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Aloin A inhibits SARS CoV-2 replication by targeting its binding with ACE2 - Evidence from modeling-supported molecular dynamics simulation

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Abstract
The current study aimed to expand on the recently published results and assess the inhibitory efficacy of aloin A against SARS CoV-2. In vitro testing of aloin A against SARS CoV-2 proteases (i.e., MPro and PLPro) showed weak to moderate activity (IC₅₀ = 68.56±1.13 µM and 24.77±1.57 µM, respectively). However, aloin A was able to inhibit the replication of SARS CoV-2 in Vero E6 cells efficiently with an IC₅₀ of 0.095±0.022 µM. Depending on the reported poor permeability of aloin A alongside its insignificant protease inhibitory activities presented in this study, we ran a number of extensive virtual screenings and physics-based simulations to determine the compound's potential mode of action. As a result, RBD-ACE2 was identified as a key target for aloin A. Results from 600ns-long molecular dynamics (MD) simulation experiments pointed to aloin A's role as an RBD-ACE2 destabilizer. Therefore, the results of this work may pave the way for further development of this scaffold and the eventual production of innovative anti-SARS CoV-2 medicines with several mechanisms of action.
Keywords: SARS CoV-2; M\textsuperscript{Pro}, PL\textsuperscript{Pro}, RBD-ACE2, molecular dynamics simulation, virtual screening.

1. Introduction

Coronaviruses are enveloped, single-stranded RNA viruses that infect a wide range of hosts, including birds, pigs, and humans. They are also related to other coronaviridae family members [1]. While most family members only have minor effects on the human respiratory system, the 21\textsuperscript{st} century has seen the emergence of new members that cause severe respiratory disorders. In Guangdong Province, China, in 2002, SARS-CoV-1 (SARS) was the pathogen responsible for the severe acute respiratory syndrome outbreak. Ten years later, in Jordan, MERS-CoV was discovered in a patient's sputum who had previously been diagnosed with the Middle East Respiratory Syndrome (MERS). Due to worldwide travel by infected individuals, both viruses triggered an epidemic that spread across multiple countries. The epidemic was brought under control around a year after it began by instituting stringent infection control measures[2].

In December 2019, a new coronavirus known as SARS-CoV-2 was found to be the cause of the COVID-19 global outbreak of respiratory sickness, which started in Wuhan, Hubei Province, China. Despite having a fatality rate of about 2\%, SARS-CoV-2 is more contagious than its two progenitors and is thought to be responsible for greater overall death rates [3].

This circumstance compelled the World Health Organization to identify SARS-CoV-2 as a pandemic infectious disease of global concern in March 2020. As of June 2021, there were around four million confirmed deaths and over 179 million confirmed cases of COVID-19 worldwide[3], [4]. The urgent response to the COVID-19 pandemic prompted researchers to consider drug repurposing as a viable strategy to address the epidemic as soon as possible. Several hundred
clinical trials are currently being conducted to evaluate the effectiveness of various medications at various stages of the disease [5], [6]. As a result, there are now a few medications available to treat COVID-19 patients, either FDA-approved or otherwise, in clinical trials (Table 1) [7-9]. However, the rapid genetic mutation and evolution of new SARS CoV-2 variants might reduce the therapeutic efficacy of these newly developed medications. Therefore, finding and developing new specialized antiviral treatments with novel modes of action against SARS-CoV-2 is still urgently needed [10, 11].

Table 1. FDA-approved COVID-19 medications and other therapeutics in late stages of clinical trials

<table>
<thead>
<tr>
<th>Drug name (Brand name)</th>
<th>Mode of action</th>
<th>Approval state</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remdesivir (Veklury)</td>
<td>Viral RNA-dependent RNA polymerase inhibitor</td>
<td>FDA-approved</td>
<td>[7]</td>
</tr>
<tr>
<td>Baricitinib (Olumiant)</td>
<td>Immune modulator</td>
<td>FDA-approved</td>
<td>[7]</td>
</tr>
<tr>
<td>Molnupiravir (Paxlovid)</td>
<td>Viral main protease inhibitor</td>
<td>FDA-approved</td>
<td>[7]</td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>Immune modulator</td>
<td>Phase III</td>
<td>[8]</td>
</tr>
<tr>
<td>Sotrovimab</td>
<td>Monoclonal antibody targeting the viral spike protein</td>
<td>Phase III</td>
<td>[9]</td>
</tr>
</tbody>
</table>

The viral spike protein (S-protein) with its host receptor angiotensin-converting enzyme-2 (ACE-2) is among the many key targets that can be used to develop antiviral drugs. Targeting the viral S-protein with antibodies or specific small molecules has been proven to have therapeutic efficacy either in vitro or in clinical experiments [12]–[14]. However, this vital target is prone to a very rapid mutation rate that hinders the development of effective long-term binders. On the other hand, its receptor (i.e., ACE-2) is one of the normal host proteins that mutate much slower than the viral
S-protein. Hence, blocking this protein offers a better option for developing new drugs that target one of the major viral entry routes and remain stable for the long term [15]–[19].

