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Exploring *Azadirachta indica's* Potential to Combat Visceral Leishmaniasis Through GP63 Inhibition

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Abstract

Objectives: *Millions of people worldwide suffer from the parasite disease visceral leishmaniasis. Determine the naturally occurring active ingredients in Azadirachta indica, which are employed as a disease-prevention agent and have several uses in medicine. To choose the best ligand to use as a medication, absorption, distribution, metabolism, and excretion (ADME) studies and molecular docking are employed.* ***Methods:*** *To obtain the GP63 protein, the Protein Data Bank (PDB) database was accessed. The IMPPAT database was utilised to obtain phytocompounds. The pharmacokinetic characteristics of these drugs were evaluated using in silico ADME analysis. PyRx is used for molecular docking.* ***Results:*** *Molecular docking analysis revealed promising interactions between the selected Azadirachta indica phytocompound and the GP63 protein, indicating its potential as inhibitor of Leishmania parasite virulence. 6-Deacetylnimbin demonstrated significant binding affinity with GP63, warranting further investigation. Furthermore, the phytocompound exhibited favourable pharmacokinetic properties, suggesting their suitability for drug development.* ***Conclusion:*** *The phytocompound 6-Deacetylnimbin exhibits a promising binding affinity with the GP63 protein, making it a potential therapeutic candidate for the treatment of visceral leishmaniasis.*

Keywords: *Azadirachta indica*, visceral leishmaniasis, GP63, phytocompound, molecular docking, ADMET analysis

INTRODUCTION

Leishmania, a collection of parasites that afflict humans in over 20 different forms, is the cause of leishmaniasis [1]. About 1.3 million new instances of the disease are reported year, with 20,000–30,000 fatalities, mostly in some of the world's poorest nations [2]. These parasites are spread by over 90 species of sandflies and can also be carried by a variety of animals, including dogs, foxes, and rats. Leishmaniasis comes in three primary forms: mucosal, visceral, and cutaneous [3–5]. The most severe type of leishmaniasis, known as visceral leishmaniasis, can appear ten days to years following a sandfly bite. This disease, which is often ignored but has the potential to be fatal, is primarily seen in regions of Bangladesh, India, several African nations, and Latin American nations like Brazil [6, 7].

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Leishmania parasites target organs, such as the liver, spleen, and bone marrow when they penetrate the skin through sandfly bites. For the parasites to enter and remain within host cells, a protein GP63 is essential. With the help of T cells, neutrophils, and macrophages, the immune system responds, but Leishmania attempts to blunt the attack by inhibiting specific immune cells and causing regulatory chemicals. This makes it possible for the parasites to spread infection and

bring about illness [8].

Visceral leishmaniasis symptoms can be classified as either localized (enlarged spleen and liver, swelling, cough, renal difficulties, and diarrhea) or constitutional (fever, weight loss, nausea, and anemia). A variety of techniques are used in diagnosis, including molecular techniques, testing for immunological responses, and parasite examination of samples [9].

Although there are therapies for visceral leishmaniasis, they come with a lot of disadvantages. Treatment options are complicated by our inadequate understanding of the exact molecular pathways underlying medication resistance in Leishmania parasites [10]. Moreover, prompt disease diagnosis is hampered by the lack of very sensitive point-of-care diagnostic instruments. Furthermore, the challenges associated with disease prevention are made worse by the lack of a specific vaccination. Current treatment choices, including pentavalent antimonials, come with a range of side effects, such as myalgia, hepatotoxicity, cardiotoxicity, and gastrointestinal problems. In addition, their varied efficiency against different Leishmania species and dose-dependent toxicity requires judicious administration to minimize hazards. Their lengthy treatment plans and high expense further restrict their usefulness. Comparably, over-the-counter drugs like Amphotericin B come with a price tag and a host of adverse effects include nephrotoxicity, fever responses, and gastrointestinal intolerance [11]. Therefore, it is imperative to investigate new treatment pathways, and *Azadirachta indica* as shown in Figure 1 appears to be a viable option that merits more research.

