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Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies

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Abstract

Leishmaniasis is a vector-borne parasitic disease caused by *Leishmania* spp., for which there is no vaccine and an urgent need for better drugs. The zinc metalloprotease gp63 of *Leishmania* has been identified as an antigenic structure for vaccine design and a promising target for new antileishmanial agents. In this study, immunoinformatics was used to design a full vaccine construct with the cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes of gp63 from Old and New World *Leishmania* spp. The vaccine construct comprising of these epitopes, with suitable adjuvant and linker sequences, was found to be thermostable, highly antigenic and non-allergenic. A total of 13 linear B-cell epitopes, and 12 continuous and four discontinuous B-cell epitopes, were further identified using the BepiPred and ElliPro prediction programs, respectively. In addition, molecular docking and molecular dynamics simulation studies were performed to identify new antileishmanial molecules with the potential to target gp63. Nareline - a phytomolecule from the antileishmanial plant *Alstonia scholaris* - showed the best predictive binding affinity for gp63, forming stable interactions with key residues in the active site of this protein. This study highlights the promising role of gp63 in the search for new vaccines and therapeutic agents to combat leishmaniasis.

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Keywords: Immunoinformatics; *Leishmania*; leishmaniasis; molecular docking; molecular dynamics

1.Introduction

It has been estimated that 700,000 to 1 million new cases of leishmaniasis occur annually worldwide. This neglected tropical disease, endemic in countries where there is poverty and malnutrition, is transmitted by phlebotomine sand flies infected with the *Leishmania* protozoan parasite. It can manifest as cutaneous leishmaniasis (CL) - the most prevalent form of the disease -, mucocutaneous leishmaniasis, or visceral leishmaniasis (VL) also known as Kala-azar - the most fatal form of the disease. *Leishmania tropica*, *L. major*, *L. infantum* and *L. donovani* are the major etiologic agents in the Old World (the Eastern Hemisphere). In the New World (i.e. the Western Hemisphere/American continent), the main species are from the *L. mexicana* complex (*L. mexicana* and *L. amazonensis*) and the subgenus *Viannia* (*L. braziliensis*, *L. guyanensis*, *L. panamensis*, and *L. peruviana*). The treatment of leishmaniasis depends on the clinical manifestations, the infecting *Leishmania* spp. and the specific geographic locations where cases are encountered [1,2]. The antileishmanial drugs currently on the market (e.g. pentavalent antimonials, paromomycin, amphotericin, pentamidine, miltefosine) present serious adverse side-effects including nephrotoxicity (amphotericin), ototoxicity, impairment of liver function (paromomycin), and drug-resistant *Leishmania* strains have already emerged following exposure to these drugs [3-6]. Early trials seeking to design and develop an anti-*Leishmania* vaccine used live and pathogenic *Leishmania* (leishmaniasation), but such a hazardous immunisation method is

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no longer practiced [7]. Trials using killed and attenuated organisms have also been carried out, but with limited success [8]. The use of crude antigens with specific adjuvants has been reported, but efficacy was found to be restricted to CL and *Leishmania* spp. from specific geographic locations [9]. The use of second- and third-generation vaccines such as Leish-F1+MPL-SE, ChAd63-KH/DNA has recently been described, but these require further investigations and final approval [10,11].

The zinc metalloprotease gp63 (known as leishmanolysin) is an integral component of the *Leishmania* glycocalyx. It is a major surface antigen and virulence factor with a key role in the developmental stages of *Leishmania* promastigotes and amastigotes and their survival in the host's macrophages. As such, it has been identified as an antigenic structure for vaccine design and as a promising target for new antileishmanial drugs [12-16]. The aim of this study was twofold. The first goal was to design a full vaccine construct based on the highly immunogenic and conserved cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes of gp63 from Old and New World *Leishmania* spp using immunoinformatics. Such an approach helps predict, with high sensitivity and specificity, epitopes from antigenic proteins and characterise their interactions with the corresponding major histocompatibility complex (MHC) molecules [17]. It has also previously been used to design epitopes for vaccines against influenza, hepatitis B and C viruses [18-20]. The second goal was to identify new antileishmanial molecules with the potential to target gp63 using in silico studies such as molecular docking and molecular dynamics simulations. The molecules investigated were selected phytochemicals isolated from medicinal plants with known antileishmanial activity [21].

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2. Materials and methods

2.1. gp63 protein sequences retrieval

Nine sequences of the gp63 protein were retrieved from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/) protein database in FASTA format (Retrieval date 17/09/2019). Among these sequences, four were from Old World species (*L. donovani*, *L. tropica*, *L. infantum* and *L. major*); three were from species of the New World subgenus *Viannia* (*L. braziliensis*, *L. panamensis*, and *L. guyanensis*); and two were from the New World *L. mexicana* complex (*L. mexicana* and *L. amazonensis*) (Table S1).

2.2. Strategy for the prediction of CTL and HTL epitopes

This was carried out as described below, first using the gp63 protein of *L. infantum*, *L. braziliensis* and *L. mexicana*. Here, *L. infantum* served as the representative of the Old World *Leishmania* cluster, while *L. braziliensis* was indicative of the New World subgenus *viannia* cluster and *L. mexicana* represented the New World *mexicana* complex cluster. Following this initial identification, the conservancy of epitopes across their corresponding cluster was determined to design a multi-epitope based vaccine construct in an approach that utilized only the 100% conserved CTL and HTL epitopes.

2.3. Prediction of CTL epitopes with MHC class-I immunogenicity

The NetCTL 1.2 (www.cbs.dtu.dk/services/NetCTL/) web server was used to predict the CTL epitopes among the gp63 protein sequences of both the Old and New World *Leishmania* spp. at the default threshold value of 0.75 [22]. Only epitopes with a combined score greater than 0.75

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were selected as CTL epitopes and further subjected to the Immune Epitope Database (IEDB) MHC class I immunogenicity prediction module (tools.immuneepitope.org/immunogenicity/) [23]. Results were obtained as scores, with a positive score indicating the likelihood of eliciting an immune response. The epitope conservancy analysis tool (tools.iedb.org/conservancy/) was employed to determine the conservancy of the epitopes in their corresponding *Leishmania* clusters [24].

2.4. Prediction of HTL epitopes with IFN- γ -inducing capacity

The freely accessible web server, NetMHCIIpan 3.2 (www.cbs.dtu.dk/services/NetMHCIIpan/) was employed to predict the HTL epitopes among the gp63 protein sequences of both the Old and New World *Leishmania* spp. [25]. This prediction, based on an artificial neural network (ann) algorithm, involved the binding of the sequences to MHC class II molecules, namely H-2-IAb and H-2-IAd. The predictions were expressed as IC₅₀ values (in nM) and as %Rank. The threshold value for strong and weak binding of the peptides was set as 0.5 and 2%, respectively. The predicted epitopes were further tested for their ability to induce interferon-gamma (IFN- γ) release using the IFN epitope server (crdd.osdd.net/raghava/ifnepitope/) and a Hybrid (Motif+SVM) approach. Subsequent high scores indicated a high probability of eliciting an immune response was [26]. Finally, the epitope conservancy analysis tool (tools.iedb.org/conservancy/) was employed to determine the conservancy of the epitopes in their corresponding *Leishmania* clusters [24].

2.5. Multi-epitope vaccine construct design and physicochemical characterisation

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The design of a multi-epitope vaccine construct followed previously published procedures [27,28]. The TLR-4 agonist 50S ribosomal protein L7/L12 (Locus RL7, UniProtKB accession no. P9WHE3; PDB ID: 4G8A) of *Mycobacterium tuberculosis* was added as an adjuvant at the N-terminal site of the vaccine construct while AAY and GPGPG linkers were used to join CTL and HTL epitopes, respectively. Another linker, EAAAK, was used to join the adjuvant sequence to the linked epitopes. Various physicochemical parameters such as amino acid composition, theoretical pI, instability index, *in vitro* and *in vivo* half-life, aliphatic index, molecular weight and grand average of hydropathicity (GRAVY) were assessed using the ProtParam web server (web.expasy.org/protparam/) [29].

2.6. Antigenicity and allergenicity of the multi-epitope construct

The antigen potential of the multi-epitope construct was predicted using the online server ANTIGENpro (www.scratch.proteomics.ics.uci.edu/) [30]. This was also confirmed using the VaxiJen server (www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen) [31]. The allergenic potential of the construct was predicted using the AlgPred (crdd.osdd.net/raghava/algpred/) server by exploiting the SVMc + IgE epitope + ARPs BLAST + MAST algorithm [32]. This was also confirmed using the AllerTOP v. 2.0 tool (www.ddg-pharmfac.net/AllerTOP/) [33].

