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In Silico and *in Vitro* Studies: Tryparedoxin Peroxidase Inhibitor Activity of Methotrexate for Antileishmanial Activity

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ABSTRACT

In order to understand the mechanism of molecular interactions at the active site of Tryparedoxin Peroxidase (Try P), homology modeling and docking studies were performed. We generated a Three-Dimensional (3D) model of target protein based on the Crystal structure of *Leishmania Major* Try PI (PDB ID: 3TUE) using modeler software. Docking analysis was carried out to study the effects of methotrexate on Tryparedoxin Peroxidase (Try P). Inhibition of the Tryparedoxin peroxidase interaction has become a new therapeutic strategy in treating leishmaniasis. Docking analysis was carried out to study the effects of methotrexate on Tryparedoxin Peroxidase (TryP). Tryparedoxin peroxidase of Trypanosomatidae family functions as antioxidant through their peroxidase and peroxynitrite reductase activities. The theoretical docking study, conducted on a sample previously reported for anti-cancer properties of Methotrexate at the binding site of 3D models of Tryparedoxin Peroxidase of *Leishmania braziliensis* (*L. braziliensis* Try P) examine interaction energy. Our studies indicate that Methotrexate displays potent activity against Try P with lowest binding energy and RMSD values to be -14.5879 Kcal/Mol and 2.0 A. The results of the present study clearly demonstrated the Tryparedoxin Peroxidase inhibitory activity by methotrexate in *in silico* docking analysis and *in vitro* assay which contributes towards understanding the mechanism of antileishmanial activity.

Keywords: Homology Modeling, Molecular Docking, *Leishmania Braziliensis*, Tryparedoxin Peroxidase, Methotrexate

1. INTRODUCTION

Over the past 60 years very limited number of drugs has been developed for the treatment of Leishmaniasis and the use of available drugs has been hampered by high cost, adverse side effects, development of resistance by the parasite and also due to low efficacy (Croft *et al*., 2006). Some experimental as well as *in silico* attempts have been made to identify inhibitors or subversive substrates for various molecular targets (Krauth-Siegel and Inhoff, 2003; Perez-Pineiro *et al*., 2009). The enzyme Tryparedoxin Peroxidase (Try P) of *Leishmania braziliensis* is a 199 amino acid enzyme with a molecular weight of approximately 22.5 kDa. Try P belongs to the protein family of peroxiredoxins was selected in this investigation as molecular target for *in-silico* screening of anti-leishmanial activity. This enzyme cascade involves Trypanothione Reductase (Try R), Tryparedoxin (TXN) and trypanotione (N^1, N^8-bis (glutathionyl)-spermidine) serving as a mediator for

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transfer of reducing equivalents (Fairlamb and Cerami, 1985; Fairlamb *et al*., 1985; Shames *et al*., 1986). The first enzyme of the cascade is homologous to glutathione reductase and thioredoxin reductase (Krauth-Siegel *et al*., 1987) which is involved in NADPH-dependent hydroperoxide reduction in other species (Tamura *et al*., 1995). The other components of the trypanosomatid system also belong to protein families occasionally constituting peroxidase systems. Preliminary amino acid sequencing data indicated that tryparedoxin is phylogenetically related to thioredoxin, whereas the tryparedoxin peroxidase belongs to the peroxiredoxins (Nogoceke *et al*., 1997) comprising the thioredoxin peroxidases of yeast and mammals (Cha and Kim, 1995) and the alkyl hydroperoxide reductases of bacteria (Tartaglia *et al*., 1990).

Folates are essential vitamins which play a critical role in nucleotide biosynthesis. There is considerable difference in folate metabolism of protozoa parasite and humans (Anderson, 2005). Parasitic protozoa utilize the endogenous folate pathway for its biosynthesis whereas in humans it is done via a membrane bound folate carrier as humans cannot synthesize folate de novo (Tenter *et al*., 2000). Hence, the enzyme Dihydrofolate Reductase (DHFR) and Thymidylate Synthase (TS) plays an important role in folate metabolism and therefore are important targets against leishmaniasis. In most organisms DHFR and TS exist as separate molecular entities. Whereas, in *Leishmania*, these enzymes are a part of a bi-functional DHFR-TS complex (Beverley *et al*., 1986). Studies have shown that it is possible to achieve inhibition selectivity of the parasite against the human by targeting the DHFR-TS complex of the *Leishmania major*. Hence, the selectivity issue concerning the parasitic diseases is supposed to be resolved with the inhibition of DHFR-TS enzyme complex.

Methotrexate (MTX) (**Fig. 1**) is an inhibitor of enzyme dihydrofolate reductase which plays an important role in thymidylate synthesis (Hitchings and Smith, 1980). MTX is a chemotherapeutic agent which does not directly damage DNA (Panozzo *et al*., 1996). It is a folic acid antagonist and has been widely used as anti-cancer drug since 1953. Methotrexate has also been used in various diseases like choriocarcinoma, psoriasis, sarcoidosis and trophoblastic tumors. It has been used in treatment of autoimmune diseases and prevention of graft versus host disease (Budzynska and Zakliczynska, 2004; Longo-Sorbello and Bertino, 2001; Bertino, 1993; Tjaden and Bruijn, 1990; DIUSP, 2004). In 1960 MTX was used to treat rheumatoid arthritis and psoriasis (Ward, 1985). Currently MTX is used as a key component in treating HIV related lymphomas and other germ cell neoplasias (Lichtman *et al*., 1993; Walsh *et al*., 1993).