Strong antibacterial qualities enable neem to effectively combat a variety of bacteria, fungi, and viruses [12]. Its essential oils and extracts have been used to treat a variety of microbiological illnesses, including fungal infections and skin disorders including eczema and acne [13]. Studies indicate that it demonstrates antiviral properties against many viruses, such as hepatitis B virus (HBV) [14], human immunovirus (HIV) [15], and herpes simplex virus (HSV). Neem's potential for antiviral therapy has been highlighted by compounds found in its leaves and seeds, which have been demonstrated to have inhibitory effects on viral propagation. It is well recognized to strengthen the body's defenses against illness by strengthening the immune system. It also aids in preventing scarring and promotes wound healing. In conclusion, *Azadirachta indica*, also known as neem, is a veritable gold mine of medicinal qualities, providing a homeopathic remedy for a wide range of ailments [16].

There is currently a significant vacuum in the research about *Azadirachta indica'*s precise function in treating VL by blocking GP63 activity, a crucial Leishmania parasite virulence factor [17]. The antibacterial qualities of *Azadirachta indica* are well known, but nothing is known about its ability to target GP63, a protein that is vital to the survival and virulence of parasites. By examining the inhibitory effects of *Azadirachta indica* extracts or compounds on GP63 activity, our study seeks to close this gap.

Using molecular docking techniques, this study report explores *Azadirachta indica'*s potential to treat visceral leishmaniasis (VL). This work investigates the relationships between *Azadirachta indica* phytocompounds and the GP63 protein, a crucial Leishmania parasite virulence component [18].

Figure 1. *Azadirachta indica*.

Methodology

Protein Extraction

The protein structure corresponding to PDB ID 1LML was retrieved from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/). Upon extraction, the resultant protein structure was identified as Leishmanolysin. The retrieved structure was then downloaded and saved in the standard PDB format for subsequent analyses and computational investigations.

Secondary Structure Prediction

The protein's secondary structure was predicted using the PDBsum web server (http://www.ebi.ac.uk/pdbsum). The distribution of secondary structural features, such as alpha helices, beta strands, beta hairpins, psi-loops, and beta bulges, was revealed by this approach [19].

Additionally, PROCHECK, a program linked on the PDBsum server, was used to do the Ramachandran plot analysis. This technique made it possible to assess the overall structural integrity and conformational quality of the protein [20].

Retrieval of Phytocompounds Using IMPPAT Database

Azadirachta indica, a medicinal plant, was queried in the IMPPAT database (https://cb.imsc.res.in/imppat/) to identify related phytocompounds [21]. Subsequently, a selection process yielded twenty-five phytocompounds.

In Silico Absorption, Distribution, Metabolism, and Excretion (ADME) Analysis

Swiss ADME (http://www.swissadme.ch/) was a key instrument in the analysis of the pharmacokinetics study, which made considerable use of parameters pertaining to ADME in order to assess the behavior of the ligand [22]. This examination includes permeability, toxicity, biological, and physiological qualities, as well as bioavailability, all of which are critical factors in the drug discovery process. It ensured a comprehensive assessment of drug candidature by using Lipinski's five guidelines, as shown in Table 1, which added an extra degree of scrutiny to find potential therapeutic agents [23].

A thorough analysis was conducted to evaluate the physiochemical properties, taking into account important factors like molecular weight, saturation (i.e., Sp3 hybridization or fraction Csp3), flexibility (as determined by the number of rotational bonds), polarity (as determined by topological polar surface area (TPSA)), and lipophilicity (often expressed as xLogP).

Table 1. Lipinski rule specifications.

