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Azadirachta Indica Phytoconstituents as Novel Inhibitors for Main Proteases of COVID-19: Molecular Dynamics and Simulation Study

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ABSTRACT

Objective: This study aimed to evaluate the therapeutic potential of Azadirachta indica phytoconstituents as inhibitors of SARS-CoV-2 main protease (Mpro) and papain-like protease (PLpro) using computational approaches. **Methods:** Twenty phytochemicals were screened using molecular docking with AutoDock Vina to determine binding affinities against Mpro (PDB: 6YB7) and PLpro (PDB: 7LBR). Molecular dynamics simulations were performed using the iMODS server to evaluate protein-ligand complex stability. ADMET analysis using SwissADME assessed pharmacokinetic properties, including gastrointestinal absorption, lipophilicity, and compliance with Lipinski's Rule of Five. **Results:** The highest binding affinities were observed with 7-deacetyl 7-benzoyl gedunin for Mpro (-9.7 kcal/mol) and PLpro (-8.2 kcal/mol). ADMET analysis showed satisfactory pharmacokinetics, with most ligands demonstrating good GI absorption and no blood-brain barrier permeability. Molecular dynamics confirmed stability, with low Eigenvalues (Mpro: 1.282, PLpro: 6.226). **Conclusion:** The phytoconstituents of Azadirachta indica demonstrated significant potential as inhibitors of SARS-CoV-2 proteases, supporting their role in antiviral drug development. Further experimental validation is recommended.

INTODUCTION

SARS-CoV-2, a positive-sense RNA virus, first emerged in the Huanan market of Wuhan, China, causing a global health crisis of unprecedented proportions (1, 2). The disease caused by this virus, termed COVID-19 by the World Health Organization, was identified and isolated in December 2019 from patients linked to a seafood market in Wuhan (3, 4). SARS-CoV-2 belongs to the family of coronaviruses, named for their crown-like spikes on the viral surface, which consist of single-stranded RNA and cause a spectrum of illnesses in humans and animals, ranging from respiratory and gastrointestinal disorders to neurological and immune system dysfunctions (5-7). Coronaviruses are classified into four genera: α-CoVs, β-CoVs, γ-CoVs, and δ-CoVs, with SARS-CoV-2 being a member of the β-CoVs genus (8, 9). Its genome, the largest among RNA viruses, spans approximately 29.9 kb and exhibits high genetic similarity with bat-derived coronaviruses such as bat-SL-CoVZXC21 and bat-SL-CoVZC45 (88%) and moderate similarity with MERS-CoV (50%) (10-15). SARS-CoV-2 also shares 79% genomic similarity with SARS-CoV, with both viruses exploiting the angiotensin-converting enzyme 2 (ACE2) receptor for cellular entry (16, 17).

Upon entry into the host cell via receptor-mediated endocytosis, SARS-CoV-2 utilizes its RNA genome to encode two polyproteins, pp1a and pp1b, which are

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subsequently processed into 16 non-structural proteins (NSPs) essential for viral replication (18-22). Among these, papain-like protease (PLpro) and main protease (Mpro) play pivotal roles in cleaving polyproteins and facilitating viral replication. PLpro, a critical cysteine protease, mediates the proteolytic cleavage of NSP1-4 and contributes to immune evasion through deubiquitination of host proteins (23-25). Similarly, Mpro, also known as 3CL protease, is responsible for processing NSP5 to NSP16, activities indispensable for viral transcription and replication (25-27). Due to their essential roles in viral propagation and the absence of homologous proteins in human cells, PLpro and Mpro have been identified as promising therapeutic targets for SARS-CoV-2 drug development (28, 29).

COVID-19 disproportionately affects individuals with weakened immune systems, including those with pre-existing conditions or long-term use of immunosuppressive therapies, leading to complications such as liver cirrhosis, severe respiratory symptoms, myalgia, and fever (30-32). The disease has resulted in staggering global statistics, infecting over 770 million people and causing 6.95 million deaths as of September 2023 (33). These alarming figures underscore the urgency of developing effective therapeutic agents, including direct inhibitors targeting the virus's conserved enzymes such as PLpro and Mpro (34). In this regard, computational approaches have gained traction, enabling the identification of drug candidates through molecular modeling and in silico screening techniques.