In the present study, the 3-D structure of *L. braziliensis* Try P has been constructed using the templates *L. Major* Try PI (PDB ID: 3TUE). The resulting FASTA sequence was used to build the 3-D structures. Further, the molecular models of *L. braziliensis* Try P was constructed using Modeller9v9 package for homology modeling. The model quality was assessed using PROCHECK, PROSA, ERRAT, VERIFY 3D and WHAT-IF. The overall scores were used to choose the final model. Protein-ligand docking was performed between the molecular model of *L. braziliensis* Try P and methotrexate. The detailed analyses of probable inhibition as well as interaction of the models were performed for binding affinity.

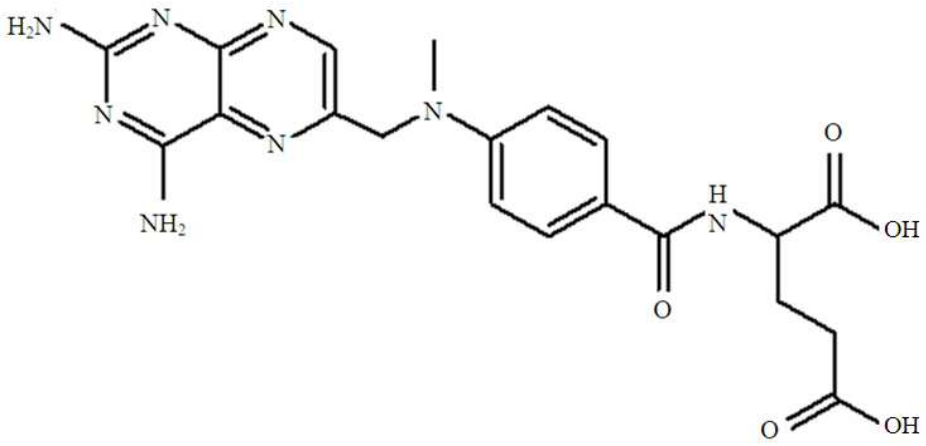


Fig. 1. Structures of methotrexate

However, there is no conclusive report as to whether the inhibition of Try P activity is due to methotrexate. In the present study, the structural models of the methotrexate in the Try P binding sites have been carried out, which may facilitate further development of more potent antileishmanial agents as inhibitors of Try P.

2. MATERIALS AND METHODS

2.1. Target and Template Sequence Alignments

The sequence of *L. braziliensis* Try P (gi No. 154334618) was retrieved from National Centre for Biotechnology Information (NCBI). The homology sequences were searched against the Protein Data Base (Berman *et al*., 2002) using NCBI-BlastP (Altschul *et al*., 1990; 1997). The most homologous structure obtained was considered as the potential template for modeling the structure of target. The atomic coordinates for modeling the structure was obtained from the protein databank.

2.2. Homology Modeling of Tryparedoxin Peroxidase

The 3D structure of the Try P from *L. braziliensis* was generated by using PDB ID: 3TUE as the potential template structure. The alignment of the target sequence and template co-ordinate files was refined by using clustalW (Thompson *et al*., 1994) with default parameters. The 3D structure was generated by applying the spatial restraints using Modeler9v9 (Sali and Blundell, 1993). A bundle of 50 models were randomly generated. From the initial model the best model with the least RMSD value was selected by superimposing the model with its template using SUPERPOSE (Maiti *et al*., 2004). This model was subjected for energy minimization by applying 20 steps of each steepest descent and conjugate gradient using GROMOS (Walter *et al*., 1999) using SwissPDB viewer for further analysis.

2.3. Validation of the Homology Model

After the construction of the model, its quality was assessed by considering the both the geometric and energetic aspects using PROCHECK (Laskowski *et al*., 1993) ERRAT (Colovos and Yeates, 1993), WHAT-IF (Vriend, 1990), PROSA 2003 (Sippl, 1993) and VERIFY 3D (Bowie *et al*., 1991; Luthy *et al*., 1992) for internal consistency and reliability. The Ramachandran plot computed with PROCHECK provided the residue position in particular segment based on the dihedral angles. Finally, the best-quality models were subjected to further calculations and molecular modeling studies, binding site analysis and other calculations.

2.4. Prediction of Binding Site

To determine the interactions between methotrexate and Try P, the amino acids in the binding site of the model was predicted through Q-site Finder (Laurie and Jackson, 2005) and the same was confirmed by the conserved residues observed in the template binding site.

2.5. Ligand Preparation

The methotrexate molecule was drawn in ACD-Chemsketch and their SMILES notation was obtained. They were converted into SDF files using 'Online SMILES convertor and Structure file generator' (Weininger, 1988).

2.6. Flexible Docking

Molecular docking analysis is carried out between the target protein active site with ligand of methotrexate. The developed SDF structures were docked within the binding site of *L. braziliensis* Try P using FlexX (Rarey *et al*., 1996) with following parameters (i) default general docking informations, (ii) base placement using triangle matching, iii) scoring of full score contribution and threshold of 0,30 and No score contribution and threshold of 0,70 (iv) chemical parameters of clash handling values for protein ligand clashes with maximum allowed overlap volume of 2.9 A^{03} and intra-ligand clashes with clash factor of 0.6 and considering the hydrogen in internal clash tests (v). Default docking detail values of 200 for both the maximum number of solutions per iteration and maximum number of solutions per fragmentation.

