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### Article

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# Amentoflavone, New Hope against SARS-CoV-2: An Outlook through its Scientific Records and an *in silico* Study

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## ABSTRACT

**Background:** The plant-derived bioflavonoid amentoflavone has many important biological activities, among them remarkable antiviral effects, even against severe acute respiratory syndrome Coronavirus (SARS-CoV). It inhibits severe acute respiratory syndrome coronavirus (SARS-CoV) with an  $IC_{50}$  value of 8.3  $\mu$ M. TMPRSS-2 activity is now thought to be the only factor necessary for cell entry and viral pathogenesis). In comparison, 3CL<sup>PRO</sup> is needed for COVID-19 replication and maturation during its life cycle. **Aim:** This study aims to perform an *in silico* study on amentoflavone activity against structural and non-structural severe acute respiratory syndrome coronavirus (SARS-CoV)-2 3-chymotrypsin-like protease (3CL<sup>PRO</sup>) and human transmembrane protease serine 2 (TMPRSS-2) proteins. **Materials and Methods:** Molecular docking studies were carried out using compounds against 3CL<sup>PRO</sup> and TMPRSS-2 proteins through the Swiss model, Uniport, PROCHECK, Swiss PDB viewer, PyMol, PyRx, and Desmond (Schrödinger package) computerized software. **Results:** Amentoflavone showed strong interactions -9.5 and -7.4 kcal/mol with 3CL<sup>PRO</sup> and TMPRSS2 proteins, respectively. In any case, it had higher binding affinities than currently approved antiviral drugs, which are underutilized in coronavirus disease (COVID-19). **Conclusion:** Amentoflavone may be one of the potential leads (drug candidate) to fight human coronavirus, including SARS-CoV-2. Further *in vivo* studies are needed to support the findings of this study.

**Key words:** Phenolic compounds, Amentoflavone, Antiviral potential, SARS-CoV-2, COVID-19, Molecular docking, 3CL<sup>PRO</sup>, TMPRSS-2.

## INTRODUCTION

Amentoflavone (C<sub>30</sub>H<sub>18</sub>O<sub>10</sub>) is a bioflavonoid naturally occurring in more than 120 plants throughout the world.<sup>[1]</sup> It has many important biological effects, mostly acting as an antioxidant,<sup>[2]</sup> anti-inflammatory,<sup>[3]</sup> anticancer,<sup>[4]</sup> anti-senescence,<sup>[5]</sup> antibacterial,<sup>[6,7]</sup> antifungal,<sup>[8]</sup> antiviral,<sup>[9]</sup> neuroprotective,<sup>[10]</sup> cardioprotective,<sup>[11]</sup> antidiabetic,<sup>[12]</sup> and so on.

Scientific reports suggest that amentoflavone is evident to act against dengue,<sup>[9]</sup> where it has been found to inhibit the viral NS5 RNA-dependent RNA polymerase (RdRp). It has also activity against coxsackievirus B3 (CVB3),<sup>[13]</sup> human immunodeficiency virus (HIV),<sup>[14]</sup> respiratory syncytial virus (RSV),<sup>[15]</sup> herpes simplex virus 1 (HSV-1) and acyclovir (ACV)-resistant strains (e.g., HSV-1/106, HSV-1/153, and HSV-1/Blue).<sup>[16]</sup> The amentoflavone bioavailability after intravenous administration is >77% in a rat model.<sup>[17]</sup> The plasma half-life (t<sub>1/2</sub>) and maximum plasma concentration (T<sub>max</sub>) of amentoflavone following oral administra-

tion were 2.06-3.34 h and 1.13-4.00 h, respectively, in normal rats.<sup>[18]</sup> However, it is evident to interact with CYP3A4 and CYP2C9, thereby reducing the metabolism of some drugs in our body.<sup>[19]</sup>

Coronaviruses (CoVs) are RNA viruses with medical and veterinary importance.<sup>[20,21]</sup> The 3CL<sup>PRO</sup> has gained much attention for the discovery, development, and design of new drugs for SARS-CoVs as a valuable target. It is also termed as 'the Achilles heel' of coronaviruses.<sup>[22,23]</sup> Generally, human coronaviruses (HCoVs) are single-stranded and positive-sense (length: 30,000 bp) RNA viruses, containing two types of proteins: (i) Structural proteins (e.g., Spike (S), Nucleocapsid (N), Matrix (M), and Envelope (E) and (ii) Non-structural proteins (e.g., 3CL<sup>PRO</sup>, Papain-like protease (PL<sup>PRO</sup>) and RNA-dependent RNA polymerase (RdRp).<sup>[20]</sup> The CoV polyprotein encodes two proteases, responsible for translating the non-structural

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proteins (nsps).<sup>[24]</sup> Briefly, the S protein, present on the outer surface of the virion, helps to attach and entry of the virus-cell into host cells.<sup>[25,26]</sup> On the other hand, RdRp, an important viral enzyme that is required in the RNA viruses' life cycle.<sup>[27]</sup>

The nsp12 polymerase binds to the cofactors, nsp7 and nsp8, which helps to replicate and transcribe the viral genome.<sup>[28]</sup> PL<sup>PRO</sup> cleaves the nsp1/2, nsp2/3, and nsp3/4 borderlines and works with 3CL<sup>PRO</sup> to cleave the polyproteins into naps.<sup>[29]</sup> The nsp13 (helicase) catalyzes the loosen of duplex oligonucleotides into single strands in a nucleoside 5'-triphosphate (NTP)-dependent manner, and, thus, can be considered as a target to develop antiviral drugs.<sup>[30]</sup> On the other hand, the nsp14 (N-terminal exoribonuclease and C-terminal guanine-N7 methyltransferase) of CoV is also crucial for HCoV's replication and transcription,<sup>[31]</sup> while nsp15 (uridylyte-specific endoribonuclease) forms a hexameric endoribonuclease that preferentially cleaves 3' of uridines, also named as uridylyte-specific endoribonuclease. The later one is one of the RNA-processing enzymes encoded by the CoV,<sup>[32]</sup> while nsp16(2'-O-methyltransferase) is an S-adenosyl methionine (SAM) dependent on the nucleoside-2'-O methyltransferase. The nsp16 is only activated following nsp10 binding.<sup>[33]</sup> Thensp10 is also an essential co-factor which forms complexes with nsp14 and nsp16.<sup>[34]</sup>