2.7. Homology modelling and 3D structure validation of the multi-epitope construct

Homology modelling of the construct was carried out using the Protein Homology/analogy Recognition Engine V 2.0 (Phyre2) server (www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi) [34]. The GalaxyRefine (galaxy.seoklab.org/) server was employed to refine the tertiary structure of the

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construct [35]. The quality of the refined model (i.e. stereochemistry and tertiary structure validation) was evaluated using PROCHECK (servicesn.mbi.ucla.edu/PROCHECK/) which provided a Ramachandran plot, and ProSA-web (prosa.services.came.sbg.ac.at/prosa.php) which provided a Z-score [36,37].

2.8. Identification of B cell epitopes

The BCPREDS (B cell epitope prediction) server (ailab.ist.psu.edu/bcpred/) was used to predict 20-mer linear B-cell epitopes at a 75% classifier identity with the default methods. ElliPro (tools.immuneepitope.org/toolsElliPro/) from IEDB analysis resource was used to predict the continuous and discontinuous conformational B-cell epitopes with a minimum score value set at 0.70 and a maximum distance was set as 6 Å [38].

2.9. Ligands and protein preparation for molecular docking

The chemical structures of the selected ligands, α -asarone [CID 636822], nareline [CID 6443592], tetrahydroalstonine [CID 72340], berberine [CID 2353] and berberastine [CID 442180] were retrieved from the PubChem compound database (pubchem.ncbi.nlm.nih.gov/). ChemOffice version 15.1 was used to visualise the chemical structures of all ligands and perform geometry optimization using the MM2 energy minimization method [39]. Drug-likeness of the five ligands was estimated using the Lipinski's Rule of Five (RO5) filters (www.scfbio-iiitd.res.in/software/drugdesign/lipinski.jsp) [40] (Table 1). All rotatable bonds on the ligands were treated as non-rotatable. The Gasteiger charge calculation method was used and partial charges were added to the ligand atoms prior to docking [41]. The crystal structure of the gp63 protein

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(PDB ID-1LML; chain A) was retrieved from the RCSB Protein Data Bank (PDB) database (www.pdb.org). All water molecules were removed using BIOVIA Discovery Studio Visualizer (v.4.5) (Accelrys), but the Zn^{2+} ion in the active site of the protein was kept.

2.10. Identification of the gp63 binding site residues and grid box preparation for docking

The gp63 binding site residues were identified based on previous reports [42,43]. The grid box parameters (**Table 2**) were determined using AutoDock Tools (v1.5.6rc3) in such a way as to allow for a suitably-sized cavity space, large enough to accommodate each compound within the binding site of gp63 [44]. Molecular docking calculations were performed using AutoDockVina (v1.1.2) [45]. Different orientations of the ligands were searched and ranked based on their energy scores. Only binding poses with the lowest value of RMSD (simply root-mean-square deviation) (threshold < 1.00 Å) were considered. Each ligand conformation was ranked using a scoring function to predict the best gp63 protein-ligand binding affinities (calculated as the predicted binding free energies ΔG_{bind} in kcal/mol). The lowest binding free energy (i.e. best score of the docking pose with the least root mean square deviation) indicated the highest predicted ligand/protein affinity. Only molecules showing the lowest binding energy (most favourable docking interactions) and ranking better than the control (amphotericin) were selected. Specific intermolecular interactions of ligands with the gp63 target were visualised using BIOVIA Discovery Studio Visualizer (v.4.5) (Accelrys).

2.11 Molecular dynamics simulation

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Molecular dynamics (MD) simulation of the docking complex was conducted using GROMACS 2021 software [46,47]. The ligand topologies were generated using Chem3D and the SwissParam server, while the protein topologies were generated employing the *gmx pdb2gmx* tool with the CHARMM36 force field [48]. The complex was positioned at the center of a dodecahedron box, ensuring a 1 nm distance between the complex and the box, and then solvated with TIP3P water molecules. Counter ions were added to neutralize the system. Subsequently, energy minimization was performed employing the steepest-descent method with a force tolerance of 1000 kJ mol⁻¹ nm⁻¹, followed by 1 ns of NVT and NPT equilibration steps. During productive MD simulations, the temperature was regulated at 300 K using the velocity-rescaling thermostat, while the Parrinello–Rahman isotropic barostat maintained a pressure of 1 bar. Periodic boundary conditions were applied in all directions throughout the simulation, which ran for a total of 150 ns. The stability of the docked complex was predicted through Root Mean Square Deviation (RMSD) and hydrogen bonding analysis using GROMACS.

3. Results

3.1. Identification of CTL epitopes

A total of 16 peptide sequences of 9-mer length with prediction scores greater than 0.75 were obtained as CTL epitopes from the gp63 protein of *L. infantum* and other Old World *Leishmania* species (**Table S2**). Only five CTL epitopes showing a high MHC-I immunogenic score and 100% conservancy were further selected. A total of 17 peptide sequences of 9-mer length with prediction scores greater than 0.75 were obtained as CTL epitopes from the gp63 protein of *L. braziliensis*

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and other species of the New World subgenus *Viannia* cluster (**Table S3**). Of these, only three CTL epitopes showing a high immunogenicity score and 100% conservancy were further selected. A total of 15 peptide sequences of 9-mer length were obtained as CTL epitopes from the gp63 protein of *L. mexicana* and other species of the New World mexicana complex cluster (**Table S4**). Of these, only seven CTL epitopes showing a high immunogenicity score and 100% conservancy were further selected. The latter contained two epitopes “LTDPAYHCA” and “PAANIASRY” that were also present in the *L. infantum* gp63 protein. This meant that altogether 13 CTL epitopes were selected for the vaccine construct.

3.2. Identification of HTL epitopes

A total of 25 peptide sequences of 15-mer length were obtained as HTL epitopes from the gp63 protein of *L. infantum* and associated species with percentile ranks ranging from 0.02–0.5 and IC₅₀ values ranging from 36–210 (**Table S5**). Only one epitope, showing a high IFN- γ -inducing capability and 100% conservancy, was selected for the vaccine construct. A total of 20 peptide sequences of 15-mer length were obtained as HTL epitopes from the gp63 protein of *L. braziliensis* and associated species with percentile ranks ranging from 0.1–0.5 and IC₅₀ values ranging from 54–206. Only six HTL epitopes, showing a high IFN- γ -inducing capability and 100% conservancy, were further selected (**Table S6**). A total of 25 peptide sequences of 15-mer length were obtained as HTL epitopes from the gp63 protein of *L. mexicana* and associated species with percentile ranks ranging from 0.02–0.5 and IC₅₀ values ranging from 37–206. Only seven HTL epitopes, showing a high IFN- γ -inducing capability and 100% conservancy, were further selected (**Table S7**).

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3.3. Multi-epitope vaccine construct design and physicochemical characterisation

Overall, 13 CTL and 14 HTL epitopes were selected from the studied leishmanial gp63 proteins for the design of the multi-epitope vaccine construct. The latter consisted of an adjuvant, linker, CTL and HTL epitopes from the N-terminal to the C-terminal sites. The construct had a molecular weight of 56.3 KDa. Its theoretical isoelectric point (PI) was 5.41, showing its acidic nature. Its aliphatic index was 81.94, indicating it was thermostable. Its GRAVY values was calculated to be -0.259, indicating that its hydrophilic nature (**Table 3**).

3.4. Antigenicity and allergenicity of the multi-epitope construct

The predicted antigenicity score of the vaccine construct using ANTIGENpro was 0.83 which shows a good antigenic nature. The score obtained with the VaxiJen server was 0.59 at the default threshold level of 0.4, which confirmed antigenicity. The construct was found to be non-allergenic with a score of -0.79 from AlgPred at the default threshold level of -0.4. It was also predicted as non-allergenic and safe for human use in the AllerTOP online server (**Table 3**).

3.5. Homology modelling and 3D structure validation of the multi-epitope construct

The best output produced following homology modelling was found to be PDB ID: 1GIY. A portion of the construct (22% of the residues) was modelled with >90% confidence and 54% identity. The remaining residues were modelled with lower confidence. The refinement of this model is presented in **Figure 1a**. The Ramachandran plot of the modelled protein revealed that 81.8% of residues were located in the most favored region followed by 12.5% and 3.2% in the additional and generously-allowed region, respectively. Only 2.5% of the residues were in the

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disallowed region (**Figure 1b**). The Z-score of -4.72 indicated that the model was in the range of the native protein conformation (**Figure 1c**).

3.6. Identification of B cell epitopes

A total of 13 peptide sequences of 20-mer length were obtained as B cell epitopes with prediction scores between 0.85–1.00. A total of 12 epitopes were predicted as continuous B cell epitopes with residue length ranging from 4–34 amino acids. The score of these epitopes ranged from 0.701 to 0.944. Four discontinuous B cell epitopes were predicted with residue length ranging from 4–98. The score of these epitopes ranged from 0.731 to 0.928 (**Table S8**). The mapping of the conformational continuous and discontinuous B cell epitopes in the tertiary structure of the modelled vaccine construct is illustrated in **Figure 2a** and **Figure 2b**, respectively.