Property	Optimal Range
Molecular weight	Less than 500 daltons [24]
MlogP	Less than 4.15 [24]
Hydrogen Donors	Less than 5 [24]
Hydogen acceptors	Less than 10 [24]
Molecular Refractive Index	40-130 [24]

Visualization

The detailed analysis of receptor-ligand interactions utilized BIOVIA Discovery Studio 2021 v21.1.0.20298 (https://discover.3ds.com/discovery-studio-visualizer-download).

This study looked closely at both 2D and 3D interactions to understand the molecular binding mechanisms and structures [25]. The research aimed to reveal the specific molecular interactions that drive the formation of receptor-ligand complex.

Docking

Phytochemical compounds extracted from *Azadirachta indica* underwent molecular docking analysis against the ILML protein target. Utilizing the integrated AutoDock Vina software and PyRx

Virtual Screening Tool (https://pyrx.sourceforge.io/), virtual screening of the compounds was conducted. Grid parameters were set to align with the target protein structure (PDB ID: ILML) to optimize docking simulations. The binding energies of phytochemical-protein interactions were calculated and organized into CSV format for further analysis. Subsequently, BIOVIA software was employed to visualize the ligand-protein complexes, elucidating the structural basis of their interactions.

RESULTS

Target Extraction and Purification

The protein structure linked to PDB ID 1LML was sourced from the RCSB Protein Data Bank. Following retrieval, it was identified as Leishmanolysin. Afterwards, this structure was downloaded and preserved in the standard PDB format. The downloaded PDB structure of Leishmanolysin was loaded on the BIOVIA Discovery studio software, where the cleaning of the downloaded protein structure took place. The specific residues coordinating the Zinc ions were identified and removed carefully using the software's editing tools. Thus, the new modified protein structure, as shown Figure 2, was saved and used for further computational analysis.

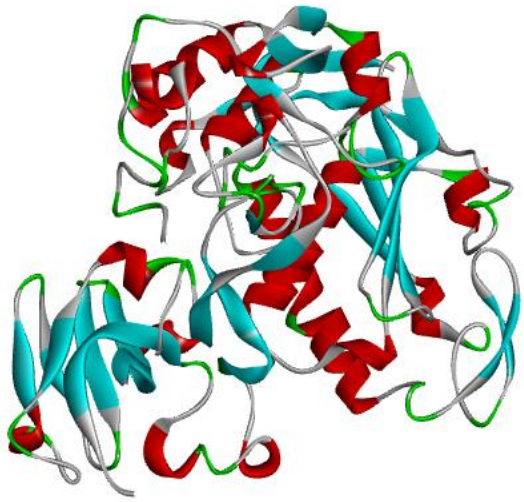

Figure 2. 3D model of purified protein.

Structure Validation of the Protein

Using PDBSUM webserver, the target protein's secondary structure (PDB ID: 1LML) was predicted. There are 570 residues in the structure altogether. The secondary structure of the protein has 10 sheets,11 beta-hairpins,1 psi-loop, 8 beta bulge, 28 strands, 20 helices, 8 helix interactions, 48 beta turns, 8 gamma turns, and 9 di sulphides deduced by an analysis of the amino acid sequence stored within its structure as shown in Figure 3.

Ramachandran Plot

Based on their torsion angles ψ and Π, amino acid conformations were graphically mapped by the Ramachandran plot, highlighting locations that are energetically favorable. 400 residues out of 465 total residues were examined, eliminating glycine and proline, using PDBsum and PROCHECK. Most of the highly preferred areas (A, B, and L) included 359 residues (89.8%) and were shown in red. Furthermore, the brown-colored permitted sections (a, b, l, and p) with 39 residues (9.8%) were depicted. A generous allowance (~a, ~b, ~l, ~p) showing 1 residue (0.2%) was shown in yellow. One residue (0.2%) came from the prohibited areas, denoted by XX in light yellow. Additionally, end-residues (Gly and Pro) were examined individually and the plot statistics showed 35 residues of Gly and 24 residues of Proline, as shown in Figure 4.