Thus, the development of new drugs for the CoVs focuses on two main strategies: (a) Blocking virus cell entry into the host cells, and (b) Halt transcription and replication of virus-cell inside the host. Therefore, the S protein, 3CL<sup>PRO</sup>, PL<sup>PRO</sup>, and nsps may be attractive targets for anti-SARS-CoV drug design. Besides, human angiotensin-converting enzyme 2 receptor (hACE2R), the calcineurin nuclear factor of activated T-cells (calcineurin-NFAT), Abelson murine leukemia viral oncogene homolog 1 (ABL1), and transmembrane protease serine (TMPRSS)-2 and -4 are also some other mentionable target proteins in anti-CoV drug discovery and development.<sup>[35,36]</sup>

According to recent data, SARS-CoV-2 reaches cells via the hACE2 receptor, which acts in tandem with the host's TMPRSS-2.<sup>[37]</sup> TMPRSS-2, in particular, cleaves the viral S glycoprotein, promoting viral activation and acting as one of the primary host factors for SARS-CoV-2 pathogenicity.<sup>[38]</sup> TMPRSS-2 activity is now thought to be the only factor necessary for cell entry and viral pathogenesis.<sup>[39]</sup> In comparison, 3CL<sup>PRO</sup> is needed for COVID-19 replication and maturation during its life cycle.<sup>[40]</sup> Additionally, nature is the best resource of lead compounds. Among them, flavonoids and phenolic compounds are a more potent class compound found in nature. A bioflavonoid, amentoflavone inhibited SARS-CoV with an IC<sub>50</sub> value of 8.3 μM,<sup>[41]</sup> possibly through inhibition of 3CL<sup>PRO</sup>. Furthermore, FDA-approved antiviral drugs Camostat mesylate used in the clinical trial against COVID-19.

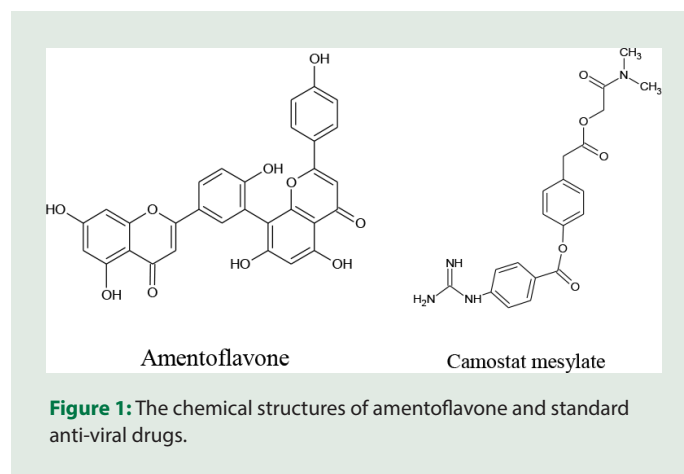
In this sense, this study aimed to address the *in silico* potential of amentoflavone against 3CL<sup>PRO</sup> and TMPRSS-2 proteins. Additionally, some host proteins interacting with HCoV-2 were also taken into account.

## MATERIALS AND METHODS

### Homology model and Macromolecule preparation

Homology modeling of 3CL<sup>PRO</sup> and TMPRSS-2 was performed by Swiss-model.<sup>[42]</sup> Before modeling, the sequence was collected from UniProt,<sup>[43]</sup> followed by BLAST Analysis using NCBI BLAST,<sup>[44]</sup> program to choose the template. PROCHECK was employed for Homology Model validation.<sup>[45]</sup> Molecular docking of amentoflavone was performed to shed light on the binding mode of these proteins.

Amentoflavone activity was assessed against the active inhibitory site of HCoV-2 3CL<sup>PRO</sup>,<sup>[46]</sup> and TMPRSS2.<sup>[47]</sup> For that, a simple docking method explored the properties of GSF, i.e. a Quasi-Newton solid body optimization of the ligand location from random starting positions near the



**Figure 1:** The chemical structures of amentoflavone and standard anti-viral drugs.

receptor site.<sup>[48]</sup> For energy minimization of protein structure, the Swiss-PDB Viewer software package (v. 4.1.0) was used, and then all heteroatoms and water molecules of proteins were removed by using PyMOL (version 1.7.4.5) before docking.<sup>[49]</sup>

### Ligand preparation

The chemical structure of amentoflavone (PubChem ID: 5281600), as well as of the FDA-approved antiviral drugs Camostat mesylate (PubChem ID: 5284360) are shown in Figure 1. Amentoflavone and Camostat mesylate were downloaded from the PubChem (a database of chemical molecules) in the 'sdf' file format. All internal energies of the ligands were optimized by using Chem3D Pro12.0 program packages.<sup>[50]</sup>

### Docking protocol

Molecular docking is a computational method for drug design in medicinal chemistry. This method is used for predicting the drug candidate's pharmacodynamics profile by scoring and orienting them to the receptor binding sites,<sup>[51]</sup> by PyRx-virtual screening tool. The docking result determines the measure of ligand interaction to the active site of the targeted protein. The active sites are the coordinates with the ligand in the original target protein grids (25Å × 25 Å × 25Å grid size),<sup>[52]</sup> with PyMol, PyRx, and Drug Discovery Studio (v.4.5) being used for scrutinizing these active binding sites of the target protein.<sup>[53]</sup>

### Molecular dynamic simulation (MD) study

We performed MD simulations via utilizing Desmond (Schrodinger package).<sup>[54]</sup> The selected ligand-protein complexes were first soaked into TIP3 water box, extending 10 Å beyond any of the complex's atoms. Counter ions of sodium and chloride were included to neutralize charges. We set salt concentration to 0.15 M sodium, and chloride ions to approximate physiologic condition. We implemented the MD in the NPT ensemble at temperature of 300 K and 1.63 bar pressure over 100 ns. Simulations were passed with the OPLS-3e forcefield. Plots were depicted with Maestro tool.

