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REVIEW

Phytonutrient Inhibitors of SARS-CoV-2/NSP5-Encoded Main Protease (M^{pro}) Autocleavage Enzyme Critical for COVID-19 Pathogenesis

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ABSTRACT

The genomic reshuffling, mutagenicity, and high transmission rate of the SARS-CoV-2 pathogen highlights an urgent need for effective antiviral interventions for COVID-19 control. Targeting the highly conserved viral genes and/or gene-encoded viral proteins such as main proteinase (M^{pro}), RNA-dependent RNA polymerase (RdRp) and helicases are plausible antiviral approaches to prevent replication and propagation of the SARS-CoV-2 infection. Coronaviruses (CoVs) are prone to extensive mutagenesis; however, any genetic alteration to its highly conserved M^{pro} enzyme is often detrimental to the viral pathogen. Therefore, inhibitors that target the M^{pro} enzyme could reduce the risk of mutation-mediated drug resistance and provide effective antiviral protection. Several existing antiviral drugs and dietary bioactives are currently repurposed to treat COVID-19. Dietary bioactives from three ayurvedic medicinal herbs, *18 β-glycyrrhetinic acid* ($\Delta G = 8.86$ kcal/mol), *Solanocapsine* ($\Delta G = 8.59$ kcal/mol), and *Vasicoline* ($\Delta G = 7.34$ kcal/mol), showed high-affinity binding to M^{pro} enzyme than the native N3 inhibitor ($\Delta G = 5.41$ kcal/mol). Flavonoids strongly inhibited SARS-CoV-2 M^{pro} with comparable or higher potency than the antiviral drug, remdesivir. Several tannin hydrolysates avidly bound to the receptor-binding domain and catalytic dyad (His_{41} and Cys_{145}) of SARS-CoV-2 M^{pro} through H-bonding forces. Quercetin binding to M^{pro} altered the thermostability of the viral protein through redox-based mechanism and inhibited the viral enzymatic activity. Interaction of quercetin-derivatives with the M^{pro} seem to be influenced by the 7-OH group and the acetoxylation of sugar moiety on the ligand molecule. Based on pharmacokinetic and ADMET profiles, several phytonutrients could serve as a promising redox nutraceutical for COVID-19 management.

KEYWORDS

Antiviral;
Ayurvedic Medicine;
COVID-19;
M^{pro};
Phytonutrients;
Quercetin;
Redox nutraceutical;
SARS-CoV-2

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Introduction

Coronaviruses (CoVs) are a large family of enveloped viruses with a positive-sense single-stranded RNA genome (about 26 to 32 kilobases) and a nucleocapsid with helical symmetry (1). Generally, CoVs are zoonotic pathogens that cause mild to moderate upper-respiratory tract illnesses, like the common cold. However, three new CoVs emerged from animal reservoirs over the past two decades have inflicted severe morbidity and mortality in human populations worldwide. The SARS-CoV emerged in November 2002 has caused *severe acute respiratory syndrome* (SARS) and disappeared by 2004 (2). The MERS-CoV transmitted from camel reservoir caused *Middle East respiratory syndrome* (MERS) in September 2012, which continues to wreak sporadic and localized outbreaks (3). Emergence of the third novel SARS-CoV-2 from China in December 2019 has manifested into *Coronavirus Disease 2019* (COVID-19), declared a global pandemic by the World Health Organization on March 11, 2020 (4).

The SARS-CoV-2 pathogen shares a significant gene sequence homology with its CoV predecessors, the SARS-CoV and the MERS-CoV. The transcription of SARS-CoV-2 genome results in *a* ~790 kDa polypeptide, which undergoes a proteolytic autocleavage to generate various functional proteins that regulate the viral life cycle. The SARS-CoV-2 *replicase* gene harbors two open reading frames (ORFs), the ORF1a and the ORF1b, wherein ORF1a encodes two cysteine proteases, a *papain-like protease* (PL^{pro}) and a 3-*chymotrypsin-like protease* ($3CL^{pro}$) also known as *main protease* (M^{pro}) (5). The PL^{pro} slices three cutting sites on the *pp1a* polyprotein, whereas the M^{pro} cleaves at least eleven inter-domain sites on the *pp1a* and *pp1ab* polyproteins and release sixteen nonstructural proteins (NSPs) (6) (Figure 1). This autocleavage involves complex

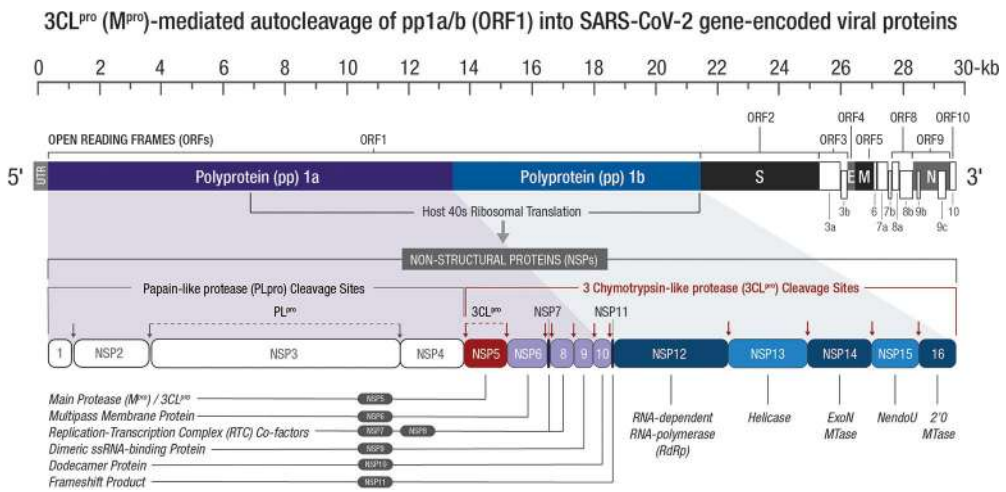

Figure 1. The polypeptide gene transcript of SARS-CoV-2 undergoes an enzymatic cleavage to generate several functional proteins to support the viral life cycle. The SARS-CoV-2 replicase gene harbors two open reading frames (ORFs), the ORF1a and the ORF1b, wherein ORF1a encodes two cysteine proteases, a papain-like protease (PL^{pro}) that slices three cutting sites on the pp1a polyprotein; and a 3-chymotrypsinlike protease ($3CL^{pro}$) also known as main protease (M^{pro}) that cleaves at least eleven inter-domain sites on the pp1a and pp1ab polyproteins and release sixteen nonstructural proteins (NSPs).

enzymatic processing of *replicase polyproteins*, critical for viral life cycle; therefore, M^{pro} is a potential antiviral target to combat SARS-CoV-2, as well as other CoV pathogens such as SARS-CoV and MERS-CoV (7, 8).

Global demand for effective antiviral interventions for clinical management of COVID-19 is aggravated with emerging incidences of highly transmissible SARS-CoV-2 variants (9). The viral evasion of host immune defenses (10), and the newly evolved SARS-CoV-2 single nucleotide polymorphism (SNP) alleles, have seriously undermined the widely anticipated global immune protection from anti-COVID vaccination programs (11, 12). Therefore, well-conserved (less variable) genomic domains and gene clusters in SARS-CoV-2 may serve as promising antiviral targets

Nonstructural protein (NSP)-5 (M^{pro} or $3CL^{pro}$) is the main protease that cleaves the large replicase polyproteins (13). The NSP12 (*RNA-dependant RNA polymerase*, RdRp) and the NSP13 (*helicase* (NSP13) (14), are integral components of the 'replication and transcription complex' (RTC) required for viral assembly and release. These essential viral enzymes are highly conserved genomic domains in the RNA of *Coronaviridae* family. The life cycle of SARS-CoV-2 is strictly regulated by these viral enzymes. In the past, several pharmaceutical drugs, and phytonutrient inhibitors effective against these CoV enzymes have been widely used in the clinical management of SARS and MERS outbreaks (15). Based on current constrains in the global management of COVID-19 crisis, identification and/or repurposing of anti-CoV phytonutrients with better therapeutic index against viral enzymes is highly warranted. Evidence-based investigations of important medicinal plants may uncover novel phytonutrients with significant potential to inhibit M^{pro} of SARS-CoV-2 and initiate the development effective natural inventions for COVID-19 management.

