

# Studies on key enzyme(s) of leishmania parasite



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Award of Degree

**Doctor of Philosophy**

By

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**Dedicated to my parents,  
extended family, and all my  
mentors**

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## List of Abbreviations

TCA	Tricarboxylic Acid
VL	Visceral
CL	Cutaneous
MCL	Mucocutaneous
FDA	Food and Drug Administration
WHO	World Health Organization
AMR	American Region
AFR	African Region
EMR	Eastern Mediterranean Region
SEAR	South-East Asia Region
SbV	Pentavalent Antimony
Sb+3	Trivalent Antimony
AQP1	Aquaporin 1
ABC	ATP-binding Cassette
AmB	Amphotericin B
HIV	Human Immunodeficiency Virus
ABCC3	ATP binding Cassette Subfamily C Member 3
SSG	Sodium Stibogluconate
PR	Paromomycin
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
NADH	Nicotinamide Adenine Dinucleotide
Acetyl-CoA	Acetyl Coenzyme A
FADH2	Flavin Adenine Dinucleotide
ETC	Electron Transport Chain
IDH	Isocitrate Dehydrogenase
OGDC	oxoglutarate dehydrogenase complex
SCS	Succinyl-CoA synthetase

GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
ATP	Adenosine Triphosphate
CS	Citrate synthase
CIC	Citrate Carrier
ACC	Acetyl CoA Carboxylase
NAD	Nicotinamide Adenine Dinucleotide (oxidized form)
OXPPOS	Oxidative Phosphorylation
PK	Pyruvate Kinase
PFK1	Phosphofructokinase-1
FBPase1	Fructose 1,6-bisphosphatase
FBPase2	Fructose 2,6-bisphosphatase
PFK2	Phosphofructokinase-2
PDH	Pyruvate Dehydrogenase
SDH	Succinate Dehydrogenase
ACLY	ATP Citrate Lyase
FAO	Fatty Acid Oxidation
CPT1	Carnitine Palmitoyl Transferase 1
IFN- $\gamma$	Interferon Gamma
NF- $\kappa\beta$	Nuclear Factor Kappa B
PGE2	Prostaglandin E2
NO	Nitric Oxide
HIF-1 $\alpha$	Hypoxia-Inducible Factor 1-Alpha
IL-1 $\beta$	Interleukin 1 Beta
FASN	Fatty Acid Synthase
TLR4	Toll-like Receptor 4
LTB4	Leukotriene B4
iNOS	Inducible Nitric Oxide Synthase
NRF2	Nuclear Factor Erythroid 2- related Factor 2 f
ATF3	Activating Transcription Factor 3

TNF $\alpha$	Tumor Necrosis Factor Alpha
IFN $\beta$	Interferon Beta
GSH	Glutathione
KEAP1	Kelech-like ECH Associated Protein 1
ACSS	Acetyl-CoA Synthetase
DC	Dendritic Cell
NTDs	Neglected Tropical Diseases
LdCS	<i>Leishmania donovani</i> citrate synthase
MMPBSA	Molecular Mechanics Poisson- Boltzmann Surface Area
ADMET	Adsorption-Distribution- Metabolism- Excretion- Toxicity
MD Simulation	Molecular Dynamic Simulation
NCBI	National Centre for Biotechnology Information
I-TASSER	Iterative Threading Assembly Refinement
RCSB PDB	Research Collaboratory for Structural Bioinformatics Protein Data Bank
C Score	Confidence Score
Tm Score	Template Modelling Score
SDF	Spatial Data File
PDBQT format	Protein Data Bank, Partial Charge, Atom Type format
LGA	Lamarckian Genetic Algorithm
NVT	Number of particles, system Volume, and Temperature
NPT	Number of particles, system Pressure, and Temperature
PME	Particle Mesh Ewald
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
H-bond	Hydrogen Bond
Rg	Radius of Gyration
PB	Poisson- Boltzmann
SASA	Solvent Accessible Surface Area
HsCS	Human Citrate Synthase
CNS	Central Nervous System