Natural products are an intriguing source of compounds for the development of small antiviral molecules. Several natural products are now under clinical trials to evaluate their effectiveness against COVID-19 [20], [21]. With an IC$_{50}$ value of less than 1 µM, many plant terpenoids and phenolics proved effective antivirals against SARS-CoV-1 and 2 [22]–[24]. More recently, different flavonoids were also effective inhibitors of SARS-CoV-2 replication in vitro [25].

Aloin A (aka barbaloin) is a bitter, yellowish-colored compound noted in the exudate of at least 68 Aloe species. This natural anthrone glycoside is a well-known laxative agent, and recently it was reported to inhibit SARS CoV-2’s M$^{\text{Pro}}$ and PL$^{\text{Pro}}$ in vitro with IC$_{50}$ values of 96 and 13.16 µM, respectively [26], [27]. However, the inhibitory activity of this compound against the virus has not been reported yet.

Our team has conducted a comprehensive screening program in the quest for potential antiviral agents since the spread of this virus worldwide in 2020. As part of our ongoing efforts to identify potential anti-SARS CoV-2 agents and develop new in silico approaches to facilitate this, we decided in the present work to build on these recently reported findings and to evaluate the inhibitory activity of aloin A against SARS CoV-2 in vitro. The present work also explains the possible alternative antiviral modes of action of aloin A depending on a series of physics-based simulations. Accordingly, the present study shed light on further optimization of the scaffold of this molecule to produce novel anti-SARS CoV-2 drugs with multiple mechanisms.

2. Material and methods

2.1. Chemicals
All used chemicals and solvents were of analytical grade. Aloin A was purchased from Sigma Aldrich (cat No. PHL89558-10MG, Cambridge, UK) with a purity of ≥ 97%.

2.2. In vitro Biological Study

2.2.1. Enzyme Assay

Enzyme inhibition assays were carried out according to the manufacturer's protocols using commercial SARS-CoV-2 proteases assay kits (M<sup>Pro</sup> and PL<sup>Pro</sup>, Catalog #: 79955-1 and 79995, respectively, BPS Bioscience, Inc., Allentown, PA, USA) and human ACE2 assay kit (Catalog #: ab273373, Abcam, USA). Positive controls were GC376, GRL0617, and MLN-4760 for M<sup>Pro</sup>, PL<sup>Pro</sup>, and ACE2, respectively. When either enzyme cleaves the substrate, fluorescence is produced, which can be measured using a microplate reader at 460 and 360 nm for emission and excitation wavelengths, respectively (Tecan Spark, Switzerland). Various test drug concentrations (10 µL) were applied to a 96-well plate, followed by 30 µL of diluted protease (15 g/mL). The produced mixes were then incubated for 30 minutes at room temperature. Then, a mixture of 10 µL of the substrate and the reaction buffer was added to the wells to achieve a final volume of 50 µL and a final concentration of 40 µM. The resulting combination was incubated for four hours at 20 °C. By utilizing a TECAN spark microplate-reading fluorimeter, the fluorescence produced was measured.

2.2.2. Antiviral Assay

2.2.2.1. Virus and Cells

Vero-E6 cells were kept alive at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's medium (DMEM), which contains 2% penicillin/streptomycin and 10% bovine serum (BS) (Invitrogen). Cells were placed into tissue culture flasks 24 hours prior to infection with the hCoV-
19/Egypt/NRC-3/2020 isolate in an infection medium containing 4% BS and 1% trypsin treated with L-1-tosylamido2-phenylethyl chloromethyl ketone (TPCK) in DMEM with 2% penicillin/streptomycin to produce the virus stock. The virus inoculum-containing infection media was replaced with a newly infected medium two hours later, and the incubation period was extended to three days. The cell supernatant was collected after purification and centrifuged for 5 min at 2500 rpm. A plaque infectivity assay was used to titrate the supernatant after it had been aliquoted and transferred to a new 50 mL falcon tube.