2.7. Predictions of Ligand-Receptor Interaction

The interactions of methotrexate with *L. braziliensis* Try P in the docked complex were analyzed by the pose-view of LeadIT (Stierand *et al*., 2006).

2.8. Cytotoxicity Assay

Promastigotes were harvested in the exponential growth phase, resuspended in fresh medium to achieve 1×10^6 parasites/ml and were seeded in 96 well culture plates. Methotrexate was added to the plates in different dilutions and the plates were incubated at 26°C for 48 hrs and promastigote viability was evaluated by using the quantitative colorimetric MTT assay. The conversion of MTT to the formazan product by the mitochondrial electron transport chain is an indicator of cell viability and a decrease in the amount of MTT converted indicates toxicity to the cell. Briefly, the MTT labeling reagent (final concentration, 0.25 mg mL^{-1}) was added to

each well and after a incubation 4hr at 37°C, DMSO was added to dissolve the formazan crystals and obtain a homogeneous blue solution suitable for measurement of the absorbance with an enzyme-linked immunosorbent assay plate reader (wavelength, 540 nm). The percentage of surviving promastigotes versus the number of surviving control promastigotes were assessed by the formula 100× (absorbance of treated cells/absorbance of control cells). The 50% inhibitory concentration (IC_{50}) was evaluated from the graph and the results were expressed as the mean and standard deviation of three independent experiments.

2.9. Scanning Electron Microscopy

The parasites were harvested by centrifugation, washed twice in a phosphate buffer and fixed for 2 to 3 hr at 4°C in 2.5% (v/v) glutaraldehyde in phosphate buffer, with pH7.4. Meanwhile, the cells were pelleted, postfixed with 1% (w/v) osmium tetroxide for 30 min and then processed in ethanol propylene oxide series. Finally, the samples were point dried utilizing liquid CO_2 and coated with gold particles. They were observed and imaged through a scanning electron microscope (Li *et al*., 2009).

3. RESULTS AND DISCUSSION

3.1. Target and Template Sequence Alignments

The BLASTp result of *L. braziliensis* Try P showed 82.4% sequence similarity to *L. Major* Try PI (PDB ID: 3TUE). The structure Tryparedoxin Peroxidase (PDB ID: 3TUE) were selected as templates for *L. braziliensis* Try P. The most significant step in homology modeling process is to obtain the correct sequence alignment of the target sequence with the homologues. The sequence alignment was performed using the ClustalW and the alignment was shown in **Fig. 2**.

3.2. Homology Modeling of *L. Braziliensis* Try P

The sequence alignment file was used as input to build the initial model of *L. braziliensis* Try P using Modeler 9v9 by applying spatial restraints from the initial structure, a bundle of 50 models were developed using random generation and the best model was selected for further analysis based on its structural compatibility (structure with lowest DOPE score). The modeled structure was shown in **Fig. 3a**. The quality of the model was accessed by the results of the server SUPERPOSE. SUPERPOSE compared the predicted structure of *L. braziliensis* Try P to crystal structure of *L. Major* Try PI (PDB ID: 3TUE) via superposition and was shown in **Fig. 3b**. Among the generated models, the model having lowest Root Mean Square Deviation (RMSD) value of 0.87 Å when superposed onto the template 3TUE was chosen for further analysis.

3.3. Model Quality Assessment

The geometry of the main chain and side chains have been analyzed using the program PROCHECK (Tamura *et al*., 1995). Ramachandran plot for the derived model of *L. braziliensis* Try P is shown in **Fig. 4a**.

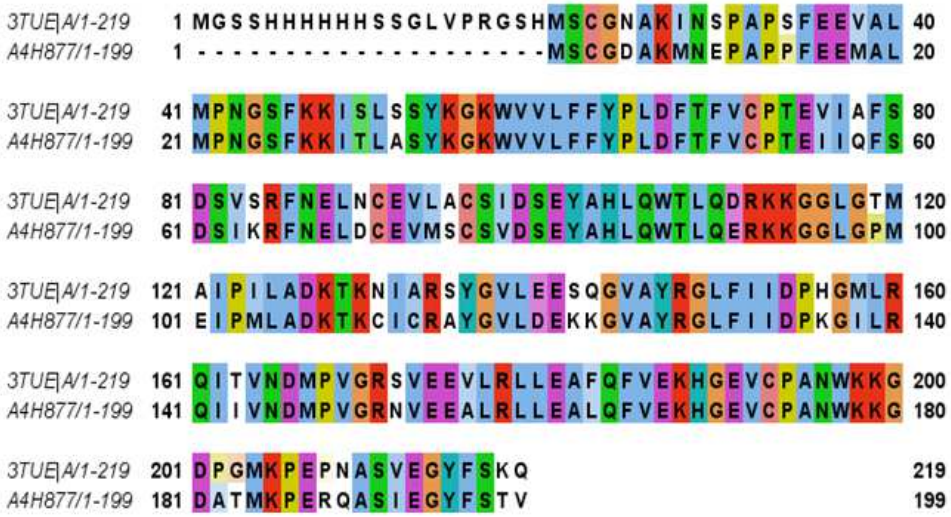
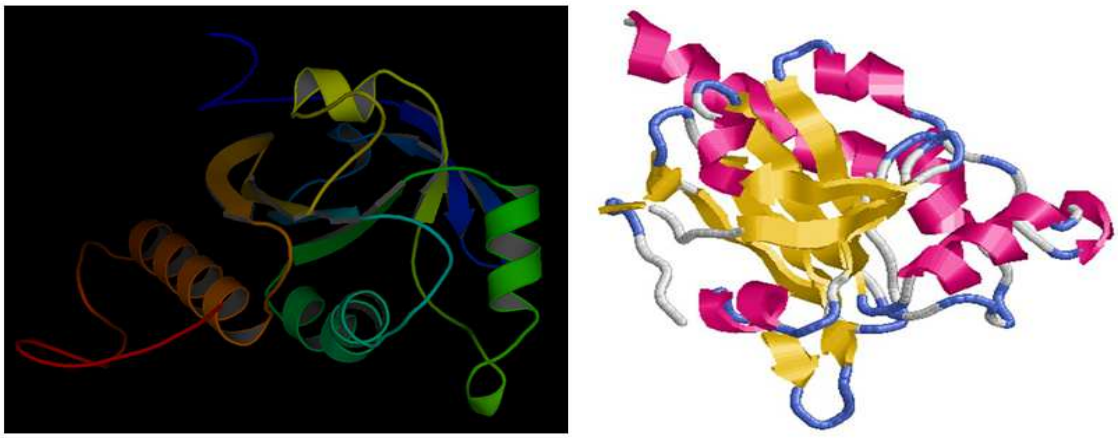