### ADME prediction

ADME (Adsorption, Distribution, Metabolism, and Excretion) is important to analyze the pharmacodynamics of the proposed molecule that could be used as a drug. SWISS-ADME tool is a website (<https://www.swissadme.ch>) which allows the user to draw their respective ligand or drug molecule or include SMILES data from PubChem and provides the parameters, such as lipophilicity (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT, Log P0/w), water solubility-Log S (ESOL, Ali, SILICOS-IT), drug-likeness rules (Lipinski, Ghose, Veber, Egan, and Muegge) and

Medicinal Chemistry (PAINS, Brenk, Leadlikeness, Synthetic accessibility) methods.<sup>[55]</sup> Data from PubChem, which consists of SMILES of amentoflavone (<https://pubchem.ncbi.nlm.nih.gov/compound/amentoflavone>) was entered into the search bar and was analyzed.

### Toxicity prediction

Toxicology prediction of small molecules is important to predict the tolerability of the small molecules before being ingested by human and animal models. pkCSM is an online database in which the small molecule can be drawn virtually or can be analyzed by submitting the SMILES of the same. The website can provide details of toxicology effects in the fields of AMES toxicity, human maximum tolerated dose, hERG-I inhibitor, hERG-II inhibitor, LD<sub>50</sub>, LOAEL, hepatotoxicity, Skin toxicity, *T. pyriformis* toxicity, and Minnow toxicity. The website was logged on and SMILES of the amentoflavone data from PubChem was searched and submitted into the website, and toxicity mode was selected.<sup>[56]</sup>

### Computer-based instrumentation for molecular docking

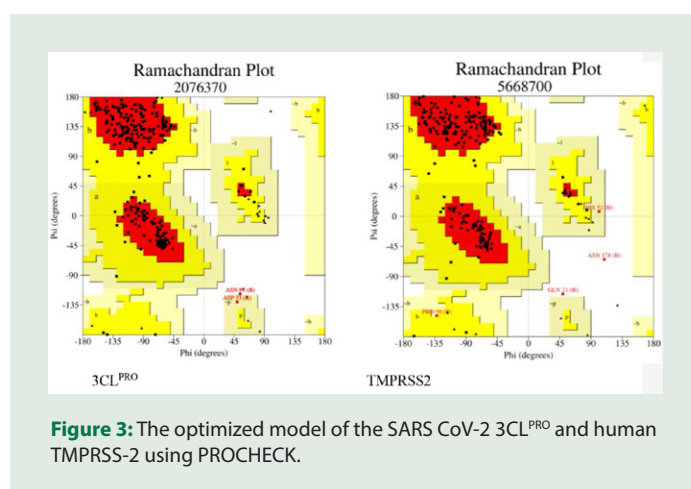
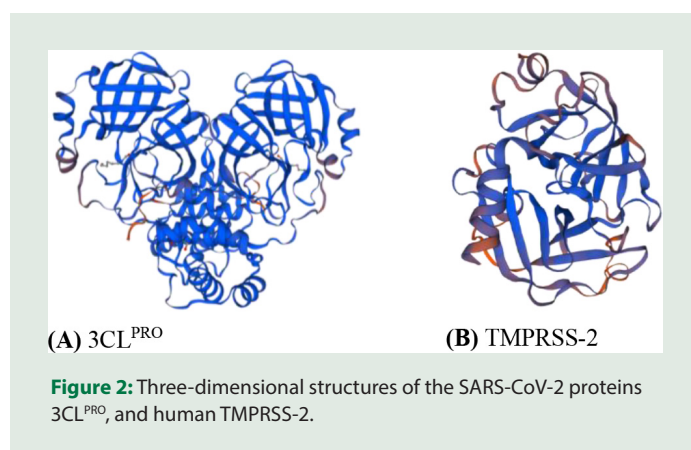
Computational drug discovery is a smart way to speed up and save money on the drug discovery and production process. Molecular docking, pharmacophore simulation and projection, de novo design, molecular similarity estimation, and sequence-based virtual scanning have all seen major advances in computational drug discovery.<sup>[57]</sup> In this study, for reducing all heteroatoms and water molecules from proteins PyMOL (version 1.7.4.5), and the Swiss-PDB Viewer software package (v.4.1.0) were used for energy minimization of protein structure. Protein and drug candidates were docked by PyRx-virtual screening tool (V.2.4), and Drug Discovery Studio (v.4.5) being used for scrutinizing these active binding sites of the target protein. For molecular dynamic simulation study Desmond (Schrödinger package).

## RESULTS

### Homology modelling of 3CL<sup>PRO</sup>, and TMPRSS2

Homology modelling has developed into an effective structural biology tool, greatly shrinking the distance between experimentally described protein structures and recognized protein sequences.<sup>[58]</sup> Using completely automated frameworks and databases, the homology modelling process is optimized and standardized, enabling even those without a specialized computational background to create accurate protein maps and have a fast and clear reference to modeling findings, representation, and evaluation.<sup>[59,60]</sup> The amino acid sequence of 3CL<sup>PRO</sup> (Uniprot accession ID: P0DTD1), and TMPRSS-2 (Uniprot accession ID: O15393) was subjected to NCBI BLAST Program for selection of the closest homologous template Homology model of 3CL<sup>PRO</sup>, and TMPRSS-2 was generated by Swiss model (Figure 2). Optimization of 3CL<sup>PRO</sup> and TMPRSS-2 was achieved by using the Swiss-PDB Viewer software package (v.4.1.0) before docking, whereas validation of *these* 3CL<sup>PRO</sup>, and TMPRSS-2 homology model was acquired through the use of Ramachandran plot performed by PROCHECK<sup>[45]</sup> and illustrated in Figure 3.