2.2.2. MTT Cytotoxicity Assay

Aloin A and test compounds were diluted to working solutions with DMEM from stock solutions in 10 percent DMSO with ddH$_2$O to determine the IC$_{50}$ used for the compounds' initial antiviral screening. The cytotoxic effect of the test compounds was assessed in Vero-E6 cells using the previously described 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique [28] with minor modifications. The cells were incubated for 24 hours at 37 °C with 5% CO$_2$ after being placed in 96-well plates (100 μL/well at a density of 3105 cells/mL). After 24 hours, cells were exposed to different concentrations of the tested agents in triplicate. After another 24 hours, the supernatant was removed, and the cell monolayers were cleaned three times with sterile 1 PBS. Each well was given 20 μL of the 5 mg/mL stock MTT solution before being incubated for 4 hours at 37 °C. The formed formazan crystals were dissolved in 200 μL of acidified isopropanol (0.04 M HCl in 100% isopropanol = 0.073 mL HCL in 50 mL isopropanol). The absorbance of formazan solutions was then calculated using a microplate reader at a maximum wavelength of 540 nm. The percentage of cytotoxicity in comparison to untreated cells was determined using the formula below:
% Cytotoxicity = \frac{(\text{absorbance of cells without treatment} - \text{absorbance of cells with treatment}) \times 100}{\text{absorbance of cells without treatment}}

The IC_{50} values were then determined using the plot of the percent cytotoxicity vs. sample concentrations. Doxorubicin was used as a positive control (IC_{50} = 23.35 \pm 0.85 \mu M).

**2.2.2.3. Viral replication assay**

After filling each well of a 96-well plate with approximately 2.4×10^4 Vero-E6 cells, the plates were incubated overnight at 37 °C with 5% CO_2. Cell monolayers were exposed to viral adsorption for one hour at room temperature after a single PBS wash. After that, the cell monolayers were covered with 50 μL of DMEM containing various concentrations of the test drug. After 72 h, the cells were fixed for 20 min in 100 μL of 4% paraformaldehyde and stained for 15 min in 0.1% crystal violet. Using an Anthos Zenyth 200 rt plate reader, the optical density of the produced colour was determined at 570 nm (Anthos Labtec Instruments, Heerhugowaard, The Netherlands). The crystal violet was then dissolved in each well with 100 μL of MeOH. The test drug's IC_{50} is the concentration at which the virus-induced cytopathic effect (CPE) is reduced by 50% when compared to virus control. Remdesivir was used as a positive control.

**2.2.3. Cellular Uptake of Aloin A**

Using the previously reported experimental procedure [29], we determined the cellular permeability of aloin A by adding 5 μM of the compound to the cellular medium (i.e., complete medium) and incubating cultured Vero-E6 cells for 4 hours. Then, both the extracellular and intracellular concentrations of aloin A were determined. Following removal of the medium, cells were washed twice with PBS to get ready for the subsequent procedures in which (i) cells were lysed with 200 μL of lysis buffer and wells were washed with 200 μL of PBS; and (ii) cellular uptake evaluation, in which cells were collected by gently scraping with PBS, centrifuged at 1500
rpm for 5 min, and added to 500 μL of 80% v/v methanolic solution in water/0.1% v/v acetic acid to recover the intracellular content. After 15 minutes on ice, we sonicated and centrifuged the samples at 10000 rpm for 10 minutes to remove any excess liquid. The supernatant solutions were collected for HPLC analysis, while the precipitated proteins were discarded. An unglycosylated anthraquinone derivative named emodin was used as a control. The HPLC equipment (Agilent Corporation, Germany) with the Photodiode Array Detector was used for the chromatographic analyses. Chromatographic separation was achieved using a SymmetryRP C18 column that was 4.6 mm in diameter and 3.5 m in thickness (Waters Corporation, Milan, Italy). A linear methanolic gradient was applied with a flow rate of 1 mL/min, beginning at 20% v/v and progressing to 80% v/v in 15 minutes (A: water with acetic acid 0.5% v/v and B: methanol with acetic acid 0.5% v/v). This approach was adapted from [29]. 210–400 nm was the range where peaks were detected. Retention time and spectral matching with aloin A and emodin standards were used for compound qualitative identification. For the quantification of each compound in both the medium and the cell lysate, standard stock solutions were made in methanol, filtered, and diluted to the relevant concentrations (1.0, 5.0, 10.0, and 20.0 μM) for the creation of the calibration curves. By comparing the results to the reference calibration curves, the concentrations of the compounds in the extracellular and intracellular solutions were determined. After doing a protein assay according to the previously described method [29] to determine how much protein was in the lysate, we were able to acquire our final results, which were expressed as nmol of aloin or emodin per mg of protein.

