protein structure was converted into ".pdbqt" file format, which also included the automatic addition of hydrogens.

The AutoDock Vina plugin which is a part of the PyRx virtual screening tool was used for docking purpose. The process was initiated by choosing the ligand and the protein molecules followed by grid box selection. The amino acid residues identified to be in interaction with the co-crystallized ligand (O6K) were used as pin for setting up the grid box, however, binding site prediction protocol present within AutoDock Vina further aids in the selection of the exact binding site

for the respective ligand. The grid box was set to fit in the following residues; His-41, Val-42, Cys-44, Ser-46, Met-49, Tyr-54, Tyr-118, Asn-119, Phe-140, Leu-141, Asn-142, Cys-145, His-163, His-164, Met-165, Glu-166, Leu-167, Pro-168, His-172, Asp-187, Arg-188, and Gln-189. The results obtained after successful docking were saved and sorted using Microsoft Excel. The interactions between protein and ligand were analyzed using PyMOL molecule visualizing software from Schrodinger LLC.[12] The ligand molecules were scored based on the binding affinity values and subsequently the ligand that showed the best interaction was selected for further study with molecular dynamic simulation.

Molecular dynamic simulation

Procyanidin B2 molecule which demonstrated the best enzyme inhibitory potential was chosen for further study using GROMACS molecular dynamic simulation software version 2018.1–1 run in UBUTNU. In this "CHARMM-36, all-atom force field (2019)" was used for setting the force field parameters for MD simulation.

The protein and the ligand structures were converted into gro file format individually and both were combined appropriately to generate the complex co-ordinate file. The structure file of the ligand was first generated using the CGenFF server (https://cgenff.umaryland.edu/) further converted into Gromacs structure ".gro" file using the python script file. A topology of the protein was generated using the "CHARMM36 all-atom force field" with recommended TIP 3P water model, stored in ".top" file format to which the ligand topology and bond parameters were manually added using a text editor tool. The enzyme-inhibitor complex was enclosed inside a dodecahedron box and further stabilizations were done by adding the solvent molecules as well as by the addition of an equivalent number of positively charged sodium ions. The position restraints for the ligand were also included in the topology file and further energy minimizations were done. To stabilize the biomolecular system to a greater extent, "nvt" and "npt" ensemble were used. Finally, the simulation was set up for a period of 20 ns (20,000ps). Procyanidin B2 was used as test ligand and "O6K" the co-crystallized ligand with 6Y2G was used as the reference standard. The results of the MD simulations were analyzed using relative mean square deviation (RMSD), relative mean square fluctuation (RMSF), H-Bonds, and potential energy plots and trajectory analysis.

RESULTS

Survey for Siddha medicinal plants

A "Siddha" medicine practitioner was contacted and ethnobotanical and traditional medicine knowledge regarding the plants that are used in the treatment of COVID19 symptoms and general antiviral plants information were retrieved to narrow down the plant of study. A total of 20 plants were identified that could be used to treat the symptoms of COVID19 and are also known to be antiviral in nature. These medicinal plants were further analyzed in detail for their scientific reports on phytochemical composition and bioactive secondary metabolites. A total of 430 phytochemical secondary metabolites were identified to be components of these 20 medicinal plants. The chemical structures of these 430 phytochemicals were retrieved from PubChem database and a dataset was created.

Virtual drug screening

Virtual drug screening procedure was performed for the 430 ligand molecules against the main protease enzyme of COVID19, retrieved from PDB database (PDB ID: 6Y2G). The ligand docking was performed at the binding pocket of the co-crystallized ligand (O6K). Among the 430 phytochemical ligands, the highest significance was demonstrated by procyanidin B2 that is present in *C. fistula*. The ligands that were analyzed form *C. fistula* and their binding affinity toward the protease enzyme are tabulated in Table 1. Procyanidin B2 demonstrated the highest affinity to the protein with a free binding energy of −9.7 kcal/mol which was higher than the reference molecule "O6K" which demonstrated free binding energy of −7.3 Kcal/mol. It also formed eight hydrogen bonds and 19 hydrophobic interactions with the protein. Based on the observed results, it was identified that among the 430 investigated phytochemicals, procyanidin B2 has significant potential as a protease enzyme inhibitor. This molecule was further subjected for molecular dynamics simulation to study the possibility of its real-time protease inhibition.