The Ramachandran plot is a simple way to see how a protein structure's torsion angles are distributed. It also gives an overview of the allowed and disallowed regions of torsion angle values, which is useful when evaluating the quality of protein three-dimensional structures. The phi-psi torsion angles for all residues in the structure are seen in the Ramachandran plot (except those at the chain termini). Glycine residues are denoted by triangles since they are not limited to the plot regions allocated for the other side chain forms. The coloring/shading on the plot represents the different regions described: the darkest areas (here shown in red) correspond to the "core" regions representing the most favorable combinations of phi-psi values. Ideally, one would hope to have over 90%



of the residues in these "core" regions. The percentage of residues in the "core" regions is one of the better guides to stereochemical quality.

From Ramachandran plot statistics, it has been found that residues in the most favored regions are about 92.1% (614 amino acid residues) and 90.1% (236 amino acid residue) for 3CL<sup>PRO</sup> and TMPRSS-2 proteins, respectively.

### Molecular Docking Interaction with 3CL<sup>PRO</sup>

Amentoflavone and Camostat mesylate showed binding energies by -9.5 and -7.4 kcal/mol with SARS-CoV-2 3CL<sup>PRO</sup>, respectively (Table 1). Amentoflavone revealed a good binding energy with 3CL<sup>PRO</sup> through interacting with Thr26, Cys145, Glu166, Gly143, His41, and Met165 amino acid residues. It also mediated hydrogen bond at a distance of 2.37Å with the hydroxyl group of Thr26, whereas His41 exhibiting  $\pi$ - $\pi$  interaction, Cys145  $\pi$ -S interaction, Glu166, and Gly143  $\pi$ -donor hydrogen bond, and  $\pi$ -CH<sub>3</sub> interaction with Met165. Since protein and phytoligands have hydrogen bonds, the ligands are more stable in their binding position. (Figure 4).

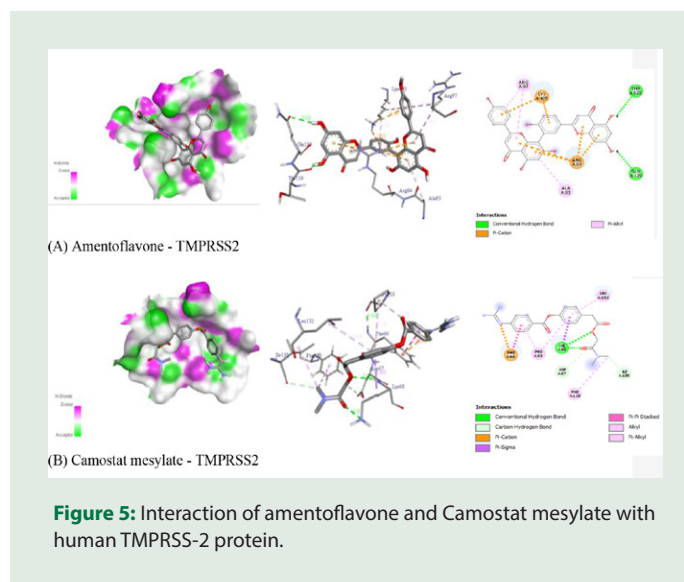
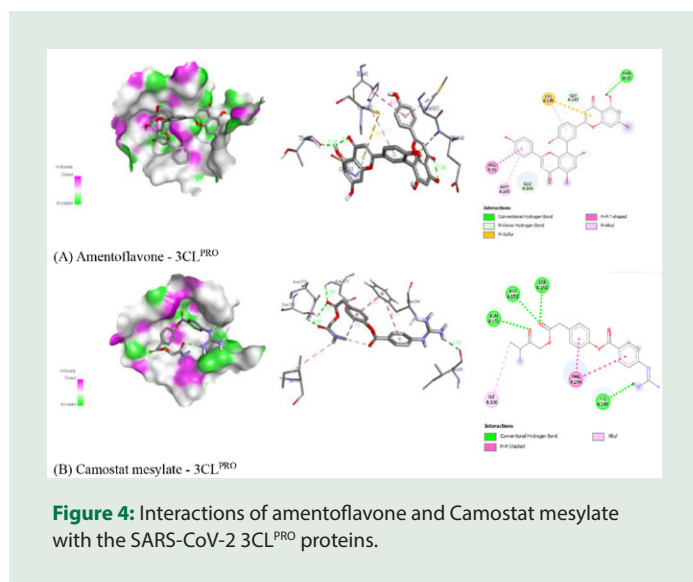
Additionally, the standard drug Camostat mesylate showed good interaction with the hydroxyl group of Asp153, Asn151, Ile249, Ser158 at a distance of 2.48, 2.84, 2.56, and 2.72Å, respectively, with the  $\pi$ - $\pi$  interaction of Phe294 and alkyl interaction of Ile106 exhibiting hydrophobic interaction. The 2D and 3D structures of non-bond interactions of amentoflavone with the target proteins are shown in Figure 4.