3.7. Molecular docking

The gp63 binding (active) site residues and the grid box parameters used in the molecular docking are reported in **Figure 3a/3b** and **Table 2**, respectively. All the tested phytochemicals, except for α -asarone, displayed scores against gp63 that ranked better than amphotericin (-6.1 kcal/mol). Nareline showed the best binding affinity towards gp63 with a score of -8.5 kcal/mol. Specific interactions were further explored to understand the nature of the intermolecular bonds formed between nareline and the binding site residues of gp63 (**Figure 3c/d**). Closer examination revealed that this ligand formed conventional and Pi-Donor hydrogen bonds with Ala346 and Ala349 (bond distance of 2.48 Å and 3.89 Å, respectively). It also formed a Pi-Alkyl bond with His264, His334, and Ala348 and a Pi-Sigma bond with Ala349 (bond distance varying between 3.7-5.05 Å). The

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molecular interactions of the other three phytochemicals, namely tetrahydroalstonine, berberine, and berberastine, with gp63 are depicted in **Figures S1-S3**. Detailed records of the docking and molecular interactions of each ligand with gp63 are summarised in **Table S9**. All five selected phytochemicals fulfilled the criteria of the Lipinski's Rule of Five (**Table 1**).

3.8. Molecular dynamics simulations

The RMSD plot revealed slight deviation during the initial phase, followed by sustained stability throughout the simulation period (**Figure 4a**). A single hydrogen bond, identified as a conventional hydrogen bond with Ala346, was observed (**Figure 4b**). The findings indicated a stable binding of the complex, with a comparable number of hydrogen bonds to those predicted during docking, affirming the reliability and consistency of both docking and molecular dynamics (MD) simulation results.

4. Discussion

Recent advancements in genomics and proteomics, together with the emergence of next-generation sequencing, have contributed to important breakthroughs in immunoinformatics, particularly related to vaccine design [49]. Studies have previously reported the use of immunoinformatics to identify secretory, membrane- and genome-derived antigenic proteins of *L. donovani* or *L. infantum* [27,28,50]. Recently, the gp63 protein has gained attention in immunoinformatics for developing a multi-epitope vaccine [51]. However, these efforts have been limited to either the Old or the New World *Leishmania* spp., which restricts their effectiveness against all species of *Leishmania* causing infections.

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The multi-epitope vaccine construct described in the present study comprises conserved epitopes against gp63 proteins of both Old and New World *Leishmania* species described in this study was designed to enhance cell-mediated as well as humoral immunity. CTLs and HTLs are CD8⁺ and CD4⁺ T-cells that recognise antigens bound to MHC class I and class II molecules, respectively [52,53]. Both CD8⁺ and CD4⁺ T cells play a key role in the cell-mediated response of the host against *Leishmania*, facilitating the differentiation of Th1 responses and producing cytokines, respectively during the early stage of VL or CL [54,55]. The identified MHC class-I immunogenic CTL and MHC class II HTL epitopes were 100% conserved in all *Leishmania* clusters and showed a high potential for inducing both CD8⁺ and CD4⁺ T-cell responses. As the host's immune response against *Leishmania* relies on the interaction of leishmanial antigens with Toll-like receptors (TLRs) such as TLR-2 and TLR-4 expressed in various immune cells (i.e. macrophages, neutrophils, T and B cells), our multi-epitope vaccine construct was designed to contain the TLR-4 agonist 50S ribosomal L7/L12 protein of *Mycobacterium tuberculosis* at its N-terminal site [56,57]. This served as an adjuvant to enhance the processing of the vaccine construct via antigen-presenting cells and potentiate the host's immune response [58]. B cells are known to play an important role in the production of neutralizing antibodies in infections including leishmaniasis [59-61]. We further identified 13 linear, 12 continuous, and 4 discontinuous conformational B cell epitopes from leishmanial gp63 proteins. Common linker amino acid sequences were used to join the different segments of the vaccine construct. These included sequences promoting α helix formation (EAAAK), triggering proteasomal cleavage (AAY), and improving protein flexibility (GPGP) [62-64]. The final vaccine construct was predicted to be highly antigenic and able to induce T and B cell responses, without involving the allergen-specific IgE response. An

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assessment of its physicochemical properties revealed that it was acidic, hydrophilic and thermostable in nature. The tertiary structure analysis gave an insight into the spatial arrangement of the amino acid residues of the protein. The Ramachandran plot showed that most of the residues of the refined model of the vaccine were present in the most favored and allowed regions, with very few residues as outliers, indicating the overall quality of the modelled multi-epitope vaccine. Alongside vaccine modelling, a molecular docking approach was used to estimate the likelihood that a given ligand would bind to the protein target, in this case gp63 [65,66].

Natural products sourced from plants have for centuries played an important role in medicine as they contain highly-diverse biologically active molecules, known as phytomolecules or phytochemicals, that can be used as templates for the development of new therapies, including antileishmanial drugs [67, 68]. The five phytomolecules selected as ligands in our study were previously isolated from three Indian medicinal plants with known antileishmanial activity. These were α -asarone from *Acorus calamus*, nareline and tetrahydroalstonine from *Alstonia scholaris*, berberine and berberastine from *Berberis aristata* [21]. The alkaloid nareline was identified as the ligand with the best predictive binding affinity for gp63, showing interactions with key amino acid residues present in the active site of gp63, notably His264, His334, Ala346, and Ala348 [42].

5. Conclusions

In this study, we report for the first time on the design of a multi-epitope vaccine construct comprising conserved epitopes against gp63 proteins of both Old and New World *Leishmania* species. A total of 13 CTL and 14 HTL epitopes were selected from the studied leishmanial gp63 proteins for the design of the vaccine construct. An additional further 13 linear, 12 continuous and

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four discontinuous B-cell epitopes were identified. Common linker amino acid sequences were used to join the different segments of the vaccine construct. The final vaccine construct was predicted to be thermostable, highly antigenic and able to induce T and B cell responses, without involving the allergen-specific IgE response. Further experimental tests are required to confirm the effectiveness of the proposed vaccine in controlling different forms of leishmaniasis caused by Old and New World *Leishmania* spp.

A molecular docking approach was employed to discover new potential antileishmanial agents by predicting the binding affinity of phytoconstituents from antileishmanial medicinal plants towards gp63. The alkaloid nareline was identified as the ligand with the best docking score towards GP63 (-8.5 kcal/mol), superior to the score obtained for the amphotericin control. This is the first report and detailed analysis of the interaction of nareline with the leishmanial gp63 protein. Further experimental studies are warranted to establish if this compound could serve as a template for the design of new antileishmanial drugs. Overall, the information gained from this study highlights the promising role of gp63 in the search for new vaccines and/or therapeutic agents to combat leishmaniasis.

Author's Contributions

Conceptualization: MTA; Data curation: MTA and MIU; Formal analysis: MTA, MIU, TR and VS; Investigation: MTA, MIU and PP; Methodology: MTA, MIU, PP and VS; Project administration: MTA, MIU and VS; Resources: MTA and MIU; Software: MTA and MIU; Validation: MTA and VS; Visualization: MTA, MIU and PP. Writing –original: PP, MIU and TR; Writing –review and editing: MTA and VS.

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377

378 **Ethics Declaration**

379 **Conflicts of interests**

380 All authors have no conflicts of interests.

381

382 **Data Availability**

383 All relevant data are included in the text, references, table, and figures.

384

385 **Ethical approval**

386 Not applicable.

387

388 **Declaration of competing interests**

389 None declared.

390

391 **Additional Information**

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See supplementary information files.

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Figure legends

Figure 1: Tertiary structure of the vaccine construct and validation. Homology model and refined 3D structure of the multi-epitope vaccine (a). The Ramachandran plot of the protein structure revealed that 81.8, 12.5, 3.2 and 2.5% of the amino acids were present in the most favored, additionally-allowed, generously-allowed and disallowed regions, respectively (b). The ProSA-Web predicted Z-score was -4.72 (c).

Figure 2: Mapping of conformational B-cell epitopes in the vaccine construct. The continuous (a) and discontinuous (b) conformational epitopes are marked with a red color in the tertiary structure of the vaccine construct.

Figure 3: Molecular interactions between nareline and gp63. Docking site of the gp63 protein with docked pose of nareline (a). Close-up surface model of the binding region highlighting the gp63 protein structure (blue), the zinc (Zn) atom in the active site (cyan) and the key amino acid residues (yellow) interacting with nareline (b). Key interacting gp63 residues forming hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with nareline (c), 2D plot of interactions (with bond lengths) between nareline and gp63 residues (d) generated by BIOVIA Discovery Studio visualizer.

Figure 4: Molecular dynamics simulation analysis of the gp63-nareline complex. (a) RMSD plot illustrating the stability of the complex over the simulation period. (b) Hydrogen bond plot demonstrating the stability of hydrogen bonding interactions between GP63 and nareline.