NSP5/main protease (M^{pro} or $3CL^{pro}$) as antiviral target

NSP5, the main protease (M^{pro}), also known as the *chymotrypsin-like protease* ($3CL^{pro}$), is one of the best characterized antiviral targets among CoVs (16). Along with the *papain-like protease* (PL^{pro}), this enzyme is essential for processing the polyproteins that are translated from the viral RNA (17, 18) (Table 1). The recognition sequence at most sites is Leu-Gln↓(Ser-Ala-Gly) (↓ marks the cleavage site) and blocking the activity of this enzyme results in the inhibition of viral replication. Unlike structural or accessory protein-encoding genes, M^{pro} is located at the 3′ end and exhibits excess variability (19). Therefore, M^{pro} is considered as one of the key antiviral targets to combat COVID-19 with competitive inhibitory substrates (20). The CoVs are prone to extensive mutagenesis; however, as a key viral enzyme, M^{pro} is highly conserved, and any genetic alteration to its protein sequence is often detrimental to the virus (21). Thus, drugs targeting the M^{pro} enzyme significantly reduce the risk of mutation-mediated drug resistance besides displaying broad-spectrum antiviral activity (22).

Structure-Function of M^{pro} enzyme

The SARS-CoV-2 M^{pro} enzyme is a 33.8-kDa polypeptide with 306 amino acid residues. M^{pro} contains a highly conserved substrate-binding domain (His_{41}, Met_{49}, Gly_{143}, Ser_{144},

Table 1. Nonstructural Proteins (NSPs) of SARS-CoV-2-encoded ORF1a/b domain and their function.

NSP	AA Residues	Gene-encoded Protein	Proposed structure-function activity
Papain-like protease (PL^{pro}) autocleavage enzyme activity			
NSP1	180	Leader Protein at 5′	Inhibits host translation by binding to 40S ribosomal subunit. Degrades host mRNA by cleaving its 5′UTRs. Selectively promotes viral gene expression. Interferes with type-1 interferon (IFN)-mediated cell signaling, thereby contributes to viral evasion of host innate defense.
NSP2	638	Prohibitin (PHB)-Binding Protein	Interacts with host factors PHB-1 and PHB-2 involved in several cellular pathways including mitochondrial biogenesis. Alters intracellular milieu and perturbs host intracellular signaling.
NSP3	1945	Papain-like protease (PL^{pro})	An enzymatic membrane protein that cleaves viral polyprotein (pp)1a at 3 sites to release NSP1, NSP2, and NSP3. It has deubiquitinating and deISGylating functions, and interacts with NSP4 and NSP6.
NSP4	500	Multi-pass Membrane Protein	Induces (with NSP3) assembly and localization of double-membrane cytoplasmic vesicles critical for viral replication.
Main protease (M^{pro}) autocleavage enzyme activity			
NSP5	306	M^{pro} or 3CL^{pro}	Main protease (M^{pro}) cleaves viral polyprotein (pp) 1a/b at 11 sites to release NSP4 to NSP16. M^{pro} also helps maturation of other NSPs.
NSP6	290	Multi-pass Membrane Protein	Induces double-membrane vesicles in the infected cell along with NSP3 and NSP4. Limits autophagosome expansion and interferes with its delivery of viral factors for lysosomal destruction.
NSP7	83	RdRp (NSP12) Enzyme Co-factor	Forms hexa-decamer with NSP8 and acts as cofactor for the RdRp enzyme (NSP12). NSP7 is considered an *RNA primase* in the viral RTC.
NSP8	198	RdRp (NSP12) Enzyme Activator	Forms a hexa-decamer with NSP7 as a cofactor for the RdRp enzyme (NSP12). May also act as an *RNA primase* in the viral RTC.
NSP9	113	ssRNA-Binding Protein	Interacts with *helicase* enzyme (NSP13) and plays an important role in viral replication.
NSP10	139	mRNA Capping Co-factor	Forms dodecamer and interacts with NSP14 and NSP16 to stimulate their respective 3′-5′ exoribonuclease (ExoN) and 2′-O-methyltransferase (MTase) activities during formation of the viral mRNA capping machinery.
NSP11	13	Unknown	A cleavage product of pp1a (at the NSP10/11 intersection). For pp1ab, it is a frameshift product that becomes the N-terminal end of NSP12. The function of NSP11, if any, is still unknown.
NSP12	932	*RNA-dependent RNA polymerase (RdRp)*	Performs both replication and transcription of the viral genome. Critical for making copies of viral RNA. RdRp is central in formation and function of the viral *replication-transcription complex* (RTC).
NSP13	601	*Helicase*	This enzyme (with NSP12) is essential for viral replication, viral mRNA capping and uses both dsDNA and dsRNA as substrates with 5′-3′ polarity. Interacts with nucleocapsid (N) in viral membrane complex.
NSP14	527	*Exoribonuclease (ExoN) / Methyl-transferase (MTase)*	Bifunctional replicase enzyme with *3′-5′ exoribonuclease (ExoN) activity* (for proofreading during RNA replication) and *guanine N7-guanine methyltransferase* activity (for viral mRNA capping). NSP14 binds to NSP10 and promotes long-term high fidelity viral replication.
NSP15	346	*Nidoviral RNA uridylate-specific endoribonuclease (NendoU)*	Cleaves the RNA at 3′-ends of uridylates. Degrades host mRNA and plays a major role in viral evasion of host cell dsRNA sensors. Loss of NSP15 results in loss of both viral replication and virulence.
NSP16	298	*2′O-ribose-methyl-transferase (MTase)*	This enzyme is activated by NSP10 and is considered essential for viral mRNA capping. NSP16 may also act against host cell antiviral sensors.

His_{163}-Leu_{167}, Asp_{187}-Glu_{192}) (23, 24), within the active site of the enzyme. The crystal structures of M^{pro} have three structural domains, wherein domains I and II has a characteristic *chymotrypsin*-like fold with a catalytic dyad (His_{41} and Cys_{145}), linked to a third C-terminal domain by a long loop (19) (Figure 2). These scaffolds are key target sites to design M^{pro} inhibitors and to develop antiviral agents, nutraceuticals, or dietary supplements for SARS-CoV-2 management.

The M^{pro} homodimer of SARS-CoV-2 shares 96% sequence homology with SARS-CoV M^{pro}; accordingly, about 294 amino acids are common out of 306 residues between these two viral enzymes. Except for two amino acids Ser_{46} and Val_{86}, remainder of the sequence is identical in the active site pocket of SARS-CoV-2 M^{pro} (25). Therefore, most inhibitors of SARS-CoV M^{pro} are also expected to act against SARS-CoV-2 M^{pro}. Interestingly, the V_{max} of SARS-CoV-2 M^{pro} is about 2-fold higher compared to SARS-CoV M^{pro}. Furthermore, the proteolytic activity of M^{pro} from SARS-CoV-2 is slightly more efficient than that of SARS-CoV. This enhanced proteolytic activity of SARS-CoV-2 M^{pro} accelerates the viral life cycle in an infected host [Chiou *et al.* 2020]. Accordingly, the viral load in patients governs the transmission potential of SARS-CoV-2, regardless of the symptoms (26).