CYP	Cytochrome Protein
hERG	Human Ether-a-go-go-Related Gene
BBB	Blood Brain Barrier
VDs	Volume of Distribution
MRTD	Maximum Recommended Tolerated Dose
CD	Circular Dichroism
GnHCl	Guanidine Chloride
PMSF	Phenyl Methyl Sulfonyl Fluoride
ANS	8-Anilionnaphthalene-1-sulphonic acid
PCR	Polymerase Chain Reaction
NaCl	Sodium Chloride
KCl	Potassium Chloride
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
BSA	Bovine Serum Albumin
K <sub>sv</sub>	Stern-Volmer quenching constant
Ni-NTA	Nickel- Nitrilo Tri Acetic Acid
KI	Potassium Iodide
RPE	Ribulose-5-Phosphate Epimerase
T <sub>m</sub>	Melting Temperature
K <sub>i</sub>	Inhibition Constant
HR <sup>+</sup>	Hormone Receptor-Positive
HER2 <sup>-</sup>	Human Epidermal growth factor Receptor negative
CDK4 & 6	Cyclin Dependent Kinase 4 & 6
SERM	Selective Estrogen Receptor Modulator
PAR-1	Protease Activated Receptor -1
TK	Tyrosine Kinase
H2DCFDA	2',7'- Dichloro Dihydro Fluorescein Diacetate
M199	Medium 199
RPMI 1640	Rosewell Park Memorial Institute 1640
FBS	Fetal Bovine Serum

DMSO	Dimethyl Sulfoxide
DTNB	5, 5-Dithio-bis-(2-Nitrobenzoic acid)
TNB	5-thio-2-nitrobenzoate
OAA	Oxaloacetic acid
NaOH	Sodium Hydroxide
K <sub>m</sub>	Michalis-Menten Kinetics Constant
K <sub>cat</sub>	Catalytic Constant
MTT	3-(4, 5- dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffer Saline
IC <sub>50</sub>	Half-maximal Inhibitory Concentration
EC <sub>50</sub>	Half-maximal Effective Concentration
CC <sub>50</sub>	Cytotoxic Concentration
SI	Selectivity Index
ANOVA	Analysis of Variance
SEM	Standard Error of the Mean
K <sub>b</sub>	Binding Constant
MitoSOX	Mitochondrial Superoxide
DAPI	Diamidino-2-phenyl Indole
PI	Propidium Iodide
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
SDS	Sodium dodecyl Sulfate
EDTA	Ethylenediamine tetra acetic Acid
SEM	Scanning Electron Microscope
JC-1	5,5,6,6'- tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide

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## Abstract

Leishmaniasis, a neglected tropical disease caused by various species of *Leishmania*, presents a significant global health challenge, especially in tropical and subtropical regions like Latin America, Southeast Asia, and parts of Africa. Annually, there are 700,000 to 1 million new cases, and 25,000 to 26,000 deaths occur worldwide. Factors like climatic shifts, co-infections with diseases such as HIV, and population mobility have altered its distribution, exacerbating its impact. It is also termed a "poverty-associated disease" due to its prevalence among economically disadvantaged populations with limited healthcare access and minimal influence on government policies. Current treatment options are inadequate, marked by several issues such as toxicity, high cost, complex administration, and drug resistance. The pharmaceutical industry's lack of focus on this disease further hampers the development of innovative therapeutics, underscoring the need for new drug targets and the repurposing of existing drugs.

Amastigotes of *Leishmania*, which reside in the phagolysosomes of host cells, endure low pH, high temperatures, and oxidative stress, often entering a semi-quiescent state to evade host defences. They primarily utilize sugars and fatty acids for survival and growth, with the TCA cycle playing a crucial role in their metabolic pathways. Citrate synthase (CS), the first enzyme of the TCA cycle, was identified as a potential drug target due to its pivotal role in the continuation of the TCA cycle & energy production in mitochondria and cholesterol & fatty acid synthesis.