Natural products have historically been pivotal in drug discovery, offering a rich repository of bioactive compounds for managing numerous diseases. Recent studies have highlighted the antiviral potential of various plant-derived compounds such as quercetin (35, 36), curcumin (37, 38), salvianolic acid (38), glycyrrhizic acid, and epigallocatechin-3-gallate (39, 40). Azadirachta indica, commonly known as neem, holds a prominent place in traditional medicine, particularly in the Indo-Pak region, for its diverse pharmacological properties, including antimicrobial, anti-inflammatory, and antiviral activities (43-45). Neem's bioactive phytochemicals, including terpenoids, flavonoids, and alkaloids, have shown therapeutic potential against a wide array of diseases and may exhibit virucidal properties against SARS-CoV-2 (45-47).

This study leverages computational techniques to screen phytochemicals from Azadirachta indica for their inhibitory potential against SARS-CoV-2 proteases, specifically PLpro and Mpro. Using molecular docking and molecular dynamics simulations, the study aims to identify potent inhibitors that disrupt viral replication and transcription by targeting these proteases. These findings not only elucidate the antiviral potential of neem-derived phytochemicals but also contribute to the broader endeavor of developing effective therapeutic agents for COVID-19.

MATERIAL AND METHODS

The study employed a comprehensive in silico methodology to investigate the potential antiviral properties of selected ligands against the SARS-CoV-2 proteases. A total of 20 ligands were identified through an extensive literature review, and their chemical structures in structure data file (SDF) format were retrieved from the PubChem-NCBI database (https://pubchem.ncbi.nlm.nih.gov/) on March 4, 2024. These ligands were preprocessed using Discovery Studio Client 2021, which involved removing water molecules and saving the structures in Protein Data Bank (PDB) format. Concurrently, the three-dimensional structures of the target SARS-CoV-2 proteins, main protease (Mpro: PDB ID 6YB7) and papain-like protease (PLpro: PDB ID 7LBR), were downloaded from the Protein Data Bank repository (https://www.rcsb.org/) on April 6, 2024. The proteins were purified to ensure accuracy in virtual screening and docking, a process that included removing water molecules, heteroatoms, and any non-specific chemical structures from the crystal structures. Additionally, polar hydrogen atoms were added to modify tautomeric and ionization states of amino acid residues, ensuring a biologically relevant protein configuration for docking analyses.

For molecular docking, AutoDock Vina 1.5.7 was used to evaluate the binding interactions between the purified ligands and the proteases. Key preparatory steps included the addition of polar hydrogen atoms and Kollman charges to the proteins, setting up a grid box, and saving the macromolecule in PDBQT format. Ligands were similarly prepared with torsions adjusted based on their chemical structure. Each ligand underwent 50 docking runs to identify the most stable protein-ligand complexes with optimal binding energies. The docking results from AutoDock Vina were further validated using the Molecular Operating Environment (MOE), where two poses per protein-ligand complex were selected for detailed evaluation of binding affinities.

The binding poses and molecular interactions were visualized using PyMol and MOE. PyMol provided a three-dimensional perspective of the ligand-protein interactions, while MOE facilitated the analysis of two-dimensional interactions, including hydrogen bonding, hydrophobic contacts, bond lengths, and distances between the ligands and interacting amino acid residues.

Molecular dynamics simulations were performed using the iMODS server (https://bio.tools/imods) on July 26, 2024, to assess the stability and flexibility of the

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docked protein-ligand complexes. The simulations provided a wide range of outputs, including deformability, mobility profiles, variance, covariance maps, and Eigenvalues, which quantify the energy required to induce structural deformation and the stiffness of the complexes. This approach offered valuable insights into the structural dynamics of the complexes at the molecular level.