Molecular dynamic simulation (protein-ligand complex)

Protein-ligand complex was simulated using GROMACS tool for a period of 20 ns and the results are interpreted as follows.

Trajectory analysis

The trajectory analysis of the procyanidin B2 and protease simulation is graphically represented in Figure

Table 1: AutoDock Vina results of phytochemicals from *Cassia fistula* against protease enzyme

Phytochemical	PubChem CID	Binding free energy (Kcal/mol)	No. of H-bonds
Epiafzelechin	443639	−6.7	0
Epicatechin	72276	−7.0	0
Kaempferol	5280863	−7.2	0
Procyanidin B2	122738	−9.7	8
Rhein	10168	−6.8	0
O6K	Standard	−7.3	4

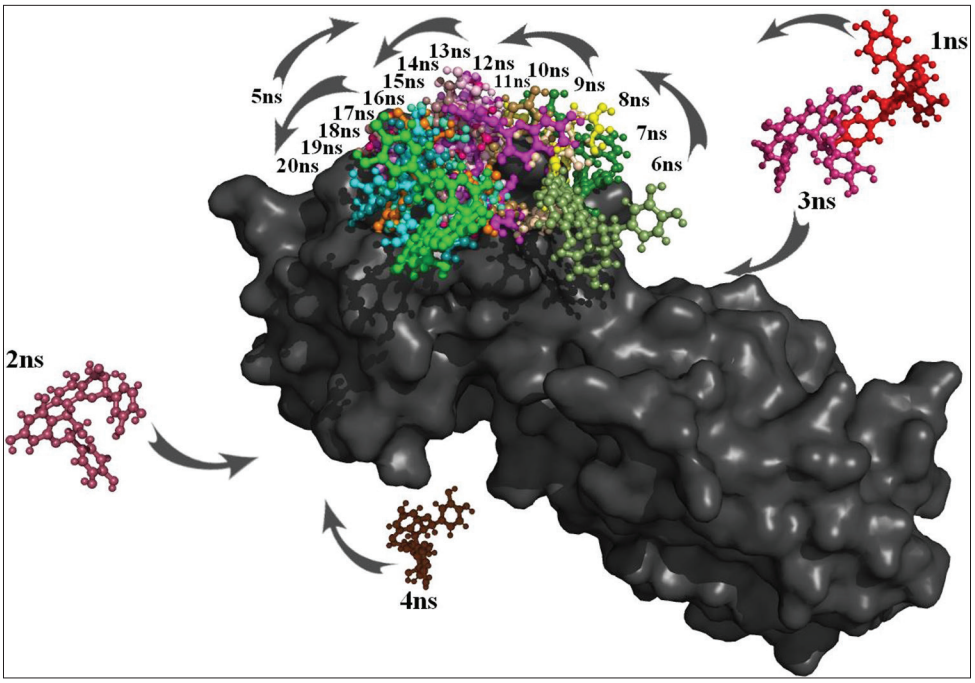

Figure 1: Trajectory movement of ligand molecule around the protein over the period of 20 ns

1 shows the trajectory of the ligand molecule, around the protein throughout the 20 ns simulation period. It is observed that the ligand demonstrated interaction with the protease enzyme from the 5th ns and was constantly in contact with the enzyme up to 20th ns. This strongly suggests that procyanidin B2 has a very significant affinity toward the protein. The interaction between the procyanidin B2 and protease was predominantly in a binding site consisting of residues that are close to the binding site of the co-crystallised ligand O6K retrieved form (PDB ID:6Y2G). This pattern was similar to that of the O6K ligand molecule simulation with protease. The initial trajectory analysis strongly suggests that the ligand procyanidin B2 has significant potential to be an inhibitor of the protease enzyme of COVID19, better than the efficacy of co-crystallized ligand O6K.

Detailed analysis of the protein-ligand complex simulation based on RMSD, RMSF, hydrogen bonds, and potential energies are as follows.

RMSD AND RMSF

RMSD analysis of the simulations shows that the ProcyanidinB2 complex had a better RMSD in comparison to the reference molecule O6K complex, suggesting the better stability of procyanidin B2 complex. The RMSD and RMSF plot of the protein-ligand complexes are graphically represented in Figure 2.