**Table 1: Binding affinities of amentoflavone, and Camostat mesylate with SARS-CoV-2 3CL<sup>PRO</sup> protein.**

Compound	Binding energy (kcal/mol)	H-bond residue	H-Bond length (Å)	No of H-Bonds	Other amino acid residue
Amentoflavone	-9.5	Thr26 (H)	2.37	1	Cys145, Glu166, Gly143, His41, Met165
	-7.4	Asp153(H) Asn151(H) Ile249(H) Ser158(H)	2.48 2.84 2.56 2.72	4	Ile106, Phe294

**Table 2: Binding affinities of amentoflavone, and Camostat mesylate with human TMPRSS-2 protein.**

Compound	Binding energy (kcal/mol)	H-Bond Residue	H-Bond length (Å)	No of H-Bonds	Other amino acid residue
Amentoflavone	-8.8	Gln129 (H)	2.58	2	Ala83, Arg84, Arg97, Lys405
		Thr 128(H)	2.70		
Camostat mesylate	-7.4	Lys68 (H)	2.60	2	Asp67, Ile135, Leu132, Phe 66, Phe118, Pro53
			2.61		



### Interaction with TMPRSS-2

Amentoflavone and Camostat mesylate were docked into TMPRSS-2 (Table 2). Results show that the hydroxyl moiety of amentoflavone mediates two hydrogen bonds with Gln129, and Thr128 at a distance of 2.58 and 2.70Å, respectively. Besides, multiple hydrophobic interactions were observed with Ala83, Arg84, Arg97, and Lys405. Similarly, the hydroxyl group of Camostat mesylate also mediates two hydrogen bond interactions with Lys68 at a distance of 2.60 and 2.61 Å.

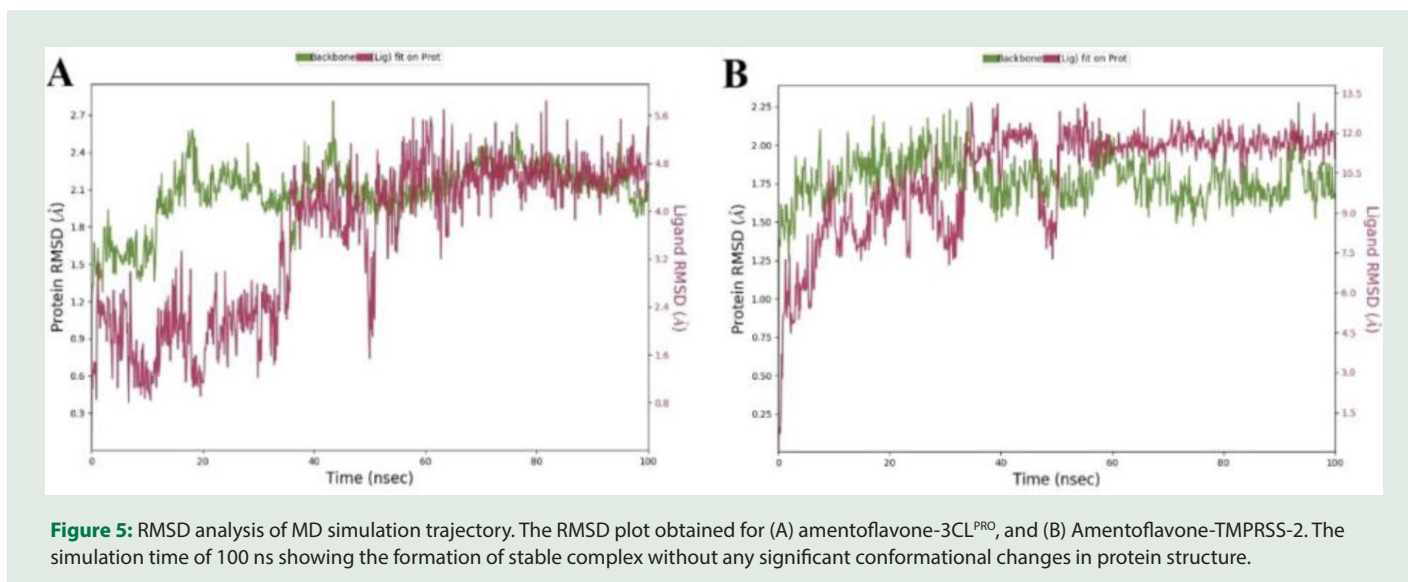
Additionally, multiple hydrophobic interactions were observed with Asp67, Ile135, Leu132, Phe 66, Phe118, and Pro53 as illustrated in Figure 5.

### Molecular dynamic simulation study

Molecular dynamics (MD) simulation Method for computing the atom movements with time by Use of Integrating-Newton's equations. Where MD simulates the dynamic style of the molecular systems and evaluate the protein ligand complex stability. RMSD Plots of 3CL<sup>PRO</sup> and TMPRSS-2 proteins on left Y-axis, while amentoflavone RMSD profiles

were depicted on the right Y-axis which were aligned on proteins backbone. The root mean square deviation (RMSD) plot in Figure 5A reveals that the amentoflavone-3CL<sup>PRO</sup> complex stabilized after 20 ns of simulation beginning. However, the fluctuations in the RMSD values of 3CL<sup>PRO</sup> were around 2.15 Å showing that the complex has not met with considerable conformational transformations. While, amentoflavone's RMSD profile when it bound to 3CL<sup>PRO</sup> showed two periods of fluctuations: RMSD is about 1 Å till 35 ns and later the RMSD jumped to 2.2 Å till the 100 ns.

The RMSD plot in Figure 6 shows the amentoflavone-TMPRSS-2 MD trajectory of 100 ns. The complex goes to be stabilized during simulation regarding the reference frame at time 0 ns. However, after arriving at the equilibrium the variation falls between 1.5-2 Å, hence, can be regarded as non-significant. Since the RMSD plots of amentoflavone and protein backbone were lying over each other, formation of a stable complex can be deduced. We can observe a minor divergence around 30 ns and 50 ns courses in the RMSD values of amentoflavone.



**Figure 5:** RMSD analysis of MD simulation trajectory. The RMSD plot obtained for (A) amentoflavone-3CL<sup>PRO</sup>, and (B) Amentoflavone-TMPRSS-2. The simulation time of 100 ns showing the formation of stable complex without any significant conformational changes in protein structure.