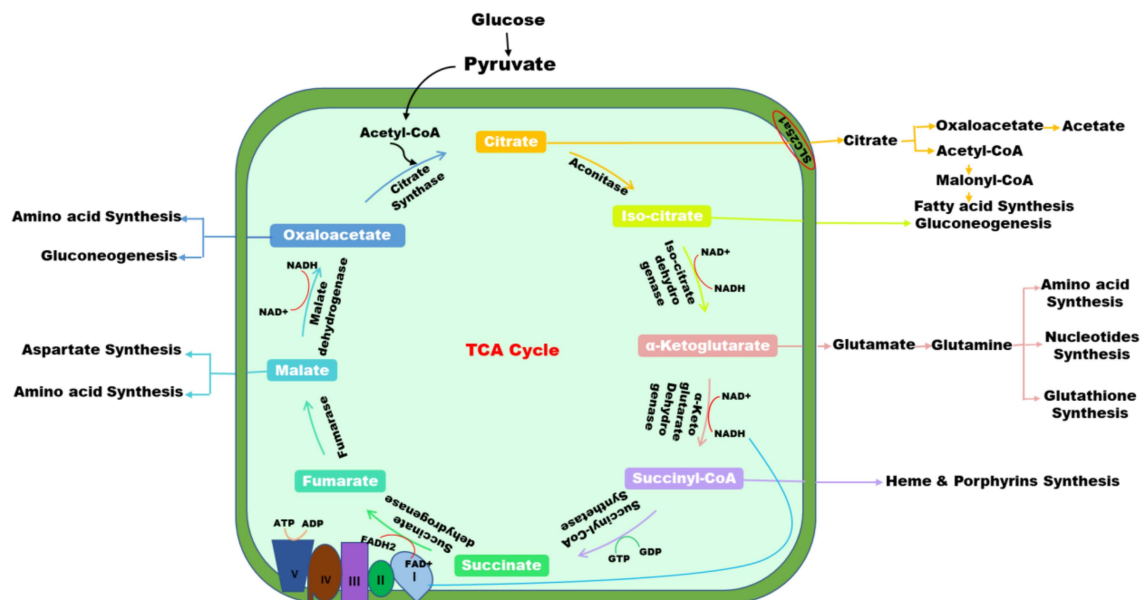


Figure 1: Biochemical role of TCA cycle intermediate.

in the cytosol and significant structural and sequential differences