Identification of Ellagitannins as Natural Inhibitors of Spike Proteins of COVID19 Virus: An *in silico*-based study for drug development

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Summary

BACKGROUND

Ellagitannins are the potential source of bioactives and found abundantly in consumable food items. S-protein is a type I fusion protein of COVID19 virus and used for initial attachment with human Angiotensin converting enzyme 2 (ACE2) receptor, which facilitates its entry into the host cell.

MATERIALS AND METHODS

S-protein is validated target and hence, we screened food borne polyphenols and FDA approved Drugs against SARS-CoV-2 spike protein by the Autodock Vina 1.1.2. and Pymol for the molecular docking and simulation studies.

RESULTS

The study showed that Sanguiin-H-6, Potentillin, Punicalagin, have excellent binding affinities for S-protein as compared to its natural N-acetyl glucosamine (NAG) ligand. Theaflavin-di-galllate and Theaflavin-mono-gallate also show comparably good binding affinities and similar binding conformations.

DISCUSSION

SH6, Casuarictin, and Potentillin compounds have shown excellent binding on the hinge of S1 and S2 subunits of spike glycoprotein. As they show hydrogen bonding interaction with S2 unit by interacting with HR region and SD2 region, and on the other side they form hydrogen bonds with amino acid residues of NTD (which is present in S1 unit). The S1 unit is responsible for the virus attachment with its host ACE2 receptor, where potential ellagitannins where found to bind with NTD, and can modulate the binding of virus with host cell.

Similarly, in S2 subunit its binding in HR region leads to disruption of membrane fusion stage, hence, ellagitannins have capability to act as potential inhibitor of virus spike protein by inhibiting both cell surface attachment as well as membrane fusion. These three compounds also show good drug likeliness properties apart from solubility which can be overcome by drug delivery advancements.

CONCLUSION

Thus, from the result it is concluded that Sanguiin-H-6, Potentillin, Casuarictin which are ellagitannins may have ability to inhibit both attachment and membrane fusion of COVID19 virus to ACE2 receptor.

RECOMMENDATION

This study needs further *in vitro* and *in vivo* testing which will provide a clear path for the development of novel compounds of natural origin that would most likely prevent the receptor binding or internalization of the SARS-CoV-2 spike protein and therefore could be potential drugs for COVID-19 preventive therapy.

Keywords: *COVID-19, Virtual Screening, Phytochemicals, Ellagitannins, Flavonoids, ADMET*

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Introduction

The corona virus outbreak has resulted in a socio-economic crisis all over the world as of 11th March, 2020 [1] when the Director General of the World Health Organization (WHO) declared COVID-19 disease to be a global pandemic. This upsurge initially started in late December 2019 as a pneumonia-associated infection, reported in Wuhan, Hubei Province of China [2].

Coronavirus is of the family of β-coronavirus (bat SARS-related coronavirus (SARSr-CoV) strain WIV1) [3], which seem to have evolved from bats and possess a dreadful threat to human health. The clinical symptoms associated with the SARS-CoV-2 generally involves the upper respiratory tract infections with its mean reproduction number (R0) 3.28 [4] and onset of pneumonia symptoms within average of 4 days (ranging from 2 to 7) [5].

The novel coronavirus is a positive single-stranded RNA that consist of majorly four proteins namely, envelope (E), membrane (M), the nucleocapsid (N), and spike (S) proteins *(see Figure 1- all tables and figures are presented at the end of this article)* [6]. These proteins function in different ways to keep the viral machinery active with E and M protein maintaining viral assembly, N protein playing role in RNA synthesis [7]. The critical glycoprotein S is a class I fusion protein found on the surface of COVID-19 virus, which majorly causes the virus' attachment to human Angiotensin converting enzyme 2 (ACE2) receptor. This is followed by its fusion with the host cells. The attachment is facilitated by the S1 subunit of S-protein via its receptor binding domain (RBD).