Fig. 2. Sequence alignment of *L. braziliensis* TryP and *L. Major* TryPI (PDB ID: 3TUE)



(a) (b)

Fig. 3. (a) Ribbon diagram of the modeled tryparedoxin peroxidase of *leishmania braziliensis*, (b) Ribbon diagram of the modeled Tryparedoxin Peroxidase of *Leishmania Major* TryPI

Table 1. Validation of the model TryP from *L. braziliensis* and its template TryPI from *L. Major*

	Ramachandran Plot quality (%)					
Procheck	Most favored	Additional allowed	Generously allowed	Dis-allowed	VERIFY-3D	ERRAT score
L. braziliensis TryP	92.4	7.0	0.6	0.0	78.90	71.123
3TUE	93.2	6.8	0.0	0.0	95.47	95.860

Table 2. Binding energies of docked methotrexate with the modeled protein *L. braziliensis*

Receptor	*L. braziliensis* TryP
Ligand (Methotrexate) with binding energy (kJ/mol)	-14.5879

The Φ and Ψ backbone torsion angles of most of the non-glycine residues are within the allowed regions of which 92.4% are in the energetically most favoured area. Only 7.0% residues are found in additional allowed regions and 0.6% in the generously allowed region. No residues are in disallowed regions; whereas the corresponding values for the *L. Major* Try PI (PDB ID: 3TUE), template were 93.2 %, 6.8 %, 0.0 % and 0.0 %, respectively (**Fig. 4b**). This ensures that the generated model was good when compared with the template structure which shows 93.2 % and our model shows 92.4% of residues in most favoured region. The total quality G-factor was -0.14. Further, the overall quality factor and compatibility of an atomic model (3D) with amino acid sequence (1D) for the modeled protein *L. braziliensis* Try P was observed as 71.123 and 78.90 % from ERRAT and Verify3D respectively (**Fig. 5 and 6a**). Whereas in the case of template *L. Major* TryPI the values for ERRAT and Verify3D were 95.866 and 95.47 % respectively (**Table 1**). The results of ERRAT and Verify3D also confirm the model was reliable and of good quality. The PROSA Z-Score indicates overall model quality. Global analysis of the model *L. braziliensis* Try P with PROSA showed a Z-Score of -7.08 (**Fig 6b**), indicating no significant deviation from typical native structures of similar size as the template when compared with Z-Scores of -6.51 for 3TUE template. The detailed secondary structural investigation of the predicted *L. braziliensis* Try P model with PDB sum, a secondary structure prediction for the model showed 6 Helices, 9 Strands, 17 Beta turns, 2 sheets, 1 Gamma turns and 3 Beta hairpins (**Fig. 7a**) indicating that the protein belongs to class alpha + beta.

The detailed secondary structural investigation of the predicted *L. Major* TryPI (PDB ID: 3TUE), template showed 7 Helices, 9 Strands, 9 Beta turns, 2 sheets, 2 Gamma turns and 3 Beta hairpins (**Fig. 7b**) indicating that the template protein also belongs to class alpha + beta.

3.4. Docking Studies with Flavonoids

This methotrexate were docked with modeled *L. braziliensis* Try P and also with its template *L. Major* Try PI using the docking program FlexX and the ligand-receptor interactions were analyzed using LeadIT. The docking interaction of the methotrexate molecule with modeled *L. braziliensis* Try P has lowest binding energy of -14.5879 kJ mol^{-1} as shown in **Table 2**.