The RMSD plot in Figure 1B shows the amentoflavone-TMPRSS-2 MD trajectory of 100 ns. The complex goes to be stabilized during simulation regarding the reference frame at time 0 ns. However, after arriving at the equilibrium the variation falls between 1.5-2 Å, hence, can be regarded as non-significant. Since the RMSD plots of amentoflavone and protein backbone were lying over each other, formation of a stable complex can be deduced. We can observe a minor divergence around 30 ns and 50 ns courses in the RMSD values of amentoflavone.

### ADME assessment

Amentoflavone revealed a molecular weight of 538.46 g/mol with hydrogen bond acceptors of 10 and hydrogen bond donors of 6. The ligand had a molar refractivity of 146.97. Besides, amentoflavone showed Log Po/w (iLOGP), Log Po/w (XLOGP3), Log Po/w (WLOGP), Log Po/w (MLOGP), Log Po/w (SILICOS-IT) and Consensus Log Po/w values of 3.06, 5.04, 5.13, 0.25, 4.61 and 3.62, respectively. Data obtained revealed that it's a poorly soluble class and the values of ESOL, Ali, and SILICOS-IT were -6.75, -8.60, and -8.70 respectively. It also showed a bioavailability score of 0.17. Our study revealed 0 alerts of PAINS and Brenk. The Comparison of amentoflavone and Camostat mesylate are summarized in Table 3.

The color space is a suitable physiochemical space for oral bioavailability. **LIPO Lipophilicity:**  $-0.7 < XLOGP3 < 5.0$ . **SIZE:**  $150\text{g/mol} < MW < 500\text{g/mol}$ . **POLAR (Polarity):**  $20\text{Å}^2 < TPSA < 130\text{Å}^2$ . **INSOLU (insolubility):**  $0 < \text{Log S (ESOL)} < 6$ . **INSATU (insaturation):**  $0.25 < \text{Fraction Csp3} < 1$ . **FLEX (Flexibility):**  $0 < \text{Num. rotatable bonds} < 9$

### Toxicity prediction

For the analysis and optimization of pharmacokinetics and toxicity profiles, the results demonstrated that the ligand displayed no AMES toxicity, with a maximum tolerated dose for the human being of 0.438. The inhibitory activity of hERG II oral rat acute toxicity (LD<sub>50</sub>) was 2.527, oral rat chronic toxicity (LOAEL) of 3.572, *T. pyriformis* toxicity of 0.285, and minnow toxicity at 2.685. However, the ligand had no hepatotoxicity neither triggered skin sensitization. The toxicity values of the ligand and positive control are shown in Table 4.

## DISCUSSION

COVID-19 outbreak started in December 2019 has triggered multiple difficulties in clinical work.<sup>[61,62]</sup> For controlling the COVID-19 outbreak,

researchers from various countries are working hard to find out effective anti-SARS-CoV-2 agents, potential preventive agents,<sup>[63]</sup> or even inhibitors or a vaccine against SARS-CoV-2.<sup>[64]</sup> Although many vaccines are in clinical trials and have been proposed by various companies using various platforms, there is currently no officially approved vaccine.<sup>[65,66]</sup>

Also, naturally-occurring bioactive molecules have been increasingly investigated as a potential source of lead compounds to combat COVID-19.<sup>[67]</sup> The bioflavonoid amentoflavone, formerly isolated by Okigawa, Hwa,<sup>[1]</sup> has gained increasing attention due to its wider range of bioactivities. The CoV S protein helps to enter through binding to host cell receptor ACE2.<sup>[68]</sup> Indeed, SARS-CoV-2 also uses hACE2R for attaching and entry into human cells.<sup>[23]</sup> In this study, amentoflavone revealed a strong affinity towards the viral S protein as well as the host hACE2R protein.

Ryu, Jeong<sup>[41]</sup> also demonstrated an interactive capability of amentoflavone with 3CL<sup>PRO</sup>. Therefore, this study is in agreement with the previously done research on this compound. On the other hand, the PL<sup>PRO</sup> catalyzes the cleavage of the site-specific peptide of viral polyprotein sites between nsp1/nsp2, nsp2/nsp3, and nsp3/nsp4. It removes both ubiquitin and IFN stimulated gene (ISG) 15 during post-translational changes.<sup>[69]</sup> This study also shows an interaction capacity of amentoflavone with the PL<sup>PRO</sup>.

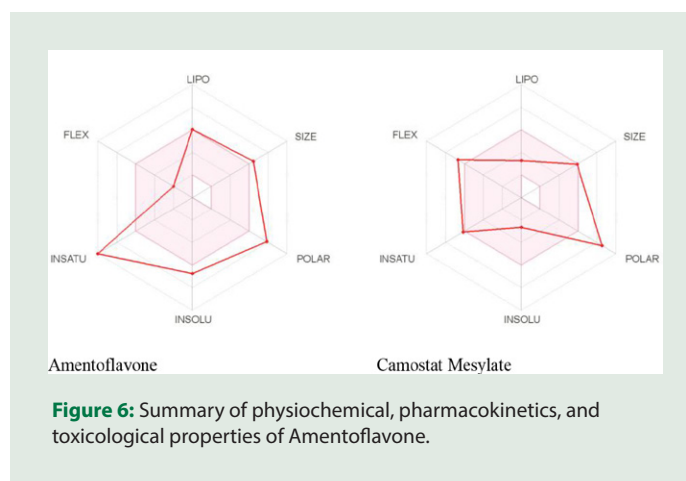
In this study, amentoflavone illustrate good to moderate binding affinity with 3CL<sup>PRO</sup> via interaction with receptor amino acids e.g., Thr26 (H), Cys145, Glu166, Gly143, His41, Met165 (Figure 6). Furthermore, 3CL<sup>PRO</sup> cleaves host polyproteins and helps to generate proteins required for viral replication.<sup>[70]</sup> It's indicate that amentoflavone interrupt SARS CoV-2 replication process through interaction with 3CL<sup>PRO</sup> protein.