The RBD is projected upward as a hairpin, and provides a structural template for ligand to bind, followed by antiparallel β-sheets (N-terminal domain) NTD; both contributing to formation of S1 domain part. The S2 domain consists of a linker which connects RBD to heptad repeat (HR), a central helix (CH) and connector region (CR) *(Figure 2)*. Two domains

are responsible for the inclusive behaviour of the virus, namely N-terminal (S1 containing RBDs) and C-terminal (S2 for fusion). Moreover, the S1 further consists of two subdomains N-terminal domain (NTD) and C-terminal domain (CTD), both functioning as RBDs.

In β-coronavirus (SARS-CoV), the receptor and virus fusion interaction takes place at C Terminal [8]. The S protein also consists of cleavage sites, fusion protein near to HR1 and a TM (transmembrane) span depicted in *(Figure 3)*. It is reported that membrane fusion and internalization of the COVID19 virus is brought about by the S2 domain of spike protein. A huge dynamism is associated with this protein as it changes its conformation from pre-fusion to post fusion state after binding and internalization [9].

Belouzard *et al*. [10] depicted the mechanism of coronavirus entry mediated by spike protein with the two different conformation structures, namely, the metastable pre-fusion and the stable post fusion. When the spike protein S1 attaches the host membrane, its trimeric pre-fusion structure gets destabilized, resulting in loss of S1, and the transition of S2 subunit to stable post fusion conformation. The virus on entering into the cell releases its genome, followed by assembly and release of virions [11] from the infected cell through the process called exocytosis. The spike glycoprotein seems to exert a potential target for inhibition of infection and treatment.

Facing, the massive casualties of this pandemic, researchers all around the world are actively engaged in finding various strategies including new and repurposed drugs to treat the SARS-CoV-2 infection. Wei *et al.* [12] reported about Digitoxin, Bisindigotin, Polygoni Tinctorii Foliu and Raltegravir having high binding affinities towards spike glycoprotein. In time of need high-throughput virtual screening is also employed, which identified KT185, KT203 GSK1838705A, BMS195614, and RS504393 as a potential binder of spike protein, thereby preventing spread of COVID19 by acting both as a entry inhibitor to cell and anti-inflammatory agents [13].

Since, the age old times, people have believed that naturally occurring substances like phytochemicals have potential to fight against infectious pathogens. Most of the polyphenols are reported to have anti-viral [14] and anti-bacterial properties [15]. It is more inspiring to know that theaflavin derivatives (Flavon-3-ols), an active constituent of black tea is found to have proteolytic activity against main protease (3-CL-Protease) of SARS-CoV established through biological assays [16].

In a similar context, we recently performed virtual screening of Phenol Explorer database against main protease of SARS-CoV2 (COVID19 virus) and strikingly similar results indicating that Theaflavin-di-gallate and Theaflavin-mono-gallate have potential to bind actively in substrate binding pocket of main protease of COVID19 virus by a characteristic covalent bond [17, 18]. This, study motivated us to perform another investigation to explore the capability of polyphenols (majorly ellagitannins) against spike protein of COVID19 virus.

In this study, we performed *in silico* screening to ascertain the most potent natural food borne compound against spike glycoprotein of COVID19 virus which can inhibit virus attachment and internalization with host cell. We found that four Ellagitannins and two flavon-3-ols can bind to (NTD) of S1 and (HR) of S2 sub unit of spike protein, with a binding interaction more stable than that of N-acetyl glucosamine (NAG) and HCQ. These natural compounds have unique features of binding to both S1 and S2 subunit and most probably prevent COVID19 virus from binding to the ACE2 receptor or internalization during fusion [10, 19]. The ADMET analysis also suggested that these ellagitannins have potential to function as effective anti-SARS-CoV-2 agents with further testing and scientific modifications.

Material and Methods

Data Set Preparation

In-house database preparation of 69 ellagitannins (comprising of hydroxybenzoic acids and urolithin) derivatives and few other polyphenols was carried out by taking these compounds mainly from Phenol Explorer [20, 21] Version 3.6. The 2D structures of these compounds were exported from ZINC database [22] in SDF format and some of them (not available) were drawn manually by ChemDraw. The database was checked for redundancy and redundant molecules, in order to avoid any repetition. These structures were further refined in Chemdraw3D ultra by performing energy minimization using Molecular Mechanics 2 (MM2) force field method and saved in .mol2 format [23].