Identifying Phytocompound Candidates

The twenty-five compounds, namely Nimbiol, 6-Deacetylnimbin, Kulinone, Methyl kulonate, Kulactone, Gedunin, Kulolactone, 6beta-Hydroxystigmast-4-en-3-one, Methyl 2,5-dihydroxycinnamate,(4bS,8aR)-2,4b,8,8-tetramethyl-7,10-dioxo-5,6,8a,9-tetrahydrophenanthrene-3-carboxylic Acid, (4aS,10aR)-7-hydroxy-1,1,4a,6-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione, (4aS,10aR)-6-hydroxy-1,1,4a,7-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione, Nimbionol, Sugiol, Nimbionone, Epoxyazadiradione, Nimbin, Nimbosone, (4aS,10aR)-6,7-dimethoxy-1,1,4a-trimethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione, Margolonone, (4bS,8aS)-3,4b,8,8-tetramethyl-10-oxo-6,7,8a,9-tetrahydro-5H-phenanthrene-2-carboxylic Acid, 6-Hydroxycyclohexa-2,4-dien-1-one, Deacetylgedunin, beta-Sitosterol, and Benzyl Alcohol were subsequently retrieved from the PubChem database and saved in structure data file (SDF) form.

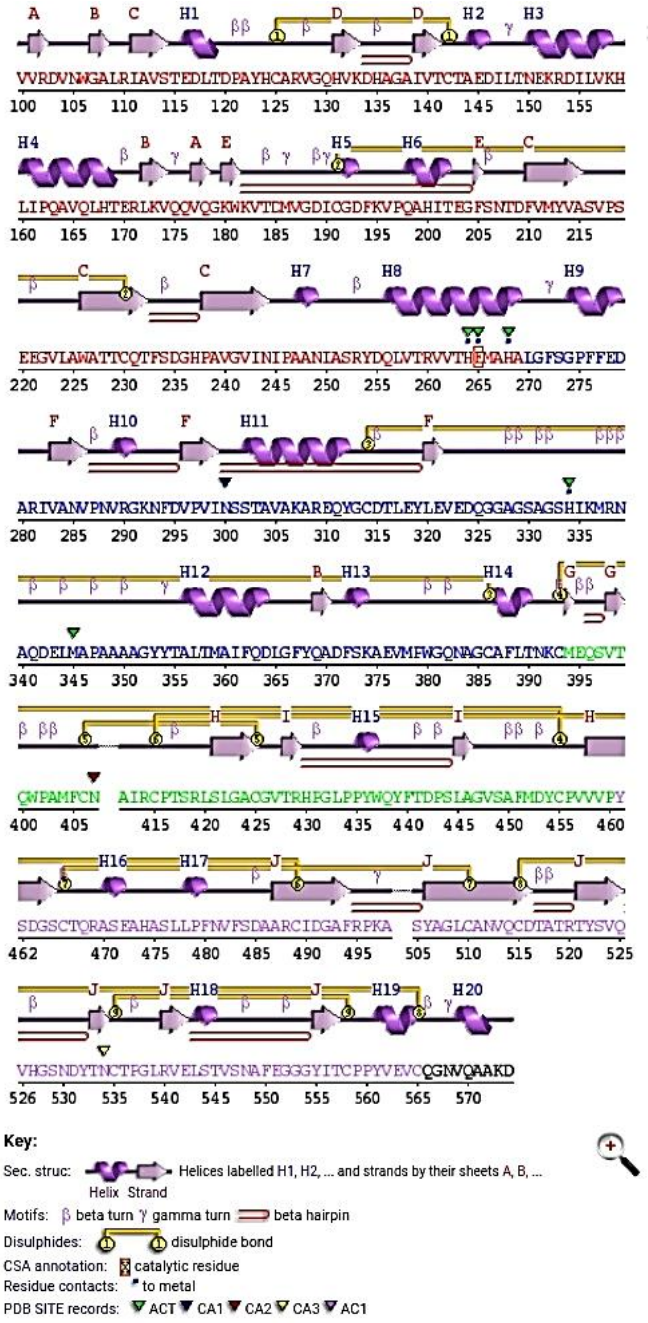

Figure 3. Secondary structure of the protein.