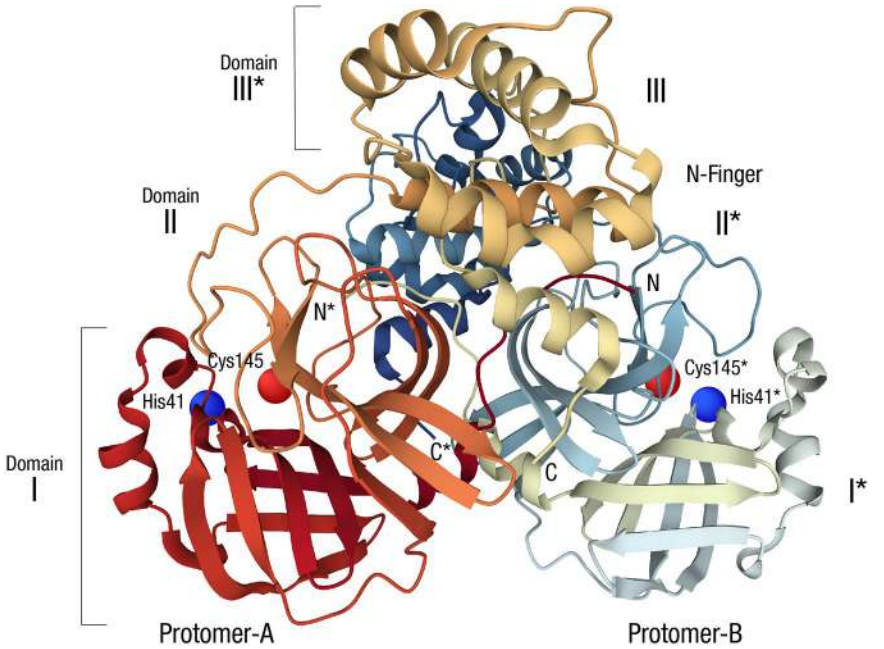

Figure 2. 3-D structure of SARS-CoV-2 $3CL^{pro}$ (M^{pro}) dimer with catalytic residues (PDB ID:6M2Q]. Protomer-A is depicted in ORANGE and GOLD with Domains (I, II & III) and carboxy (C) & amino (N) termini. Protomer-B is shown in SILVER and LIGHT BLUE with Domains (I*, II* & III*) and C* & N* termini. The dimerization of the enzyme is necessary for catalytic activity. The amino acid residues of the catalytic dyad, histidine (His_{41}: INDIGO circle) and cysteine (Cys_{145}: RED circle) are shown for each of the Protomer. The tight dimer formed by SARS-CoV-2 $3CL^{pro}$ has a contact interface, predominantly between domain II of Protomer-A and the NH_2-terminal residues ("N-finger") of Protomer-B, where both molecules are oriented perpendicular to one another.

Redox modulation of M^{pro} enzyme

The unique catalytic dyad of M^{pro} enzyme natively adopts to a zwitterionic reactive form in which Cys_{145} is in the negatively charged thiolate state and His_{41} is doubly protonated and positively charged residue (27). The hydrophilic protein structure allows M^{pro} to form hydrogen (H)-bonds with various ligands (28). Metal-conjugated compounds are potent inhibitors of M^{pro} enzyme. Accordingly, mercury-conjugates phenylmercuric acetate (K_i = 0.7 μM), thimerosal (K_i = 2.4 μM), and hexachlorophene (K_i = 13.7 μM) effectively inhibit SARS-CoV M^{pro} activity. Also, zinc (Zn^{2+})-conjugate, *1-hydroxypyridine-2-thione zinc* (K_i=0.17 μM) has a pronounced anti-M^{pro} effect than the Zn^{2+} ion alone (K_i = 1.1 μM) (29). The individual monomers of SARS-CoV M^{pro} are enzymatically inactive, and the inhibitors against this viral enzyme fall under two categories: (i) inhibitors that target the substrate binding pocket to block the catalytic activity (30), and (ii) an alternative potential therapeutic strategy to inhibit the dimerization of M^{pro} protein (31).

Anti-M^{pro} interventions during the 2002-SARS outbreak

Phenolic extracts of Chinese herb *Isatis indigotica* were frequently used as antiviral agents during the SARS outbreak in 2002. Several bioactive phytonutrients from *I. indigotica* root (*Radix isatidis*) have been identified as promiscuous chymotrypsin inhibitors (32). In later studies, *Sinigrin* (IC_{50}: 217 μM), *β-sitosterol* (IC_{50}: 1210 μM), and *Indigo* (IC_{50}: 752 μM) from *I. indigotica* root extracts were shown to inhibit the cleavage activities of SARS-CoV M^{pro} in a dose-dependent manner (33). Two phenolic compounds, *Aloe-emodin* (IC_{50}: 132 μM) and *Hesperetin* (IC_{50}: 60 μM) were also identified as anti-M^{pro} agents in cell-free and cell-based assays (33). *Sinigrin* is a powerful antioxidant that appears to modulate quinine reductase and glutathione S-transferase, the human redox enzymes (34). *Lycorine* extracted from Chinese medical herb *Lycoris radiata* also demonstrated potent anti-SARS-CoV activity (IC_{50}=15.7 nM) (35). Furthermore, two natural polyphenols from green tea, i.e. *Tannic acid* (IC_{50}= 3 μM) and *3-Isotheaflavin-3-gallate* (IC_{50}= 7 μM) strongly inhibited SARS-CoV M^{pro} (36). Alkylated *Chalcone-6*, isolated from the traditional herb, *Angelica keiskei* also showed strong inhibitory activity (IC_{50}= 11.4 μM) against SARS-CoV M^{pro} (37).

Biflavonoids with stable physico-chemical properties and optimal pharmaco-kinetics are potential anti-CoV candidates; wherein, M^{pro} is a preferred antiviral target for clinical management of SARS-CoV infections (38). Biflavonoids extracted from the *Torreya nucifera* leaves show strong inhibitory activity against SARS-CoV M^{pro} (62% at 100 μg/mL). Among these extracts, *Amentoflavone* was the first biflavonoid to elicit potent anti-M^{pro} activity (IC_{50}= 8.3 μM). Other biflavonoids *Luteolin* (IC_{50}=20.2 μM), *Quercetin* (IC_{50}=23.8 μM), and *Apigenin* (IC_{50}=280.8 μM) demonstrated moderate anti-M^{pro} effect (39). These studies identified that the benzene ring moiety at C3′ position of flavones is essential for the anti-M^{pro} activity of biflavonoids. Based on its potent antiviral activity against SAR-CoV clinical isolates from patients, a German study suggested the use of *glycyrrhizin*, an active compound of licorice roots, for the clinical management of SARS (40).

Phytonutrient inhibitors of SARS-CoV-2 M^{pro} – antiviral spectrum

The genomic reshuffling, mutagenicity, and high transmission rate of the SARS-CoV-2 pathogen highlights the urgent need for design and development of broad-spectrum antiviral interventions. Antiviral agents that target highly conserved enzymes such as M^{pro} or RdRp of various CoV pathogens provide two advantages: (i) the potential for broad-spectrum antiviral activity, and (ii) reduced risk of mutation-mediated drug resistance.

Screening of plant-derived antiviral compounds

Phytonutrients with medicinal value have been widely used for centuries in global healthcare practices based on their all-natural bio-functional spectrum and minimal side effects (41). Most plant-derived compounds undergo stringent screening protocols to comply with safety, tolerance, and reduced drug-attrition rates. The test protocols follow the Lipinski's 'Rule of Five' (42), the Veber's 'Rule of Filters' to test oral bioavailability of the compounds (43); and established the '*Adsorption, Distribution, Metabolism, Excretion and Toxicity*' (ADMET) physicochemical parameters (44). According to the 'Rule of Five, a compound is orally active if its physio-chemical properties are within safe limits (MW ≤ 500 Da, hydrogen (H)-bond donors ≤ 5, H-bond acceptors ≤ 10, and an octanol–water partition coefficient log $p \leq 5$) (42). Veber's 'Rule of Filters' states that a threshold permeation rate of a compound is prerequisite for its oral bioavailability. Thus, a compound with optimal oral bioavailability should consist of ≤10 rotatable bonds and ≤ 140 $Å^2$ topological polar surface area (12 or fewer H-bond donors and acceptors) (43). Furthermore, any potential plant-derived compounds should be orally bioactive, nontoxic, non-carcinogenic, non-mutagenic, nonirritant and show no adverse effects on the reproductive health.

