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Research Article

In Silico Studies on the Binding of Phytosterols and Derivatives to SARS-CoV-2 Main Protease

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Abstract

Background: Coronavirus disease 2019 (COVID-19) pandemic caused by the coronavirus SARS-CoV-2 has disrupted the world in many ways since it first emerged in China in late December, 2019. Since its emergence to that of May 21, 2021, the virus has been responsible for 165,833,254 infected cases in 220 countries and territories, which has led to 3,444,270 deaths.

Objective: As of May 21, 2021, there has been no discovery of any drugs against the disease. Four vaccines that of Pfizer-BioNTech, Moderna, AstraZeneca-Oxford, and Johnson and Johnson have secured emergency approval for administration up till now. However, the vaccination process is daunting because the first three vaccines need two doses to be administered requiring 15.6 billion doses for the world's 7.8 billion people, the requirement for maintenance of two of the vaccines at very cold temperatures - a facility not available in low income countries (LICs), apathy among a section of people regarding vaccination, and appropriation of most of the vaccines produced by the wealthy nations. As such, discovery of drug(s) against this virus remains a prime objective.

Methods: No drugs against this viral disease have been discovered till now, though attention is increasingly being focused on synthetic antivirals, repurposed drugs, and plant phytochemicals (particularly flavonoids) as possible therapeutics against COVID-19.

Results: In this study, we demonstrate using *in silico* techniques that phytosterols like β-sitosterol and its esters, as well as other natural phytosterols possess potential of being lead compounds or possible therapeutics against COVID-19. Our results indicate that β-sitosterol, β-sitosterol ferulate, β-stigmasterol, stigmasterol glucoside, and clionasterol have high binding affinities for the main protease Mpro of SARS-CoV-2, and so can possibly inhibit viral replication through inhibition of Mpro.

Conclusion: Although flavonoids are the prime focus for anti-COVID-19 drugs, phytosterols should not be overlooked as possible anti-COVID-19 therapeutics.

Keywords: *NCD; MS; Alcoholic Beverages; Hypertension; Ischemic; Cerebrovascular Heart Disease; Prostate and Breast Cancer; Alcoholism*

Introduction

Coronaviruses are a zoonotic group of viruses [1], which have spikes resembling a crown on their surface, leading to the name corona [2]. They are enveloped positive sense RNA viruses belonging to the family Coronaviridae, and thus far seven coronaviruses have been

discovered to cause diseases in humans [3]. Four of these seven viruses, namely HKU1, NL63, 229E and OC43 cause mild respiratory distresses in humans, which goes away in most cases by itself, and are commonly called 'viral flu' or 'viral fever' [4]. However, in the last two decades, there had been two major outbreaks of coronavirus diseases in humans, preceding the present pandemic. The first started in the Guandong province of China from bats transmitting the disease Severe Acute Respiratory Syndrome or SARS to humans via an intermediary, civet cat in 2002. SARS affected 8,422 people resulting in 916 deaths [5]. The second corona-viral emergence, named MERS for Middle East Respiratory Syndrome occurred in 2012 in Saudi Arabia, also from bats but through dromedary camels as the intermediary host. MERS affected 2,494 people and caused 858 deaths [6]. The present virus named Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) emerged in Wuhan, China at the tail end of 2019 and is causing the disease abbreviated as COVID-19 for coronavirus disease-2019. Since its emergence till that of May 21, 2021, the virus has been responsible for 165,833,254 infected cases in 220 countries and territories, which has led to 3,444,270 deaths [https://www.worldometers.info/coronavirus/].