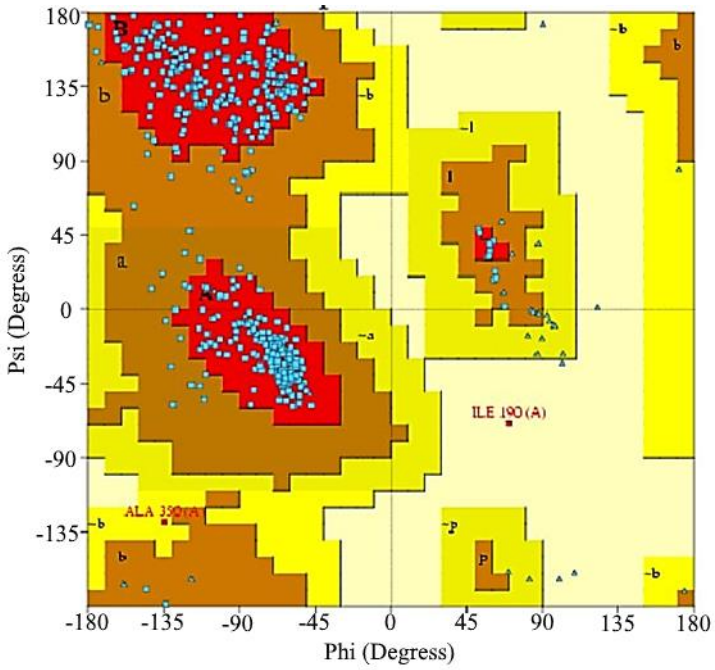

Figure 4. Ramachandran plot.

Table 2. Molecular and Structural Features of Bioactive Ligands.

Ligand	Formula	MW (g/mol)	mlogp	Hydrogen Donors	Hydrogen Acceptors	MRI	Fraction Csp3	RB	TPSA (Å²)
Nimbiol	$C_{18}H_{24}O_2$	272.38	3.44	1	2	82.44	0.61	0	37.30
6-Deacetylnimbin	$C_{28}H_{34}O_8$	498.56	1.69	1	8	129.07	0.61	6	112.27
Kulinone	$C_{30}H_{48}O_2$	440.70	5.79	1	2	137.24	0.83	4	37.30
Methyl kulonate	$C_{31}H_{48}O_4$	484.71	4.97	1	4	143.33	0.81	6	63.60
Kulactone	$C_{30}H_{44}O_3$	452.67	5.63	0	3	135.25	0.80	3	43.37
Gedunin	$C_{28}H_{34}O_7$	482.57	2.56	0	7	126.04	0.68	3	95.34
Kulolactone	$C_{32}H_{48}O_4$	496.72	5.97	0	4	145.95	0.81	5	52.60
6beta-Hydroxystigmast-4-en-3-one	$C_{29}H_{48}O_2$	428.69	5.70	1	2	133.43	0.90	6	37.30
Methyl 2,5-dihydroxycinnamate	$C_{10}H_{10}O_4$	194.18	1.00	2	4	51.48	0.10	3	66.76
(4bS,8aR)-2,4b,8,8-tetramethyl-7,10-dioxo-5,6,8a,9-tetrahydrophenanthrene-3-carboxylic Acid	$C_{19}H_{22}O_4$	314.38	2.59	1	4	87.58	0.53	1	71.44
(4aS,10aR)-7-hydroxy-1,1,4a,6-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	$C_{18}H_{22}O_3$	286.37	2.48	1	3	82.64	0.56	0	54.37
(4aS,10aR)-6-hydroxy-1,1,4a,7-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	$C_{18}H_{22}O_3$	286.37	2.48	1	3	82.64	0.56	0	54.37
Nimbionol	$C_{18}H_{24}O_4$	304.38	1.98	2	4	85.13	0.61	1	66.76
Sugiol	$C_{20}H_{28}O_2$	300.44	3.90	1	2	92.06	0.65	1	37.30