To further evaluate the pharmacokinetic properties and drug-likeness of the ligands with high binding affinities, ADMET (absorption, distribution, metabolism, excretion, and toxicity) analysis was performed using the SwissADME web tool (http://www.swissadme.ch/) on July 29, 2024. Ligand structures in SDF format were analyzed to predict parameters such as water solubility, lipophilicity, gastrointestinal absorption, P-glycoprotein substrate inhibition, blood-brain barrier permeation, CYP enzyme inhibition, and skin permeability (log Kp). Additionally, drug-likeness assessments were conducted based on Lipinski's rule of five and bioavailability scores. These analyses provided crucial insights into the pharmacological viability of the selected ligands, facilitating their potential development as therapeutic agents against SARS-CoV-2.

RESULTS

The molecular docking analysis revealed the binding affinities of selected phytochemicals from *Azadirachta indica* against the main protease (Mpro) and papain-like protease (PLpro) of SARS-CoV-2. Docking scores demonstrated the potential of these phytochemicals to bind effectively with the proteases, with lower binding energy (more negative scores) indicating stronger binding interactions. Among the tested compounds, 7-deacetyl 7-benzoyl gedunin exhibited the highest binding affinities for both proteases, with docking scores of -9.7 kcal/mol for Mpro and -8.2 kcal/mol for PLpro. The second-highest binding affinity for Mpro was observed with Neemfruitin B (-9.5 kcal/mol), while for PLpro, azadiradione displayed the second-best binding affinity (-8.1 kcal/mol). These results identified 7-deacetyl 7-benzoyl gedunin, Neemfruitin B, azadiradione, and 17-hydroxy azadiradione as the most promising compounds for further analysis. Specific binding interactions for 7-deacetyl 7-benzoyl gedunin with Mpro included Thr199, Lys137, Glu290, and Arg131 residues, with bond distances of 3.40 Å, 4.52 Å, 4.65 Å, and 3.53 Å, respectively. Similarly, interactions with PLpro involved Glu67 and Val20, forming hydrogen bonds at distances of 3.59 Å and 3.12 Å.

Figure 1

3-D binding of hit compounds against Main proteases Mpro and PLpro of SARS-CoV-2

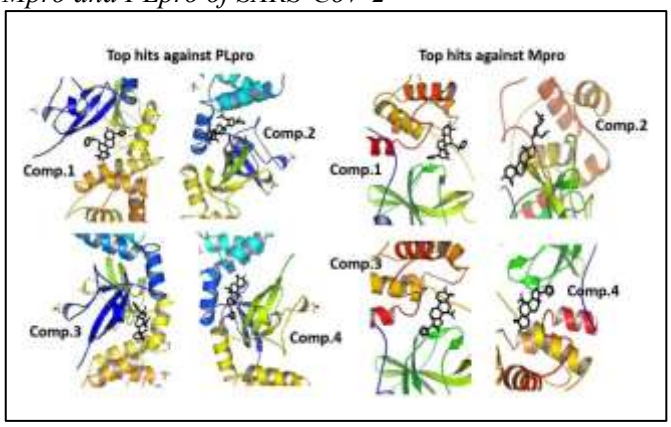

Figure 2

2-D binding and interactions of hit compounds against Main proteases Mpro and Plpro of SARS-CoV-2