As of May 21, 2021 there have been no reported discoveries of any drugs against SARS-CoV-2. The same applies to SARS and MERS. Leaving aside COVID-19 vaccines developed in China and Russia, four vaccines all against SARS-CoV-2, that of Pfizer-BioNTech, Moderna, AstraZeneca-Oxford, and Johnson and Johnson has secured 'emergency approval' for administration up till now from either United States Food and Drug Administration (USFDA), United Kingdom (UK) drug authorities, or the European Union (EU) [7-9]. Though the vaccines have done an excellent job in some countries (USA, UK and some European countries) in lowering infection rates, several problems have been noticed with the world vaccination program. First, it is a time-consuming process to vaccinate 7.8 billion people of the world twice, for apart from the Johnson and Johnson vaccine, the other three need two doses of the vaccine to be administered for full efficacy. Second, three of the four vaccines need cold units for storage, with the Pfizer-BioNTech vaccine needing ultra-cold temperature for storage (-70^{o}C) [10]; such units are not available in low income countries (LICs) and low middle income countries (LMICs), particularly in the rural and remote areas. Third, although there is a COVAX program to give vaccines to LICs and LMICs, in practice the richer countries have bought or reserved a substantial number of the vaccines produced, leaving the poorer countries stranded without vaccines or an extremely insufficient amount of vaccines with respect to their needs; the problem has been compounded by a sudden surge of COVID-19 infections in India [11], the biggest producer of AstraZeneca vaccines at its production facility Serum Institute and a major supplier of vaccines to the COVAX program, but now India has stopped exporting vaccines to other countries because it is reserving all vaccines produced for its own use. Fourth, SARS-CoV-2 has developed several 'variants of concern' like the UK, South Africa, Brazil, and the Indian variants; scientists are concerned that the present vaccines may not be able to give full protection against these variants [12].

The above scenario makes it more important to discover drugs against SARS-CoV-2. Scientists are taking a three-pronged approach towards new drug discovery. The first approach involves synthetic drugs [13]. The second approach is repurposing drugs, that is using drugs originally meant for other purposes to tackle COVID-19; such drugs can be anti-viral (used against other viruses), antibiotics, anti-malarial, or steroids [14]. The third approach is used to select bio-active compounds from generally plant sources and determine through *in silico* studies for their potential to inhibit various components of SARS-CoV-2 [15]. In all these approaches, *in silico* studies are taking a major role for the simple reason that viral laboratories and studying anti-viral activities are costly and time-consuming, but *in silico* programs can screen and determine the potential of even thousands of compounds within a matter of days. In the third approach, flavonoid-type of phytochemicals from plants have taken center stage for their *in silico* potential of binding to and inhibiting the main protein (Mpro) of SARS-CoV-2, spike protein (S), or even the human receptor ACE2 (hACE2) of the virus [16].

Most flavonoids present in plants are bound to sugar moieties and available as β-glycosides. This feature of attachment allows flavonoids to be absorbed from the small intestine rather than the colon, which increases their bio-availability. However, dietary flavonol glycosides show a wide time range of absorption in human beings [17]. Low bioavailability of flavonoids has always been a concern regarding this class of compounds despite their health benefits [18]. As such, it is also important to evaluate other groups of phytochemicals for any potential anti-COVID-19 activity *in silico* or *in vitro* and *in vivo*. Phytosterols are abundantly present in plants, easily absorbed, and have a wide range of health benefits [19]. Daily consumption of phytosterols can lower blood cholesterol and also lower atherosclerotic risks [19].

Objective of the Study

The objective of our study was to evaluate *in silico* binding of naturally obtained phytosterols as well as esters of β-sitosterol to Mpro, which plays a vital role in SARS-CoV-2 replication [20]. Phytosterol as a COVID-19 therapeutic can also lower the risks when heart disorder is also present as a comorbidity.

Materials and Methods

Three-dimensional structure of COVID-19 major protease (3C-like protease or Mpro)

We have used the pdb file (6LU7) of the main protease of SARS-CoV-2 known as Mpro as published before [21]. Inhibitor (called N3) bound to the protease was removed from the pdb file before using the protein's structure in our molecular docking studies. The reported interacting residues of N3 with the protease amino acids include His41, Met49, Phe140, Leu141, Asn142, Gly143, His163, His164, Glu166, Leu167, Pro168, Gln189, Thr190, and Ala191. The active amino acid residues of SARS-CoV-2 Mpro protease are His41 and Cys145, which forms a catalytic dyad. Monomeric form of protein was used for molecular docking. Mpro contains three domains; domain 1 contains amino acid residues 10-99, domain 2 is composed of amino acid residues 100-182, and domain 3 contains amino acid residues 198-303. The active site of Mpro is located in a cleft between domains 1 and 2. Domain 3 plays a role in the dimerization of Mpro, the resulting homodimer being reported as the active form of the enzyme [22,23].