Nimbionone	$C_{18}H_{22}O_4$	302.36	1.89	1	4	84.17	0.56	1	63.60
Epoxyazadiradione	$C_{28}H_{34}O_6$	466.57	2.54	0	6	124.96	0.68	3	86.11
Nimbin	$C_{30}H_{36}O_9$	540.60	2.04	0	9	138.81	0.60	8	118.34
Nimbosone	$C_{20}H_{28}O_2$	300.44	3.90	0	2	91.72	0.65	2	26.30
(4aS,10aR)-6,7-dimethoxy-1,1,4a-trimethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	$C_{19}H_{24}O_4$	316.39	2.13	0	4	88.64	0.58	2	52.60
Margolonone	$C_{19}H_{22}O_4$	314.38	2.59	1	4	87.58	0.53	1	71.44
(4bS,8aS)-3,4b,8,8-tetramethyl-10-oxo-6,7,8a,9-tetrahydro-5H-phenanthrene-2-carboxylic Acid	$C_{19}H_{24}O_3$	300.39	3.52	1	3	87.38	0.58	1	54.37
6-Hydroxycyclohexa-2,4-dien-1-one	$C_6H_5FO_4$	160.10	-0.47	2	5	32.00	0.17	1	66.76
Deacetylgedunin	$C_{26}H_{32}O_6$	440.53	2.23	1	6	116.31	0.69	1	89.27
beta-Sitosterol	$C_{29}H_{50}O$	414.71	6.73	1	1	133.23	0.93	6	20.23
Benzyl Alcohol	C_7H_8O	108.14	1.54	1	1	32.57	0.14	1	20.23

ADME Analysis

The phytocompounds underwent assessment using SWISS ADME, as shown in Table 2, to ascertain their physiochemical attributes and adherence to Lipinski's rule. This crucial step involved the analysis of molecular properties, such as molecular weight, lipophilicity, and polarity, which are imperative for predicting the compound's pharmacokinetic behavior. Additionally, Lipinski's rule, a widely accepted guideline in drug discovery, was applied to evaluate the compound's drug-likeness based on parameters like molecular weight, partition coefficient, hydrogen bond donors, and acceptors.

Pharmakokinetics Properties

The phytocompounds were then subjected to a thorough evaluation to determine their pharmacokinetic characteristics, which included GI absorption, P-glycoprotein (Pgp) substrate potential, and blood-brain barrier (BBB) permeability as shown in Table 3.

Table 3. ADME information acquired via Swiss ADME.

Ligand	**G.I Absorption**	**BBB Permeant**	**P-gp Substrate**
Nimbiol	High	Yes	No
6-Deacetylnimbin	High	Yes	No
Kulinone	Low	No	No
Methyl kulonate	High	No	No
Kulactone	Low	No	No
Gedunin	High	No	Yes
Kulolactone	Low	No	No
6beta-Hydroxystigmast-4-en-3-one	Low	No	No
Methyl 2,5-dihydroxycinnamate	High	Yes	No
(4bS,8aR)-2,4b,8,8-tetramethyl-7,10-dioxo-5,6,8a,9-tetrahydrophenanthrene-3-carboxylic Acid	High	Yes	Yes
(4aS,10aR)-7-hydroxy-1,1,4a,6-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	High	Yes	Yes
(4aS,10aR)-6-hydroxy-1,1,4a,7-tetramethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	High	Yes	Yes