Several natural compounds with broad-spectrum anti-CoV activity, particularly against M^{pro} enzyme have been identified (45). Natural inhibitors against SARS-CoV-2 M^{pro} are screened from extensive phytonutrient libraries, and the therapeutic efficacy of a potential phytocompound for COVID-19 management is considered after an in-depth scientific scrutiny (46).

SARS-CoV-2 M^{pro} inhibitors from ayurvedic medicinal plants

Ayurvedic system of medicine is widely practiced in the South-East Asia for management of COVID-19 infections; accordingly, we have investigated three potential ayurvedic medicinal plants, *Solanum xantocarpum*, *Glycyrrhiza glabra* (licorice) and *Justica adhatoda* for SARS-CoV-2 M^{pro} inhibitory compounds through *in silico* molecular docking studies. These plants and their derived compounds are commonly used in traditional medicine to treat a broad range of respiratory ailments (47, 48). Since SARS-CoV-2 targets the respiratory system during early phase of infection, it is apparent to repurpose these medical herbs for COVID-19 prophylaxis and therapy.

Protein preparation and active site prediction

Three-dimensional crystal structures of SARS-CoV-2 M^{pro} (PDB ID: 7BQY) were retrieved from RCSB Protein Data Bank (https://www.rcsb.org/). The M^{pro} enzyme is a single-chain peptide that consists of total 306 amino acids at 1.7 Å resolution. Further the active site prediction and 'Prepare Protein' protocol of BIOVIA Discovery Studio 4.5 (DS 4.5) was used for protein preparation at physiological pH 7.4. Water molecules and other hetero-atoms were deducted from the crystal structures.

Molecular docking of ayurvedic phytonutrients

The Indian Medicinal Plants Phytochemistry and Therapeutics (IMPPAT) database (49) was followed to screen and retrieve phytonutrient compounds from the Ayurvedic medicinal herbs. A total of 30 bioactive phytoconstituents, 10 from each plant were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in 2 D SDF format. Geometry minimization and conversion of all retrieved small molecules in 3 D PDB format was performed with DS 4.5.

In molecular docking studies, active phytonutrients from *S. xanthocarpum, G. glabra* and *J. adhatoda* demonstrated significant binding affinity to SARS-CoV-2 M^{pro}. From a total of 10 active phytonutrients identified from the above medicinal plants, three phytonutrients *Solanocapsine, 18 β-glycyrrhetinic acid* and *Vasicoline* demonstrated binding affinity (ΔG) higher than 7.0 kcal/mol with M^{pro}. The *18 β-glycyrrhetinic acid* ($\Delta G = 8.86$ kcal/mol) with the highest binding energy formed conventional and carbon (C)/H- bonds with residues Lys_{137} and Leu_{271}, alkyl and π-alkyl interactions with the residues Leu_{286}, Leu_{272}, Tyr_{239}, Leu_{287} and Met_{276} and few van der Waals interactions with remaining residues (Figure 3A). A recent study showed that glycyrrhizin binding is highly stable not only with the active pocket of M^{pro} (ΔG= −8.7 kcal/mol), but also with the PL^{pro} (ΔG= −7.9 kcal/mol), and the Nucleocapsid (ΔG= −7.9 kcal/mol) enzymes. This compound also interacts with several amino acid residues that are critical to natural substrate binding and functionality of the viral receptors (50).

In our study, *Solanocapsine* ($\Delta G = 8.59$ kcal/mol) showed the second most inhibition of M^{pro} and formed different ligand-protein interactions including the conventional and C/H-bonds with residues Gln_{110}, Thr_{111} and Asn_{151}, as well as alkyl and π-alkyl interactions with Arg_{298} and Tyr_{154} while remaining residues showed van der Waals interactions with the protein receptor (Figure 3B). Finally, *Vasicoline* ($\Delta G = 7.34$ kcal/mol), the third M^{pro} protein inhibitor exhibited C/H-bonds with the residues Gln_{110} and Thr_{111}, π-alkyl interactions with residues Val_{104}, Phe_{294} and Arg_{298}, while a few Van der Waals interactions with remaining residues (Figure 3C). Based on these data and YASARA scores, all three active phytoconstituents from Ayurvedic medicinal plants could be potential inhibitors of M_{pro} protein, with binding energies superior to the native N3 inhibitor of M^{pro} ($\Delta G = 5.41$ kcal/mol).

In our earlier study, Ayurvedic medicinal herbs *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Giloy) and *Ocimum sanctum* (Tulsi) also inhibited SARS-CoV-2 M^{pro} activity. The anti-M^{pro} phytonutrients from these Ayurvedic herbs included: *Withanoside V* (10.32 kcal/mol] and *Somniferine* (9.62 kcal/mol), from *W. somnifera; Tinocordiside* (8.10 kcal/mol) from *T. cordifolia*; *Vicenin* (8.97 kcal/mol), *Isorientin*

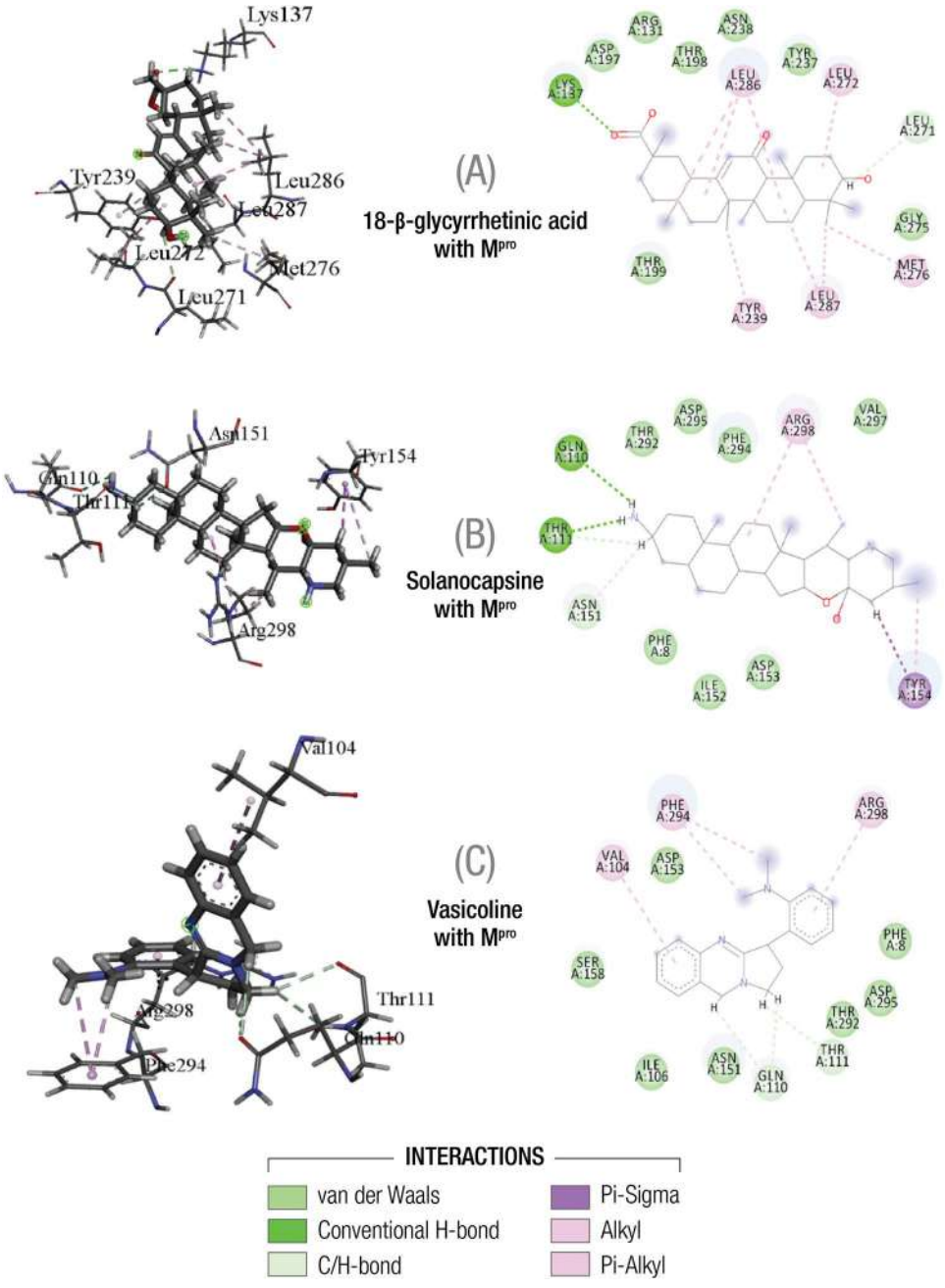

Figure 3. 3 D-2D interaction of SARS-CoV-2 M^{pro} with 18 β-glycyrrhetinic acid (*Glycyrrhiza glabra*), Solanocapsine (*Solanum xantocarpum*), and Vasicoline (*Justica adhatoda*).