Compounds used in docking studies

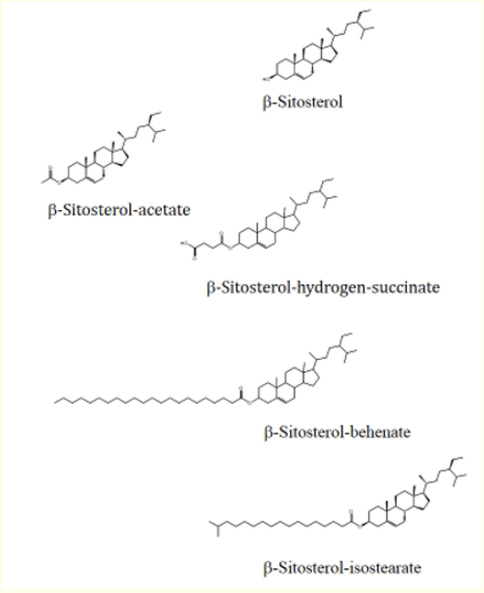
We concentrated on twelve phytosterols (including esters of β-sitosterol) in the present study, which were downloaded from Pubchem [24]. in sdf format. They were optimized with the force field type MMFF94 using Openbable softwares and saved as pdbqt format, as described before [25].

Ligand molecular docking studies

Molecular docking (blind) studies were conducted using AutoDock Vina [26]. The grid box in Autodock Vina was generated aiming to cover up all the key residues for ligand binding of the main protease, where the center was at X: -6.35, Y: 8.36, Z: 41.26 and the dimensions of the grid box were, X: 38.84, Y: 38.77and Z: 97.25 (unit of the dimensions, Å). We have used exhaustiveness '16' for better ligand and protein binding. We report ΔG values as an average of values from five independent runs of the docking program. In our figures, the pose of the compounds are shown bound to SARS-CoV-2 main protease (Mpro) as obtained from PyMOL and displayed in Discovery Studio [27].

Phytosterols and esters

The various naturally occurring phytosterols and esters of b-sitosterol were obtained from published reports [28], and PubChem. β-Sitosterol, campesterol and stigmasterol are common phytosterols in plants consumed by humans [29]. Daucosterol has been identified from roots of *Mangifera indica* [30]. Clionasterol can be found in freshwater microalgae [31]. Esters of β-sitosterol were obtained from PubChem. The structures of the β-sitosterol esters are shown in figure 1.



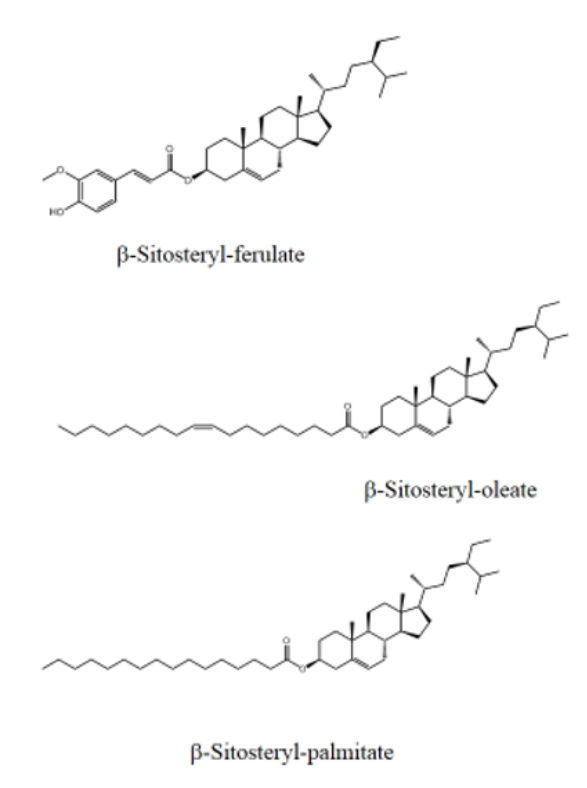


Figure 1: *Structures of β-sitosterol and its esters.*

Results and Discussion

By itself, β-sitosterol gave a predicted binding energy in molecular docking studies of -7.5 kcal/mol. The various esters of β-sitosterol with the exception of β-sitosteryl ferulate showed lower binding energies than β-sitosterol. β-Sitosteryl ferulate showed a predicted binding energy of -7.8 kcal/mol, suggesting that a possibility exists of obtaining even higher predicted binding energy with other β-sitosterol esters, which have not gone through this computational analysis in the present study. The structures of β-sitosterol and its various esters are shown in figure 1 and the binding energies of β-sitosterol and its esters, and other naturally occurring phytosterols shown in table 1. Interestingly, the other naturally occurring phytosterols also showed good binding affinities to Mpro (that is low predicted binding energies). Stigmasterol glucoside showed the lowest predicted binding energy of -8.2 among the naturally occurring phytosterols. The compound also known as stigmasterol 3-*O*-β-D-glucoside has been isolated from leaves and stem bark of *Phyllanthus reticulatus* Poir. (Phyllanthaceae) [32].