The energy minimized structures, were imported in AutoDock Vina 1.1.2 for ligand preparation [24]. Along with this, the SDF files of known 68 FDA approved anti-asthamatic drugs and A2 receptor agonist were also imported from the ZINC database for screening study.

Virtual Screening by Molecular Docking Approach

Molecular docking is an essential tool in computer-based drug design and drug discovery, which helps to predict the small ligand conformation and orientation (docking pose) within the active sites of the target receptor protein [25-30]. This technique has been highly useful in virtual screening of large libraries of ligands against suitable target proteins to identify potential drug candidates based on scoring functions and intermolecular interactions. The following were taken in conducting this study:

a. Protein Preparation

The Cryo-EM structure of spike protein of COVID19 virus co-crystallized with its ligand N-Acetyl-D-Glucosamine (NAG) was reported by Wrapp *et al.* in 2020 [31]. This protein with PDB ID: 6VSB was taken from (protein data bank) depository for virtual screening. The protein was prepared using the protein preparation wizard of Auto Dock Vina 1.1.2. This tool helps in adding hydrogens, Kollman charges, assigning AD4 type and repairing missing atoms.

b. Ligand Preparation

The 3D structural files of compounds of the in-house database along with FDA approved drugs [22] were subjected to energy minimization using MM2 by keeping a check on the connection error in the bonds. The torsions for the ligands were set by detecting the roots in AutoDock Vina 1.1.2 followed by setting aromaticity criteria of 7.5 [24].

c. Ligand Docking

First of all, the bound ligand (NAG) was extracted from spike protein and then re-docked to generate the same docking pose as found in its co-crystallized form (PDB: 6VSB) for validation of docking protocol.

The prepared sets of ellagitannins and related ligands were then docked against the spike protein using AutoDock Vina 1.1.2. based upon docking score and docked pose, the protocol validated for docking of multiple ligands.

d. Visualization

The results obtained from AutoDock Vina (1.1.2) were visualized using the academic version of Pymol software [32].

e. Prediction of ADMET by In Silico Analysis

ADMET profiling of top 3 scoring ellagitannins at pH 7 were determined using Swiss ADME, online web server (http://www.swissadme.ch/index.php) [33]. The important parameters allied with ADME properties such as Lipinski's rule of five, solubility of drug, pharmacokinetics properties, molar refractivity and drug likeliness were

deliberated. Regarding toxicity [34] analysis, Ames mutagenicity and carcinogenicity on rat and mouse models were considered. All calculated values are provided in Tables 5, 6 and 7, presented at the end of this article.

Results

The docking scores of top 14 polyphenols on spike glycoprotein of COVID19 virus is shown in Table 1 and found to be much higher in comparison to NAG (bound ligand), a natural ligand of spike protein, having docking scores of –5.3 kcal/mol. The two compounds Sanguiin-H6 (SH6) and Punicalagin (PC) found to have excellent docking scores of -9.8 kcal/mol and -9.1 kcal/mol, followed by theaflavin derivatives Theaflavin 3, 3'-O-digallate (TDG) and Theaflavin 3-O-gallate (TMG).

Out of 69 docked ellagitannins compounds, most of the compounds show binding affinities higher than that of NAG, however, we have listed top six compounds in Table 2 with their docking scores, which have been shown to be potential binders of spike glycoprotein of COVID19 virus in *in silico* drug-receptor interaction studies

This docking exercise shows that ellagitannin nucleus has more suitability to bind in the active site of spike protein as compared to Flavan-3-ol skeleton. Based on our previous reports [17, 18] TDG and TMG, were found to have excellent binding affinities towards Main Protease (Mpro) of COVID19 virus, and we were again surprised to notice that theaflavin derivatives (anti-asthmatic agents and are established bronchodilators) have potential to bind with spike glycoprotein too, based on obtained docking scores. Hence, in order to seek any relationship between anti-asthmatic and anti-COVID activity, we have also screened a number of anti-asthmatic drugs and A2-receptor agonist. Though, data from Table 3 have shown that anti-asthmatic drugs and A2-receptor agonist have poor binding affinities on Spike glycoprotein of COVID19 virus

Molecular Interactions with Spike Protein

Similarly, the ligand interactions of SH6, Casuarictin, and Potentillin with spike glycoproteins were also studied and represented in Figure 5a, 5b and 5c and their interactions with amino acid residues are presented in Table 4.