Procyanidin B2 and protease complex was stable and demonstrated the lowest RMSD value of 0.089 nm at 11.2 ns during this point of contact the complex exhibited −894359.68 KJ/mol of potential energy which is one among the lowest potential energy state of the complex, suggesting that the contact of the ligand and protein has a significant effect on overall potential energy of the complex, and the stability. The reference molecule O6K exhibited the lowest RSMD of 0.090nm at 0.06 ns after which the complex had higher RMSD values. This also suggests that procyanidin B2 has higher stability with the protease enzyme when compared to the reference molecule, in regards to the RMSD values.

Procyanidin B2 exhibited a gradual decline in the RMSD between 8.27 ns and 10.19 ns during which the molecule was observed to be positioned in between the N-terminal and C-terminal (Residues: Ser-01 and Ser-301, respectively) of the protein, which are the most flexible residues in the protein, as observed from the RMSF plot. The positioning of the ligand in between these either terminals imposed a restriction to the flexibility of free terminals, causing a drop in the RMSD for a period of almost 2 ns up to the observed 10.19 ns timeframe. After this period of contact, the ligand was observed to be pulled towards the key residues that maintained the interaction constantly from 10.19 ns up to 20 ns. The key residues include Ala-210; Ala-211; Val-212; Ile-213; Asn-214; Gly-215; Asp-216; Arg-217; Trp-218; Phe-219; Leu-202; Leu-250; Gly-251; Pro-252; Leu-253; Ser-254; Ala-255; Gln-256; Gly-258; Ile-259; and Ala-260, which were involved in both hydrogen bond formation (polar contacts) and hydrophobic interactions (non-polar interactions) periodically, but these residues retained the ligand for a prolonged period of time.