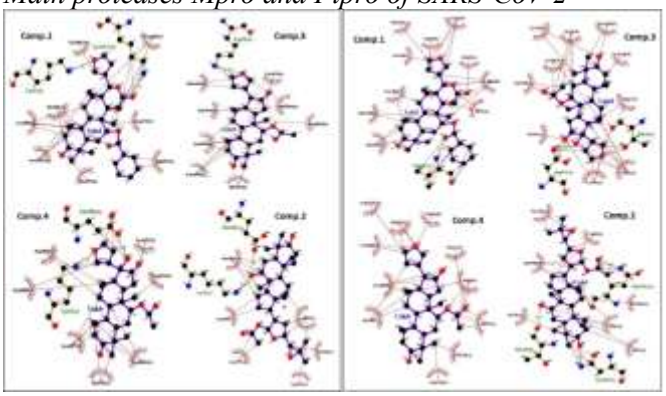

Figure 3

Predicted interactions of protein-ligand docked complexes

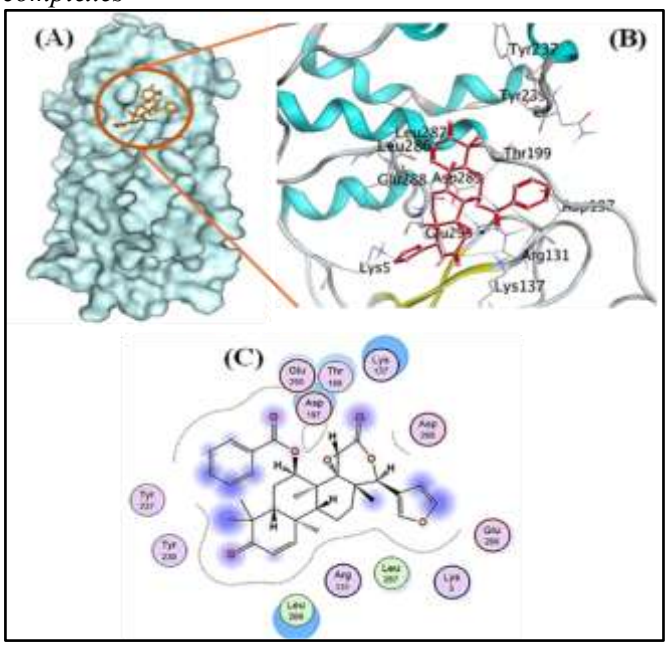

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Figure 4
Predicted interactions of protein-ligand docked complex:

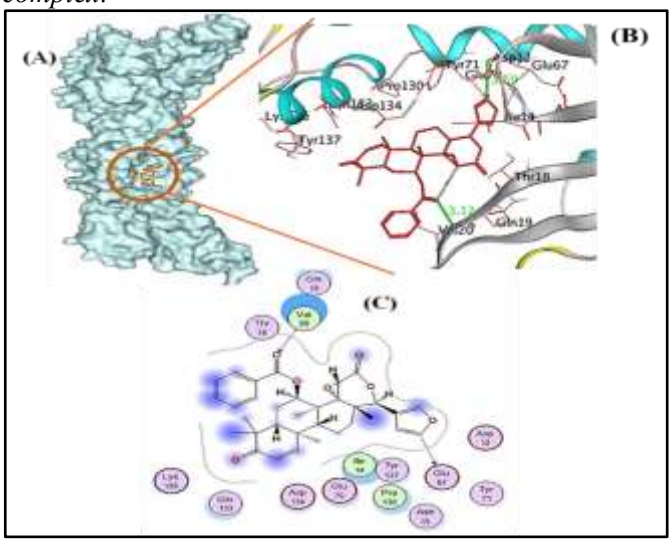


Figure 5
Prediction of binding interactions of protein-ligand docked complex:

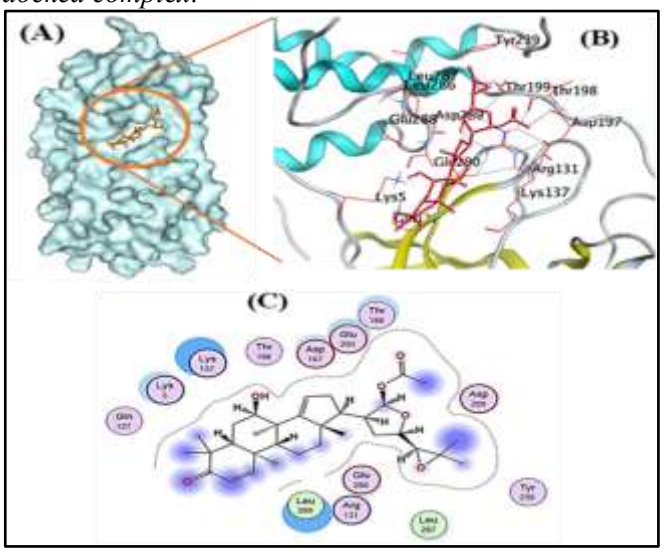


Figure 6
Predicted binding interactions of protein-ligand complex

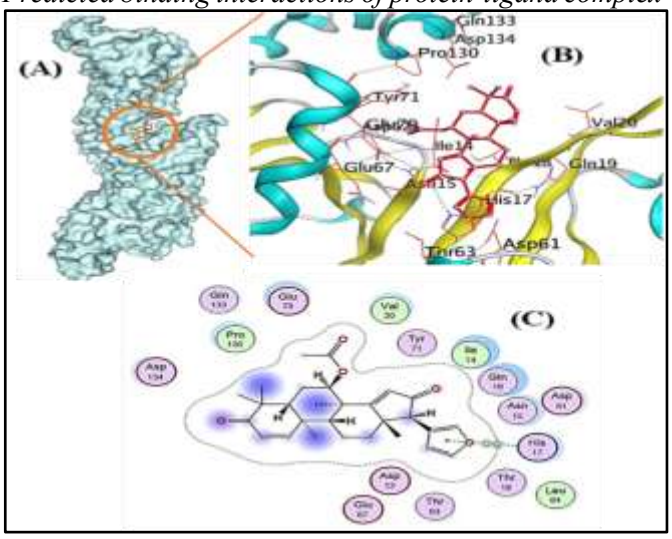


Figure 7
Molecular dynamics simulation of predicted 7-deacetyl 7-benzoyl gedunin–Mpro complex: (a)

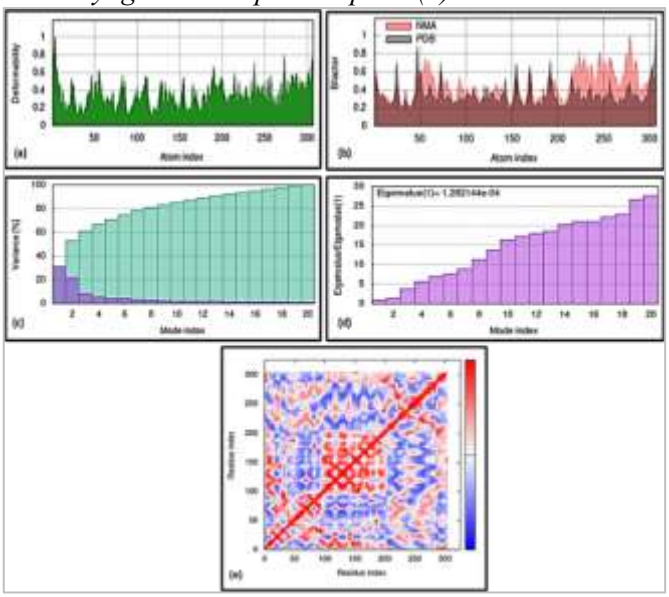
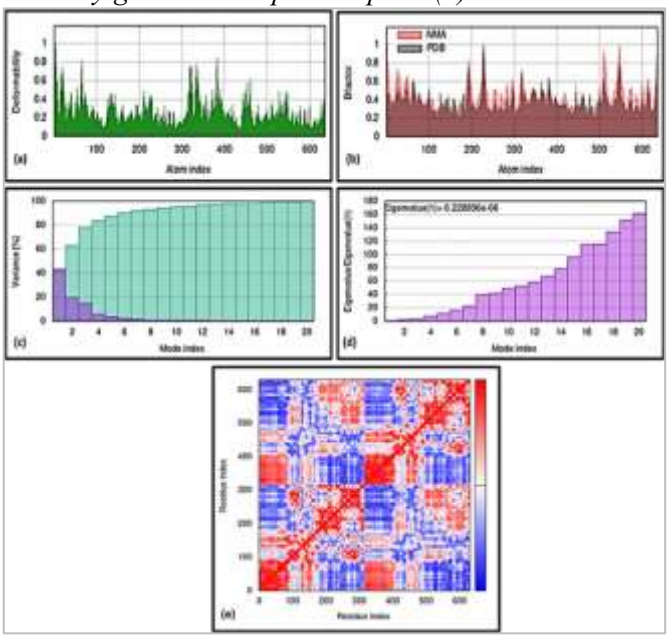


