Chapter 4

Evaluation of selected compound as a potential inhibitor of citrate synthase: an alternative chemotherapeutic option against leishmaniasis

Abstract

The World Health Organization has targeted the eradication of the contagious parasitic disease, leishmaniasis. However, millions of individuals from impoverished nations continue to suffer from this ailment, highlighting the urgent need for alternative solutions. In this context, we have identified citrate synthase as a potential drug target, given its direct and indirect involvement in parasite growth, survival, and defense against host machinery. Employing a drug repurposing approach, we conducted *in-silico* studies and selected the top four hits: Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib against *Leishmania donovani* Citrate Synthase (LdCS). These compounds exhibited strong binding affinity with LdCS in intrinsic fluorescence studies. Further enzyme inhibition studies revealed that LdCS follows a bi-bi-ordered catalytic mechanism, with competitive inhibition towards acetyl-CoA and uncompetitive inhibition towards oxaloacetic acid, exhibiting inhibition constant (Ki) values ranging from 2 to 3 μM. Additionally, in antileishmanial and cytotoxicity assessments, three compounds—Abemacilcib, Bazedoxifene, and Vorapaxar—demonstrated effective anti-leishmanial activities against both *L.donovani* promastigote and intra-macrophagic amastigote, without significantly harming macrophage cells. Overall, this study underscores the potential of these FDA-approved inhibitors as pivotal components in combating leishmaniasis.

Keywords: Leishmaniasis, Citrate synthase, Michalis-Menten, FDA-approved compound, Inhibitor, Antileishmanial drug.

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4.1 Introduction

Leishmaniasis stands among neglected tropical diseases, characterized by alarmingly high mortality and morbidity rates. Particularly prevalent in economically disadvantaged regions where populations lack influence over governmental decisions and have limited access to healthcare services. Moreover, the pharmaceutical industry's reluctance to invest in neglected diseases due to minimal commercial returns exacerbates the challenge of developing effective treatments (Ungogo, 2020). Compounding this issue is the absence of a vaccine against leishmaniasis, leaving chemotherapy as the sole treatment option. However, current chemotherapeutic options have significant drawbacks and side effects, impeding efforts to eradicate this disease (Ungogo, 2020). Given these challenges, exploring alternative treatment avenues through drug repurposing has become imperative. In this context, several FDA-approved chemotherapeutic agents, including Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib, were identified through in-silico studies. These compounds were selected based on Citrate synthase as a drug target, given its role in leishmania's carbon metabolism and contribution to their growth, survival, and defense mechanisms against host machinery (Saunders, 2010).

Abemacilcib is reported as an anticancer drug and used against a type of breast cancer cells that are hormone receptor-positive (HR^+) and human epidermal growth factor receptor negative ($HER2^-$) through inhibiting protein cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) [Corona and Generali, 2018]. Bazedoxifene is a selective estrogen receptor modulator (SERM) used to manage osteoporosis and vasomotor symptoms in postmenopausal women. This drug acts like an agonist and ensures proper regulation of bone turnover by stimulating lipid metabolism [Raina and Parmar, 2024]. It also acts as an antimalarial drug, inhibiting Hemozoin formation [Sudhakar et al., 2022]. Vorapaxar is a tricyclic himbacine-derived selective inhibitor of protease-activated receptor -1 (PAR-1) that inhibits thrombin-induced

platelet activation [Tantry et al., 2020]. Imatinib is a 2-phenylamino-pyrimidine-derived protein used to treat chronic myelogenous leukemia, gastrointestinal stromal tumors, and other malignancies. It is a tyrosine-kinase inhibitors that inhibit protein tyrosine kinases such as c-kit (gastrointestinal stromal tumors) and BCR-ABL fusion protein (Philadelphia chromosome chronic myelogenous leukemia) [Flynn and Gerriets, 2024]. As this drug is a tyrosine kinase inhibitor, it is also used as an antileishmanial drug because protein kinase is an essential enzyme in the leishmania genus. It stops the abnormal function of TK, such as BCR-ABL, through assembling into transmembrane pores in a sterol-dependent manner [Moslehi et al., 2019];[Moslehi et al., 2020]. It is also evaluated as an anti-*Trypanosoma cruzi* drug candidate [Nesic De Freitas et al., 2023]. Other than Imatinib, no reports have been found related to the antileishmanial activities of these compounds.

In this study, we employed an *in-vitro* approach to target *Leishmania donovani* Citrate synthase (LdCS) and identified FDA-approved drugs such as Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib as LdCS inhibitor, which may emerge as a new therapeutic option for the treatment of leishmaniasis.