In-Silico ADMET Studies: Pharmacokinetic Prediction

We also carried out ADME studies of three active polyphenolic compounds, using Swiss ADME, [33] a free web tool to evaluate the pharmacokinetics and drug-likeness scores of medicinally important compounds. The *in silico* pharmacokinetic prediction and toxicity data of top three polyphenolic compounds are presented in Tables 5, 6 and 7.

The results show that these compounds violate the Lipinski rule of 5, because of their higher molecular weight >500. These natural compounds have low Gastro-intestinal (GI) absorption rate which leads to their low bioavailability and subsequent low plasma concentration. However, these challenges can be overcome by use of drug delivery vehicles or microencapsulation process [35, 36].

Discussion

Molecular Docking of Ligand NAG

The molecular docking was performed for the bound ligand N-acetyl glucosamine (NAG), this ligand is able to dock inside spike glycoprotein with the binding affinity value of -5.3 kcal/mol. Figure 4, demonstrates the binding of NAG in S1 and S2 subunit with the spike glycoprotein. This inhibitor forms hydrogen bonds with key amino acid residues Asn 856, Cys743, Leu977, Arg1000, which are important for the interaction.

Screening of Flavonoids Database (Ellagitannins and other Polyphenols) against Spike Glycoprotein

In our earlier study on exploration of polyphenols against Mpro of COVID19 virus, we found that sanguiin H6 (SH6), Theaflavin-di gallate (TDG), Theaflavin-mono-gallate (TMG) and punicalagin (PC) effectively bind to S1 subunit of substrate binding pocket of Mpro [17,18]. This, led us to perform their binding energy evaluation on spike glycoprotein (S-protein, where *in silico* docking results, again suggested the potentiality of these polyphenolic compounds to bind to spike glycoprotein in association with Mpro. Thus, these polyphenols can act both as entry inhibitors and stop the virus replication by inhibiting main protease.

The top scoring compounds SH6 and PC both bear ellagitannin nucleus, which inspired us to perform molecular docking of in house prepared ellagitannin database containing 69 compounds.

Sanguiin-H-6 (hydroxybenzoic acid derivative) (SH6), is found to be the highest-scoring compound with the docking score of -9.8 kcal/mol, followed by Casuarictin and Potentillin (isomers) both at -9.4 kcal/mol followed by another hydroxybenzoic acid derivative i.e., Punicalagin with -9.1 kcal/mol binding affinity.

SH6, Casuarictin, and Potentillin compounds have shown excellent binding on the hinge of S1 and S2 subunits of spike glycoprotein. As they show hydrogen bonding interaction with S2 unit by interacting with HR region and SD2 region, and on the other side they form hydrogen bonds with amino acid residues of NTD (which is present in S1 unit).

The S1 unit is responsible for the virus attachment with its host ACE2 receptor, where potential ellagitannins where found to bind with NTD, and can modulate the binding of virus with host cell.

Similarly, in S2 subunit its binding in HR region leads to disruption of membrane fusion stage, hence, ellagitannins have the capability to act as potential inhibitor of virus spike protein by inhibiting both cell surface attachment as well as membrane fusion. This docking exercise again proves the importance of Sanguiin-H6 as compounds with potential anti-CoV activity as it shows excellent binding affinities with spike as well as Mpro of COVID19 virus.