4′-O-glucoside 2″-O-p-hydroxybenzoate (8.55 kcal/mol), and *Ursolic acid* (8.52 kcal/mol) from *O. sanctum* (51).

Due to their natural origin and therapeutic benefits, phytonutrients may be considered potent antioxidants capable of neutralizing or scavenging harmful free radicals,

modulate several oxidative stress-mediated signaling pathways and protect cellular systems. Phytonutrients from Cat's claw (*Cadambine* & *Epiafzelechin-4β-8-epicatechin*) and Ayurvedic medicinal herb 'Tulsi' (*Isorientin 4′-O-glucoside*) are potential M^{pro} inhibitors (51, 52), as well as active regulators of COX-mediated inflammatory pathways (53–55)

The Nrf2 (*nuclear factor erythroid 2-related factor 2*), referred to as the 'master regulator' of the antioxidant response. Nrf2 modulates the expression of hundreds of genes, including the most prominent antioxidant enzymes [i.e. peroxiredoxins (PRXs), glutathione peroxidases (GPXs), thioredoxin (TRX), heme oxygenase 1 (HO-1), and catalases (CAT)]; as well as large numbers of genes (i.e. *Keap1*) that control immune and inflammatory responses. The Nrf2/Keap1 signaling pathway mainly regulates anti-inflammatory gene expression, which can be deployed against SARS-CoV-2 (56). Therefore, the identification of new Nrf2-dependent anti-inflammatory phytonutrients has become a key investigative point in drug discovery (57). Anti-M^{pro} phytonutrients such as *Rutin* and *Punicalagin* (58, 59) are promising Nrf2 activators (60, 61).

The SARS-CoV-2 uses *nuclear factor-κB* (NF-κB), caveloae, clathrin, lipoxin, serine protease and proteasome pathways in addition to ACE2 to enter the target cell and initiate damage (62). Natural bioflavonoids from 'Japanese nutmeg-yew' (*Amentoflavone* & *Ginkgetin*) are not only strong anti-M^{pro} agents (63), but also inhibitors of the NF-κB activity through multiple pathways (64, 65).

Other medicinal plants such as *Isatis tinctoria, Torreya nucifera*, *Psoralea corylifolia*, and *Rheum palmatum*. demonstrate potent anti-M^{pro} activity (66). *Baicalein*, a natural compound commonly used in traditional Chinese medicine, is a potent inhibitor of SARS-CoV-2 M^{pro}. In a randomized, double-blind, single-dose clinical trial *(n = 72)*, *Baicalein* (100-2800 mg) was well tolerated in the treatment of acute, or chronic hepatitis (67). The M^{pro} inhibitor NLC-001, a phytonutrient supplement that is currently undergoing a human clinical trial in Israel, is a possible oral intervention against COVID-19 (68).

Flavonoids and anti-M^{pro} activity

Flavonoids are natural phytonutrients widely found in fruits and vegetables. These compounds form polymers (i.e. tannins) through various carbon–carbon and ether linkages. The multifunctional health benefits of flavonoids include antimicrobial, antioxidant, anti-inflammatory, anti-mutagenic, and anti-cancer effects (69). Several flavonoids demonstrate potent antiviral activity, while a few directly inhibit viral proteolytic activities (7). For example, *Baicalin* (IC_{50}: 34.7 µM), *Herbacetin* (IC_{50}: 53.9 µM), and *Pectolinarin* (IC_{50}: 51.6 µM) demonstrate potent inhibitory activity against SARS-CoV-2 M^{pro} (70). Flavonoids, especially in a glycosylated form, effectively inhibit SARS-CoV-2 proteases. Both *Quercetin-3-O-rhamnoside* and *Rutin* demonstrate high-affinity binding to the catalytic dyad (His_{41} and Cys_{145}) of M^{pro} and inhibit the M^{pro} enzyme of SARS-CoV-2 (71). In another study, three flavonols extracted from *Boerhavia diffusa*, i.e. *Biorobin* (-8.17 kcal/mol), *Bioquercetin* (-7.97 kcal/mol) and *Boerhavisterol* (-6.77 kcal/mol) effectively docked to SAR-CoV-2 M^{pro} with optimal binding energies (72). However, based on ADME profiles and Lipinski's rule, only *Boeravisterol* showed effective anti-M^{pro} activity.

Tannins and anti-M^{pro} activity

Tannins are a heterogeneous group of high molecular weight, water-soluble, polyphenolic compounds, naturally present in cereals, leguminous seeds, and predominantly in several fruits and vegetables, where they appear to provide protection against a wide range of biotic and abiotic stressors. Tannins are considered as potent antimicrobial agents against viruses, bacteria, and fungi (73). These compounds also demonstrate antioxidant and free radical scavenging activities, which may help avert several oxidative stress-related metabolic disorders (74).

In-silico studies identified several tannin hydrolysates such as *Pedunculagin*, *Tercatain*, and *Castalin* to interact with the receptor-binding domain and catalytic dyad (His_{41} and Cys_{145}) of SARS-CoV-2 M^{pro} through H-bonding forces (75). Among these tannins, *Pedunculagin* bound to the catalytic dyad with high-affinity, excellent docking score, and ADMET profile. *Pedunculagin*, *Tercatain*, and *Castalin* are commonly found in pomegranates, walnut, Indian gooseberry, oak wood, leaves of *Melaleuca quinquenervia, Terminalia catappa*, and *Combretum glutinosum* (76).

Natural phytonutrients, *Pentagalloylglucose* (PGG), and *Epigallocatechin-3-gallate* (EGCG) demonstrate potent inhibition of SARS-CoV-2 M^{pro}. In molecular docking studies, both PGG and EGCG showed avid interaction with the receptor-binding pocket of SARS-CoV-2 M^{pro}, forming H-bonds with multiple residues, including the catalytic dyad (His_{41} and Cys_{145}) (77). An earlier study, similar binding profiles for PGG and EGCG with SARS-CoV-2 M^{pro} were observed and the inhibitory activities were contingent upon their molecular accessibility to the receptor-binding site and interaction with residues in the catalytic dyad (78). Overall, PGG showed a greater inhibition of SARS-CoV-2 than EGCG. PGG is a plant-based hydrolyzable tannin, credited with anti-inflammatory and antiviral activities that could directly block viral attachment to host cells, inhibit viral gene expression and protein translation (79). Besides anti-SARS-CoV-2 M^{pro} activity of the PGG, its derivative *Tetra-O-galloyl-beta-D-glucose (TGG)* also exhibits a prominent anti-SARS-CoV M^{pro} activity (IC_{50} value: 4.5 µM) (80). EGCG is the most abundant ester of catechin and gallic acid in green tea. EGCG is a broad-spectrum antioxidant, anti-inflammatory, antibacterial, and antiviral agent (81), with a potent inhibitory activity against the NS3 serine protease of hepatitis C virus (82). The anti-SARS-CoV-2 M^{pro} activity of EGCG and *Theaflavin* had also been reported, with IC_{50} values of 7.58 and 8.44 µg/mL, respectively (83).