Phytochemicals	Predicted binding energy to Mpro (ΔG = kcal/mol)
β-Sitosterol acetate	-7.0
β-Sitosterol behenate	-4.9
β-Sitosterol hydrogen succinate	-6.6
β-Sitosterol isostearate	-5.3

β-Sitosterol	-7.5
β-Sitosteryl_ferulate	-7.8
β-Sitosteryl oleate	-6.1
β-Sitosteryl palmitate	-6.0
β-Stigmasterol	-7.9
Clionasterol	-7.6
Daucosterol	-7.2
Stigmasterol glucoside	-8.2
Lopinavir (antiviral drug)	-8.2
Nelfinavir (antiviral drug)	-8.1

Table 1: *Predicted binding energies of naturally occurring phytosterols and esters of β-sitosterol.*

The entry, release and subsequent amplification of SARS-CoV-2 can possibly be blocked through changes in membrane lipid composition and metabolism of lipids [33]. Cholesterol is an important factor in virus fusion with host cell membrane, which is necessary for release of viral genomic material and viral reproduction scaffold build-up; low cholesterol diet, use of statins, and use of phytosterols have all been recommended to inhibit SARS-CoV-2 entry and multiplication in human host cells [34]. Our study proposes an alternate mechanism through which phytosterols can bind to and inhibit main protease of SARS-CoV-2, namely Mpro, and block viral replication.

The interactions of some of the phytosterols and β-sitosterol esters and the control antiviral drug lopinavir with Mpro are shown in figure 2-4. Among the naturally occurring phytosterols, β-sitosterol interacted with amino acid residues Tyr237, Tyr239, Met276, Leu286, Leu 287, and Asp289 of Mpro, the amino acids being all domain 3 amino acids; β-stigmasterol interacted with Phe8, Val104, Ile106 and Phe294, the amino acids being mostly domain 2 amino acids of Mpro and one domain 3 amino acid; stigmasterol glucoside interacted with Pro108, Glu240, His246, Ile249, Pro252, Pro293, Phe294, and Val297, the amino acid residues being with one exception (Pro108) all Mpro domain 3 amino acids; clionasterol interacted with Val104 and Phe 294, one amino acid each from domains 2 and 3 of Mpro. The predicted binding energies of these four phytosterols were -7.5, -7.9, -8.2, and -7.6, respectively. Since a number of interactions of the phytosterols were with domain 3 amino acid residues of Mpro (which domain plays a part in the dimerization and activation of both SARS and SARS-CoV-2 main protease) [35,36]. it can be expected that binding to this domain will inhibit dimerization and subsequent catalytic activity.