2.3. In silico Study

2.3.1. Data Preparation
SARS CoV-2 protein structures were obtained from the Swiss-Model repository (https://swissmodel.expasy.org/repository/species/2697049, accessed on March 27, 2022) and String database (https://string-db.org/cgi/covid.pl, accessed on March 27, 2022) [30]. Table S1 contains a list of all the PDB codes for SARS CoV-2-relevant proteins used in this work, along with their acronyms and functions.

2.3.2. Docking and Modeling

All docking studies were conducted using AutoDock Vina software [31]. All of the gathered proteins were docked against the modeled structure of aloin A (Table S1 listed all PDB codes) using a rigid docking protocol where the ligand (i.e., aloin A) was kept flexible, but the active site was set to be rigid. Each protein's binding site was identified based on the co-crystallized ligand, which is automatically located at the center of the grid box allocated for docking. The dimensions of the grid box were set to 15 Å.

The value for exhaustion was set to 24. Proteins without co-crystallized ligands or those with predicted models were exposed to blind docking, where the entire protein structure was located within the Docking grid box. For each docking experiment, 10 poses were generated. The top generated poses in each docking trial were then examined by visual inspection using Pymol software [31].

2.3.3. Molecular dynamics simulation

The MD simulation experiments were carried out using the Desmond v. 2.2 platform [32]–[33]. The force field Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) was applied by this software. OPLS force field is optimized for simulations in a liquid medium using the TIP3P water model, and implicitly includes hydrogen atoms next to carbon in the carbon parameters that
can be used to save simulation time. This force field was also optimized for other specific functional groups and types of molecules, such as carbohydrates. The System Builder option was used to construct protein systems, which included checking the protein structure for any missing hydrogen atoms, setting the protonation states of the amino acid residues to protons at pH 7.4, and removing the co-crystallized water molecules. The more general approach includes applying the Poisson-Boltzmann equation with subsequent Monte Carlo titration over all protonatable sites in protein in a self-consisting manner, as described in the literature [34]. The entire structures were placed inside an orthorhombic TIP3P water box with 0.15 M Na\(^+\) and Cl\(^-\) ions in a solvent buffer chosen to ensure 11 Å between all protein atoms and the box edges. The constructed systems were then subjected to unrestrained minimization consisting of 1000 steps of steepest descent followed by 1000 steps of conjugate gradient minimization. The systems’ energy was then brought into equilibrium for 10ns at 310 K. The top-scoring poses for protein-ligand complexes were chosen as starting points for simulation. Desmond software automatically parameterizes input ligands during the system development phase according to the OPLS force field. The relaxed systems were then simulated in the NPT ensemble. The full particle-mesh Ewald approach was utilized for electrostatic interactions, and the SHAKE algorithm was applied to constrain all covalent bonds involving hydrogen atoms [35]. Van der Waals non-bonded interactions were truncated using a 2 fs time step and a 10 cutoff. Trajectory Interaction Analysis tools of Maestro software were used for all the structural and energetic analyses [32]–[33].

Similarly, MD simulation experiments were carried out by NAMD 3.0 software, and the protein structures were created and optimized using the QwikMD toolkit of the VMD software D [35]. Using the Ligand Reader and Modeler (http://www.charmm-gui.org/?doc=input/ligandrm, accessed on 9\(^{th}\) May 2022) and the Charmm27 force field, the compounds’ properties and
topologies were determined. [36]. The resulting parameters and topology files were then put into VMD to quickly and error-free read the protein-ligand complexes and carry out the simulation stage. All MD simulation experiments were carried out three times (n = 3) in the NPT ensemble. The resulting trajectories (60000 frames for each one) were analysed by VMD software [35].

2.3.4. Absolute Binding Free Energy Calculation

Utilizing the free energy perturbation (FEP) method, the binding free energy ($\Delta G_{\text{binding}}$) was calculated [31]. In their most recent study, Kim and his colleagues [36] went into great detail about this technique. In a nutshell, this approach determines the binding free energy $\Delta G_{\text{binding}}$ using the formula $\Delta G_{\text{binding}} = \Delta G_{\text{Complex}} - \Delta G_{\text{Ligand}}$. NAMD 2.14 software was used to estimate each $\Delta G$'s value from a separate simulation. Using the Charmm-GUI tool (https://charmm-gui.org/?doc=input/afes.abinding), all the input files needed for the NAMD simulation were created. Then, using NAMD's FEP calculation function, the necessary simulations could be generated. The equilibration (5ns long) was accomplished in the NPT ensemble at 310K and 1 atm (1.01325 bar) with Langevin piston pressure (for "Complex" and "Ligand"). Then, 10ns FEP simulations were run for each compound, and the final free energy values were determined by measuring the values from the last 5ns of the simulations [36]. Finally, VMD software was used to evaluate and show the obtained trajectories. In their recent benchmarking research, the FEP approach of determining $\Delta G_{\text{binding}}$ was shown to be the most accurate way of predicting MPro inhibitors by Ngo and colleagues [37].