Figure S1: Molecular interactions between tetrahydroalstonine and gp63. Docked pose of tetrahydroalstonine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with tetrahydroalstonine (a), 2D plot

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of interactions (with bond lengths) between tetrahydroalstonine and key residues of gp63 generated by BIOVIA Discovery Studio visualizer (b).

Figure S2: Molecular interactions between berberine and gp63. Docked pose of berberine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with berberine (a), 2D plot of interactions (with bond lengths) between berberine and key residues of GP63 generated by BIOVIA Discovery Studio visualizer (b).

Figure S3: Molecular interactions between berberastine and gp63. Docked pose of berberastine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with berberastine (a), 2D plot of interactions (with bond lengths) between berberastine and key residues of GP63 generated by BIOVIA Discovery Studio visualizer (b).

Table legends

Table 1: Drug-likeness of the five selected ligands.

Table 2: Grid box parameters selected for gp63, based on binding site residues^a.

Table 3: Amino acid sequence, physicochemical and immunological properties of the leishmanial gp63-based multi-epitope vaccine construct. The different components of the construct are shown in different colors (Adjuvant-Grey, CTL Epitopes-Yellow, and HTL Epitopes-Green) with linker sequences in bold font.

Table S1: Accession numbers and distribution of different leishmanial gp63 protein sequences.

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Table S2: CTL Epitope analysis of the *Leishmania infantum* gp63 protein.

Table S3: CTL Epitope analysis of the *Leishmania braziliensis* gp63 protein.

Table S4: CTL Epitope analysis of the *Leishmania mexicana* gp63 protein.

Table S5: HTL Epitope analysis of the *Leishmania infantum* gp63 protein.

Table S6: HTL Epitope analysis of the *Leishmania braziliensis* gp63 protein.

Table S7: HTL Epitope analysis of the *Leishmania mexicana* gp63 protein.

Table S8: BCPREDS linear B-cell epitope prediction for the final vaccine construct.

Table S9. Binding free energy, ligand efficiency and molecular interactions of selected natural compounds towards the leishmanial gp63 protein (PDB ID: 1LML).

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Table 1: Drug-likeness of the five selected ligands.

Compound name [PubChem ID]	Lipinski's filters				
	Molecular weight (g/mol)	Number of H-bond donors (HBD)	Number of H-bond acceptors (HBA)	cLogP	Molecular refractivity
α-asarone [CID 636822]	208	0	3	2.7	60.8
Nareline [CID 6443592]	352	1	6	1.7	92.6
Tetrahydroalstonine [CID 72340]	352	2	3	1.4	96.7
Berberine [CID 2353]	337	0	4	2.7	93.0
Berberastine [CID 442180]	353	1	5	2.2	94.3

Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of Leishmania for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

Table 2: Grid box parameters selected for gp63, based on binding site residues^a.

Target Protein	Binding Site Residues and Atoms	Grid Box Centre (Points in X, Y, Z-axis)	Grid Box Size (Points in X, Y, Z-axis)
gp63	Ser219, Glu220, Val223, Ala225, Trp226, His264, Glu265, Gly329, Ala346, Pro347, Ala348, Ala349, Zn	18.48, 46.03, 16.71	22 × 22 × 22

^a Spacing and exhaustiveness values were set up at 1 Å and 9, respectively in all cases.

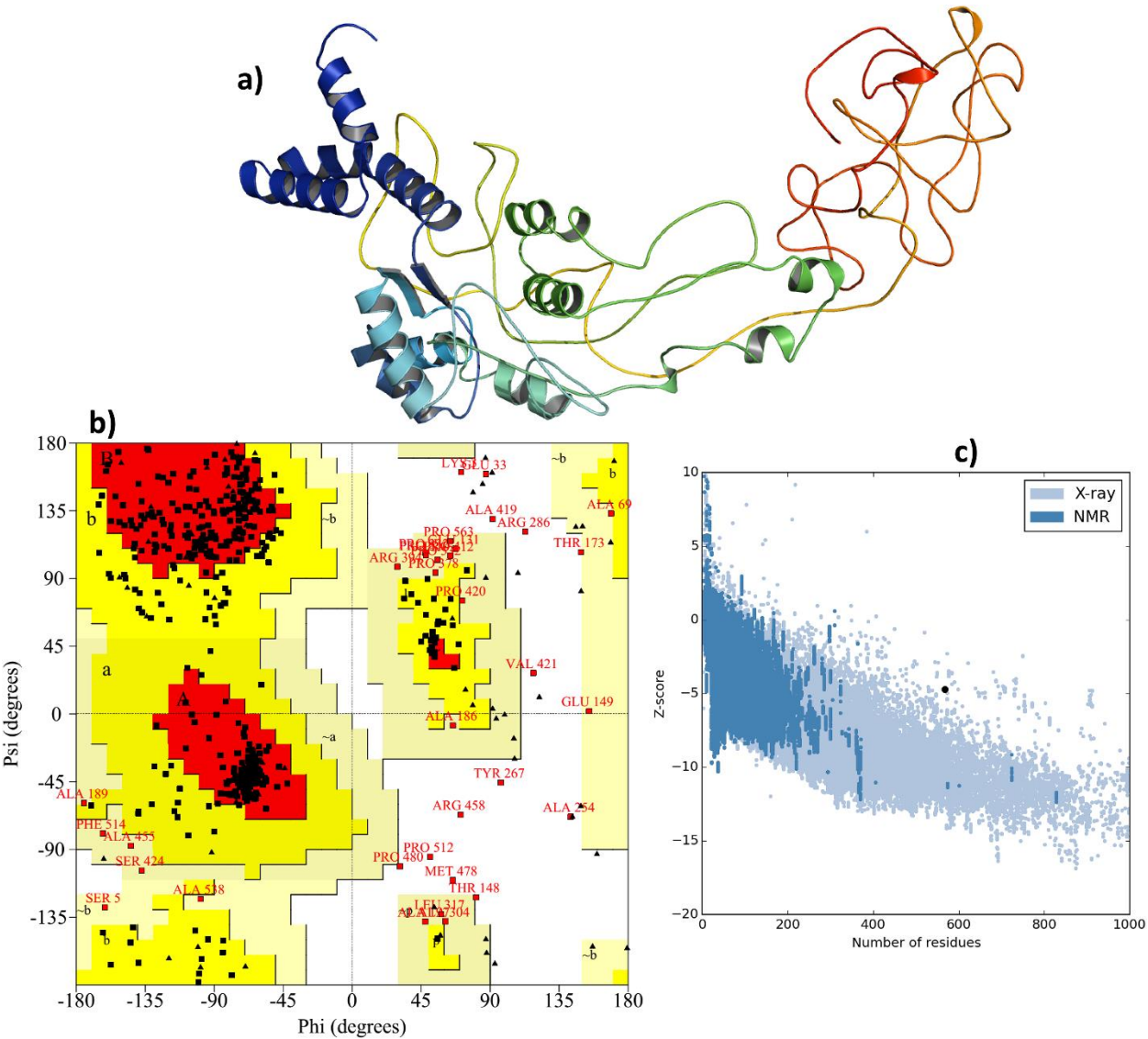
Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of Leishmania for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

Table 3: Amino acid sequence, physicochemical and immunological properties of the leishmanial gp63 -based multi-epitope vaccine construct. The different components of the construct are shown in different colors (Adjuvant-Grey, CTL Epitopes-Yellow, and HTL Epitopes-Green) with linker sequences in bold font.

Amino acid Sequence	Adjuvant + EAAAK + 13 CTL Epitopes (AA Y) + 14 HTL Epitopes (GP GP G) MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAA GAAPAGAAVEAAEEQSEFDVILEAAGDKKIGVIKVVREIVSGLGL KEAKDLVDGAPKPLLEKVAKAADADEAKAKLEAAGATVTVKEAA AK AVSAVGLPY AA Y TEDLTDPAY AA Y LTDPAYHCAA Y NTDFV MYVAA AA Y PAANIASRY AA Y PAANIVSRY AA Y MAPASAAG Y AA Y YTALTMVFA AA Y LTDEKRDIL AA Y AVFQDLGF Y AA Y FTDPFLAG SA AA Y FMDYCPVV AA Y KCDTAART Y GP GP G VAAARLVRLAAAG AAV GP GP G DFVLYVASVPSEPGV GP GP G FVLYVASVPSEPGVL G PG PG NLRGKPFAAPVINSS GP GP G LRGKPFAAPVINSST GP GP G R GKPFAAPVINSSTV GP GP G GKPFAAPVINSSTVV GP GP G SVAAQR MAPSAVSAV GP GP G VAAQRMAPSASVAVGG GP GP G AAQRMAPS AVSAVGL GP GP G AQRMAPSASVAVGL GP GP G FVLYVASVPSEE SVL GP GP G RQSVAAQRMAPSASV GP GP G QSVAAQRMAPSASVSA
Length and Molecular weight	568 amino acids and 56.3kDa
Isoelectric point (PI) and Aliphatic index	5.41 and 81.94
GRAVY value	-0.259
ANTIGEN _{pro} and VaxiJen antigenicity score	0.83 and 0.59

Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of Leishmania for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

AlgPred allergenicity score and AllerTOP prediction	-0.79 and non-allergen
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Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

Figure 1: Tertiary structure of the vaccine construct and validation. Homology model and refined 3D structure of the multi-epitope vaccine (a). The Ramachandran plot of the protein structure revealed that 81.8, 12.5, 3.2 and 2.5% of the amino acids were present in the most favored, additionally-allowed, generously-allowed and disallowed regions, respectively (b). The ProSA-Web predicted Z-score was -4.72 (c).

Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

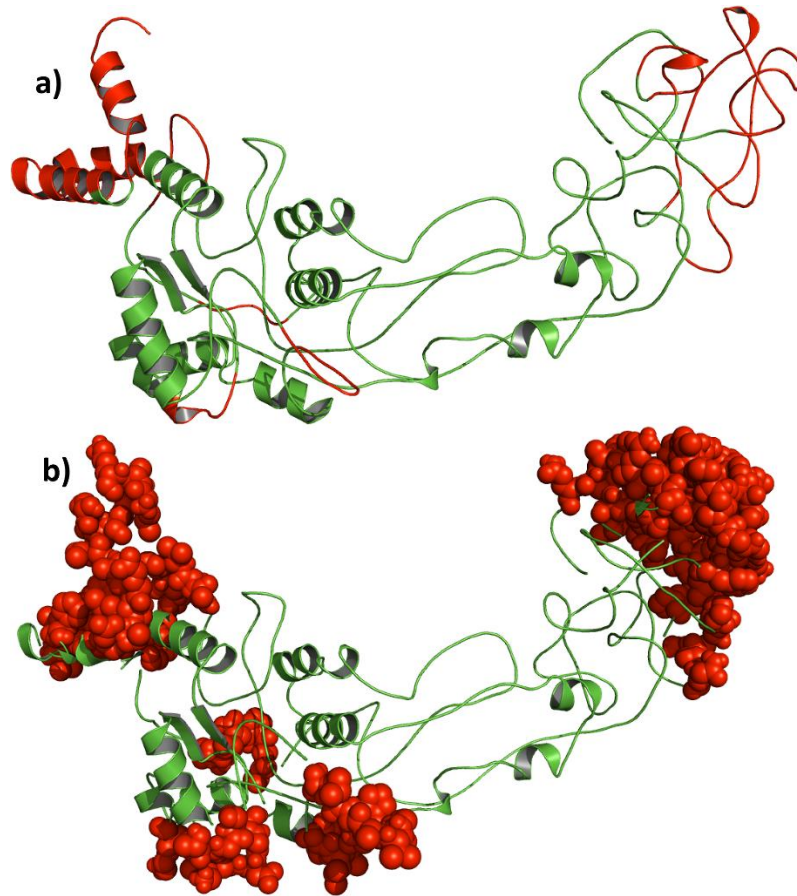


Figure 2: Mapping of conformational B-cell epitopes in the vaccine construct. The continuous (a) and discontinuous (b) conformational epitopes are marked with a red color in the tertiary structure of the vaccine construct.

Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

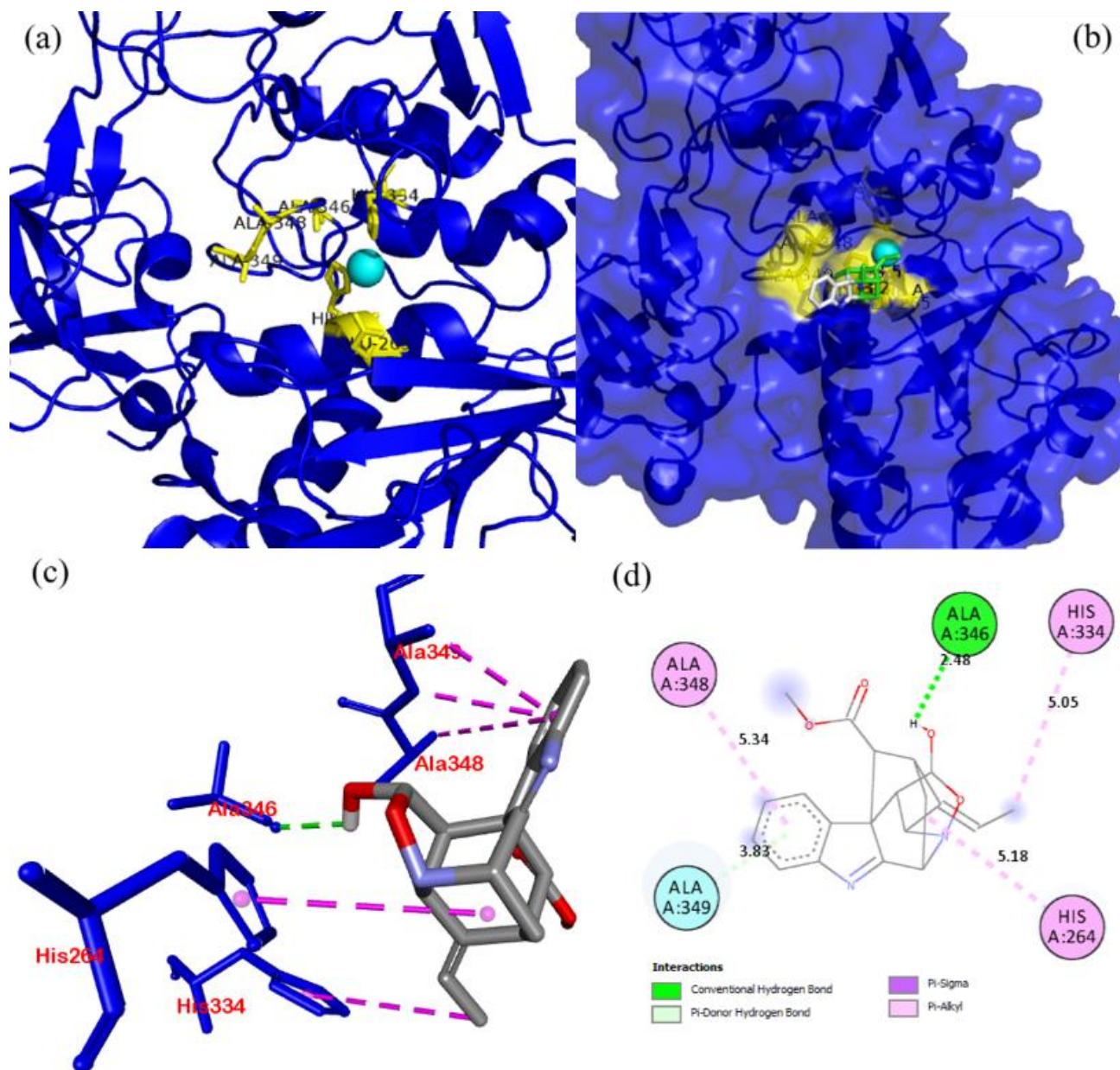


Figure 3: Molecular interactions between nareline and gp63. Docking site of the gp63 protein with docked pose of nareline (a). Close-up surface model of the binding region highlighting the gp63 protein structure (blue), the zinc (Zn) atom in the active site (cyan) and the key amino acid

Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

688 residues (yellow) interacting with nareline (b). Key interacting gp63 residues forming hydrophobic
689 (pink dashed lines) and hydrogen bonds (green dashed lines) with nareline (c), 2D plot of
690 interactions (with bond lengths) between nareline and gp63 residues (d) generated by BIOVIA
691 Discovery Studio visualizer.