Figure 8
Predicted molecular dynamics simulations of 7-deacetyl 7-benzoylgedunin – PLpro complex: (a)



Molecular dynamics simulations were employed to assess the stability and behavior of the docked protein-ligand complexes under physiological conditions. Deformability studies showed that most residues of the 7-deacetyl 7-benzoyl gedunin complexes with both proteases exhibited moderate flexibility, allowing for optimal binding. The B-factor analysis, indicating the vibrational motion of atoms, revealed greater vibrational motion and disorder in the Mpro complex compared to the PLpro complex. Variance analysis showed that the PLpro complex had higher cumulative variance (42%) compared to the Mpro complex (30%), reflecting the structural adaptability of the complexes. Eigenvalues,

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which represent the energy required for structural deformation, were lower for both complexes, indicating high stiffness and stability. The Eigenvalues for the Mpro and PLpro complexes were 1.282 and 6.226, respectively. Covariance mapping revealed moderate to high correlation between residue pairs in both complexes, supporting the stability of the protein-ligand interactions. Overall, these findings highlight the potential of 7-deacetyl 7-benzoyl gedunin as a strong inhibitor of both proteases, with effective binding and stability.

Table 1

Binding affinities of selected ligands against proteins of SARS-CoV-2.

Sr no.	Phytoconstituents	Pub Chem CID	Molecular weight (MW) (g/mol)	Binding energies against *6YB7 (kcal/mol)	Binding energies against **7LBR (kcal/mol)
1.	7-deacetyl 7-benzoyl gedunin	52952112	544.6	-9.7	-8.2
2.	Neemfruitin B	46919586	526.70	-9.5	-7.9
3.	17-hydroxy azadiradione	52951892	466.60	-9.4	-7.9
4.	Azadiradione	12308714	450.60	-9.0	-8.1
5.	Nimocinolide	6442906	500.6	-9.4	-7.4
6.	7-deacetylnimolicinol	12011153	440.5	-9.2	-7.4
7.	Naheedin	129754	528.7	-9.1	-7.4
8.	Azadirone	10906239	436.6	-9.0	-7.8
9.	Azadiradionolide	11798426	466.6	-9.0	-7.7
10.	6-deacetyl nimbinene	102285347	297.7	-8.9	-7.3
11.	Azadirachtol	23256847	580.6	-8.8	-7.0
12.	Nimolicinol	184937	482.6	-8.7	-7.6
13.	Nimbandiol	157277	456.5	-8.2	-7.4
14.	Nimbinene	44715635	482.5	-8.1	-6.9
15.	Mahmoodin	126566	526.6	-8.0	-7.3
16.	Nimolinin	180429	316.4	-8.0	-7.1
17.	Isonimbolide	139059467	466.5	-7.9	-7.0
18.	Neemfruitin A	49864006	516.7	-7.8	-7.9
19.	Nimbiol	11119228	272.4	-7.8	-6.8
20.	Nimbosone	177090	300.4	-7.6	-7.3

*6YB7: main protease; **7LBR: papain-like protease

Table 2

Physicochemical Properties, Pharmacokinetic Profiles, and Drug-Likeness of Selected Compounds