3. Results

3.1. In vitro Biological activity

3.1.1. Enzyme activity assays
Aloin A was first tested for its enzyme inhibitory activity against both M<sup>Pro</sup> and PL<sup>Pro</sup> in vitro. In the case of M<sup>Pro</sup>, aloin A was practically inactive (IC<sub>50</sub> > 50 µM), while it showed moderate inhibitory activity against PL<sup>Pro</sup> with an IC<sub>50</sub> value of 24.77±1.57 µM (Figure 1). These results were in good accordance with those previously reported [26], [27] (IC<sub>50</sub> = 96 and 13.16 µM, respectively), indicating that aloin A might inhibit SARS CoV-2 replication via inhibiting its PL<sup>Pro</sup>.

3.1.2. Virus replication assay

Based on the previous results of enzyme-based assays and the previous reports on the weak to the moderate activity of aloin A against both SARS CoV-2’ M<sup>Pro</sup> and PL<sup>Pro</sup>, it was selected for testing its viral replication inhibitory activity in Vero E6 cells. First, we evaluated its cytotoxicity against Vero E6 cells for 24 and 48 h at 50 and 100µM concentrations. Aloin A did not affect cell viability at the measured doses for up to 48 hours. Next, we used Vero E6 cells to carry out SARS-CoV-2 viral replication tests and measure SARS-CoV-2 replication. Surprisingly, we found that aloin A was able to inhibit the viral replication effectively with an IC<sub>50</sub> of 0.095±0.022 µM (IC<sub>50</sub> of the positive control, Remdesivir, was 0.013 ± 0.004 µM). This observed potent antiviral activity of aloin A appears not to be correlated to the moderate PL<sup>Pro</sup> inhibitory activity. This conclusion is supported by a recent report on several of synthesized compounds that were found to be more potent PL<sup>Pro</sup> inhibitors than aloin A (IC<sub>50</sub> values ranged from 2.3 µM to 16.8 µM) with efficacy against viral replication IC<sub>50</sub> did not exceed 1.4 µM [38].

Moreover, aloin A has poor permeability to the cellular membrane and needs specific carrier proteins (e.g. sodium-dependent glucose co-transporter (SGLT1), glucose transporter (GLUT2)) that are present only in certain tissues like the small intestine [39], [40].

Aloin A’s cellular permeability was evaluated in the present study by adding 5 µM of the compound to the Vero-E6 following the incubation for 4 hours, as described in a recent experimental report.
The levels of aloin A were then measured both outside of cells and inside of them. The level of aloin A outside the cells was $4.53 \pm 0.35 \mu M$. Aloin A concentration intracellularly was below the detection limit. Since aloin has only been found in the extracellular environment, it is likely that this molecule cannot cross cellular membranes.

This poor cellular uptake of aloin A is likely due to the hydrophilic carbohydrate part, where its close unglycosylated derivative emodin was obviously detected intracellularly with a concentration of $0.36 \pm 0.17 \text{ nmol/mg protein}$.

Based on these observations, aloin A will not be able to pass through the cellular membrane of the SARS CoV-2 infected cells efficiently to bind with and inhibit PL$_{Pro}$, which is essentially expressed intracellularly. Accordingly, aloin A might exert its potent anti-SARS CoV-2 activity by another extracellular mechanism(s) that can be putatively elucidated by running a number of comprehensive physics-based simulation experiments.
Figure 1. Inhibitory activity of aloin A against SARS CoV-2 replication (E) and its derived proteases (i.e. M<sup>Po</sup> and PL<sup>Pro</sup>; A and C, respectively) expressed as IC<sub>50</sub> values. GC376 and GRL0617 were used as positive controls for M<sup>Po</sup> and PL<sup>Pro</sup> assays (B and D, respectively), while the positive control for the viral replication assay (F) was Remdesivir.