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Ali, M. T., Rahman, T., Palit, P., Uddin, M. I., & Seidel, V. (2025). Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatics, molecular docking and molecular dynamics simulation studies. *Experimental parasitology*, 277, Article 109009. <https://doi.org/10.1016/j.exppara.2025.109009>. For the purpose of open access, a CC BY 4.0 licence has been applied

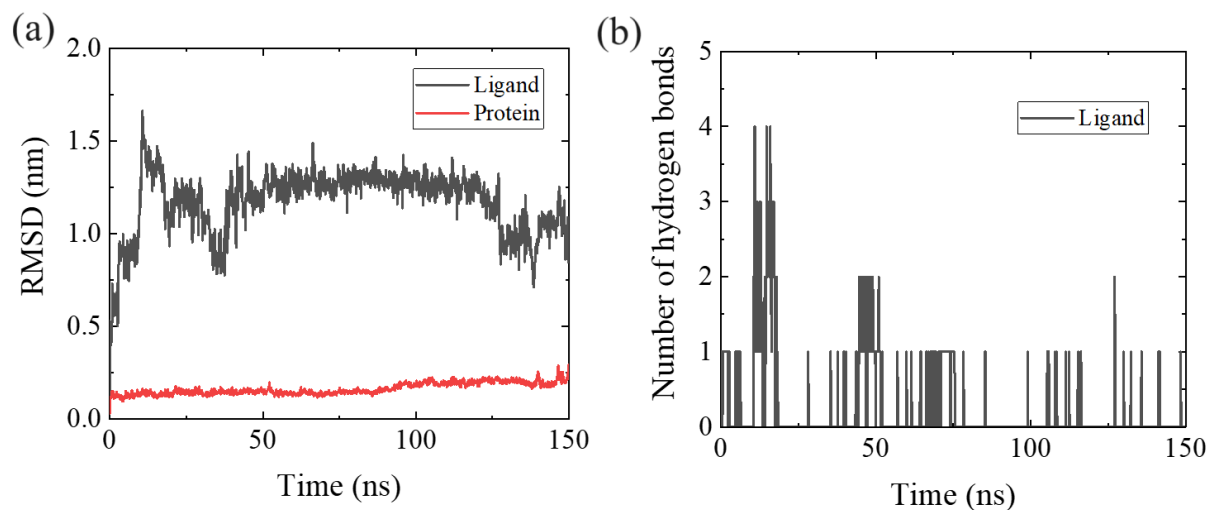


Figure 4: Molecular dynamics simulation analysis of the gp63-nareline complex. (a) RMSD plot illustrating the stability of the complex over the simulation period. **(b)** Hydrogen bond plot demonstrating the stability of hydrogen bonding interactions between gp63 and nareline.

Supplementary material

Targeting the zinc metalloprotease gp63 of *Leishmania* for vaccine design and new drug discovery using immunoinformatic and molecular docking studies

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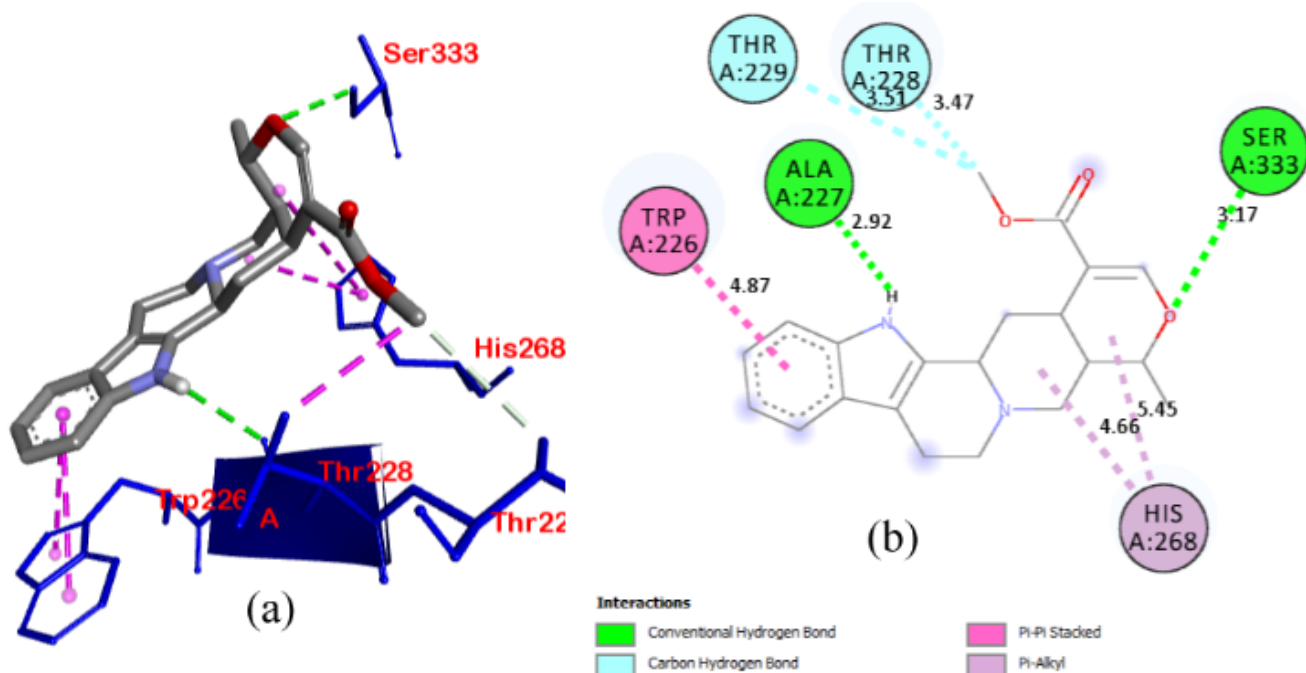


Figure S1: Molecular interactions between tetrahydroalstonine and gp63. Docked pose of tetrahydroalstonine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with tetrahydroalstonine (a), 2D plot of interactions (with bond lengths) between tetrahydroalstonine and key residues of gp63 generated by BIOVIA Discovery Studio visualizer (b).

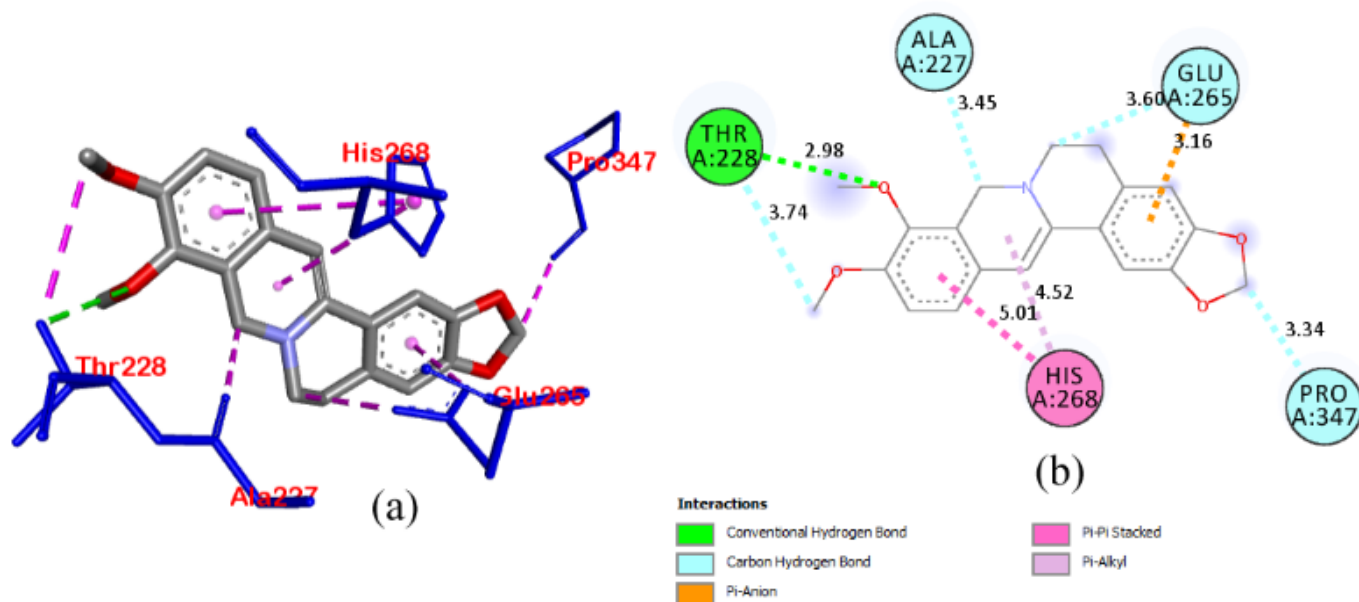


Figure S2: Molecular interactions between berberine and gp63. Docked pose of berberine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with berberine (a), 2D plot of interactions (with bond lengths) between berberine and key residues of gp63 generated by BIOVIA Discovery Studio visualizer (b).