Compounds	Water solubility	Lipophilicity	Pharmacokinetics	*Lipinski's RO5	**Bioavailability score
	*LogS (ESOL)	Class	**LogS (SILICOS-IT)	Class	***Log Po/W
7-deacetyl 7-benzoyl gedunin	-6.86	poorly soluble	-7.81	poorly soluble	4.86
Naheedin	-6.12	poorly soluble	-4.61	moderately soluble	4.65
Azaidradione	-5.58	moderately soluble	-6.31	poorly soluble	4.34
Azadirone	-6.07	poorly soluble	-6.43	poorly soluble	5.02
Azadirachtol	-3.15	soluble	-0.17	soluble	-0.1
Nimolicinol	-5.17	moderately soluble	-5.66	moderately soluble	3.72
17-hydroxy azadiradione	-5.09	moderately soluble	-5.93	moderately soluble	3.47
Neemfruitin A	-5.42	moderately soluble	-4.74	moderately soluble	3.95
Neemfruitin B	-6.16	poorly soluble	-5.01	moderately soluble	4.78
6-deacetyl nimbinene	-3.33	soluble	-5.05	moderately soluble	2.99
Isonimbolide	-4.2	moderately soluble	-5.27	moderately soluble	3.12
Mahmoodin	-5.14	moderately soluble	-6.16	poorly soluble	3.72
Nimbandiol	-3.06	soluble	-4.23	moderately soluble	2.36
Nimbinene	-3.83	soluble	-5.66	moderately soluble	3.41
Nimbiol	-4.81	moderately soluble	-5.22	moderately soluble	4.03
Azadiradionolide	-5.1	moderately soluble	-5.05	moderately soluble	3.8
Nimolinin	-4.57	moderately soluble	-5.29	moderately soluble	3.87
Nimocinolide	-4.17	moderately soluble	-3.16	moderately soluble	2.44
7-deacetylnimolicinol	-4.69	moderately soluble	-5.05	moderately soluble	3.24
Nimbosone	-5.3	moderately soluble	-5.98	moderately soluble	4.62

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In silico ADMET analysis evaluated the pharmacokinetics, drug-likeness, and toxicity profiles of the selected phytochemicals. Parameters such as water solubility, lipophilicity, gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, and interactions with P-glycoprotein (P-gp) and cytochrome P450 enzymes (CYP450) were assessed. Most ligands exhibited moderate water solubility and lipophilic properties, except for Azadirone. Ligands with the lowest binding affinities, excluding Azadirachtol, demonstrated high GI absorption, indicating their potential for effective oral administration. Only Nimbiol, Nimolinin, and Nimbosone were predicted to permeate the BBB, which could have implications for treating neurological manifestations of SARS-CoV-2. Drug-likeness was evaluated using Lipinski's Rule of Five, and all ligands with favorable binding affinities adhered to these rules. The bioavailability scores supported the potential of these compounds for further development. These ADMET results provide critical insights into the pharmacological profiles of the phytochemicals, facilitating their prioritization for preclinical testing. Together, these findings suggest that 7-deacetyl 7-benzoyl gedunin, along with other high-affinity ligands, represents promising candidates for therapeutic intervention against SARS-CoV-2.

DISCUSSION

The COVID-19 pandemic initiated a global race among scientists to develop effective vaccines, which undoubtedly played a crucial role in reducing viral transmission and preventing millions of deaths. However, concerns surrounding vaccine-related side effects have emerged, with issues such as thrombosis with thrombocytopenia syndrome (TTS) linked to viral vector vaccines (50) and myocarditis reported at higher rates among recipients of mRNA vaccines (51). Additionally, common adverse reactions, including fever, headache, and myalgia, affected more than 60% of vaccinated individuals (52, 53). Despite these achievements, the virus continued to evolve, with the emergence of new variants and the possibility of vaccine resistance posing persistent challenges. These factors highlighted the necessity of exploring alternative therapeutic options, as vaccines alone could not completely curb the spread of SARS-CoV-2.

Several drugs, including Remdesivir, Ritonavir, Molnupiravir, and Ebselen, have demonstrated anti-COVID-19 potential, yet more efficient and precise therapeutics remain highly anticipated (54, 55). The absence of universally effective antiviral agents motivated researchers to investigate the therapeutic potential of natural compounds, particularly phytochemicals derived from medicinal plants. Over the years, bioactive compounds from plants have consistently exhibited anti-inflammatory, antioxidative, and antiviral properties, offering significant promise in combating various diseases, including viral infections. Plants such as *Glycyrrhiza glabra*, *Curcuma longa*, and *Moringa oleifera* have shown notable efficacy against SARS-CoV-2 in vitro, supporting the continued evaluation of plant-based therapeutics (56-60). Leveraging computational tools for drug discovery, which allow the rapid screening of numerous compounds while conserving resources and avoiding animal testing, became a vital strategy in addressing the urgent need for effective COVID-19 treatments.