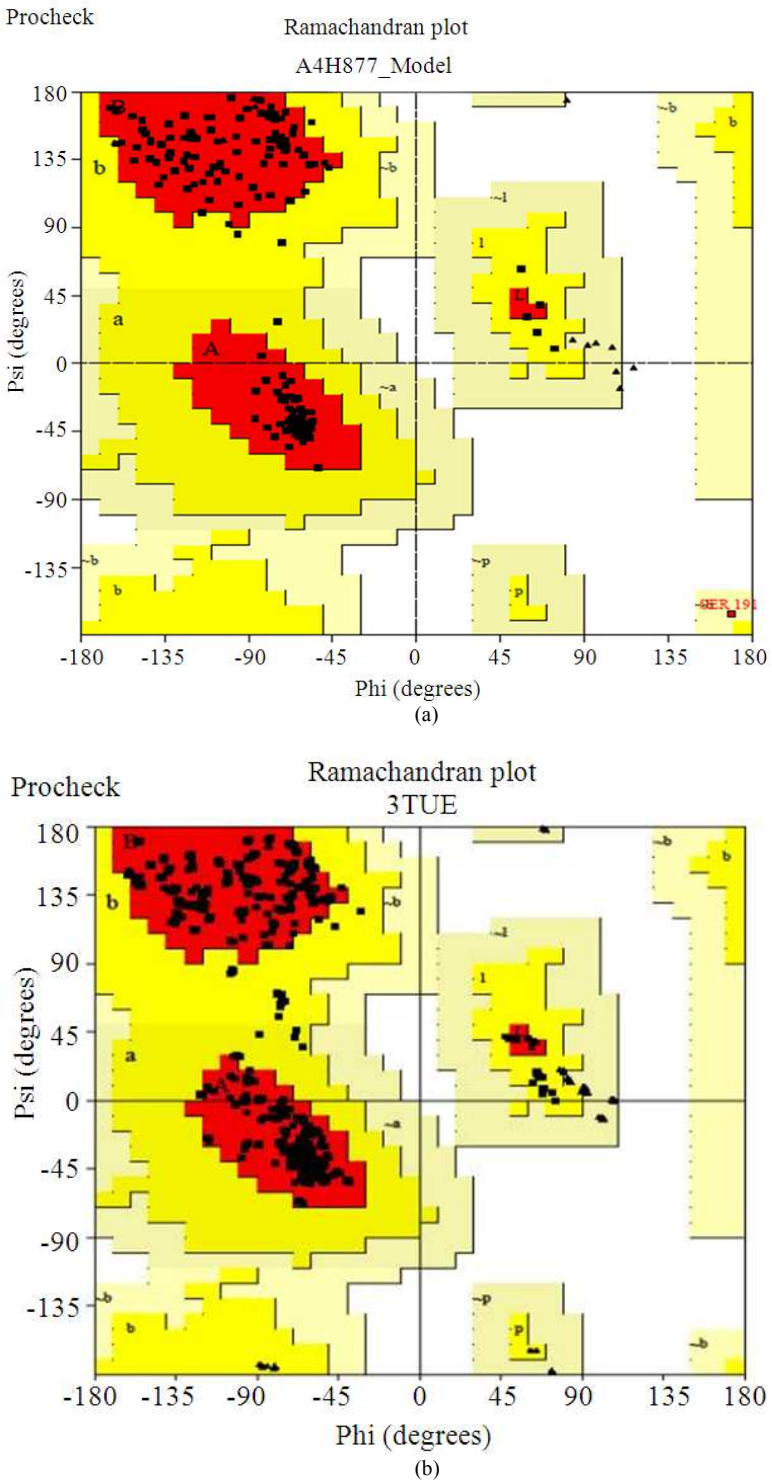


Fig. 4. (a) Ramachandran plot for the Modeled protein TryP from *L. braziliensis*, (b) Ramachandran plot for the template protein TryPI from *L. Major*

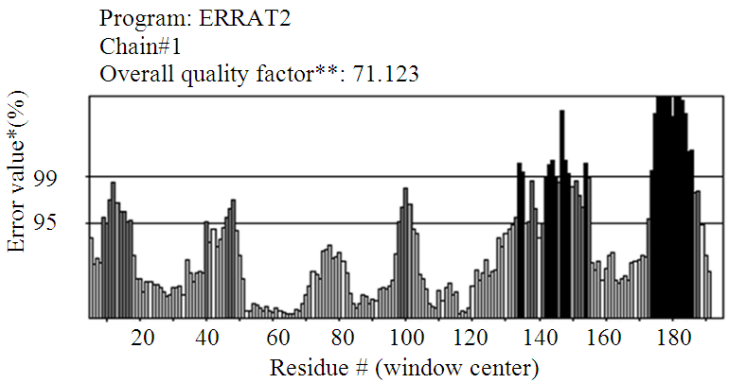


Fig. 5. ERRAT plot for Model assessment of Modeled protein TryP from *L. braziliensis*