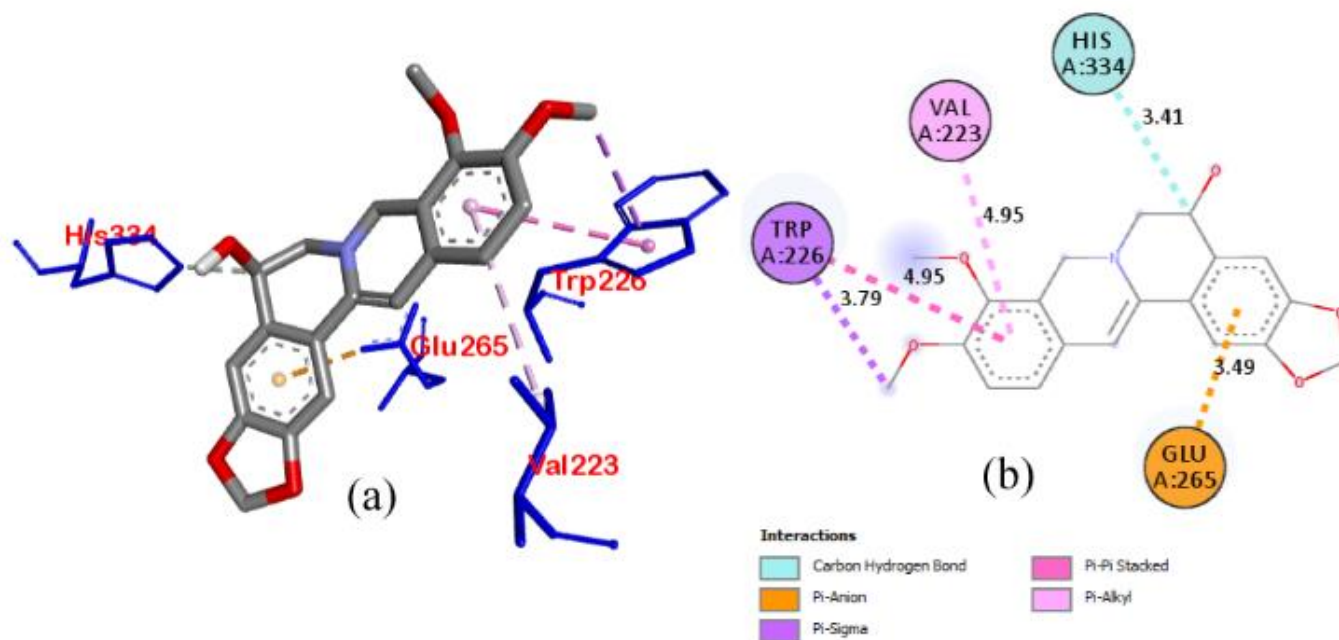


Figure S3: Molecular interactions between berberastine and gp63. Docked pose of berberastine in the binding site of gp63. Key interacting residues that form hydrophobic (pink dashed lines) and hydrogen bonds (green dashed lines) with berberastine (a), 2D plot of interactions (with bond lengths) between berberastine and key residues of gp63 generated by BIOVIA Discovery Studio visualizer (b).

Table S1: Accession numbers and distribution of different leishmanial gp63 protein sequences

Species name	NCBI Accession number	Distribution*
<i>L. infantum</i>	CAB06018.1	OLD World <i>Leishmania</i> (Eastern Hemisphere) <i>L. chagasi</i> is generally considered synonymous with <i>L. infantum</i>
<i>L. donovani</i> MHOM/SU/1984/Marz-Krim	CAD42816.1	OLD World <i>Leishmania</i> (Eastern Hemisphere)
<i>L. tropica</i> MHOM/SU/1974/K27	CAD42817.1	OLD World <i>Leishmania</i> (Eastern Hemisphere)
<i>L. major</i> MRHO/IR/75/ER	ACL01096.2	OLD World <i>Leishmania</i> (Eastern Hemisphere)
<i>L. braziliensis</i> MHOM/BR/75/M2904	XP_001562827.1	NEW World (Western Hemisphere) Belongs to the <i>Leishmania</i> subgenus Viannia
<i>L. panamensis</i> MHOM/PA/94/PSC-1	XP_010696763.1	NEW World (Western Hemisphere) Belongs to the <i>Leishmania</i> subgenus Viannia
<i>L. guyanensis</i> MHOM/BR/75/M4147A	AAA29240.1	NEW World (Western Hemisphere) Belongs to the <i>Leishmania</i> subgenus Viannia
<i>L. mexicana</i> MHOM/GT/2001/U1103	XP_003872886.1	NEW World (Western Hemisphere) Belongs to the <i>L. mexicana</i> complex species
<i>L. amazonensis</i> MHOM/BR/73/LV78	Q27673.1	NEW World (Western Hemisphere) Belongs to the <i>L. mexicana</i> complex species

*According to the provided information on CDC Resources for Health Professionals (www.cdc.gov/parasites/leishmaniasis/health_professionals/index.html)(Accessed 21/11/23).

Table S2: CTL Epitope analysis of the *Leishmania infantum* gp63 protein.

Serial No.	Position	9-mer CTL Epitope	Combined score (Threshold: 0.75)	MHC-I Immunogenicity score*	Percent of epitope conservancy <= 100%**
1	70	AVSAVGLPY	1.894	0.042	100% (4/4)
2	112	TEDLTDPAY	0.765	0.068	100% (4/4)
3	115	LTDPAYHCA	0.982	0.026	100% (4/4)
4	145	LTDEKRDIL	0.958	0.043	75% (3/4)
5	202	LSNTDFVMY	2.713	0.101	50% (2/4)
6	204	NTDFVMYVA	1.012	0.021	100% (4/4)
7	243	PAANIASRY	1.332	0.063	100% (4/4)
8	301	AVAKAREQY	0.943	-0.101	100% (4/4)
9	308	QYGCDTLEY	0.809	0.064	50% (2/4)
10	342	MAPAAAAGY	0.964	0.164	75% (3/4)
11	351	YSALTMAIF	1.328	-0.015	50% (2/4)
12	357	AIFQDLGFY	0.802	0.030	75% (3/4)
13	437	FTDPSLAGI	1.113	-0.123	25% (1/4)
14	495	TSHGIIKSY	0.999	0.021	25% (1/4)
15	511	RCDTATR TY	1.485	0.187	75% (3/4)
16	544	SSAFEEGGY	1.974	0.371	50% (2/4)

*Epitope with positive MHC-I Immunogenic Score will be considered for analysis.

** Epitope conservancy was determined using the GP63 protein sequences of the all four old world *Leishmania* species: *L. infantum*, *L. donovani*, *L. major* and *L. tropica* . Only 100% conserved epitopes among all four species were considered for the multi-epitope vaccine modelling.

Table S3: CTL Epitope analysis of the *Leishmania braziliensis* gp63 protein.

Serial No.	Position	9-mer CTL Epitope	Combined score (Threshold: 0.75)	MHC-I Immunogenicity score*	Percent of epitope conservancy <= 100%**
1	66	SVSALGLPY	2.107	0.197	66.67% (2/3)
2	101	AADWGTLRI	0.778	-0.101	100.00% (3/3)
3	113	TADLTDPDY	2.175	0.247	33.33% (1/3)
4	203	VSNADFVLY	2.444	0.321	66.67% (2/3)
5	244	PAANIVSRY	1.283	0.117	100.00% (3/3)
6	302	VVAKAREQY	0.929	-0.009	100.00% (3/3)
7	309	QYGCATLEY	0.904	-0.036	33.33% (1/3)
8	343	MAPASAAGY	0.997	0.065	100.00% (3/3)
9	352	YTALTMAVF	1.428	0.146	100.00% (3/3)
10	358	AVFEDLGFY	0.856	-0.069	100.00% (3/3)
11	427	TYSTPMPTY	0.778	-0.149	33.33% (1/3)
12	428	YSTPMPTYF	1.159	0.097	33.33% (1/3)
13	439	FTNAFLGGF	1.049	0.081	33.33% (1/3)
14	450	FLDYCPFIV	0.950	0.058	33.33% (1/3)
15	513	MCDTAARTY	2.079	0.189	66.67% (2/3)
16	546	SAAFVNGSY	1.899	0.188	66.67% (2/3)
17	552	GSYITCAPY	0.763	-0.177	66.67% (2/3)

*Epitope with positive MHC-I Immunogenic Score will be considered for analysis.

** Epitope conservancy was determined using the GP63 protein sequences of the all three New world's subgenus *Viannia* species: *L. braziliensis*, *L. panamensis* and *L. guyanensis* . Only 100% conserved epitopes mong all four species were considered for multi-epitope vaccine modelling.

Table S4: CTL Epitope analysis of the *Leishmania mexicana* gp63 protein.

Serial No.	Position	9-mer CTL Epitope	Combined score (Threshold: 0.75)	MHC-I Immunogenicity score	Percent of epitope conservancy <= 100% **
1	118	LTDPAYHCA	1.013	0.026	100% (2/2)
2	148	LTDEKRDIL	0.945	0.043	100% (2/2)
3	185	DMAADVCSY	1.179	-0.030	50% (1/2)
4	205	VSNTDFVLY	2.729	0.197	50% (1/2)
5	246	PAANIASRY	1.332	0.063	100% (2/2)
6	304	AVAKAREQY	0.940	-0.101	100% (2/2)
7	311	QYGCNSLEY	0.885	-0.156	100% (2/2)
8	345	MAPAASAGY	0.974	-0.029	50% (1/2)
9	354	YTALTMAVF	1.444	-0.069	100% (2/2)
10	360	AVFQDLGFY	0.886	0.030	100% (2/2)
11	431	QALPPYLQY	0.914	-0.106	100% (2/2)
12	440	FTDPFLAGS	0.821	0.152	100% (2/2)
13	451	FMDYCPVVV	0.860	-0.001	100% (2/2)
14	498	TTHGVIKSY	1.217	-0.069	50% (1/2)
15	514	KCDTAARTY	1.266	0.188	100% (2/2)

*Epitope with positive MHC-I Immunogenic Score will be considered for analysis

** Epitope conservancy was determined using the GP63 protein sequences of the two New world's *L. mexicana* complex species: *L. mexicana* and *L. amazonensis* . Only 100% conserved epitopes among all four species were considered for multi-epitope vaccine modelling.