Quercetin and anti-M^{pro} activity

Quercetin is a naturally occurring plant flavanol from the flavonoid group of polyphenols. These compounds are ubiquitous in foods, including vegetables such as onions, garlic, and ginger; fruit such as apples; and in tea and wine. *Quercetin* is a powerful scavenger of free radicals (i.e. ROS and RNS), hence classified as a redox nutraceutical known to reduce oxidative stress and inflammation. *Quercetin* inhibits NF-κB activation and down-regulates cytokine production *via* this transcription factor (84).

Quercetin and its derivatives inhibit proteases of other CoVs including SARS-CoV (M^{pro} and PL^{pro}) that share 97% homology with M^{pro} of COVID-19 (85), as well as the M^{pro} of MERS-CoV (86). *Quercetin* inhibits both proteases at micromolar doses

in vitro (87); where, SARS-CoV and MERS-CoV share 82.45% and 69.58% genomic similarity with SARS-CoV-2 (88). *Quercetin* also inhibits cellular entry of SARS-CoV-2 by blocking the host ACE2 receptor; and in earlier studies this flavanol also reduced interleukin (IL)-6 levels in SARS and MERS patients (80, 89). Also, *Quercetin* inhibits both the SARS-CoV-2 proteases, M^{pro} and PL^{pro}, with a docking binding energy corresponding to −6.25 and −4.62 kcal/mol, respectively. Thus, *Quercetin* could strongly interfere with SARS-CoV-2 replication (90, 91). *Quercetin* also binds to the 'receptor-binding domain (RBD)' of 'spike' (S)-protein, which suggests its viral receptor blocking activity, alongside the virion neutralizing effect on SARS-CoV-2 (92). Based on well-documented pharmacokinetic and ADMET profiles, *Quercetin* could serve as a promising redox nutraceutical for COVID-19 management.

Quercetin as redox phytonutrient

Quercetin demonstrates a unique redox-based mechanism to inhibit M^{pro} enzyme activity. The monomeric M^{pro} is inactive and becomes functional in the homodimer state (93). Highly specific amino acid residues Arg_4, Ser_{10}, Gly_{11}, Glu_{14}, Asn_{28}, Ser_{139}, Phe_{140}, Ser_{147}, Glu_{290}, and Arg_{298} are involved in M^{pro} protein dimerization (94, 95). Based on the pH milieu, M^{pro} could form dimers, tetramers, or even highly active octamers (28). *Quercetin* inhibits the SARS-CoV-2 M^{pro} (Inhibition constant K_i: ~7 µM) by destabilizing and altering thermostability of the viral enzyme. Its interaction with M^{pro} measures at a dissociation constant (K_D) of 2.7 µM and 10 µM, in the absence and presence (150 mM) NaCl, respectively. The low-affinity interactions at high ionic strength indicates that *Quercetin* interaction with M^{pro} is coupled to the release of salt ions (96). Therefore, *Quercetin* is a promising scaffold to engineer new functional groups for the development of novel inhibitor compounds against SARS-CoV-2 M^{pro} (Figure 4).

Several *Quercetin*-derivatives have been developed as potent anti-M^{pro} agents during the 2002-SARS outbreak. The interaction of such derivatives with the M^{pro} are influenced by the 7-hydroxy group on the *Quercetin* moiety and the acetoxylation of its sugar moiety (97). Accordingly, *Quercetin-3-β-galactoside* was identified as an inhibitor of SARS-CoV M^{pro} enzyme. Recent molecular dynamics simulation studies have confirmed that *Quercetin-3-D-xyloside* (-9.1 kcal/mol), and *Quercetin 3-O-α-L-arabinopyranoside* (-9.0 kcal/mol) are promising candidates for SARS-CoV-2 M^{pro} inhibition, where both molecules show desirable properties with regards to stability, flexibility and binding energy (59).

Numerous studies have reported synthetic competitive drug inhibitors against M^{pro} enzyme; however, increased dosages often reduce their effectiveness. On the other hand, allosteric inhibition by natural phytonutrients could provide safe and effective intervention strategy by alleviating this limitation. Quercetin is an effective allosteric inhibitor against SARS-CoV-2 M^{pro}, and molecular docking studies demonstrated consistent binding of this natural flavonoid at a domain other than the active site of M^{pro} enzyme (ΔG= − 8.31 kcal/mol) forming 6 H-bonds with residues $Q_{127}/C_{128}/K_{137}/D_{289}/E_{290}$ (99).. Also *Quercetin-3-rutinoside-7-glucoside* (ΔG= −12.27 kcal/mol; H-bonds: T_{24}-$T_{26}/S_{46}/N_{142}/E_{166}/H_{164}$) (Figure 4), and *Rutin* (ΔG= −10.16 kcal/mol; H-bonds: $T_{24}/T_{25}/N_{142}/G_{143}/H_{164}$) phytocompounds derived from *W. somnifera* demonstrate superior