4.2 Materials & Methods

4.2.1 Materials

M199 media, The Thiazolyl Blue Tetrazolium Bromide dye, were procured from Sigma-Aldrich. RPMI-1640 Media and Giemsa were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from Hi-media. All compounds were purchased from med chem express. All the other chemicals used in the experiments were of the highest grade.

4.2.2 *L. donovani* and J774 A.1 cell culture maintenance

L. donovani cultures AG83 (MHOM/IN/1983/AG83) were maintained in M199 media (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic at 25°C. The parasites consistently passaged every 3-4 days. J774A.1

cell line (murine macrophage cells) was cultivated in RPMI-1640 media (pH 7.4) with 10% FBS, 1% penicillin-streptomycin, and gentamicin antibiotic, at 37°C and 5% CO_2. The chemical stock was prepared in DMSO, followed by blank M199 media.

4.2.3 Intrinsic fluorescence measurement of selected compounds.

The purified LdCS (0.5 mg/ml) was mixed with increasing concentration (1.56 µM to 25 µM) of selected compounds at 25°C and incubated for half an hour, and then its change in fluorescence intensity was measured using a Cary Eclipse spectrofluorometer with excitation of 280 nm and emission of 300 to 500 nm. The relative fluorescence intensity was calculated using this formula **[(F0 – F)/F0]**, where F0 and F were the fluorescence intensities in the absence and presence of the selected compound, and the graph was plotted against the selected compound concentration and its quenching and binding constants were calculated using Stern-Volmer and modified Stern-Volmer plots respectively [Bhat and Qureshi, 2021].

4.2.4 Enzymatic assay and kinetics studies of LdCS

The standard mixture with a total volume of 200 µl, containing 0.25 mM oxaloacetic acid (OAA), 0.15 mM acetyl- CoA, 0.2 mM DTNB, and an appropriate volume of enzyme solution in 25 mM Tris-HCl buffer (pH 8.0) was used for the determination of citrate synthase activity which is based on the measurement of the amount of production of 5-thio-2-nitrobenzoate (TNB) that is formed as a result of the reaction between 5-5'-dithio-bis-2-nitrobenzoate (DTNB) and free -SH group of acetyl CoA and its absorbance was measured at 412 nm using a microtiter plate reader [Srere, 1966]. Using a linear regression analysis of raw data, the change in absorbance during the first 120 seconds was used to determine the initial reaction rate. The enzyme concentration utilized in the kinetic assay was determined by monitoring the reaction time needed for substrate depletion at different enzyme concentrations of 0.5 µM, 1 µM, and 2 µM in the presence of 0.25 mM OAA and 0.15 mM acetyl-CoA. The effect of pH on the activity of purified LdCS was examined using 50 mM of each buffer sodium acetate (pH

4-5), Potassium phosphate (pH 6-7), Tris-HCl (pH 8-9), and Glycine-NaOH (pH 10) under 0.5 μM LdCS enzyme and saturating concentration of OAA and acetyl-CoA. The optimal pH was ascertained by determining the residual activity, with the maximum value taken as 100, and then kinetic experiments were performed [Ortiz-Ramírez et al., 2022]. In this experiment, the apparent Km of OAA was established by varying the concentration from 0.39 to 50 μM at the saturating concentration of 0.1mM acetyl-CoA. Similarly, the apparent Km of Acetyl-CoA was ascertained by adjusting the concentration from 6.25 to 150 μM at the saturating concentration of OAA (0.25 mM). Using the non-linear regression method, which is accessible in Origin 2021, the Michalis-Menten kinetics constant (Km), the catalytic constant (Kcat), and the catalytic efficiency (Kcat/Km) of both substrates, OAA and acetyl-CoA, were determined. Each and every measurement was carried out in triplicate.

4.2.5 Inhibition Studies of LdCS

Based on their affinity for the LdCS catalytic site and free binding energy (-11 kcal/mol), the four compounds Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib were chosen from *in-silico* studies to assess their *in-vitro* inhibition of LdCS activity [Ranjan and Dubey, 2023b]. The enzyme activity was assessed with 100 μM of compounds, and its % inhibition was computed with respect to the untreated enzyme, and then their type of inhibition was computed at a varying concentration of compound (0 to 25 μM) by keeping one substrate at varying concentration, and the other at constant concentration, and then Ki was calculated using non-linear least squares regression analysis with origin 2021 [Teixeira et al., 2019].