3.2. Proposing the aloin A mode of action via modeling and in silico investigation

3.2.1. Virtual screening

To identify a potential molecular target for aloin A, its modeled structure was docked against all known proteins involved in the SARS CoV-2 life cycle (https://swissmodel.expasy.org/repository/species/2697049, accessed on 14<sup>th</sup> April 2022; https://www.genome.jp/kegg-bin/show_pathway?hsa05171+H02398, accessed on 14<sup>th</sup> April 2022; Table S1). For proteins with co-crystalized ligands, the grid box of docking was located around these co-crystalized molecules. In contrast, docking against proteins without co-crystalized ligands was carried out against the whole protein structure (i.e., blind docking). Subsequently, the top-scoring binding poses generated for each docking experiment were selected. A cut-off value of -7.0 kcal/mol was set to select the best target for aloin A. Seven proteins were found to be probable targets for aloin A (Table 1), of which RBD-ACE2 was the best-scoring target. Four of these selected proteins were of viral origin (nonstructural proteins; nsp), and the remaining three were human proteins. ACE2 showed two potential binding sites: the first (site 1) was a deep pocket inside it (docking score = -9.45), and the second (site 2) was a shallow, superficial pocket at the RBD-ACE2 binding interface (docking score = -7.35) (Figure 2).

Further refinement of the docking results was achieved by subjecting these seven docking poses to absolute binding free energy (ΔG<sub>binding</sub>) calculation using the Free Energy Perturbation (FEP) method [37]. This step was carried out to tentatively determine the affinity of aloin A structure toward these selected proteins using a series of molecular dynamics simulation-based experiments. As a result, five out of the seven top targets showed a very good affinity with aloin A (ΔG<sub>binding</sub> <
-7.0 kcal/mol) (Table 1). Four of these proteins are viral nonstructural proteins expressed inside
the host cells (i.e., Helicase, PL\textsuperscript{Pro}, M\textsuperscript{Pro}, and CatL), while one of them occurs as a transmembrane
protein (i.e., ACE2) (Table 2).

As proposed in the previously discussed findings, aloin A is an interesting anti-SARS CoV-2
molecule that is likely to exert its activity via an extracellular route due to its poor cellular
permeability. The main extracellular target(s) for aloin A is either the viral S-protein or the host
receptor ACE-2. Although there are other viral entry routes inside the host cells, ACE-2-mediated
entry is considered the major one [12]. Accordingly, ACE2 was proposed in the present study to
be the most probable target for aloin A. Hence, its interaction with aloin A was further explored
using several physics-based simulations.

<table>
<thead>
<tr>
<th>Protein target</th>
<th>PDB code</th>
<th>Origin</th>
<th>Docking Score</th>
<th>(\Delta G_{\text{binding}}) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBD-ACE2</td>
<td>6LZG</td>
<td>Human</td>
<td>-9.45*, -7.35**</td>
<td>-10.35 ± 0.13*, -7.89 ± 0.18**</td>
</tr>
<tr>
<td>Helicase (nsp13)</td>
<td>5RL6</td>
<td>Viral (Nonstructural protein)</td>
<td>-8.45</td>
<td>-7.81 ± 0.45</td>
</tr>
<tr>
<td>ADP ribose phosphatase (nsp3)</td>
<td>6W02</td>
<td>Viral (Nonstructural protein)</td>
<td>-8.28</td>
<td>-5.36 ± 0.27</td>
</tr>
<tr>
<td>PL\textsuperscript{Pro} (nsp11)</td>
<td>6WX4</td>
<td>Viral (Nonstructural protein)</td>
<td>-7.87</td>
<td>-8.19 ± 0.14</td>
</tr>
<tr>
<td>M\textsuperscript{Pro} (nsp5)</td>
<td>6LU7</td>
<td>Viral (Nonstructural protein)</td>
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<td>-7.56 ± 0.22</td>
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<tr>
<td>Transmembrane protease, serine 2 (TMPRSS2)</td>
<td>Deposited model in Swiss-Mode</td>
<td>Human</td>
<td>-7.36</td>
<td>-3.46 ± 0.93</td>
</tr>
<tr>
<td>Cathepsin L (CatL)</td>
<td>2YJC</td>
<td>Human</td>
<td>-7.12</td>
<td>-7.74 ± 0.33</td>
</tr>
</tbody>
</table>

*, **, ## and ### are the Docking and \(\Delta G_{\text{binding}}\) scores of aloin A at 2 binding sites on RBD-ACE2 (i.e. Site1 and Site2).
Figure 2. Structure of RBD-ACE2 complex. ACE2 is the green-colored structure, while S-protein-derived RBD is the pink-colored structure. Docking poses of aloin A on ACE2 (i.e., sites 1 and 2) are encircled. Site 1 coordinates: x = -31.354, y = 19.072, z = -11.998; Site 2 coordinates: x = -28.852, y = 26.251, z = 2.164.