from the human version, making it a viable candidate for selective drug targeting.

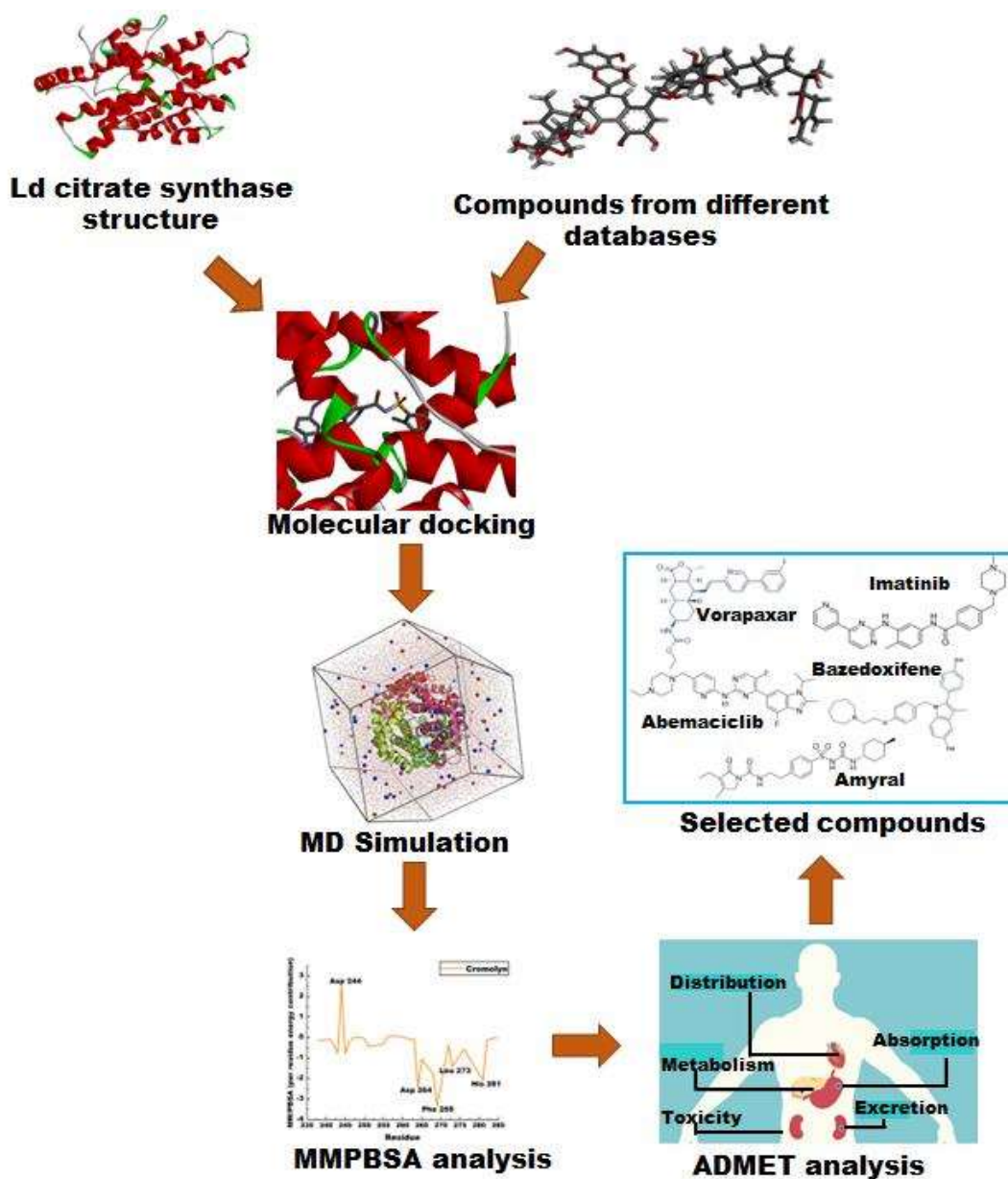
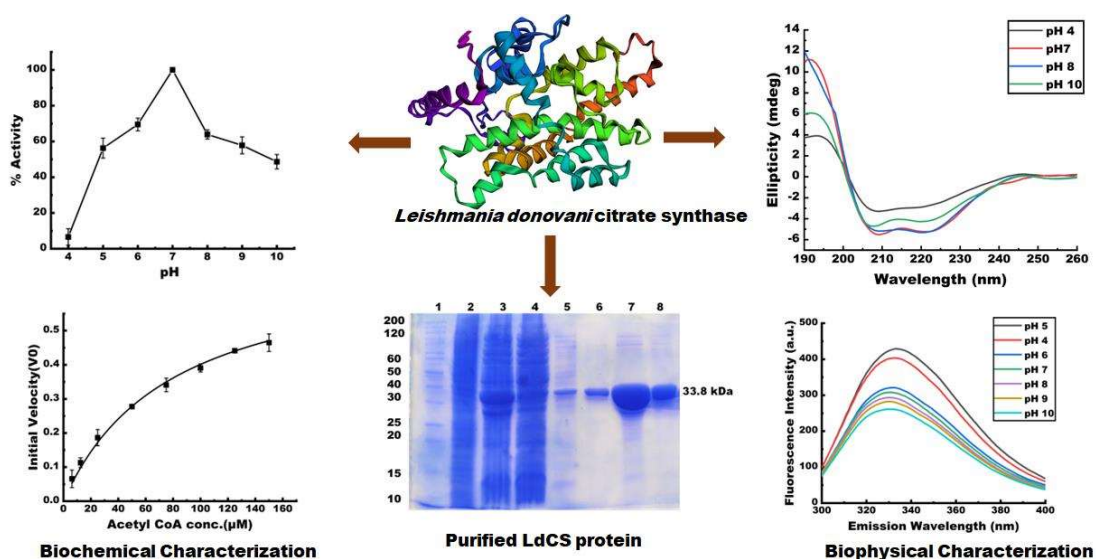