Nimbionol	High	Yes	Yes
Sugiol	High	Yes	No
Nimbionone	High	Yes	Yes
Epoxyazadiradione	High	No	Yes
Nimbin	High	No	No
Nimbosone	High	Yes	No
(4aS,10aR)-6,7-dimethoxy-1,1,4a-trimethyl-3,4,10,10a-tetrahydrophenanthrene-2,9-dione	High	Yes	Yes
Margolonone	High	Yes	Yes
(4bS,8aS)-3,4b,8,8-tetramethyl-10-oxo-6,7,8a,9-tetrahydro-5H-phenanthrene-2-carboxylic Acid	High	Yes	Yes
6-Hydroxycyclohexa-2,4-dien-1-one	High	No	No
Deacetylgedunin	High	No	Yes
beta-Sitosterol	Low	No	No
Benzyl Alcohol	High	Yes	No

Molecular Docking of the Target Protein and Selected Phytocompounds

2.5- dihydoxycinnamate, Nimbosone, and 6-Hydroxycyclohexa-2,4-dien-1-one, exhibited the highest binding affinity with the protein and were consequently selected for further docking studies, as shown in Table 4.

Binding Affinity

Out of the selected five phytocompounds, 6 – Deacetylnimbin showed the highest binding affinity, as shown in Table 4, and was subsequently chosen as the purpoted ligand to be visualized. Following molecular docking in PyRx, ligand-protein interactions were analyzed using BIOVIA Discovery Studio 2021 v21.1.0.20298. The amino acid residues within the macromolecule were scrutinized for 2D interactions with the ligands, encompassing conventional hydrogen bonds, halogen interactions, and unfavorable donor-donor interactions. Through 2D analysis, each amino acid's identity, position within the protein chain, distance from the ligand, bond type, and interaction category were delineated. Specifically, the docking interactions of 6-Deacetylnimbin were thoroughly examined and interpreted based on these parameters.

Table 4. Data for the binding affin.

Ligand	**Binding Affinity**
Nimbiol	–7.1
6-Deacetylnimbin	–7.5
Methyl2,5-dihydroxycinnamate	–5.7
Nimbosone	–6.8
6-Hydroxycyclohexa-2,4-dien-1-one	–5.8

The Molecular Interaction and Visualization of the 6 – Deacetylnimbin with GP63

The ligand was interacting with GLU A:376 and ARG A:520 amino acids as shown in Table 5. The ligand showed a binding affinity of –7.5 in Table 4.

Table 5. Docking interactions of 6 – Deacetylnimbin-protein complex.

Name	**Category**	**Type**
A: GLU 376	Hydrogen	Conventional hydrogen bond
A: ARG 520	Hydrogen	Conventional hydrogen bond

The resultant 2D interactions were visualized using the Discovery Studio software as shown in Figure 5.