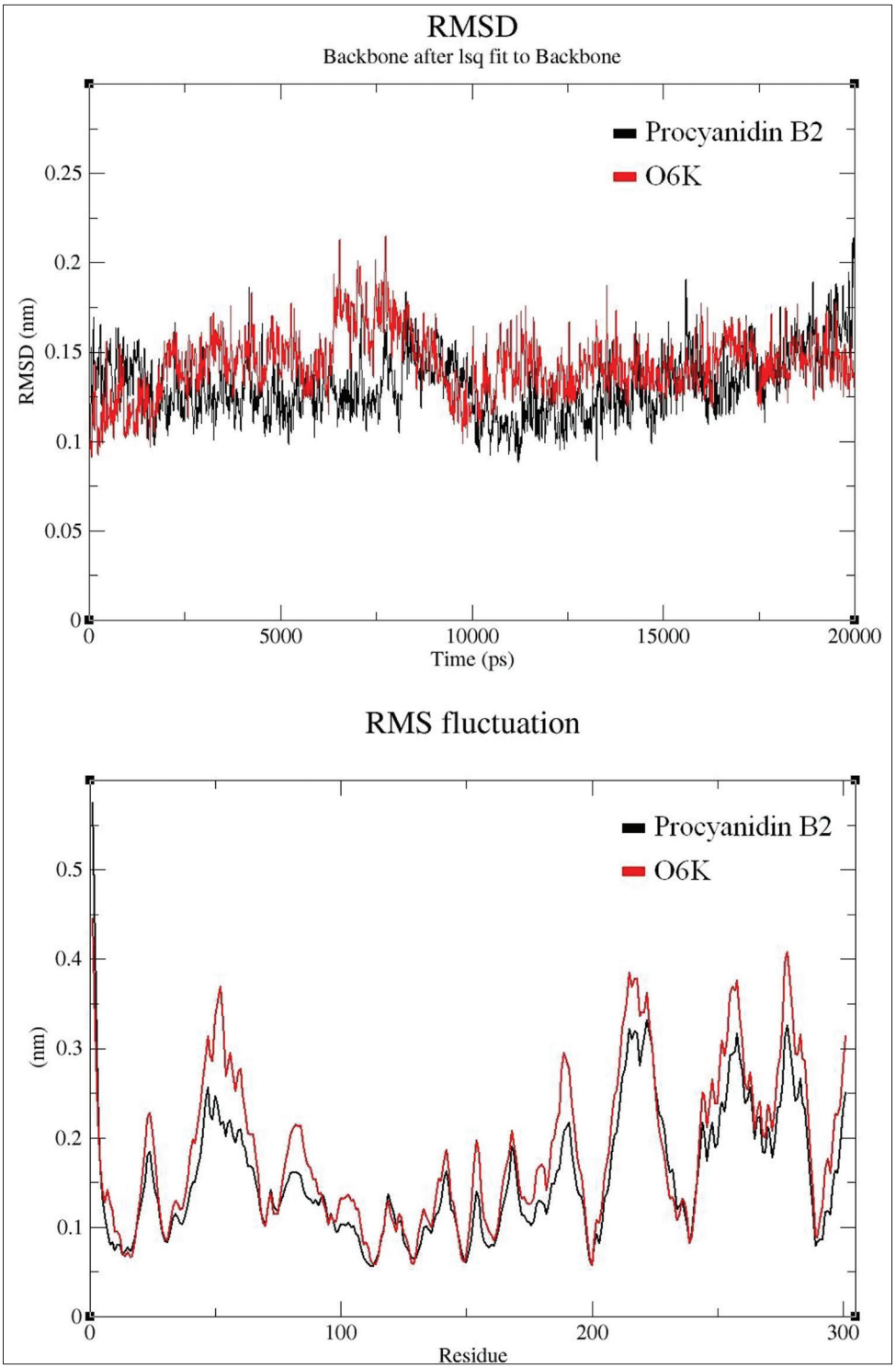

Figure 2: Relative mean square deviation and relative mean square fluctuation of protein-ligand complex simulation

The RMSF plot strongly suggests that the procyanidin B2 molecule is exerting higher rigidity to the individual residues in comparison to the reference molecule. The key interacting residues between 1 to 3; 210 to 220; and 250 to 260 all exhibited a much lower RMSF during simulation with procyanidin B2, suggesting that the flexibility of the interacting residues is strongly restricted by this molecule. This further confirms that procyanidin B2 and protease enzyme complex are more stable than the reference molecule.

H-bond analysis

The hydrogen bond plot of procyanidin B2 and O6K molecules is shown in Figure 3. The MD simulation of procyanidin B2 and protease enzyme showed constant polar interactions (hydrogen bonds) from 6.18 ns up to 20.0 ns. The highest number of h-bonds observed during the simulation is six bonds, with an average number of 2~3 hydrogen bonds at the predominant stages of the simulation. The simulation between the reference