In recent years' awareness about the use of natural products over the synthetic consumables has revived the scientists' attention to conduct research, which will support the concept of chemicals derived from a natural source [37] which are practically considered to be much safer. Nowadays phytochemicals or bioactive compounds mainly comprising of flavonoids derived from plant sources are being marketed as preventive medicines under the class of "nutraceuticals" [38-40]. The flourishing market of nutraceuticals products has created awareness among the consumer to use more natural ways to treat an ailment [41]. Therefore the knowledge of pharmacokinetics and drug-likeness properties of natural compounds is crucial as these compounds can be a future drug candidate/pharmaceutical adjuvant.

Since the drug development process involves absorption, distribution, metabolism and excretion, profiling it is very important to access the knowledge about drug targets, its sufficient concentration and the plasma concentration to bring about the effective concentration (EC). Thus, the Swiss ADME tools works on principle of molecular fingerprint over a particular structure to report details on pharmacokinetic data.

Apart from the solubility issue the three compounds SH6, Casuarictin, and Potentillin show good drug likeliness and fair pharmacokinetic profile with good data on synthesis accessibility.

Conclusion

This *in-silico* investigation to identify a potent novel natural compound that can interfere in COVID19 virus attachment/internalization is decisively a seamless therapeutic option in the prevailing pandemic period. Bioinformatics based drug design studies have been effectively used by many for a fast and more or less accurate prediction of potential drugs or inhibitors against their targets.

In this study, we have virtually screened ellagitannins, polyphenols and FDA approved drugs (only anti-asthmatic and A2 agonist) to identify potent natural compounds that targets and binds to the spike protein of COVID19 virus. We have identified about 3 ellagitannins (Sanguiin-H-6, Potentillin, Casuarictin) out of the whole pool capable of binding both S1 and S2 subunit of spike protein of COVID19 virus.

Our results suggest that all of these identified compounds, abundantly present in "*berries*", have the potential to inhibit COVID19 virus attachment/internalization and should be explored further as preventive therapeutics for COVID-19.