The HCoV-2s use the RdRp enzyme in their life cycle.<sup>[27]</sup> CoVs RNA replication and transcription occur through nsp encoded by the open reading frames (ORF) 1a and 1b. The nsp encoded in ORF1a and ORF1b are nsp1 to nsp11 and nsp12 to nsp16, respectively.<sup>[71]</sup> On the other hand, the ABL1 and calcineurin-NFAT play important roles in SARS-CoV-2 infection. Amentoflavone showed strong interaction abilities with the RdRp and nsp that linked to ORF1a (e.g., nsp10) and ORF1b (e.g., nsp12, nsp13, nsp14, nsp15, nsp16).

In this study, amentoflavone is evident to interact with TMPRSS2 through Gln129 (H), Thr 128(H) Ala83, Arg84, Arg97 and Lys405. The TMPRSS2 facilitates hCoVs, including SARS-CoV-2 infections via two independent mechanisms: (i) proteolytic cleavage of hACE2R which promotes viral uptake, and (ii) CoV Spike proteins cleavage which

**Table 3: Comparison of amentoflavone and Camostat mesylate.**

Compounds	Amentoflavone	Camostat mesylate
<b>Pharmacochemical properties</b>		
Formula	C <sub>30</sub> H <sub>18</sub> O <sub>10</sub>	C <sub>21</sub> H <sub>26</sub> N <sub>4</sub> O <sub>8</sub> S
Molecular weight	538.46 g/mol	494.52 g/mol
Hydrogen bond acceptors	10	9
Hydrogen bond donors	6	3
Num. rotatable bonds	3	10
TPSA	181.80 Å <sup>2</sup>	200.06 Å <sup>2</sup>
Fraction Csp-3	0.00	0.24
Molar Refractivity	146.97	123.31
<b>Lipophilicity</b>		
Log Po/w (iLOGP)	3.06	1.92
Log Po/w (XLOGP3)	5.04	0.24
Log Po/w (WLOGP)	5.13	1.57
Log Po/w (MLOGP)	0.25	1.28
Log Po/w (SILICOS-IT)	4.61	1.47
Consensus Log Po/w	3.62	1.30
<b>Watersolubility</b>		
Log S (ESOL)	-6.75	-2.66
Log S (Ali)	-8.60	-4.00
Log S (SILICOS-IT)	-8.70	-4.46
<b>Druglikeness</b>		
Lipinski	No; 2 violations: (MW>500, NHorOH>5)	Yes; 1 violation: NorO>10
Ghose	No; 2 violations: MW>480, MR>130	No; 1 violation: MW>480
Veber	No; 1 violation: TPSA>140	No; 1 violation: TPSA>140
Egan	No; 1 violation: TPSA>131.6	No; 1 violation: TPSA>131.6
Muegge	No; 3 violations: XLOGP3>5, TPSA>150, H-don>5	No; 1 violation: TPSA>150
Bioavailability Score	0.17	0.55
<b>Medicinal Chemistry</b>		
PAINS	0 alert	0 alert
Brenk	0 alert	4 alerts: imine_1, imine_2, phenol_ester, sulfonic_acid_2
Leadlikeness	No; 2 violations: MW>350, XLOGP3>3.5	No; 2 violations: MW>350, Rotors>7
Synthetic accessibility	4.27	3.46

**Figure 6:** Summary of physiochemical, pharmacokinetics, and toxicological properties of Amentoflavone.**Table 4: Toxicity prediction for amentoflavone.**

Model Name	Predicted Value		Unit
	Amentoflavone	Camostat mesylate	
AMES toxicity	No	No	Categorical (Yes/No)
Max. tolerated dose (human)	0.438	0.133	Numeric (log mg/kg/day)
hERG I inhibitor	No	No	Categorical (Yes/No)
hERG II inhibitor	Yes	No	Categorical (Yes/No)
Oral Rat Acute Toxicity (LD <sub>50</sub> )	2.527	2.319	Numeric (mol/kg)
Oral Rat Chronic Toxicity (LOAEL)	3.572	2.81	Numeric (log mg/kgbw/day)
Hepatotoxicity	No	No	Categorical (Yes/No)
Skin Sensitisation	No	No	Categorical (Yes/No)
<i>T. pyriformis</i> toxicity	0.285	0.285	Numeric (log ug/L)
Minnow toxicity	2.685	0.524	Numeric (log mM)

triggers glycoprotein activation for host cell entry.<sup>[72]</sup> It has also been suggested that the gut is a potential site of SARS-CoV-2 replication. Besides, TMPRSS2 and TMPRSS4 were seen to facilitate SARS-CoV-2 spike fusogenic activity, thereby promoting virtual entrance into the host.<sup>[73]</sup> Therefore, molecular docking suggested that amentoflavone interact and inhibit TMPRSS-2.