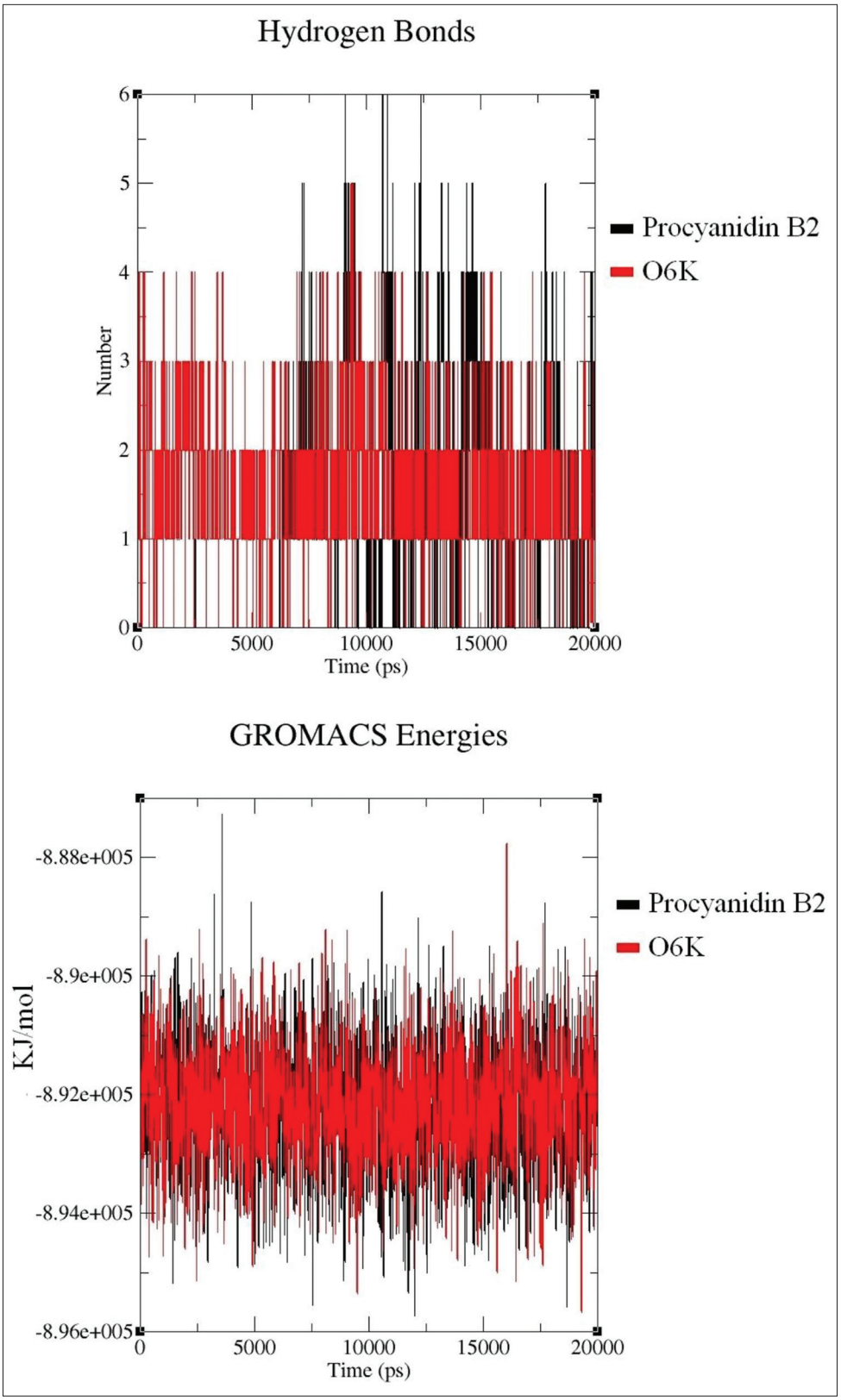

Figure 3: Hydrogen bond plot and potential energy plot of protein-ligand complex simulation

molecule O6K and protease enzyme shows slightly lesser polar interactions, with a maximum number of five hydrogen bonds, and an average of two hydrogen bonds throughout the simulation. Procyanidin B2 is exhibiting significantly higher polar interactions with the protease enzyme, suggesting that the stability of the protein-ligand complex is stronger than the interactions with O6K reference ligand. The trajectory analysis of the 12363ps of the simulation (procyanidin B2 and protein)

which is one of the states with the highest number of hydrogen bonds showed that the ligand is producing six hydrogen bonds with Val-212 (1.7Å); Ile-213 (2.0Å); Arg-217 (1.7Å, 2.1Å, 2.2Å); and Gln-299 (2.2Å) in addition to seven non-polar interactions with Ser-001; Phe-003; Asn-214; Gly-215; Asp-216; Thr-257; and Cys-300. This H-bond analysis and trajectory analysis of the protein-ligand interaction strongly suggests that procyanidin B2 is capable of exhibiting both polar and non-polar interactions with the protease enzyme simultaneously, which is significantly higher than the reference molecule, which indicates that the procyanidin B2 and protease complex is stronger than the interaction between O6K and protease.

Potential energy

The potential energy plots of procyanidin B2 and O6K molecules are shown in Figure 3. Procyanidin B2 exhibited an average of −892210.88 KJ/mol of potential energy, while O6K exhibited an average of −892167.021 KJ/mol, and it was observed to be in equilibrium throughout the simulation. Procyanidin B2 demonstrated a lower potential energy average than the reference molecule suggesting that the complex is more stable. However, the lowest potential energy state of the protein-ligand complex was attained at five different timeframes, i.e. 12010 ps; 18660 ps; 7550 ps; and 11710 ps all of which are attained, while procyanidin B2 was interacting with the enzyme. This strongly suggests that interaction of the procyanidin B2 with the protein significantly reduces the potential energy, suggesting that the protein-ligand complex is very stable. Procyanidin B2 exhibited the lowest potential energy state at 12010 ps during which the complex exhibited potential energy of −895733.56 KJ/mol. During this state, the ligand exhibited one hydrogen bond formation with Cys-300 (1.8Å) and formed six hydrophobic interactions with Ser-001; Gly-002; Phe-003; Ile-213; Asn-214; and Gly-215. The reference molecule O6K demonstrated the lowest potential energy at 19280 ps during which the potential energy was −895659.0 KJ/mol. Procyanidin B2 had a much lower potential energy, in comparison to O6K, further confirming that the procyanidin B2 complex is more stable compared to the reference molecule.