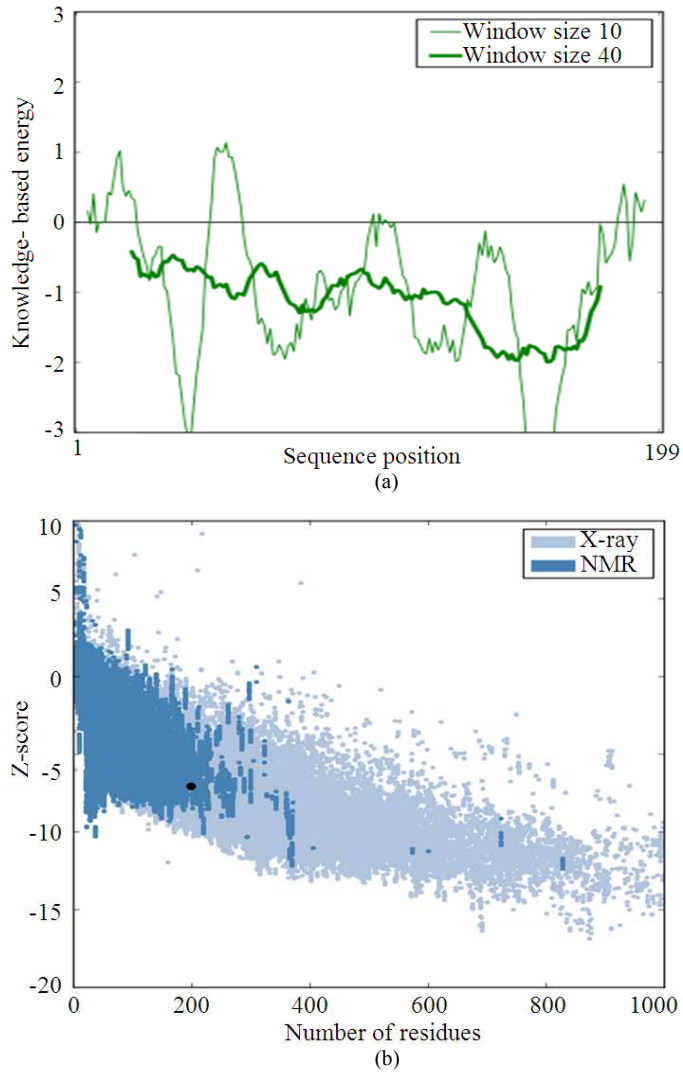
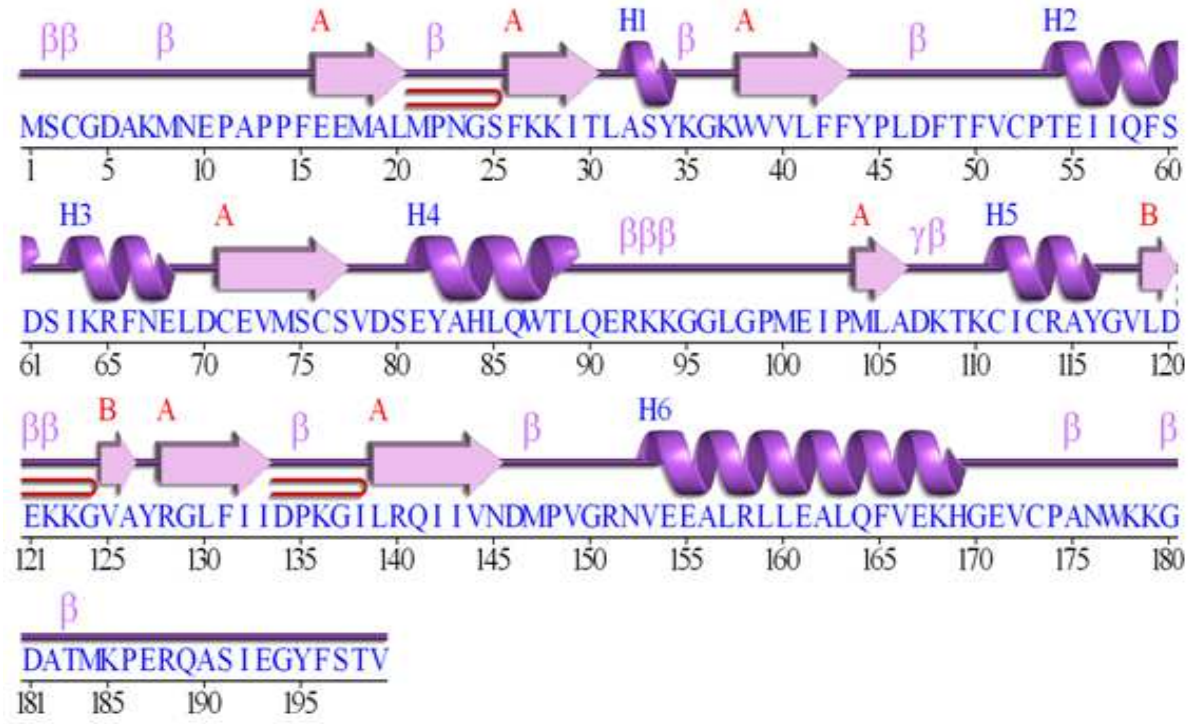
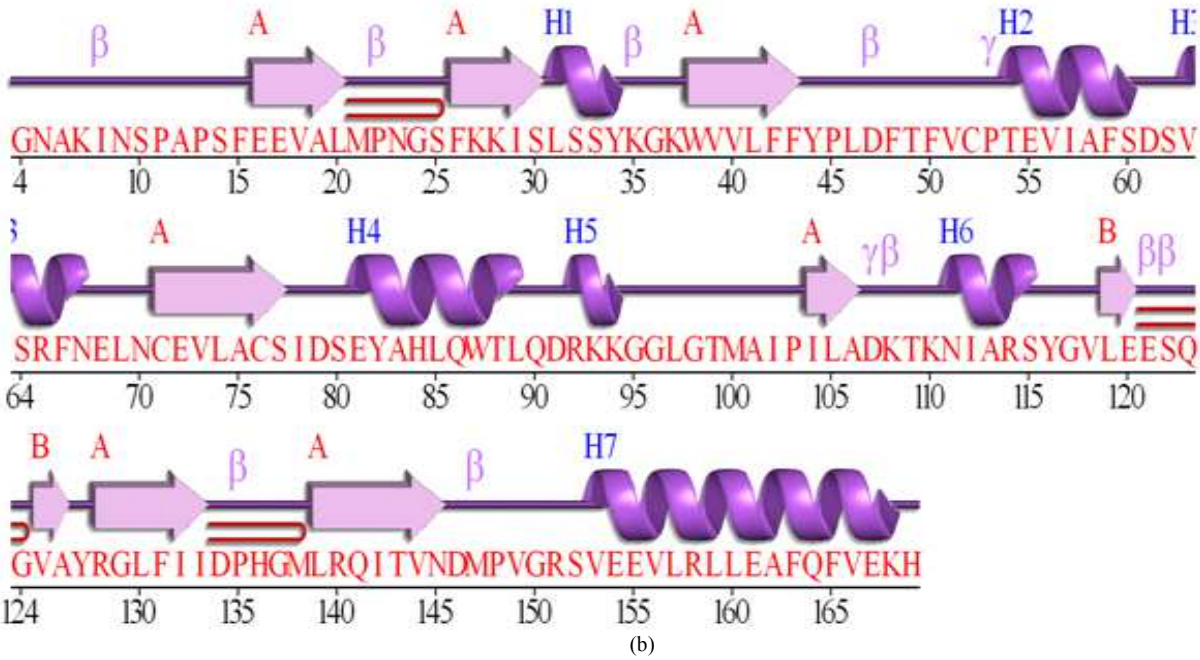