4.2.6 Promastigote viability assay

To assess the antileishmanial capability of all selected compounds from *in silico* studies on the promastigote stage of *L. donovani*. The logarithmic phase of *L. donovani* promastigotes ($2*10^6$ parasites/ml) was grown in complete M199 media for 48 hours at 25°C with or without different concentrations of all selected compounds (0-100 μM), and MTT assay was performed

[Saudagar et al., 2013]. Miltefosine concentration displayed complete inhibition and was used as a positive control, while culture without drug treatment served as the negative control. The inhibition values were normalized against the positive control values and cell viability and IC_{50} were determined using the non-linear regression method of dose-response curve using Origin 2021 software.

4.2.7 Intra-macrophagic amastigotes viability assay

In order to evaluate the anti-amastigote efficacy, J774 A.1 cells ($2*10^5$/ml) were grown in a complete RPMI medium in 6-well culture plates. After 24 hours, Cells were treated with *L. donovani* in a 1:10 ratio for 12 hours at 37°C and 5% CO_2. After removing unbound parasites, infected cells were exposed to Abemaciclib (0.625 µM to 10 µM), Bazedoxifene (0.312 µM to 5 µM), Vorapaxar (2.5 µM to 40 µM) and Imatinib (5 µM to 40 µM) for 48 hours. Cells were washed twice or three times with 1x PBS after the incubation period, fixed with cooled methanol for five minutes, and then dried by air. Giemsa dye was applied to the cells for 15 minutes, after which again underwent two times 1x PBS washes. Using a 40x magnification of a light microscope, 100 infected macrophages were counted in a randomly selected region of the sample and compared with untreated infected cells, and IC_{50} was determined using a non-linear regression method of the dose-response curve using Origin 2021 software [Saudagar and Dubey, 2014] [Ilaghi et al., 2021].

4.2.8 Cell toxicity assay in J774A.1 macrophage

J774A.1 cells ($5*10^5$/ml) were grown in 96-well microplates with complete RPMI-1640 medium at 37 °C and 5% CO_2. The cells were exposed to various doses (0-100 µM) of chosen compounds after 24 hours and then incubated for 48 hours. Following incubation, an MTT assay was performed. The origin 2021 was used to create a dose-response curve using a non-linear regression technique to calculate the 50% cytotoxic concentration (CC_{50}). The CC_{50}/IC_{50} ratio value was used to determine the selectivity index (SI) [Saudagar and Dubey, 2014] .

4.2.9 Statistical analysis

Each quantitative experiment was carried out in triplicate with three different experiments. The data were presented as the mean of the triplicate ± SEM. The statistical analysis was done using Origin Pro 2021 software, and groups were compared using one-way ANOVA, followed by Tukey's test. We considered p values > 0.05 to be statistically significant.

4.3 Results

4.3.1 Analysis of the interaction of the selected compound with *L. donovani* citrate synthase by fluorescence spectroscopy

As tyrosine residues are involved in the active site region of LdCS, thus, to evaluate the binding affinity of the LdCS with selected compounds Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib, intrinsic fluorescence spectroscopy was used. The fluorescence spectra of LdCS with increasing concentrations of selected compounds are shown in Figure 4.1 (A, D, G, J). The findings showed that the LdCS protein exhibited a significant fluorescence emission peak at 333 nm, which decreased in a concentration-dependent manner following the binding of a selected compound. The decrease in LdCS fluorescence emission upon binding of selected compound might result in protein-inhibitor complex formation. Abemacilcib and Imatinib did not show a shift, whereas the compounds Bazedoxifene and Vorapaxar exhibited a red shift of the maximum emission spectra. The fluorescence redshift for Vorapaxar and Bazedoxifene showed that the tryptophan residues were more exposed to a polar environment. To assess the binding affinity of LdCS with the selected compounds Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib, intrinsic fluorescence spectroscopy was employed. The fluorescence spectra of LdCS with increasing concentrations of the selected compounds are presented in Figure 4.1 (A, D, G, J). The results revealed a notable fluorescence emission peak at 333 nm for the LdCS protein, which exhibited a concentration-dependent decrease upon binding of the selected compound. This decline in LdCS fluorescence emission suggests the formation of a protein-

inhibitor complex. Abemacilcib and Imatinib did not induce a spectral shift, whereas Bazedoxifene and Vorapaxar caused a red shift in the maximum emission spectra. The fluorescence redshift observed with Vorapaxar and Bazedoxifene indicates that the tryptophan residues became more exposed to a polar environment upon compound binding. Further, the Stern-Volmer equation was utilized to determine the quenching constant of the selected compounds. The plots of F0/F against [Q] for each compound with LdCS were depicted in Figure 4.1 (B, E, H, K). Notably, a strong linear correlation between F0/F and [Q] was observed. The quenching constants (Ksv) for Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib were determined as 2.58 X 10^4 M^{-1}, 2.32 X 10^4 M^{-1}, 1.60 X 10^4 M^{-1}, and 3.05 X 10^4 M^{-1}, respectively, derived from the slope of the curves as illustrated in Figure 5 (B, E, H, and K).