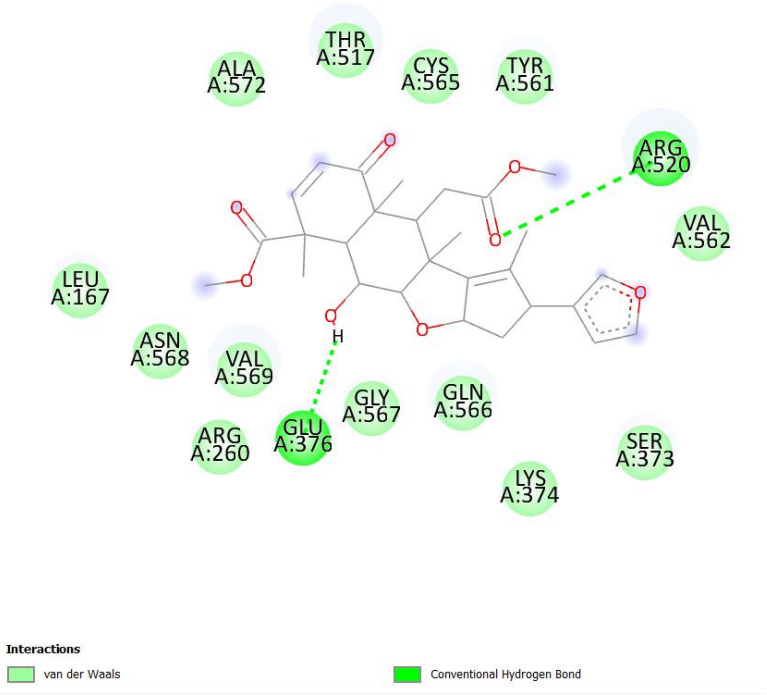

Figure 5. 2D ligand interaction of Nimbiol, 6-Deacetylnimbin with the protein.

DISCUSSION

The research offers a thorough analysis of *Azadirachta indica'*s possible medical uses in the treatment of visceral leishmaniasis (VL), a condition brought on by the Leishmania parasite. Even though *Azadirachta indica* has long been known to possess antibacterial, antiviral, and immunomodulatory qualities [26], little is known about how efficient it is specifically against Leishmania parasites, especially when it comes to targeting the GP63 protein that is essential to the parasites' virulence. By examining the inhibitory effects of several *Azadirachta indica* phytocompounds on GP63 activity, such as nimbiol, 6-Deacetylnimbin, methyl 2.5-dihydoxycinnamate, nimbobosone, and 6-Hydroxycyclohexa-2,4-dien-1-one, this study seeks to close this gap in knowledge.

The study investigates the interactions between these phytocompounds and the GP63 protein using molecular docking techniques [27]. The results show that the GP63 protein and particular phytocompounds from *Azadirachta indica* interact favorably, indicating that these phytocompounds may have the ability to reduce the virulence of Leishmania parasites. The chemical 6-Deacetylnimbin is of great interest because of its remarkable affinity for GP63.

Using molecular docking techniques, the study not only examines the interactions between *Azadirachta indica* phytocompounds and the GP63 protein, but also looks at the structural dynamics of these interactions. The results imply that the beneficial patterns of interaction between the GP63 protein and particular phytocompounds may interfere with important virulence pathways that Leishmania parasites use [28, 29]. Additionally, the study explores the molecular mechanisms that underlie these phytocompounds' inhibitory effects on GP63 function, offering important insights into how they work. The study looks at the possible synergistic effects of mixing different *Azadirachta indica* phytocompounds to improve their effectiveness against Leishmania parasites [30]. Finally, the study assesses the bioavailability and metabolic stability of the identified phytocompounds to determine their appropriateness by evaluating their pharmacokinetic features [31].

Additionally, the pharmacokinetic evaluation of the compounds under investigation reveals encouraging traits, suggesting that these compounds may be developed further as VL therapies [32–34]. The significance of looking at natural substances as possible treatments for neglected tropical diseases like VL is highlighted by these findings. Through illuminating the ways in which *Azadirachta indica* phytocompounds block GP63 action [35], this study adds significant context to the current attempts to prevent leishmaniasis.

CONCLUSIONS

Through this study, it has been observed that *Azadirachta indica* has a compound called 6-Deacetylnimbin, which possesses amazing antiparasitic, anticancer, and antioxidant effects. 6-Deacetylnimbin has been demonstrated via molecular docking studies to exhibit a substantial binding affinity with the GP63 protein, suggesting that it could serve as an appropriate candidate for future medication development.

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Abbreviations

- BBB: Blood-brain barrier
- GI: Gastrointestinal
- HBV: Hepatitis B Virus
- HIV: Human Immuno Virus
- HSV: Herpes Simplex Virus
- MRI: Molecular Refractive Index
- MW: Molecular Weight
- PDB: Protein Data Bank
- PGP: Permeability Glycophorin
- RB: Rotatable Bond
- SDF: Structural Data File
- SMILES: Simplified Molecular Input Line Entry System
- VL: Visceral Leishmaniasis

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