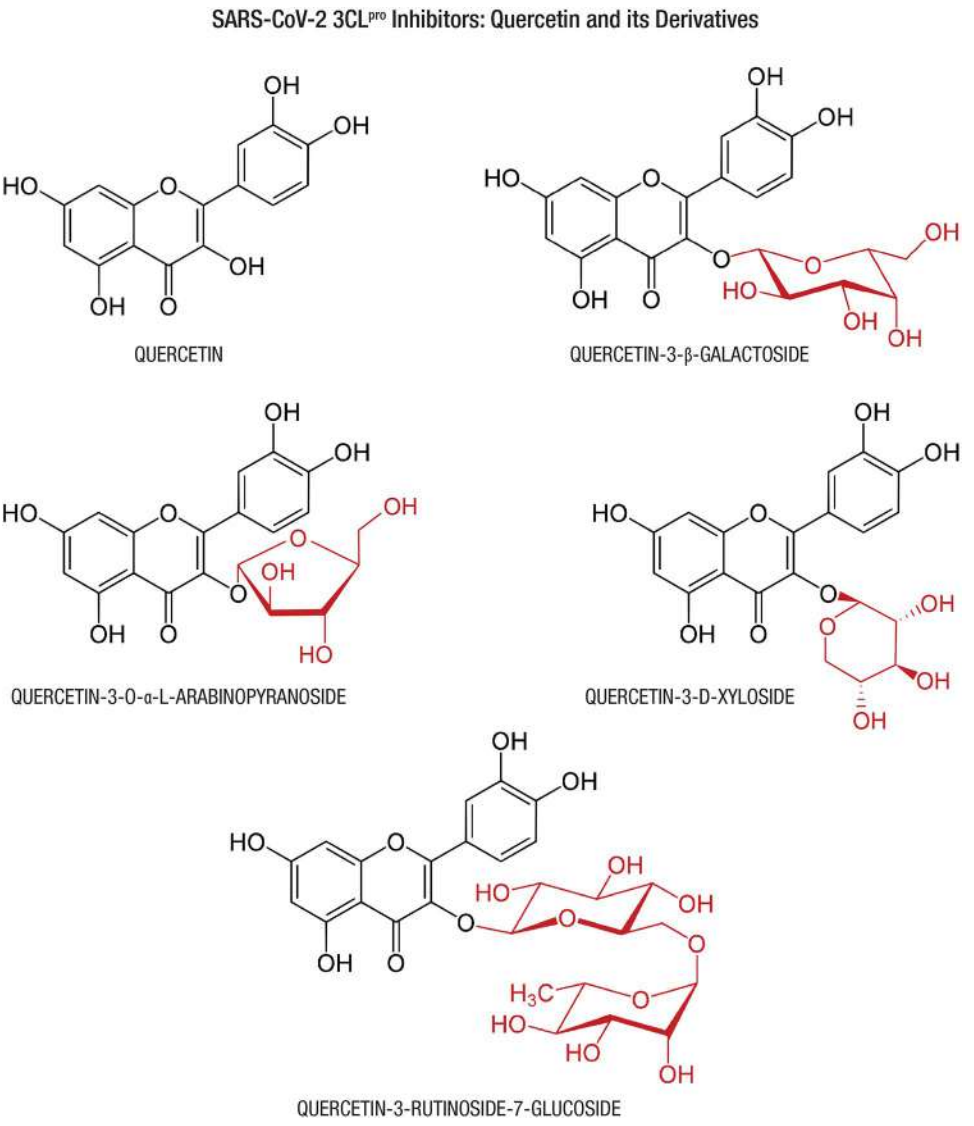

Figure 4. Quercetin-derivatives are potent anti-M^{pro} agents and their interaction with viral main protease is influenced by the '7-OH group' on the Quercetin moiety and the 'acetoxylation' of its sugar moiety (97). Accordingly, Quercetin-3-β-galactoside, Quercetin 3-O-α-L-arabinopyranoside, and Quercetin-3- D-xyloside, are effective M^{pro} inhibitors (59). Quercetin-3-rutinoside-7-glucoside demonstrated superior SARS-CoV-2 M^{pro} binding with more H-bonds and higher inhibitory potential than the standard antiviral drugs nelfinavir and lopinavir (98).

M^{pro} binding with more H-bonds and higher inhibitory potential than the standard antiviral drugs nelfinavir (ΔG= −6.16 kcal/mol; H-bonds: N_{142}/H_{41}) and lopinavir (ΔG= −5.33 kcal/mol; H-bonds: C_{145}/E_{166}) (98). In recent molecular docking studies, *Quercetin-3-rhamnoside* (ΔG= −10.90 kcal/mol with drug-likeness rating, 0.82) and the anti-HIV protease inhibitor drug, darunavir (ΔG= −10.25 kcal/mol with drug-likeness rating, 0.60), demonstrated avid binding to SARS-CoV-2-M^{pro} enzyme (100). The

combined *Quercetin* + darunavir docking complex interacted with eight strong H-bonds with stability, and higher binding energy compared to individual compounds against the SARS-CoV-2-M^{pro}. Therefore, a combination therapy of *Quercetin* with the anti-HIV-drug has been proposed for COVID-19 intervention (100).

Anti-M^{pro} spectrum of phytonutrients compared to antiviral drugs

In SARS-Cov-2-infected patients, elevated oxidative stress, exacerbated proinflammatory cytokines release ('cytokine storm') and loss of T lymphocytes ('leukopenia') characterize the most aggressive presentation of COVID-19 (101). SARS-Cov-2 infection causes local and systemic inflammation mediated by pro-inflammatory cytokines and cyclo-oxygenase (COX)-mediated eicosanoid products with metabolic dysfunction and tissue damage. SARS-CoV-2 also stimulates secretion of TNF, IL-6 and other cytokines, a pro-inflammatory response that could lead to cytokine storm (102). Treatment of COVID-19 symptoms with nonsteroidal anti-inflammatory drugs (NSAIDs) pose several risks. NSAIDs inhibit the enzymes COX-1 and COX-2, which are critical for the generation of prostaglandins (PGs) and thromboxanes (TXs) with diverse roles in homeostasis and inflammation. Inhibition of PG synthesis by NSAIDs; therefore, has many adverse implications on COVID-19 pathogenesis. NSAID treatment could reduce both the antibody and pro-inflammatory cytokine response to SARS-CoV-2 infection. It also raises the possibility that NSAIDs may alter the immune response to SARS-CoV-2 vaccination (103). The conventional NSAIDs are associated with gastric, cardiac, and renal side-effects. Targeting the viral genome and/or gene-encoded viral enzymes such as M^{pro}, RdRp and helicases are plausible antiviral approaches to prevent replication and propagation of the SARS-CoV-2 infection. Interestingly, several potential phyto-nutrient inhibitors of SARS-CoV-2 M^{pro} enzyme also show strong antioxidant and free radical scavenging effects, as well as anti-inflammatory activity, which could be a tremendous supplemental health advantage (Table 2).

Natural compounds such as flavonoids are potential inhibitors of SARS-CoV-2 with comparable or higher potency as that of antiviral drug, remdesivir. The interaction of *Rutin* (~106 M-1) with the substrate-binding pocket of M^{pro} of SARS-CoV-2 is much higher than chloroquine (~103 M-1) and hydroxychloroquine (~104 M-1), and the reference antiviral drug remdesivir (~105 M-1) (129). Bioactive alkaloids and terpenoids derived from plants of African origin were also reported as potential inhibitors of SARS-CoV-2 M^{pro} (130). Four plant-derived alkaloids (*10-Hydroxyusambarensine*, and *Cryptoquindoline*) and terpenoids (*6-Oxoisoiguesterin* and *22-Hydroxyhopan-3-one*), showed avid binding to the receptor-binding site and catalytic dyad of SARS-CoV-2 M^{pro} based on predictive ADME/tox and Lipinski filter analysis. The docking scores of these compounds were comparable with M^{pro}-referenced inhibitors, lopinavir and ritonavir. Phytonutrients, *Silybin* B ($\Delta G = -10.96$ kcal/mol), a standardized extract of milk thistle seeds, and *Cianidanol* ($\Delta G = -9.52$ kcal/mol), an antioxidant flavonoid, demonstrated better binding and ADME profile compared to the antiviral drugs, hydroxychloroquine and lopinavir. Following amino acid residues from the M^{pro} protein structure: Met_6-Ala_7-Phe_8-Pro_9, Asp_{295}, Gly_{302}, Val_{303} and Thr_{304} seem to participate in protein-ligand interactions *via* H-bonds and Vander Waals forces (131).

Table 2. Phytonutrient inhibitors of SARS-CoV-2 M^{pro} enzyme and their supplemental health attributes.

Phytonutrient inhibitor	PubChem CID	ΔG (kcal/mol)	Supplemental Health Attributes
Plant-based natural compounds as M^{pro} Inhibitors (59)			
Peonidin 3-O-glucoside	443654	−9.4	Anti-inflammatory via TNF-α signaling (104).
Kaempferol 3-O-β-rutinoside	25201364	−9.3	Anti-inflammatory via NF-κB/MAPK pathways (105).
Rutin	52808	−9.2	Lowers oxidative stress via Nrf2 signaling pathway (60).
Quercetin-3-D-xyloside	5320863	−9.1	Antioxidant & potent free r adical scavenger (59).
Quercetin-3-O-α-L-arabinopyranoside	329766687	−9.0	AMP-activated protein kinase (AMPK) activity (106).
Kaempferol 3-rutinoside 4′glucoside	329824889	−8.9	Anti-inflammatory, analgesic & antipyretic activities (107).
Apigenin 7-O-neohesperidoside	24891380	−8.9	Free radical scavenger & antibacterial agent (108).
Quercetin 3-O-β-D-glucoside	329751366	−8.8	Antithrombotic agent with anticoagulant activity (109).
Idaein	44256700	−8.8	Inhibits epidermal growth-factor receptor (110).
Callistephin	44256621	−8.8	Neuroprotectant that reduces nitrosative stress (111).
Malvin	41765	−8.7	Neutralize hydroxyl and NO• radicals (112).
Luteolin 7-rutinoside	44258082	−8.6	Free radical scavenging & antimutagenic activity (113).
Cyanin	441688	−8.5	Blocks renin-angiotensin system (114).
Apiin	5280746	−8.5	Inhibits iNOS expression & nitrite production (115).
Mearnsitrin	6918652	−8.5	Inhibits aldose reductase & α-glucosidase (116).
Hispidulin 7-glucuronide	44258434	−8.5	Potent antioxidant activity (117).
N3 Inhibitor (Reference)	146025593	*−7.9*	*Native co-crystal ligand*
Isatin-derivative as Mpro inhibitor (118)			
Ursodeoxycholic acid	31401	−9.4	Inhibits endothelin-1 production in vascular endothelia (119).
Hydroxychloroquine (Reference)	3652	*−7.4*	*Anti-malarial drug*
Chloroquine (Reference)	2719	*−7.0*	*Anti-malarial drug*
Favipiravir (Reference)	492405	*−4.7*	*Antiviral drug*
***Uncaria tomentosa* (Cat's Claw)-derived M^{pro} Inhibitors** (52)			
Cadambine	21723831	−8.6	Inhibits inflammatory mediators: COX-2, IL-1β, and TNF-α (55).
Proanthocyanidin B2	130556	−9.2	Inhibits brain inflammation, reduces astrocytosis & gliosis (120).
Epiafzelechin-4β-8-epicatechin	16131425	−8.9	Anti-inflammatory with COX-1 inhibitory activity (53).
Proanthocyanidin B4		−9.2	Prevents DNA fragmentation & inhibits apoptosis (121).
Proanthocyanidin C1	169853	−8.8	Anti-HIV activity (122).
N3 Inhibitor (Reference)	146025593	*−8.1*	*Native co-crystal ligand*
Lopinavir (Reference)	92727	*−8.0*	*Antiviral drug*
***Torreya nucifera* (Japanese nutmeg-yew)-derived bioflavonoid inhibitors** (63)			
Amentoflavone	5281600	−9.2	NOS inhibitor, blocks NF-κB activation (65).