Table S5: HTL Epitope analysis of the *Leishmania infantum* gp63 protein.

Serial No.	Position	15-mer HTL Epitope	IC50 in nM	Rank	IFN- γ epitope prediction score	Percent of epitope conservancy <= 100%**
MHC-II allele: H-2-IAb						
1	15	AARLVRLAAAGAAVI	138.32	0.25	4	50% (2/4)
2	16	ARLVRLAAAGAAVIA	98.38	0.09	4	50% (2/4)
3	17	RLVRLAAAGAAVIAA	85.8	0.07	3	50% (2/4)
4	18	LVRLAAAGAAVIAAV	104.42	0.12	1	50% (2/4)
5	19	VRLAAAGAAVIAAVG	131.76	0.2	1	50% (2/4)
6	20	RLAAAGAAVIAAVGT	186.27	0.4	1	50% (2/4)
7	33	GTAAAWAHAGAVQHR	206.88	0.5	1	50% (2/4)
8	338	QDELMAPAAAAGYYYS	207.58	0.5	0.41	50% (2/4)
9	339	DELMAPAAAAGYYSA	210.41	0.5	0.34	50% (2/4)
10	579	RRGPRAAATALLVAA	187.50	0.4	5	50% (2/4)
MHC-II allele: H-2-IAc						
1	8	THRHSVAARLVRLA	85.41	0.4	11	50% (2/4)
2	9	HRHSVAARLVRLAA	78.94	0.4	10	50% (2/4)
3	10	RHSVAARLVRLAAA	62.89	0.17	9	50% (2/4)
4	11	HRSVAARLVRLAAAG	78.95	0.4	6	50% (2/4)
5	12	RSVAARLVRLAAAGA	69.22	0.25	5	50% (2/4)
6	13	SVAARLVRLAAAGAA	52.91	0.09	4	50% (2/4)
7	14	VAARLVRLAAAGAAV	44.77	0.05	4	100% (4/4)
8	15	AARLVRLAAAGAAVI	39.21	0.03	4	50% (2/4)
9	16	ARLVRLAAAGAAVIA	36.52	0.02	4	50% (2/4)
10	17	RLVRLAAAGAAVIAA	38.33	0.03	3	50% (2/4)
11	18	LVRLAAAGAAVIAAV	49.90	0.07	1	50% (2/4)
12	19	VRLAAAGAAVIAAVG	74.52	0.3	1	50% (2/4)
13	25	GAAVIAAVGTAAAWA	74.68	0.3	0.4	50% (2/4)
14	26	AAVIAAVGTAAAWAH	74.61	0.3	0.42	25% (1/4)
15	27	AVIAAVGTAAAWAHA	78.32	0.4	1	50% (2/4)

** Epitope conservancy was determined using the GP63 protein sequences of the all four old world *Leishmania* species: *L. infantum*, *L. donovani*, *L. major* and *L. tropica*. Only 100% conserved epitopes among all four species were considered for multi-epitope vaccine modelling.

Table S6: HTL Epitope analysis of the *Leishmania braziliensis* gp63 protein.

Serial No.	Position	15-mer HTL Epitope	IC50 in nM	Rank	HTL-γ epitope prediction score	Percent of epitope conservancy <= 100%*
MHC-II allele: H-2-IAb						
1	69	ALGLPYVSAGTISSA	124.16	0.17	0.51	33.33% (1/3)
2	70	LGLPYVSAGTISSAH	111.55	0.12	0.51	33.33% (1/3)
3	71	GLPYVSAGTISSAHT	106.91	0.12	0.49	33.33% (1/3)
4	72	LPYVSAGTISSAHTV	114.62	0.15	0.51	33.33% (1/3)
5	73	PYVSAGTISSAHTVD	158.12	0.3	0.49	33.33% (1/3)
6	207	DFVLYVASVPSEPGV	206.44	0.5	3	100.00% (3/3)
7	208	FVLYVASVPSEPGVL	174.49	0.4	3	100.00% (3/3)
8	286	NLRGKPF AAPVINSS	189.82	0.4	2	100.00% (3/3)
9	287	LRGKPF AAPVINSST	161.3	0.3	2	100.00% (3/3)
10	288	RGKPF AAPVINSSTV	137.37	0.2	2	100.00% (3/3)
11	289	GKPF AAPVINSSTVV	154.29	0.3	2	100.00% (3/3)
MHC-II allele: H-2-IAd						
1	9	PRRSVAARLMRLAA	84.5	0.4	15	33.33% (1/3)
2	10	RRRSVAARLMRLAAA	56.32	0.12	15	33.33% (1/3)
3	11	RRSVAARLMRLAAAG	64.49	0.17	5	33.33% (1/3)
4	12	RSVAARLMRLAAAGL	64.57	0.17	3	33.33% (1/3)
5	13	SVAARLMRLAAAGLV	68.87	0.25	2	33.33% (1/3)
6	14	VAARLMRLAAAGLVM	65.06	0.2	2	33.33% (1/3)
7	15	AARLMRLAAAGLVMA	54.39	0.1	3	33.33% (1/3)
8	16	ARLMRLAAAGLVMAV	57.24	0.12	3	33.33% (1/3)
9	17	RLMRLAAAGLVMAVG	69.86	0.25	4	33.33% (1/3)

*Epitope conservancy was determined using the GP63 protein sequences of the all three New world's subgenus Viannia species: *L. braziliensis*, *L. panamensis* and *L. guyanensis* . Only 100% conserved epitopes among all four species were considered for multi-epitope vaccine modelling.

Table S7: HTL Epitope analysis of the *Leishmania mexicana* gp63 protein.

Serial No.	Position	15-mer HTL Epitope	IC50 in nM	Rank	IFN- γ epitope prediction	Percent of protein sequence matches at identity <= 100%*
MHC-II allele: H-2-IAb						
1	15	AARLVRLAAAGAAVT	133	0.2	4	50% (1/2)
2	16	ARLVRLAAAGAAVTV	96.26	0.09	4	50% (1/2)
3	17	RLVRLAAAGAAVTVA	78.05	0.05	3	50% (1/2)
4	18	LVRLAAAGAAVTVAV	89.53	0.07	1	50% (1/2)
5	19	VRLAAAGAAVTVAVG	112.32	0.12	2	50% (1/2)
6	20	RLAAAGAAVTVAVGT	163.65	0.3	2	50% (1/2)
7	60	SVAAQRMAPSAVSAV	153.8	0.3	1	100% (2/2)
8	61	VAAQRMAPSAVSAVG	160.09	0.3	1	100% (2/2)
9	62	AAQRMAPSAVSAVGL	158.91	0.3	1	100% (2/2)
10	63	AQRMAPSAVSAVGLP	190.65	0.4	1	100% (2/2)
11	210	FVLYVASVPSEESVL	206.79	0.5	1	100% (2/2)
12	581	GRRGPRAAATALVVA	174.95	0.4	6	50% (1/2)
13	582	RRGPRAAATALVVAA	141.36	0.25	5	50% (1/2)
14	583	RGPRAAATALVVAAL	163.19	0.3	6	50% (1/2)
MHC-II allele: H-2-IAc						
1	12	RCVAARLVRLAAAGA	88.16	0.5	4	50% (1/2)
2	13	CVAARLVRLAAAGAA	58.98	0.15	4	50% (1/2)
3	14	VAARLVRLAAAGAAV	44.77	0.05	4	50% (1/2)
4	15	AARLVRLAAAGAAVT	38.67	0.03	4	50% (1/2)
5	16	ARLVRLAAAGAAVTV	37.22	0.02	4	50% (1/2)
6	17	RLVRLAAAGAAVTVA	39.46	0.03	3	50% (1/2)
7	18	LVRLAAAGAAVTVAV	52.69	0.09	1	50% (1/2)
8	19	VRLAAAGAAVTVAVG	86.84	0.5	2	50% (1/2)
9	58	RQSVAAQRMAPSAVS	87.43	0.5	2	100% (2/2)
10	59	QSVAAQRMAPSAVSA	85	0.4	2	100% (2/2)
11	60	SVAAQRMAPSAVSAV	75.68	0.3	1	100% (2/2)
12	61	VAAQRMAPSAVSAVG	93.5	0.5	1	100% (2/2)
13	295	TVAVINSSTAVAKAR	93.36	0.5	2	50% (1/2)

*Epitope conservancy was determined using the GP63 protein sequences of the two New world's *L. mexicana* complex species: *L. mexicana* and *L. amazonensis*. Only 100% conserved epitopes among all four species were considered for multi-epitope vaccine modelling.