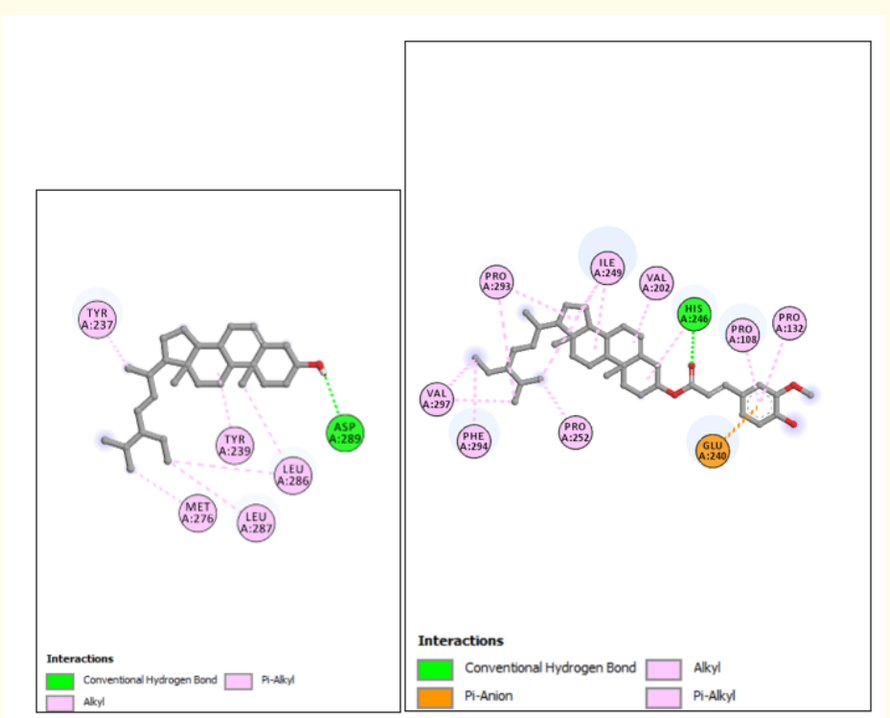


Figure 2: *Interaction of β-sitosterol (left) and β-sitosterol ferulate (right) with Mpro (2D diagram).*

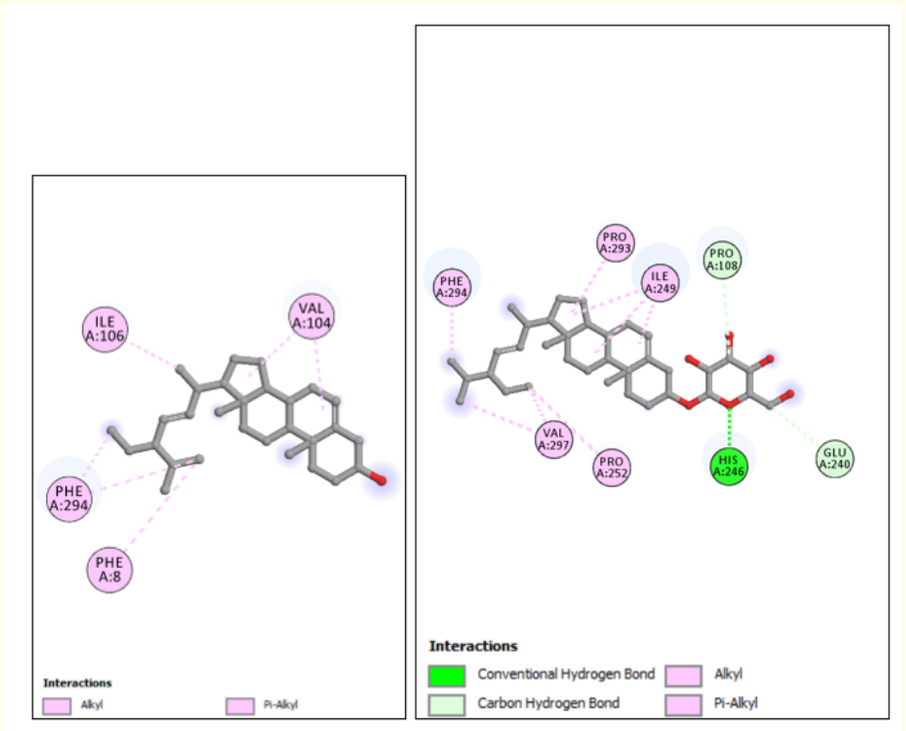


Figure 3: *Interaction of β-stigmasterol (left) and stigmasterol glucoside (right) with Mpro (2D diagram).*