Fig. 6. (a) Energy plot for the predicted TryP from *L. braziliensis*, (b) ProSA plot for Modeled protein TryP from *L. braziliensis*



(a)



(b)

Fig. 7. (a) Secondary structure of Tryparedoxin Peroxidase of *Leishmania braziliensis*, (b) Secondary structure of Template Tryparedoxin Peroxidase (3TUE)

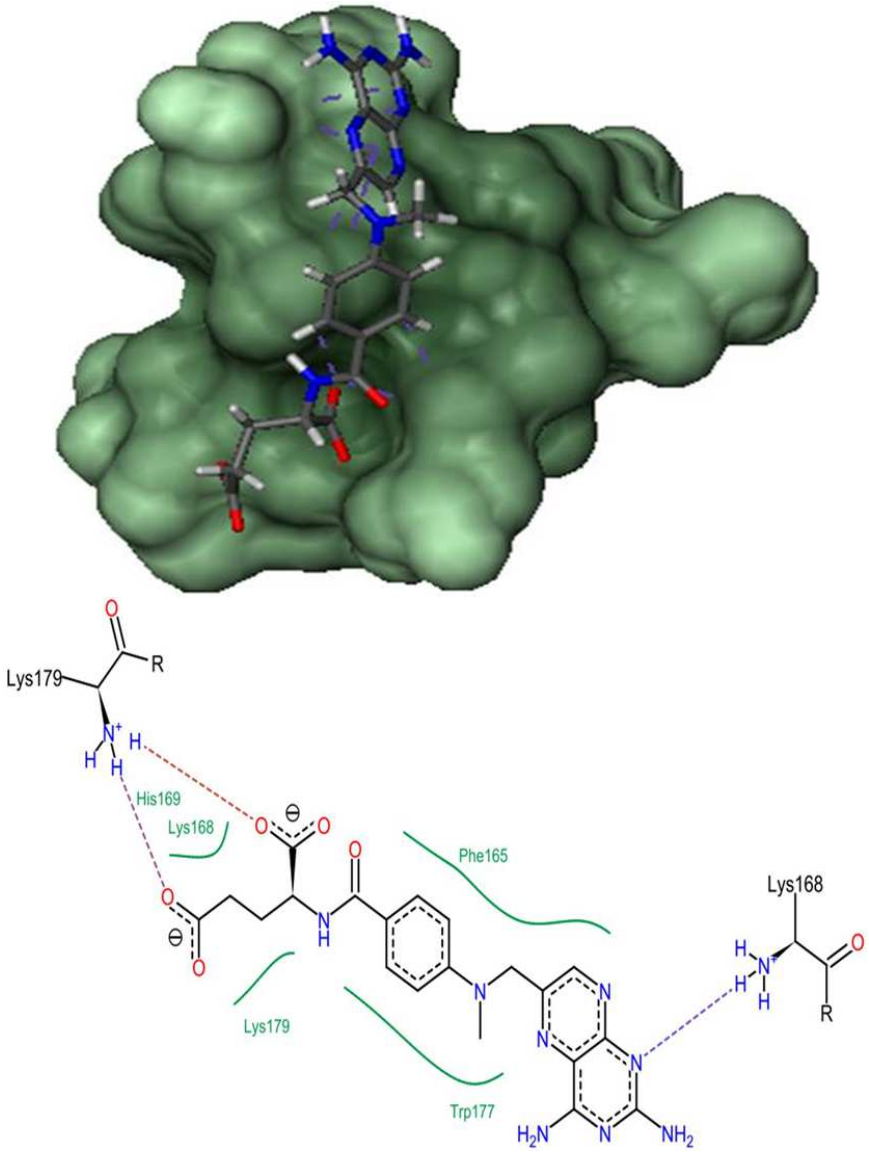


Fig. 8. Interaction of methotrexate with Tryparedoxin Peroxidase from *L. braziliensis*

Table 3. Binding site residues and their type of interactions in TryP from *L. braziliensis*

Protein	*L. braziliensis* TryP
Ligand	Methotrexate
Amino acids involved in bonded interactions	
Phe-165	
Lys-168	
His-169	
Trp-177	
Lys-179	

Figure 8 show the docking results of modeled *L. braziliensis* Try P proteins and **Table 3** shows the corresponding amino acids with their specific binding energies favoring the interactions. The binding energy scores of the methotrexate molecules with *L. braziliensis* Try P was -14.5879kJ mol^{-1}. The amino acids that interacted with methotrexate were found to be Phe-165, Lys-168, His-169, Trp-177 and Lys-179 in *L. braziliensis* Try P. These findings suggested that the amino acids Phenylalanine (Phe), Histidine (His).