Figure 2: *In silico* studies for selecting top hits using LdCS as a drug target.

In this study, the crystal structure of *Leishmania donovani* citrate synthase (LdCS) was not available; thus, a model was generated using the ITASSER server. The best model, based on C and TM scores, was energy minimized and validated using RAMPAGE and ProSA servers. This validated model was used for molecular docking studies with 1065 natural and 1565 FDA-

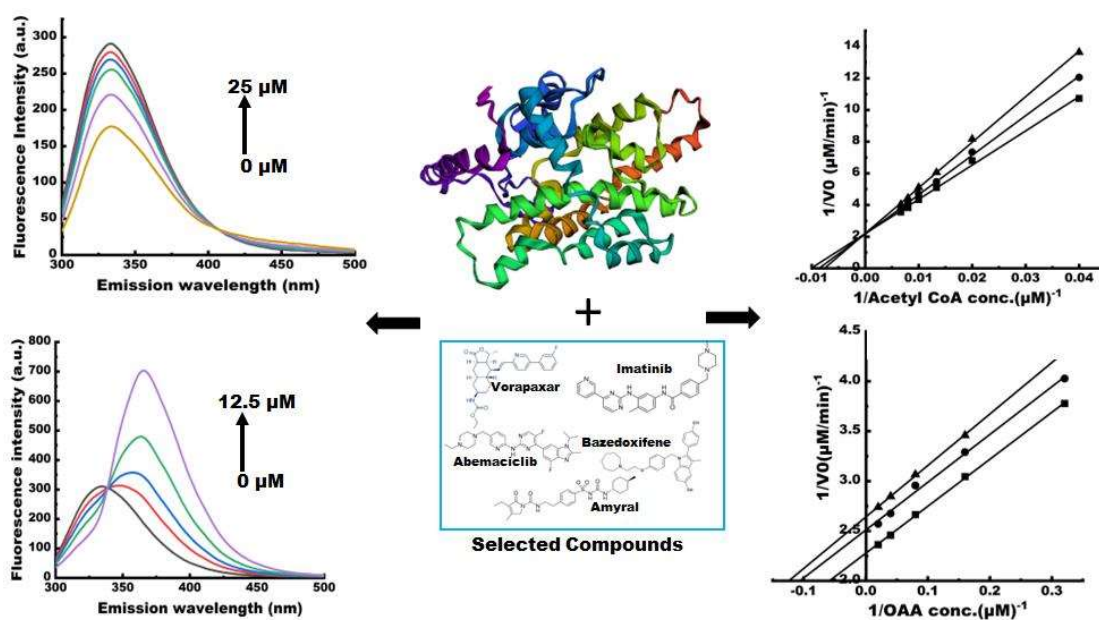
approved compounds from the ZINC 15 database. The top five hits—Abemaciclib, Bazedoxifene, Vorapaxar, Imatinib, and Amyral—were selected based on their lower binding energies with LdCS compared to human citrate synthase (HsCS) and interactions with the active site residues of LdCS which analysed through discovery studio analyser. All selected compounds adhered to Lipinski's rule, indicating favourable oral bioavailability. Molecular dynamics (MD) simulations were conducted with GROMACS to assess the stability of the protein-ligand complexes, considering parameters like RMSD, RMSF, radius of gyration, and hydrogen bond analysis. The simulations demonstrated that the ligands formed stable, robust interactions with LdCS. MM-PBSA analysis further confirmed the lower binding energies of these complexes, predominantly driven by hydrophobic contacts. Residue energy decomposition studies identified key residues—Tyr 238, His 242, His 245, Asp 264, Phe 269, Leu 273, and Asn 285—crucial for ligand interactions. Pharmacokinetic studies based on ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis showed that all selected ligands had good water solubility, strong CaCO-2 permeability, good intestinal absorption, and minimal toxicity. These properties suggest favourable pharmacokinetic profiles and potential for effective therapeutic application.



**Figure 3: Biochemical and Biophysical characterization of LdCS.**

To further characterize LdCS, biophysical and biochemical studies were performed. The Ldcs gene was cloned and expressed in a bacterial system, and its protein, purified through affinity chromatography, was confirmed to be pure and of the correct size via SDS-PAGE. Circular dichroism fluorescence spectroscopy revealed that LdCS is predominantly alpha-helical and

maintains a highly folded structure at physiological pH, with changes in secondary structure at extreme pH levels. Thermal denaturation studies indicated that LdCS resists structural changes upon unfolding, with a melting temperature of  $44 \pm 0.31^\circ\text{C}$ . Fluorescence spectroscopy also indicated that LdCS contains tryptophan residues buried in its core, which are sensitive to environmental changes. Quenching studies with potassium iodide and acrylamide confirmed that these residues are accessible to quenchers, suggesting their strategic location within the protein structure. Urea and GnHCl-mediated unfolding studies provided insights into the stability of LdCS, with transition concentrations indicating a relatively stable protein structure. Functional characterization of LdCS included enzyme activity assays, revealing optimal catalytic activity at pH 7.0. Kinetic studies showed a higher  $K_m$  for acetyl-CoA than OAA, indicating a lower affinity for acetyl-CoA.



**Figure 4:** *In vitro* studies with selected compound.

Binding studies with selected compounds through fluorescence spectroscopy show good binding affinity, and inhibition studies with selected compounds demonstrated competitive inhibition for acetyl-CoA and uncompetitive inhibition for OAA, with inhibition constants in the micromolar range. Antileishmanial and cytotoxicity studies on promastigote and amastigote forms of *Leishmania*, as well as murine macrophages, revealed that the selected compounds effectively reduced cell viability and infection rates, with  $IC_{50}$  and  $EC_{50}$  values lower than those of the standard drug Miltefosine.