Additionally, the binding constant (Kb) and binding site (n) of the complexes were determined using a modified Stern-Volmer equation. The graph of log[(F0-F)/F] vs. log[Q] for each compound with LdCS was presented in Figure 4.1 (C, F, I, L). This graph exhibited a straight line with a slope equal to n and an intercept on the Y-axis equal to log Kb. The results indicated that all complexes had values of n nearly equal to 1, suggesting the presence of a single binding site. Moreover, compounds with high binding affinities typically possess binding constant values greater than 10^4 M^{-1}, while those with lower affinities have values less than 10^3 M^{-1} (Zarger, 2020). Here, the binding constant (Kb) values for Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib were found to be 2.28 X 10^4 M^{-1}, 2.87 X 10^3 M^{-1}, 5.66 X 10^3 M^{-1}, and 5.24 X 10^5 M^{-1}, respectively, as depicted in Figure 4.1 (C, F, I, L). These results suggest that the compounds bind to LdCS in the following order: Imatinib > Abemacilcib > Vorapaxar > Bazedoxifene. This validates both the high potency and robust binding interactions.

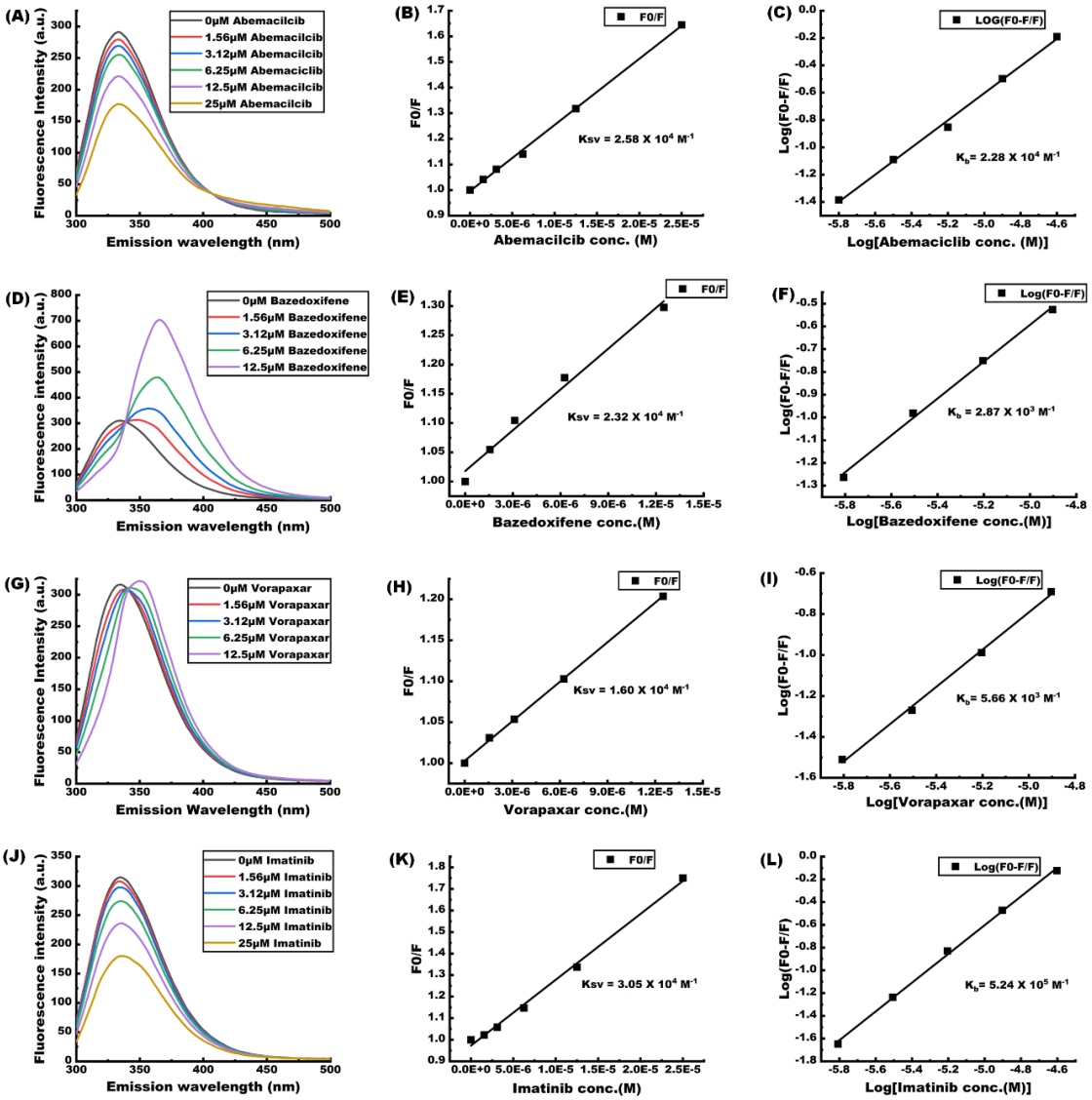

Figure 4.1: Binding association of selected compound with the LdCS. (A-L) Fluorescence spectra of the LdCS with potent inhibitors Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib along with their Stern-Volmer and modified Stern-Volmer plots showing binding association of these inhibitors with the LdCS.

4.3.2 Kinetic characterization of *L. donovani* citrate synthase activity

The citrate synthase enzymatic reaction was monitored by an increase in absorbance at 412nm. It displayed a linear profile that continued for 180 s after the reaction started when using 0.5 μM of the enzyme. This indicates that the assays reached a steady-state condition for at least the first 30 seconds.