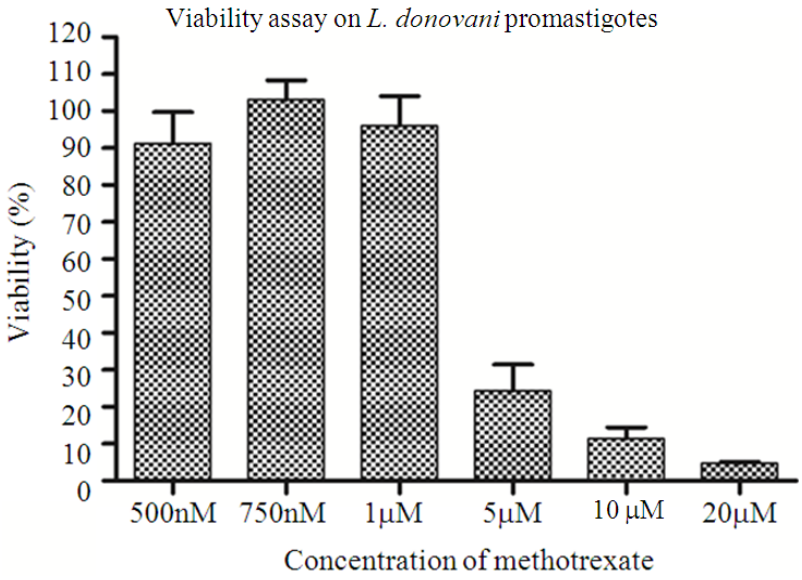


Fig. 9. Estimation of IC_{50} of methotrexate using MTT assay. The graph represents the data of three independent experiments with SD. The bars show the percentage (%) viability cells at different concentrations of the extracts

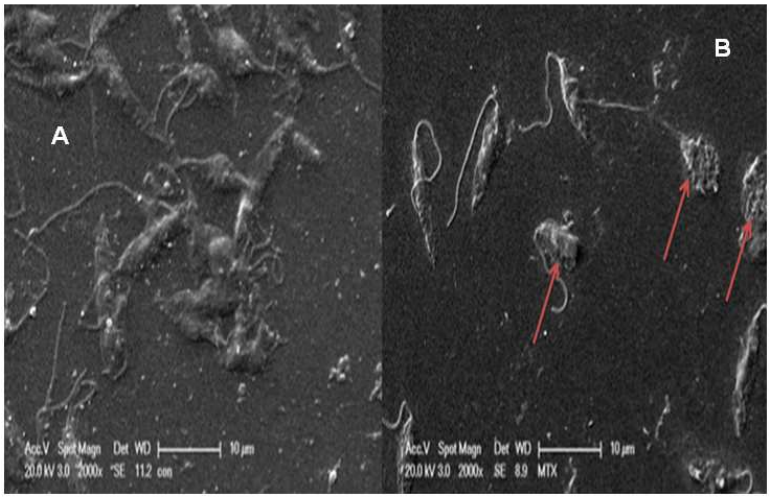


Fig. 10. Assessment of treated and untreated *L. donovani* promastigote morphology using scanning electron microscope at 48 h incubation. A indicate control parasites and B indicates methotrexate treated parasites

Tryptophan (Trp) and Lysine (Lys) in the active site of Try P of modeled protein were conserved and favoring the interactions with the ligand.

3.5. Cytotoxicity Assay

MTT assay is widely used for determining the cytotoxicity of any given compound or drug on the parasites based on the viability. Promastigotes were treated with different concentrations of methotrexate for 48 hrs and viability of these treated parasites were determined by comparing with the control parasites. Till 1µM concentration of methotrexate had no effect on the parasites as the viability of the parasites were about 95%. From 2µM concentration the viability of the parasites went down drastically to 25% (**Fig. 9**). The 50% inhibitory concentration of methotrexate on the

promastigotes was determined to 1.65µM. So we can conclude that methotrexate has anti promastigote activity on the *L.donovani* parasites.

3.6. Scanning Electron Microscopy

Morphology of treated parasites can be assessed by SEM by comparing with the control parasites. Parasites were treated with methotrexate at IC_{50} concentration was incubated for 48 h and samples were processed for SEM analysis. Compared to the control parasites, in treated parasites the morphology was deformed from the usual spindle shape of the promastigotes (**Fig. 10**). So methotrexate can induce morphological deformalities in the promastigote stage of *L.donovani* parasites.

4. CONCLUSION

In this study, we have developed a very accurate 3D model of *L. braziliensis* Try P by using crystal structure of *L. Major* Try PI (PDB ID: 3TUE) as template in Modeller9v9. This model is validated by using several validation methods, including PROCHECK, PROSA2003, ERRAT and VERIFY-3D. All evidences suggest that the geometric quality of the backbone conformation, the residue interaction, the residue contact and the energy profile of the structure is well within the limits established for reliable structures. In conclusion, this study clearly demonstrated the tryparedoxin peroxidase inhibitory activity of the selected anticancer compound, methotrexate by *in silico* docking analysis and *in vitro* assay. *In silico* docking analysis is actually an added advantage to screen the tryparedoxin peroxidase inhibition. These results clearly indicate that methotrexate has an excellent binding interaction with *L. braziliensis* Try P.

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