On the other side, it has been reported that the acceptable range of molecular weight to a drug should be <500. Here, the molecular weight of amentoflavone is a little bit higher (538.46 g/mol). Hydrogen bond acceptors and hydrogen-bond donors with a range of ≤10 and ≤5 are adaptable.<sup>[74]</sup> It has also been reported that molar refractivity ranging from 40-130 is suitable, with an acceptable range of high lipophilicity (LogP) of <5.<sup>[74]</sup> Indeed, the numerous achievements in drug development are highly facilitated by the use of soluble molecules.<sup>[75]</sup> In



our study, the ESOL, Ali, SILICOS-IT, and class.<sup>[76,77]</sup> were determined. Drug-likeness evaluates the probability for a molecule to turn an oral drug concerning bioavailability. The Lipinski,<sup>[78]</sup> is the pioneer rule-of-five and the Ghose,<sup>[79]</sup> Veber,<sup>[80]</sup> Egan,<sup>[81]</sup> and Muegge,<sup>[82]</sup> were performed in case of drug-likeness. No value was stated for Lipinski, Ghose, Veber, Egan, and Muegge. Bioavailability Score pursues to compute the probability of a compound to have oral bioavailability in rat or measurable Caco-2 permeability.<sup>[83]</sup> Here, PAINS are the molecules carrying substructures exhibiting optimal response in assays irrespective of the protein target.<sup>[84,85]</sup> Brenk, Schipani<sup>[84]</sup> reported a list of 105 fragments for the structural alert. Our study revealed 0 alerts of PAINS and Brenk. The lead likeness is subjected to chemical modifications which can enhance the size and lipophilicity of the compound and the leads are requisite to be lesser and small hydrophobicity.<sup>[86]</sup> Synthetic accessibility (SA), in the selection of the suitable virtual molecules, is a chief factor. Medicinal chemists, for a reasonable number of molecules, are the best able to determine SA. The SA Score ranges from 1-10 (very easy-very difficult to synthesize), after normalization.<sup>[87]</sup> Moreover, it has no hepatotoxicity and skin sensitization. From the several test, the results have been shown that amentoflavone is a good candidate for COVID-19 treatment.

An ideal anti-SARS-CoV-2 drug must have four basic criteria: (i) restriction ability of viral entrance, thereby inhibiting cellular attachment; (ii) inhibition of viral replication in the host cells; (iii) cytotoxic effects on the existing viruses; and (iv) protect the host normal cells from the viral origin oxidative stress and inflammatory responses. Amentoflavone is evident to work through all of these pathways. Moreover, it has anti-oxidant,<sup>[2,88]</sup> and anti-inflammatory,<sup>[3,89,90]</sup> activities.

## CONCLUSION

Amentoflavone (a biflavonoid) previously illustrated anti-SARS CoV activity in SARS-CoV experimental system, additionally it exerted antiviral effect in a plethora of viruses. In this molecular docking and dynamic simulation study, it has been displayed that amentoflavone strongly interacts with SARS-CoV-2 non-structural 3CL<sup>PRO</sup> protein, and also showed strong binding affinities with host proteins responsible for SARS-CoV-2 entrance and replication in humans body. Interestingly, the binding affinities evidenced by amentoflavone were even greater than those observed in the clinical trial antiviral drugs (Camostat mesylate), currently used in many countries for the treatment of COVID-19. Other than that, pharmacokinetics tests expose to view good parameters when compare with Camostat mesylate. From this study, amentoflavone can be conceived as a potential lead compound against SARS-CoV-2 infection. Although further *in vivo* studies are needed to establish the findings observed here, our findings will be helpful for further non-clinical, pre-clinical, and clinical studies with these compounds, at the same time that will inspire medicinal chemistry scientists to conduct adequate research on this hopeful natural lead compound and its derivatives.

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## CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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None.

## ABBREVIATIONS

**ABL1:** Abelson murine leukemia viral oncogene homolog 1; **BLAST:** Basic Local Alignment Search Tool; **Calcineurin-NFAT:** Calcineurin nuclear; factor of activated T-cells; **COVID-19:** Coronavirus disease 2019; **3CLPRO:** 3-chymotrypsin-like protease; **HIV:** Human immunodeficiency virus; **hACE2R:** Human angiotensin-converting enzyme 2 receptor; **NCBI:** National Center for Biotechnology Information; **RdRp:** RNA-dependent RNA polymerase; **RSV:** Respiratory syncytial virus; **TMPRSS-2:** Transmembrane protease serine 2; **PLPRO:** Papain-like protease; **SAM:** S-adenosyl methionine; **SARS-CoV:** Severe acute respiratory syndrome coronavirus.

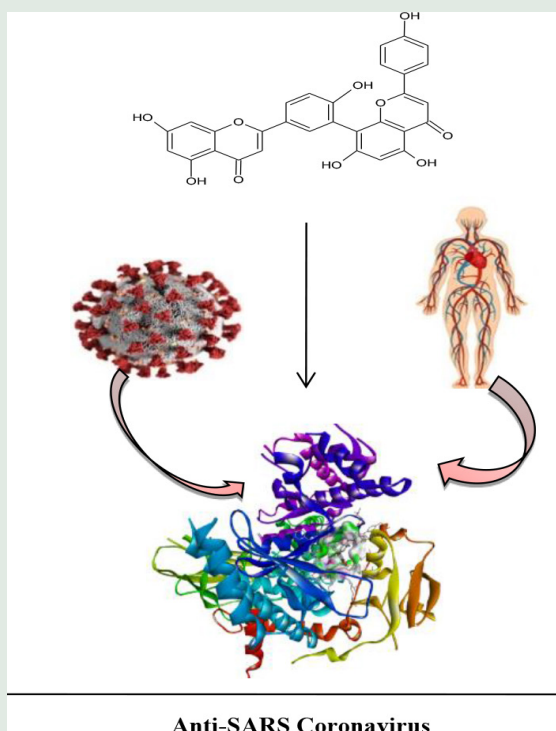
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## GRAPHICAL ABSTRACT



## SUMMARY

A bioflavonoid, amentoflavone inhibited SARS-CoV with an  $IC_{50}$  value of 8.3  $\mu$ M, possibly through inhibition of 3CL<sup>PRO</sup>. Furthermore, FDA-approved antiviral drugs Camostat mesylate used in the clinical trial against COVID-19. In this sense, this study aimed to address the *in silico* potential of amentoflavone against 3CL<sup>PRO</sup> and TMPRSS-2 proteins. Additionally, some host proteins interacting with HCoV-2 were also taken into account.

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