DISCUSSION

Bioinformatic studies have huge advantages of saving years of time in pharmaceutical research, which has never been more needed than the current COVID19 pandemic. Molecular dynamic simulation and virtual drug screening have proven to possess impeccable applications in pharmaceutical companies by saving an enormous amount of money and time requirement.[13] Similar approaches are employed in this study to address the current pandemic scenario. This study is also in accordance with the study done by Dayer *et al*., where a similar approach has been employed for predicting the activity of a known protease inhibitor of HIV as a potential drug to combat SARS coronavirus.[14]

Pharmaceutical industries are greatly investing in "repurposing" of medicines for better pharmacological activities with lesser side effects. This study gives one such proposal, where the traditionally well-known *C. fistula* an antimicrobial medicinal plant, is proposed to be a suitable treatment option for COVID19. "Golden shower" is an ideal plant that is commonly available in many parts of the country and is also available in several online markets that can be easily accessed by common public and researchers.

The plant parts have been popularly used by people for various health benefits and have not been reported anywhere for either toxic or undesirable side-effects; hence, it can be proposed that the plant is safe to be consumed under controlled quantities to expect an antiviral effect. The plant also possesses the laxative property to treat constipation; hence, overdosage of the plant could probably induce loose stools/diarrhea; hence, the quantity at which it is administered has to be under control. This plant is prescribed as a laxative even for pregnant women in "Siddha" medicine; hence, it is also suggested by "Siddha" practitioners that the plant is safe to be consumed by pregnant women and children.[15]

One of the well-known medicinal property of *C. fistula* is that it helps in the treatment of respiratory disorders such as asthma, pulmonary disease, and also to treat respiratory tract microbial infections. This property greatly adds to the benefit of COVID19 treatment, as the complexity of COVID19 includes respiratory disorders like severe acute respiratory syndrome. In addition, *C. fistula* is well known as an antimicrobial agent with properties to kill all different kinds of microbes such as virus, bacteria, fungi, and parasites; hence, it is considered as a very significant antibiotic source to treat any type microbial infections,[16-18] according to "Siddha" medicine. The plant is also known as an antipyretic agent; hence, it is clear that *C. fistula* can not only control the multiplications of the COVID19 but also helps in addressing of all symptoms of the patients along with prevention of the complications in the respiratory tract.

Siddha practitioners suggest the following protocol for administration of this *C. fistula* fruit pulp; the fruit pulp is to be mixed with hot water for 6 h until it is evenly macerated, followed by filtration and concentration under mild temperature that results in a pasty crude essence of the pulp. This crude essence extract can be administered up to 10 g a day for patients (maximum of 5 g intake per dose [morning and night]) for a positive response. It is also suggested that the crude extract could be mixed with an equal volume of "Tirikaṭukam" powder or equal volume of "Ghee," to make the extract more effective and also to ease the gastrointestinal tract preventing from discomfort due to the strong flavor of the pulp extract.

This study is based on advanced structural bioinformatics protocol, using MD simulation as the primary mode of analysis for confirming the given proposal. The MD simulation of

procyanidin B2 strongly suggested that the molecule could be a significant protease inhibitor of COVID19, causing an impairment in the multiplication of virus within the host. This approach is already in use for treatment of HIV, hence, the mode of action and the process of screening is also well established. Hence, the study can be cross-verified with many other standard reports that follow similar protocols combining chemistry and biological studies.

CONCLUSION

Based on the molecular dynamic simulation studies, it is concluded that the procyanidin B2 molecule present at the highest concentration in the fruit pulp of *C. fistula* is a potential protease inhibitor of COVID19. Since the plant *C. fistula* has already been proven through several *in-vitro* and *in-vivo* studies that it has antiviral and other medicinal properties addressing to the complications and symptoms of COVID19 infection and also most importantly, that the plant bark has been already patented for antiviral applications in human, it is definitely one of the best options to try to tackle the current COVID19 pandemic. The study proposes that the fruit pulp of *C. fistula* to be an effective treatment measure for COVID19 to prevent the multiplication of the virus in the host. The dosage of *C. fistula* fruit pulp, however, has to be under control and can be accompanied with other medical procedures, to help recover the COVID19 infected patients.

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CONFLICTS OF INTEREST

No known conflicts of interest.

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