3.2.2. Molecular dynamics Simulation

From the previous virtual screening and binding free energy calculation step, ACE2 was proposed to be one of the possible key targets that mediate the antiviral activity of aloin A. Two binding sites (sites 1 and 2; Figure 2) for aloin A were designated in this step. It is important to note that ACE2 is an enzyme. However, in our case, it acts as a receptor to mediate SARS CoV-2 entry into the host cells. Hence, aloin A binding with ACE2 does not guarantee the inhibition of the viral entry process. The binding of aloin A with ACE2 at Site 2, located at the RBD-ACE2 interface (Figure 2), is likely more convenient to inhibit the RBD-ACE2 association or even induce their
dissociation after binding (i.e., destabilize the interaction between RBD and ACE2). This suggestion can be concluded by visually inspecting the whole complex (Figure 2).

To confirm this assumption, several long MD simulations (Table S2) were conducted (600ns long) using the RBD-ACE2 complexes with aloin A in each site. As depicted in Figure 3, the binding of aloin A to ACE2-RBD at site 2 destabilized the RBD-ACE2 complex, leading to a complete dissociation after 468 ± 98 ns ($n = 3$), while binding at site 1 did not influence the RBD-ACE2 complex stability over 600ns of MD simulation similarly to the ligand-free complex. Moreover, the binding of two aloin molecules to ACE2-RBD complex, one molecule at site 1 and another one at site 2 led also to a complete dissociation after 484 ± 75 ns ($n = 3$) (Figures 3 and 4).

Aloin A established three H-bonds with ALA-387, PHE-390, and TYR-505 inside the binding site 2 over ~ 224ns of MD simulation (Figure 4). Additionally, the 12 H-bonds keeping RBD-ACE2 intact [41] were also preserved during this period, including those inside site 2 (i.e., H-bonds between LYS-353 and GLY-469, HIS-34 and TYR-453, and ASP-30 and LYS-417; Figure 5). At 225 ± 73 ns, the structure of aloin A started to change slightly, forming an additional two H-bonds with two of the RBD-ACE2-interacting residues (HIS-34 and LYS-353), making them unavailable for RBD-ACE2 stabilization (Figures 3 and 5). This transition also weakened the interaction between ASP-30 and LYS-417 (the H-bond length between them increased to 3.7). Thus the complex became less stable, with both RBD and ACE2 gradually losing their binding until complete dissociation at 468 ± 87 ns (Figures 3 and 5). These findings provide a putative explanation for aloin A mode of action against SARS CoV-2 and present good proof that site 2 was likely the effector binding site.
It is worth noting that either site 1 or site 2 is not the ACE2’s active site or probably an allostery site (Figure S3), and hence, binding of aloin A as suggested herein should not affect the enzyme's activity, and this is what was found upon ACE2 in vitro activity assay, where aloin A was not able to inhibit ACE2 up to the concentration of 100 µM.

Figure 3. Stability of the RBD-ACE2 complex upon binding of aloin A over 600 ns of MD simulation (i.e., one of the key interacting residues in ACE2) and TYR-453 (i.e. one of the key interacting residues in RBD). The binding of aloin A to ACE2 at site 1 did not influence the stability of the RBD-ACE2 complex throughout 600ns of MD simulation (i.e., no significant change in the calculated distance between HIS-34 and TYR-453). Binding of aloin A to ACE2 at site 2 induced complex dissociation starting at 225 ± 73 ns ($n = 3$). RBD appears in cyan, while ACE2 appears in light brown color. Aloin A detached from ACE2 at 489 ns. The movie of the dissociation event can be found on the zenodo website at the following link: https://zenodo.org/record/7423134#.Y5UVz8tBy3A.
Figure 4. RMSFs (A-D) and RMSDs (E-H) of ACE2-RBD complex in the presence of: (i) aloinA bound to site 2 (A and E), (ii) aloinA bound to site 1 (B and F), (iii) 2 aloin A molecules, one bound to site 1 and the other bound to site 2 (C and G), (iv) ligand-free ACE2-RBD complex (D and H). ACE2 and RBD domains are highlighted, including amino acids of site 2 in the RMSFs (A). The MD simulation results were obtained from three independent runs.
Figure 5. Binding modes of aloin A inside ACE2’ site 2 during the course of MD simulation. From the beginning until ~198 ns, aloin A was able to establish a number of H-bonds with ALA-387, PHE-390, and TYR-505. RBD-ACE2 complex was still intact over the course of this period via 12 H-bonds, including those between LYS-353 and GLY-469, HIS-34 and TYR-453, and ASP-30 and LYS-417 (A). Starting from ~225 ns, aloin A changed its binding mode a bit, establishing two additional H-bonds with two of the RBD-ACE2 interacting residues (HIS-34 and LYS-353), making them unavailable for RBD-ACE2 stabilization. This transition also made the interaction between ASP-30 and LYS-417 weaker (H-bond length between them became 3.7 Å) (B). RBD appears in magenta, while ACE2 appears in green color.