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Conflict of Interest

None to declare

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Appendix

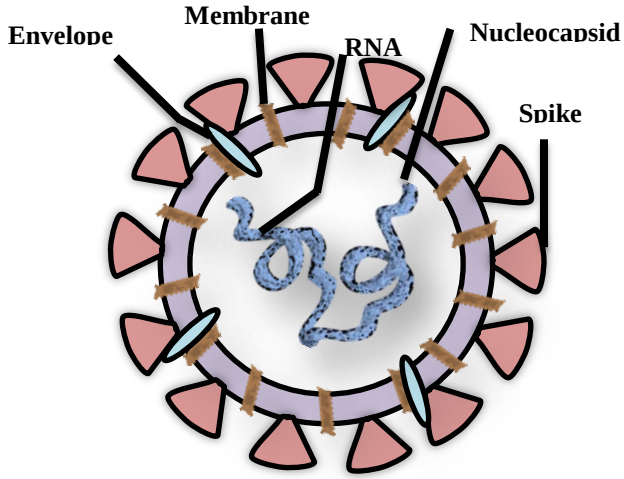


Figure 1: Structural details of coronavirus

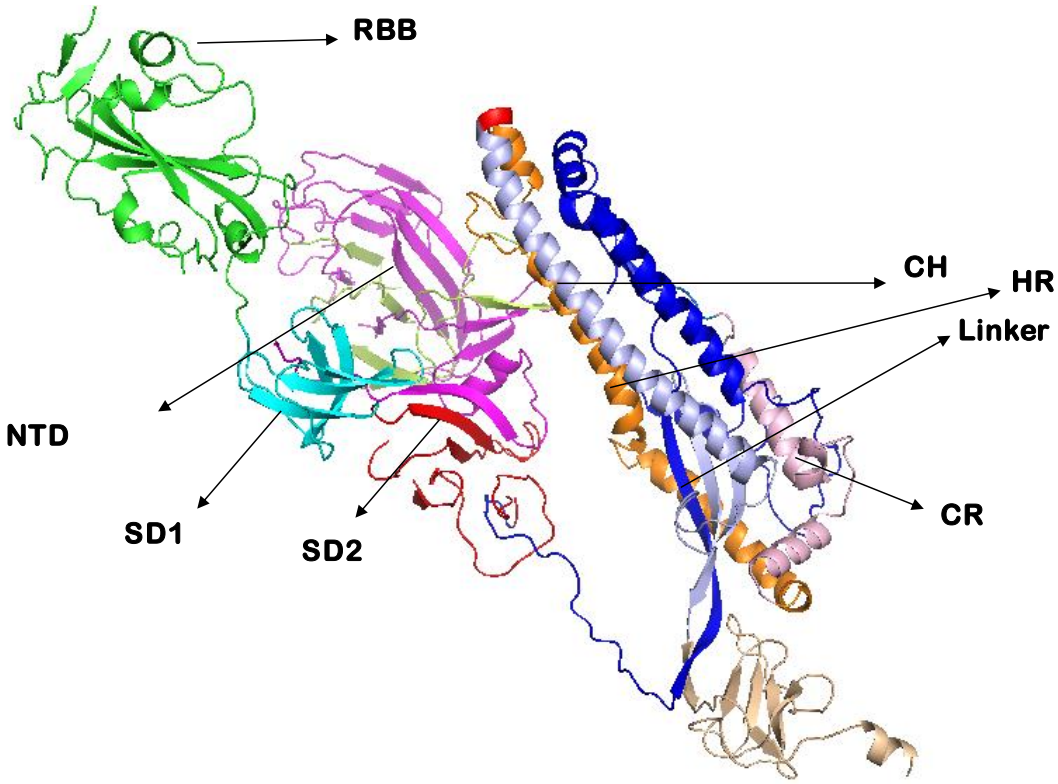


Figure 2: The 3D structure of spike protein is composed of multiple domains. RBD (residues 8–101) (shown in green); NTD (residues 102–184) (shown in magenta, have antiparallel β-sheets structure); SD1 (residues 201–303) (shown in cyan); SD2 (shown in red) These four domains form a part of S1 unit. However, S2 unit comprise of HR (shown in orange); CR (shown in light pink); CH (shown in violet); and a linker connecting S1 and S2 in blue.