Since the medium pH influences the protonation states of amino acids associated with catalysis and/or intermolecular interaction, it was essential to assess the effect of pH on the catalytic activity to select the optimal assay conditions for LdCS kinetic characterization and inhibition. The pH range of 4 to 10 affects the catalytic activity of the LdCS. Of the pH ranges assessed, the maximum catalytic activity was observed at pH 7.0. After the reaction conditions were standardized, we moved towards the kinetic characterization of the LdCS, as characterization of kinetic parameters is employed for the determination of Inhibitors' efficacy and mechanism. The substrate concentration near to Km apparent in the assay required for the identification of various inhibitor modalities, such as competitive, non-competitive, and uncompetitive [Teixeira et al., 2019]. The Km and Vmax values of acetyl-CoA were found to be 57.03 ± 0.94 μM and 0.48 ± 0.13 μM/min, respectively, while those for oxaloacetic acid were 1.14 ± 0.07 μM and 0.37 ± 0.01 μM/min respectively as depicted in Table 4.1. This demonstrated that the LdCS had a lower affinity for acetyl-CoA as compared to oxaloacetic acid. Since the acetyl-CoA affinity value is lower than the oxaloacetic acid, this characteristic can be advantageous for developing inhibitors intended to bind in the acetyl-CoA site and compete with acetyl-CoA. This is because a minor inhibitor concentration would be needed to extract the acetyl-CoA from the enzyme site, as shown by the acetyl-CoA's decreased affinity. The Kcat values of both substrates, i.e., acetyl-CoA and oxaloacetic acid, were found to be 0.97 ± 0.26 s^{-1} and 0.75 ± 0.02 s^{-1} while their catalytic efficiencies were determined to be 0.016 ± 0.004 and 0.66 ± 0.01$\mu M^{-1}s^{-1}$ respectively. Since oxaloacetic acid has an excellent catalytic efficiency value compared to acetyl-CoA, LdCS can recognize oxaloacetic acid more effectively than acetyl-CoA.

4.3.3 Analysis of inhibition kinetics of *L. donovani* citrate synthase

To evaluate the mechanism of action of selected compounds Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib from *in- silico* studies into LdCS, its Km and Vmax values were