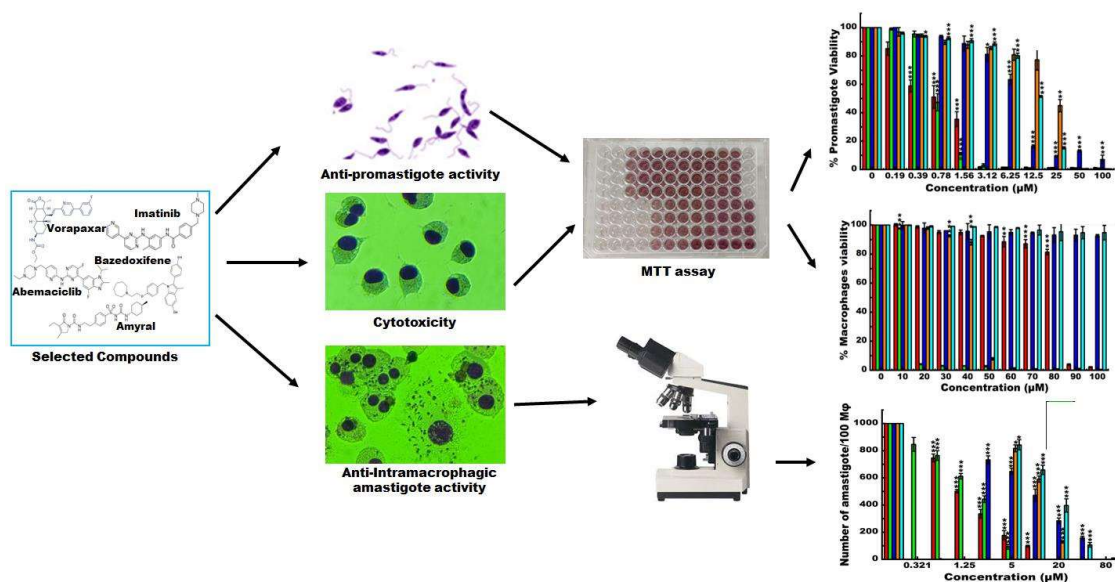


Figure 5: Antileishmanial and cytotoxicity studies with selected compounds.

Vorapaxar showed, till 500  $\mu\text{M}$ , no cytotoxic effects on macrophages, while other compounds exhibited low cytotoxicity, indicating a favourable therapeutic index. Further investigations into the mechanism of action of abemaciclib, based on its potent *in-silico* and *in-vitro* activity, revealed morphological alterations in promastigotes, mitochondrial damage, increased ROS production, depolarization of the mitochondrial membrane, nuclear condensation, increase in Sub G1 cell population in cell cycle assay and DNA fragmentation, indicating its action on mitochondria and an apoptotic mode of death. The apoptotic mode of death was further confirmed with Annexin V and PI, indicating that 64% of cells are in an apoptotic state.

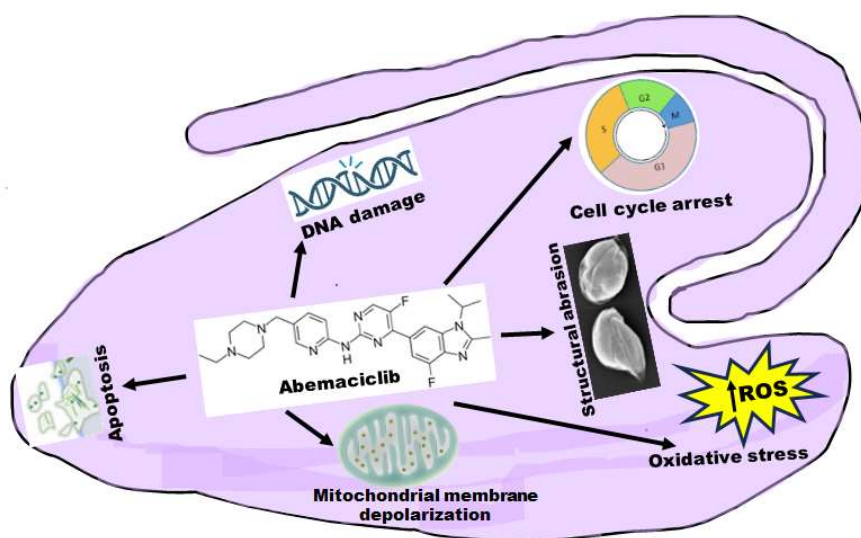


Figure 6: Mode of action of Abemaciclib on *Leishmania donovani*.

These findings suggest that Abemaciclib and other selected compounds are promising therapeutic agents for leishmaniasis, warranting further development and clinical evaluation.