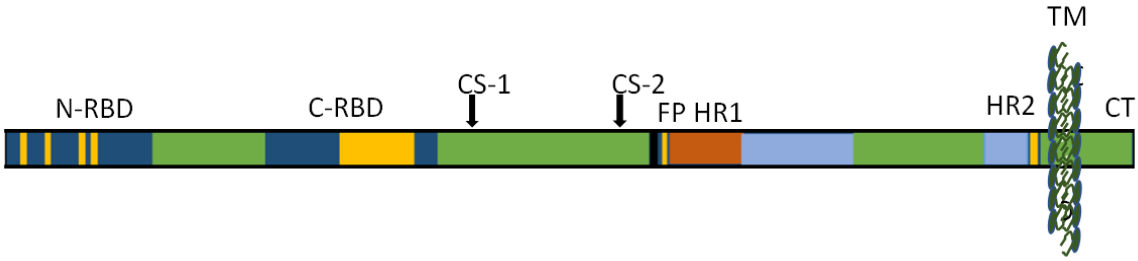


Figure 3: Genome sequence of Spike Protein

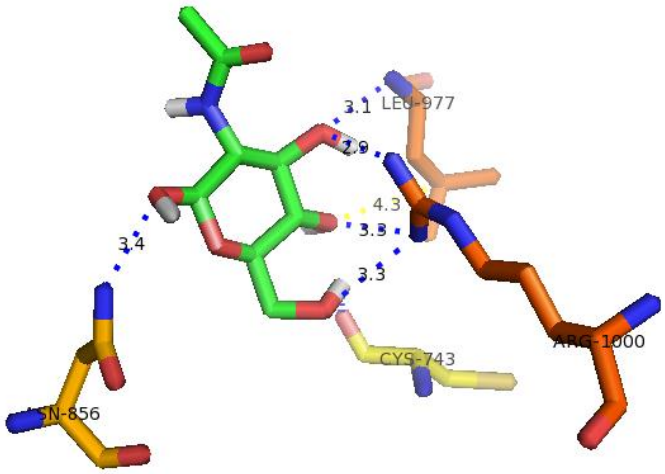
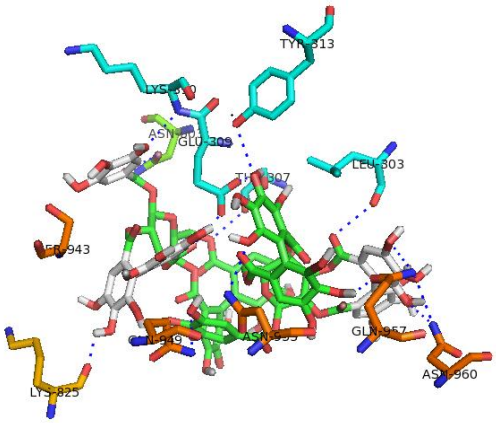
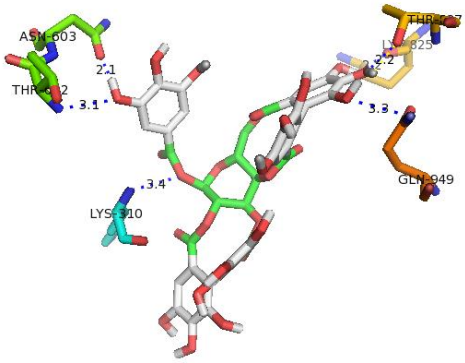
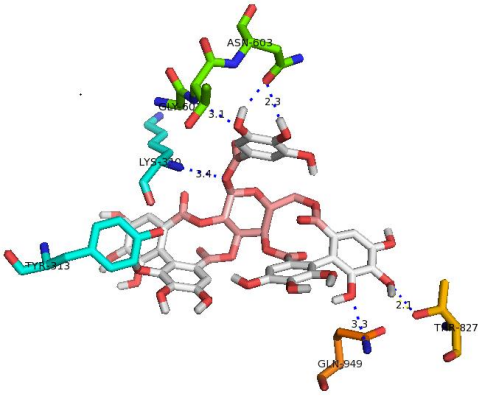


Figure 4: Interactions of NAG with the residues of Spike glycoprotein

5a







5b **5c**

Figure 5a: Interaction of SH6 with the spike glycoprotein amino acid residues that forms hydrogen bonds. 5b: Casuarictin forming hydrogen bonds with SARS-CoV-2 spike glycoprotein. 5c: Potentillin interaction with spike glycoprotein.

Table 1: Docking scores of top 14 polyphenols (screened as per our previous study on Main protease) [17, 18] on spike glycoprotein of COVID19 virus

S.No.	Polyphenol Class	Polyphenol Sub-Class	Compound Name	Docking Score on Spike Protein (kcal/mol)
1	Flavonoids	Anthocyanins	Cyanidin 3-O-galactoside	-6.6
2	Flavonoids	Anthocyanins	Peonidin 3-O-rutinoside	-7.3
3	Flavonoids	Anthocyanins	Peonidin 3-O-(6"-p-coumaroyl-glucoside)	-6.7
4	Flavonoids	Anthocyanins	Peonidin 3-O-sophoroside	-6.2
5	Flavonoids	Anthocyanins	Peonidin 3-O-sambubioside	-6.8
6	Flavonoids	Anthocyanins	Isopeonidin 3-O-sambubioside	-6.8
7	Flavonoids	Flavanols	Theaflavin 3-O-gallate	-8.3
8	Flavonoids	Flavanols	Theaflavin 3,3'-O-digallate	-8.5
9	Flavonoids	Flavanols	Procyanidin dimer B5	-7.4
10	Flavonoids	Flavonols	Kaempferol 3-O-glucuronide	-7.5
11	Flavonoids	Flavonols	Kaempferol 3-O-(2"-rhamnosyl-6"-acetyl-galactoside) 7-O-rhamnoside	-6.3
12	Phenolic acids	Hydroxybenzoic acids	Protocatechuic acid 4-O-glucoside	-6.2
13	Phenolic acids	Hydroxybenzoic acids	Sanguiin H-6	-9.8
14	Phenolic acids	Hydroxybenzoic acids	Punicalagin	-9.1
15	--	--	NAG (bound ligand)	-5.3

Table 2: Docking scores (in kcal/mol) of top 6 polyphenolic compounds against Spike glycoprotein of SARS-CoV-2 virus.