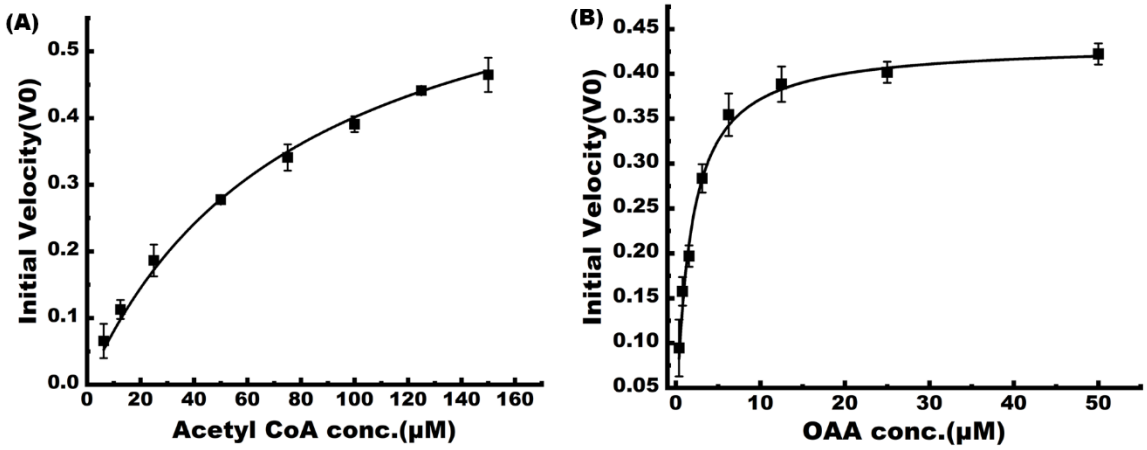

Figure 4.2: Kinetics studies of LdCS. (A, B) Determination of kinetics parameters of LdCS using various concentrations of substrate **(A)** Acetyl-CoA (12.5μM to 150μM) and **(B)** Oxaloacetic acid (3.12μM to 50μM).

Table 4.1: Kinetics Parameters of *Leishmania donovani* citrate synthase.

Parameters	Acetyl-CoA	Oxaloacetic acid
Km(μM)	57.03±0.94	1.14 ± 0.07
Vmax (μM/min)	0.48 ± 0.13	0.37 ± 0.01
Kcat (s^{-1})	0.97 ± 0.26	0.75 ± 0.02
Catalytic efficiency (Kcat/Km) ($\mu M^{-1} s^{-1}$)	0.016 ± 0.004	0.66 ± 0.01

calculated under various inhibitor concentrations using the Lineweaver-Burk plot. The effect of the selected compound under the saturating concentration of oxaloacetic acid and varying concentration of acetyl-CoA is represented by a set of lines that cross the X-axis (-1/Kmax) at

various places and intercept the Y-axis at the same point (1/Vmax) as shown in Figure 4.3 (A, C, E, G). This action points to the competitive method of inhibition, in which binding to the protein's active site involves competition with the substrate. The inhibition constant (Ki) of Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib was found to be 2.90 ± 0.49, 3.25 ± 0.45, 2.78 ± 0.06 and 2.98 ± 0.04, respectively as shown in Table 4.2. The enzyme LdCS follows a bi-bi-ordered catalytic mechanism, which means it will also show an uncompetitive method of inhibition with varying concentrations of other substrates. In order to verify this theory, enzyme inhibition studies were conducted at saturating concentrations of acetyl-CoA with varying concentrations of oxaloacetic acid. As predicted, the value of both Km and Vmax decreases proportionally with increasing concentration of the selected compound. The reciprocal double graph displays parallel lines, as shown in Figure 4.4 (A, C, E, G), a characteristic of uncompetitive inhibition. The Inhibition constant (Ki) of Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib with respect to oxaloacetic acid were found to be 2.11 ± 0.09, 2.17 ± 0.30, 3.61 ± 0.48 and 2.15 ± 0.43 respectively as shown in Table 4.2.

4.3.4 Effect of the Selected compound on promastigote viability

Except for Amyral, namely Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib, all compounds notably suppressed the viability of promastigote parasites in a dose-dependent manner compared to untreated parasite control, as depicted in Figure 4.5. Amyral did not exhibit a significant inhibitory effect on promastigote cells, with an IC_{50} value exceeding 100 µM. Conversely, the other compounds demonstrated substantial inhibition at lower concentrations. Abemaciclib and Bazedoxifene achieved over 90 % inhibition at 3 µM, while Vorapaxar achieved this at 10 µM, and Imatinib and Miltefosine (standard drug) achieved over 90 % inhibition at 50 µM each. A dose-response curve was generated plotting percent viability against compound concentration, yielding IC_{50} values of 0.92 ± 0.02 µM for Abemaciclib, 0.65 ± 0.09 µM for Bazedoxifene, 6.1 ± 0.91 µM for Vorapaxar, 23.9 ± 1.27 µM for Imatinib,