(*Continued*)

Table 2. (Continued)

Phytonutrient inhibitor	PubChem CID	ΔG (kcal/mol)	Supplemental Health Attributes
Bilobetin	5315459	−9.1	Inhibits IGF-1-induced sebum production (123).
Ginkgetin	5271805	−9.0	Modulates NF-κB/p53 signaling pathway (64).
N3 Inhibitor (Reference)	146025593	*−7.0*	*Native co-crystal ligand*
Lopinavir (Reference)	92727	*−7.3*	*Antiviral drug*
***Prunica granatum* (Pomegranate)-derived tannin inhibitors** (58)			
Punicalagin	44584733	−9.0	Activates Keap1-Nrf2 antioxidant defense system (61).
Punicalin	5388496	−8.6	Suppresses ROS/NLRP3 pathway (124).
N3 Inhibitor (Reference)	146025593	*−5.6*	*Native co-crystal ligand*
Dexamethasone (Reference)	5743	*−7.7*	*Antiviral drug*
***Withania somnifera* (Ashwagandha) / *Ocimum sanctum* (Tulsi)-based inhibitors** (51)			
Withanoside V	10700345	10.3	Attenuates Aβ-induced neurodegeneration (125).
Somniferine	14106343	9.6	Antibacterial *via* penicillin-binding protein-4 (126).
Vicenin	3084407	9.0	Inhibits NF-κB activation, TNF-α and IL-6 synthesis (127).
Isorientin 4′-O-glucoside	44257986	8.6	Selective inhibitor of COX-2 pathway (54).
Ursolic acid	64945	8.5	Modulates cell-mediated immune responses (128).
N3 Inhibitor (Reference)	146025593	*8.5*	*Native co-crystal ligand*

Binding Energy (ΔG: kcal/mol) represents the inhibitory activity of the phytonutrient against SARS-CoV-2 M^{pro}. The cutoff ΔG value was set at -8.5 kcal/mol or above.

A select group of phytonutrients (with binding energies): *18-Hydroxy-3-epi-α-yohimbine* (−8.1 kcal/mol), *Alloyohimbine* (−8.0 kcal/mol), *Gummadiol* (−7.8 kcal/mol), *Asparagamine A* (−7.6 kcal/mol), and *Vincapusine* (−7.5 kcal/mol); were tested for inhibitory activity against three CoV enzyme targets, i.e. SARS-CoV-2-M^{pro}, SARS-CoV-M^{pro} and MERS-CoV-M^{pro} and compared with prominent FDA-approved antiviral drugs (with binding energies): *simeprevir* (−9.7 kcal/mol), *ledipasvir* (−9.3 kcal/mol), *paritaprevir* (−9.3 kcal/mol), *glecaprevir* (−9.3 kcal/mol), and *daclatasvir* (−9.2 kcal/mol) (132). All the tested phytonutrients and antiviral drugs showed stable interactions with M^{pro} target through a good number of H-bonds as well as hydrophobic interactions except for *Asparagamine A* which exhibited only hydrophobic interactions. Two phytonutrients, *Gummadiol* (from the heartwood of *Gmelina arborea*) and *Vincapusine* (from *Vinca pusilla)*, as well as the drug *Glecaprevir*, showed a potent antiviral activity *via* H-bond interactions with either His_{41} or Cys_{145} residues in the catalytic dyad of SARS-CoV-2 M^{pro} enzyme.

Phytonutrients used in the traditional Chinese medicine, *5,7,3′,4′-tetrahydroxy-2′-(3,3-dim ethylallyl) isoflavone* from *Psorothamnus arborescens* showed high-affinity interaction with the receptor-binding residues of M^{pro}. This isoflavone displayed higher docking score, stronger binding energy, and closer interactions with the conserved catalytic dyad residues (Cys_{145} and His_{41}) of M^{pro} than antiviral drugs nelfinavir, prulifloxacin and colistin (133).

The emerging evidence from several model systems suggests potential applications of an array of phytonutrients that exhibit antiviral properties. These properties are important to understand in the context of the current SARS-CoV-2 pandemic, and the regulatory guidelines for drug evaluation and research (https://www.fda.gov/about-fda/fda-organization/center-drug-evaluation-and-research-cder). Within the United States, drugs are defined as "articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals" [21 U.S. Code § 321(g)]. To comply with this statute, drug producers must follow established protocols intended to evaluate the toxicology and clinical safety of the substance. Templates for these protocols are provided through the International Conference on Harmonization (ICH; https://www.ich.org/) and the Organization for Economic Co-operation and Development (OECD; https://www.oecd.org/) guidelines for toxicological testing of chemicals. In addition, the producers must submit an Investigational New Drug Application (IND) and provide an extensive dossier on the chemical manufacturing and controls (CMC) of the substance according to FDA guidelines (https://www.fda.gov/vaccines-blood-biologics/general-biologics-guidances/cmc-and-gmp-guidances). It is important to note that within the United States, there is a drug exclusion statute that states that once a substance is the active ingredient of an approved new drug — or the active ingredient of a new drug in clinical trials that have been made public — a food containing that substance cannot be shipped in interstate commerce [21 U.S.C. § 331 (ll)]. Therefore, a drug cannot be a component of the food supply within the United States.

Conclusions

Several existing antiviral drugs are currently being repurposed worldwide to treat COVID-19 patients. Recent emergence of more transmissible variants of SARS-CoV-2 raises the high possibility that this viral pathogen could accumulate adaptive mutations that modulate drug susceptibility and hamper viral antigenicity. Thus, it is critical to predict potential non-synonymous mutation sites and estimate the progression of protein structural modifications that may lead to drug tolerance. Two FDA-approved antiviral drugs, boceprevir, and telaprevir, could effectively inhibit SARS-CoV-2 M^{pro}; however, several mutants exhibited reduced binding affinity to these drugs, out of which hotspot residues having a strong tendency to undergo positive selection (134).

The SARS-CoV-2 M^{pro} is an attractive target for antiviral therapy; however, most of the M^{pro} inhibitors are peptidomimetic and bind to the active-site cysteine *via* a covalent adduct, which is unfavorable from a pharmacokinetic standpoint (135). Therefore, there is a clinical need for direct-acting antivirals targeting SARS-CoV-2, to complement current therapeutic strategies. The phytochemical M^{pro} inhibitors with their stable phenolic ring structures could more effectively target the large surface area of viral enzyme dimers (38, 39). These natural plant-based inhibitors have additional advantages over classic antiviral drugs due to their greater chemical diversity, high specificity, low toxicity, possibility of rational design, low accumulation in tissues, and stability toward proteolytic cleavage. However, the regulatory hurdles, including classic toxicology studies and clinical trials, remain to be executed.

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