4. Discussion

Over the past two years, many antiviral screening campaigns have been established in the quest for effective medication for COVID-19. Both synthetic and natural products were extensively investigated, and the rapid characterization of SARS CoV-2 proteins facilitated the virtual screening process. The main viral proteases (e.g., MPro and PLPro) were the most investigated targets for developing possible anti-SARS CoV-2 drugs [42]. Recently, paxolvid, an MPro inhibitor, has been approved as an oral pill [43]. However, more drugs acting via other mechanisms are essential to face this rapidly mutating virus.

Aloin A is a natural yellow pigment produced by many plants and has proven its efficacy as a laxative agent [44]. Moreover, it has also exhibited diverse biological activities, ranging from anti-inflammatory to anti-tumor ones [44]–[47]. Recently, this compound has exhibited inhibitory
activities against different SARS CoV-2 proteases, particularly PL$_{Pro}$. However, its efficacy against the virus itself is yet to be investigated. Building on these findings, we decided to evaluate the antiviral potential of this compound against SARS CoV-2 in vitro.

Our current findings showed very good inhibitory activity for aloin A in the direct antiviral assay. However, its proteases (i.e., M$_{Pro}$ and PL$_{Pro}$) inhibitory activities were weak to moderate. Accordingly, we conducted a series of modeling and physics-based simulations to understand the possible mode of action of this compound.

Testing for drugability and cellular permeability should not be overlooked during the screening and development of potential drugs for SARS-CoV-2, where intracellular and extracellular targets are present. Hence, upon testing of aloin A’s cellular permeability, it showed poor cellular permeability, and this finding was in very good accordance with the previously reported permeability profiles. As a result, RBD-ACE2 appeared as a potential extracellular key target for aloin A (i.e., got the best affinity score) that showed poor cellular permeability. Findings extracted from 600 ns-long MD simulation experiments suggest that aloin A might act as an RBD-ACE2 destabilizer, where it can bind inside a binding site located at the RBD-ACE2 interfaces, and hence such unique binding initiated a dissociation event started with a gradual loss of essential H-bonds between the two proteins.

On the other hand, aloin A was tested in vitro for its possible inhibitory activity against human ACE2 and was found to be inactive, indicating that the proposed binding site in the present study did not affect the catalytic activity of ACE2.
A few recent studies have reported small molecules, proteins, and antibodies capable of destabilizing RBD-ACE2 binding [48]–[51]. Almost all of these studies explored the stabilizing and destabilizing potential of these agents via extensive MD simulations, and many showed similar experimental outcomes. Despite the observed high rate of S-protein mutation, RBD was less affected, particularly at its ACE2 binding interface [51], [52]. Hence, this protein complex is both novel and unexplored by the widespread drug screening campaigns.

However, experimental proof for the dissociation event should have been conducted. The capabilities for running such an experiment are currently unavailable in our lab. Alternatively, long MD simulations were able to provide acceptable computational proof for here suggested mode of action. Moreover, the lack of in vivo evaluation of aloin A was also another limitation. However, we aim to prepare a nano-formulation of aloin A (e.g., inhaled powder or spray) to overcome its possible poor bioavailability and enhance its activity at the site of action utilizing the selectivity of this molecule in being unable to interfere with the ACE2 function and its low cellular toxicity.

In conclusion, our study presents new results on the anti-SARS CoV-2 activity of the natural product aloin A, suggesting an alternative mode of action (RBD-ACE2 destabilization). Further in-depth experimental work alongside in vivo evaluation could lead to a new anti-SARS-CoV-2 lead.

Data and Software Availability

Coordinates of the three-dimensional structures used in this investigation were retrieved from the Protein Data Bank (https://www.rcsb.org/). MD simulations were performed using NAMD 3.0
software (version 2020.11). Visual Molecular Dynamics (VMD) program version 1.9.3 was used to conduct the structural analyses of trajectory models. Figures were generated using the VMD and pymol software version 2.5. Authors will release MD trajectories upon article publication using the Zenodo repository (https://zenodo.org/).

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**CRediT authorship contribution statement**

Hani A. Alhadrami: Formal analysis, Investigation, Resources. Ahmed M. Sayed: Conceptualization, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. Hossam H. Hassam: Investigation, Resources. Mostafa E. Rateb: Conceptualization, , Resources, Writing – review & editing.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Supplementary Data**

Supplementary data to this article can be found online at XXXXXXX.
References