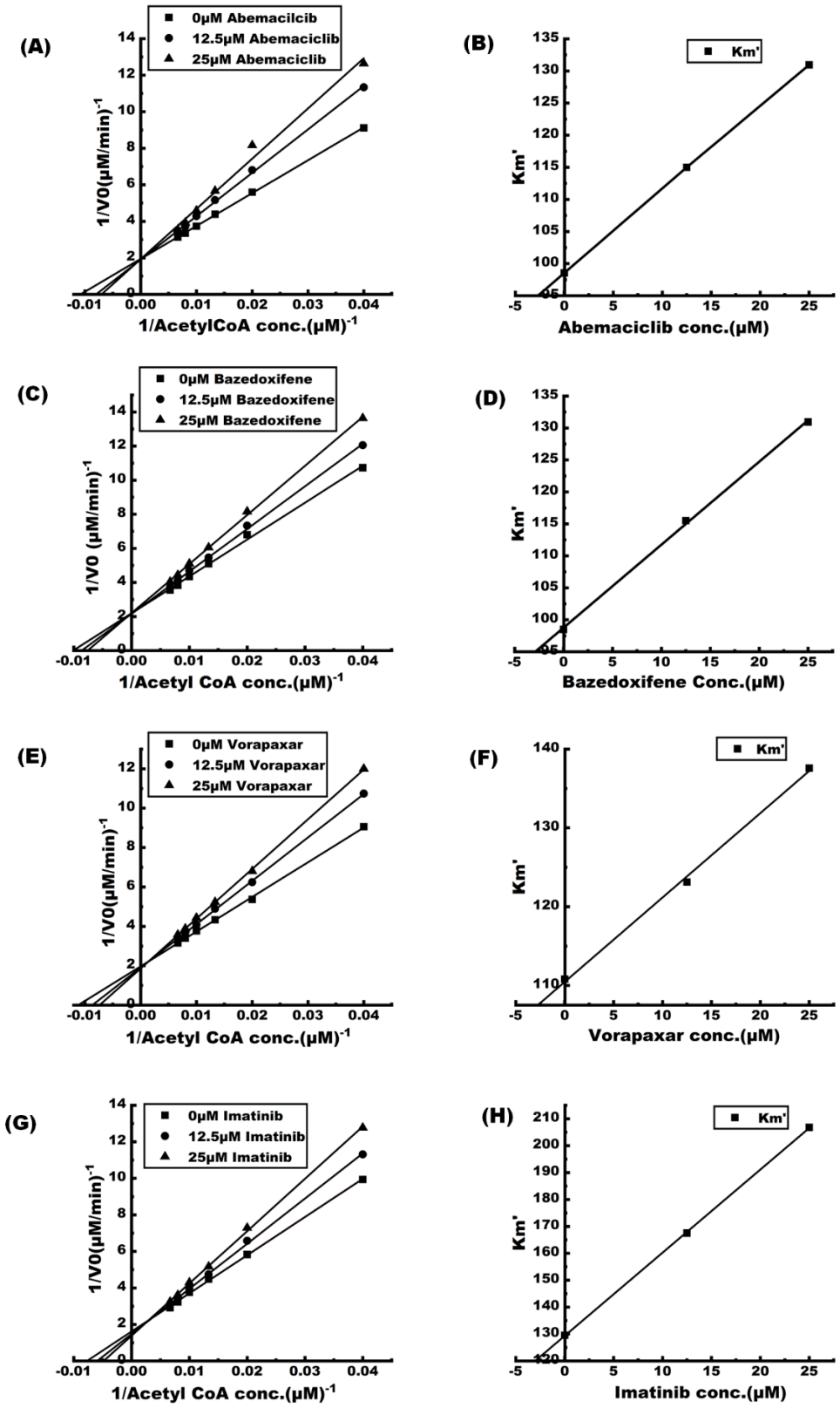

Figure 4.3: Inhibition studies with selected compound. (A, C, E & G) represent the double reciprocal plot demonstrating the competitive inhibition of the LdCS w.r.t to Acetyl CoA in the presence of selected compounds. **(B, D, F, & H)** represent the secondary plot with the selected compound for the calculation of the inhibition constant (ki).