In the present study, the phytoconstituents of *Azadirachta indica* were screened for their ability to inhibit the essential proteases of SARS-CoV-2, namely the main protease (Mpro) and papain-like protease (PLpro). Molecular docking studies revealed high binding affinities of selected ligands, with affinities ranging from -9.9 kcal/mol to -7.6 kcal/mol for Mpro and from -8.2 kcal/mol to -6.8 kcal/mol for PLpro. Interestingly, the compounds exhibited stronger binding to Mpro compared to PLpro. Among the screened compounds, 7-deacetyl 7-benzoyl gedunin demonstrated the highest binding affinity for both proteases, indicating its potential to disrupt viral replication by targeting these key enzymes. These findings align with prior studies where natural products exhibited promising antiviral activity, reinforcing the utility of *Azadirachta indica* as a viable source for anti-COVID-19 drug development.

Molecular dynamics simulations further confirmed the stability of the docked protein-ligand complexes. The evaluation of mobility, flexibility, stability, and deformability, along with Eigenvalues, indicated favorable molecular interactions. The ADMET analysis of selected ligands provided additional insights, showing satisfactory pharmacokinetic properties and adherence to Lipinski's Rule of Five, with most compounds demonstrating good gastrointestinal absorption and minimal blood-brain barrier permeability. However, some ligands displayed minor violations of the drug-likeness criteria, suggesting the need for chemical modifications to enhance their pharmacological profiles.

The findings of this study underscored the strengths of combining molecular docking and dynamics simulations with ADMET profiling for identifying potential therapeutic candidates. The results demonstrated that phytochemicals such as 7-deacetyl 7-benzoyl gedunin, Neemfruitin B, 17-hydroxy azadiradione, and Azadiradione have substantial potential to inhibit SARS-CoV-2 proteases effectively. Nevertheless, this study also had limitations. Computational approaches, while powerful, are inherently predictive and require experimental validation to confirm in vitro and in vivo efficacy. The study relied on in silico tools, which do not account for complex physiological interactions, potential off-target effects, or toxicity under real-world conditions.

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Additionally, clinical trials are essential to translate these findings into viable therapeutic applications.

Future studies should focus on experimental validation of these compounds using cellular and animal models, followed by clinical trials to evaluate their safety and efficacy in humans. The inclusion of structural optimization and the synthesis of derivatives could further enhance the drug-likeness and potency of these phytochemicals. Moreover, combining computational approaches with high-throughput experimental techniques could accelerate the discovery of novel antiviral agents.

In summary, this study demonstrated the therapeutic promise of *Azadirachta indica* phytochemicals in targeting SARS-CoV-2 proteases. The findings provide a foundation for the development of plant-based antiviral drugs, contributing to the ongoing efforts to combat COVID-19. Despite the challenges and limitations, the potential of these compounds as effective therapeutics warrants further investigation and validation in clinical settings.

CONCLUSION

In conclusion, this study highlighted the therapeutic potential of phytoconstituents from *Azadirachta indica*, particularly 7-deacetyl 7-benzoyl gedunin, Neemfruitin B, 17-hydroxy azadiradione, and Azadiradione, as promising inhibitors of SARS-CoV-2 proteases. Through computational analyses, these compounds demonstrated strong binding affinities, molecular stability, and favorable pharmacokinetic properties, supporting their role as potential anti-COVID-19 therapeutics. The findings underscore the value of plant-based natural compounds in drug discovery, offering a sustainable and accessible avenue for developing antiviral treatments. These results hold significant implications for human healthcare by addressing the ongoing need for effective and safe therapeutics to complement vaccination efforts in mitigating the global burden of COVID-19. Further experimental validation and clinical trials are essential to translate these computational insights into practical healthcare solutions.

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