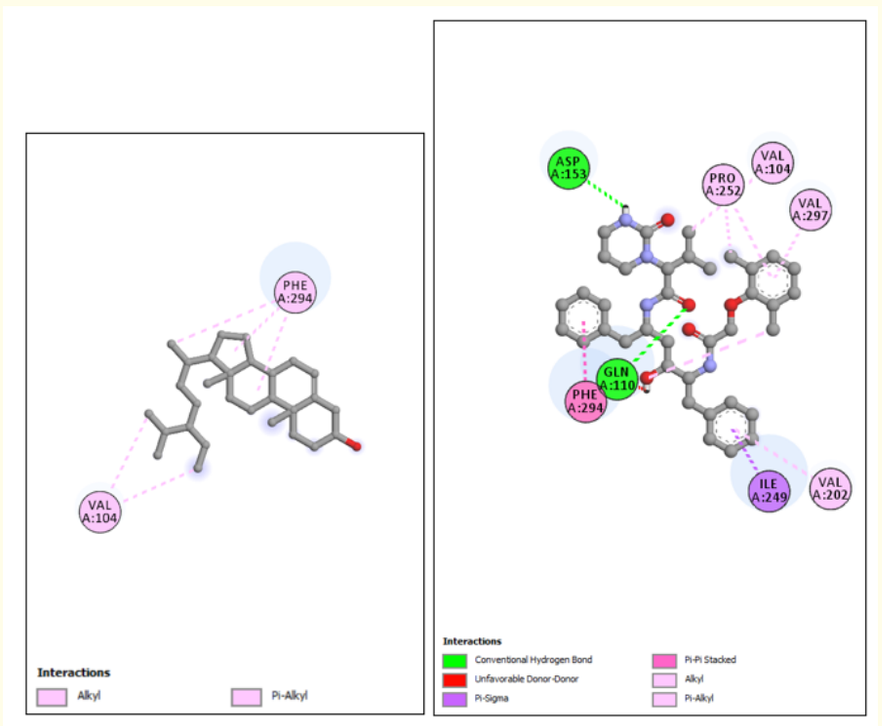


Figure 4: *Interaction of clionasterol (left) and lopinavir (right) with Mpro (2D diagram).*

The results from Lipinski's Rule of 5 (Ro5) [37]. are shown in table 2. The rule mentions that poorly absorbed molecules by intestinal wall would present two or more of these characteristics: molecular weight over than 500, lipophilicity (log P > 5), hydrogen-bond (HB) donor groups (expressed as the sum of OHs and NHs groups) more than 5, more than 10 HB acceptor groups (expressed as the sum of Os and Ns atoms), and molar refractivity outside a range of 40-130 [38]. According to Ro5, molecules demonstrating less than 2 violations have good probability of being drugs because of their higher absorption from the intestine. It can be seen from table 2 that all the naturally occurring phytosterols and a number of the β-sitosterol esters do not violate Ro5 more than 2 times and so possess the potential of being good therapeutics.

Compounds Name	Molecular weight g/mol	Number of H-Bond Acceptors	Number of H-Bond Donors	Log P	Molar Refractivity	Number of Violation
β–Sitosterol acetate	456.74	2	0	5.19	142.97	1
β-Sitosterol behenate	737.27	2	0	10.10	239.11	2
β-Sitosterol hydrogen succinate	514.78	4	1	4.67	154.35	2
β-Sitosterol isostearate	681.17	2	0	8.80	219.88	2
β-Sitosterol	414.71	1	1	4.79	133.23	1
β-Sitosteryl ferulate	590.88	4	1	6.49	181.09	2
β-Sitosteryl oleate	679.15	2	0	9.08	219.40	2
β-Sitosteryl palmitate	653.12	2	0	8.67	210.26	2
β-Stigmasterol	412.69	1	1	4.96	132.75	1
Clionasterol	414.71	1	1	4.79	133.23	1
Daucosterol	576.85	6	4	4.98	165.61	1
Stigmasterol glucoside	574.83	6	4	4.23	165.14	1
Lopinavir (antiviral drug)	628.80	5	4	3.44	187.92	1
Nelfinavir (antiviral drug)	567.78	5	4	3.87	166.17	1

Table 2: *Physico-chemical properties of the naturally occurring phytosterols and β-sitosterol esters and violations of Lipinski's Ro5.*

Conclusion

Although small molecule inhibitors of SARS-CoV-2 are being intensively studied (flavonoids being the prime example), phytosterols have not attracted the attention of researchers to any large extent. In this study, we demonstrate two things. First, although this is not an all-inclusive study of all phytosterols present in plants, we demonstrate that phytosterols can strongly bind to Mpro of SARS-CoV-2 and possess the potential of being strong inhibitors of the viral main protease. The second finding coming out of this study is that one of the most known phytosterol, namely β-sitosterol, and so possibly other naturally occurring phytosterols may be esterified with various acids to increase their binding affinity for Mpro and as such have the potential to serve as COVID-19 therapeutics.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

MR, RJ, AP, VN, KD, TM and MLP conceptualized, supervised, and checked out the first draft of the manuscript written by MR. TAB, AH and KJ did the *in silico* studies. All authors took part in editing the first draft and approved the final manuscript.

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