S.No.	Compound Name	Docking Score on spike glycoprotein (kcal/mol)
	Sanguiin H-6	-9.8
	Punicalagin	-9.1
	Casuarictin*	-9.4
	Potentillin	-9.4
	Ellagitannin	-8.8
	Sanguiin h2	-8.7

*isomer of potentillin

Table 3: Molecular docking scores of top 2 scoring Anti-asthmatics and A2 agonist drugs against Spike glycoproteins of COVID19 virus (for more detail please see (Table S2 and S3, supporting information)

S.No.	Compound Name	Docking score on Spike glycoprotein (kcal/mol)
ANTI-ASTHMATICS		
	Nepadutant	-8.2
	Revefenacin	-7.9
A2 AGONIST		
	Defibrotide Sodium	-8.2
	Rolofylline	-6.4

Table 4: Molecular Interactions of high Docked Scoring Compounds with Spike Glycoprotein (6VSB)

S. No.	Compound Name	Hydrogen Bond
1.	Sanguiin H-6	Tyr313, Leu303, Asn960, Gln957, Asn953, Gln949, Lys825, Ser943, Lys310, Glu309, Asn603
2.	Casuarictin	Asn603, Thr602, Lys310. Gln949, Thr827, Lys825
3.	Potentillin	Asn603, Gly60, Lys310, Try313, Gln949, Thr827

Table 5: In Silico Pharmacokinetics Prediction (Lipinski parameters)

Compound Name	MW[a]	n-rotb[b]	n-ON[c]	n-OH, NH[d]	MR[e]	TPSA[f]	iLOGP[g]	Lipinski #violations	Lead likeness #violations	Synthetic Accessibility
Potentillin	936.65	3	26	15	212.26	444.18	1.62	3	1	7.35
Casuarictin	936.65	3	26	15	212.26	444.18	1.62	3	1	7.35
Sanguiin H-6	1871.27	12	52	29	423.8	877.36	2.33	3	3	10

[a]Molecular weight (MW), [b]Number of rotatable bonds (n-rotb), [c]Number of hydrogen bond acceptors (n-ON), [d]Number of hydrogen bond donors (n-OH,NH),[e]molar refractivity (MR), [f]Topological polar surface area (TPSA), [g]partition coefficient calculated between n-octanol and water by considering solvation energies (iLog P)

Table 6: Swiss ADME Based Pharmacokinetics Prediction

Zinc ID	Ali Class	Silicos-IT class	GI absorption	BBB permeation	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	Synthetic Accessibility
Potentillin	Insoluble	Soluble	Low	No	Yes	No	No	7.35
Casuarictin	Insoluble	Soluble	Low	No	Yes	No	No	7.35
Sanguiin H-6	Insoluble	Moderately soluble	Low	No	Yes	No	No	10

Table 7: Toxicity Prediction for the Three Potential Polyphenolic Inhibitors of Spike Glycoprotein

S. No.	ZINC ID	TA100_ 10RLI	TA100 _NA	TA1535_ 10RLI	TA153 5_NA	Ames test	Carcino_ Mouse	Carcin o_Rat	hERG_in hibition
1.	Sangui in H-6	negative	negativ e	negative	negativ e	non-muta gen	positive	Positive	ambiguous
2.	Casuar ictin	negative	negativ e	negative	negativ e	non-muta gen	positive	positive	ambiguous
3.	Potent illin	negative	negativ e	negative	negativ e	non-muta gen	positive	positive	ambiguous

TA100_10RLI/1535_10_RLI = TA100/TA1535 strain with metabolic activation by rat liver homogenate,

TA100_NA/1535_NA = TA100/1535 strain with no metabolic activation.