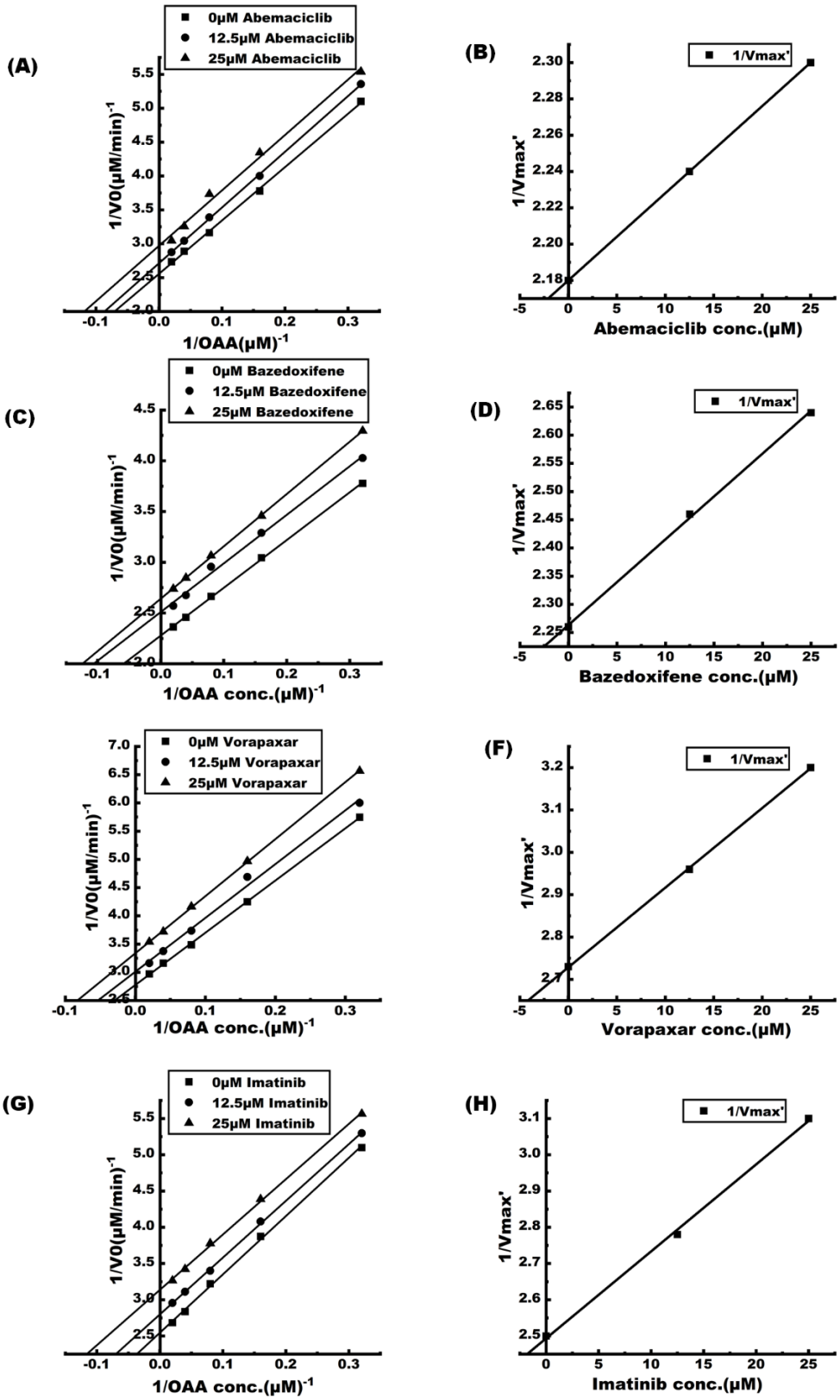

Figure 4.4: Inhibition studies with selected compound. (A, C, E & G) represent the double reciprocal plot demonstrating the Uncompetitive inhibition of the LdCS w.r.t to oxaloacetic acid in the presence of selected compounds. **(B, D, F, & H)** represent the secondary plot with the selected compound for the calculation of the inhibition constant (ki).

Table 4.2: Kinetic studies of inhibitor with LdCS in the presence of substrate Acetyl-CoA and Oxaloacetic acid by keeping one substrate constant at a time.

Inhibitor	With respect to Acetyl CoA		With respect to Oxaloacetic acid	
	Type of Inhibition	Inhibition Constant(ki)	Type of Inhibition	Inhibition Constant (ki)
Abemacilcib	Competitive	2.90 ± 0.49	Uncompetitive	2.11 ± 0.09
Bazedoxifene	Competitive	3.25 ± 0.45	Uncompetitive	2.17 ± 0.30
Vorapaxar	Competitive	2.78 ± 0.06	Uncompetitive	3.61 ± 0.48
Imatinib	Competitive	2.98 ± 0.04	Uncompetitive	2.15 ± 0.43

and 13.6 μM for Miltefosine, as indicated in Table 4.3 [Antwi et al., 2019] [Ali et al., 2021].

4.3.5 Effect of Selected Compounds on intra-macrophagic amastigote viability

A marker of pathogenesis involves the transformation of flagellated promastigotes into nonmotile aflagellated amastigotes within macrophage parasitophorous vacuoles and their subsequent survival [Islamuddin et al., 2022]. Therefore, evaluating the impact of selected compounds on intramacrophage-resident amastigotes is crucial. Treatment of amastigotes with drugs such as Abemaciclib, Vorapaxar, Bazedoxifene, and Miltefosine resulted in a dose-dependent reduction in their burden within macrophages, as illustrated in Figure 4.6. The EC_{50} was determined using the dose-response curve plotted between the percentage of infection and drug concentration [Sousa et al., 2023].

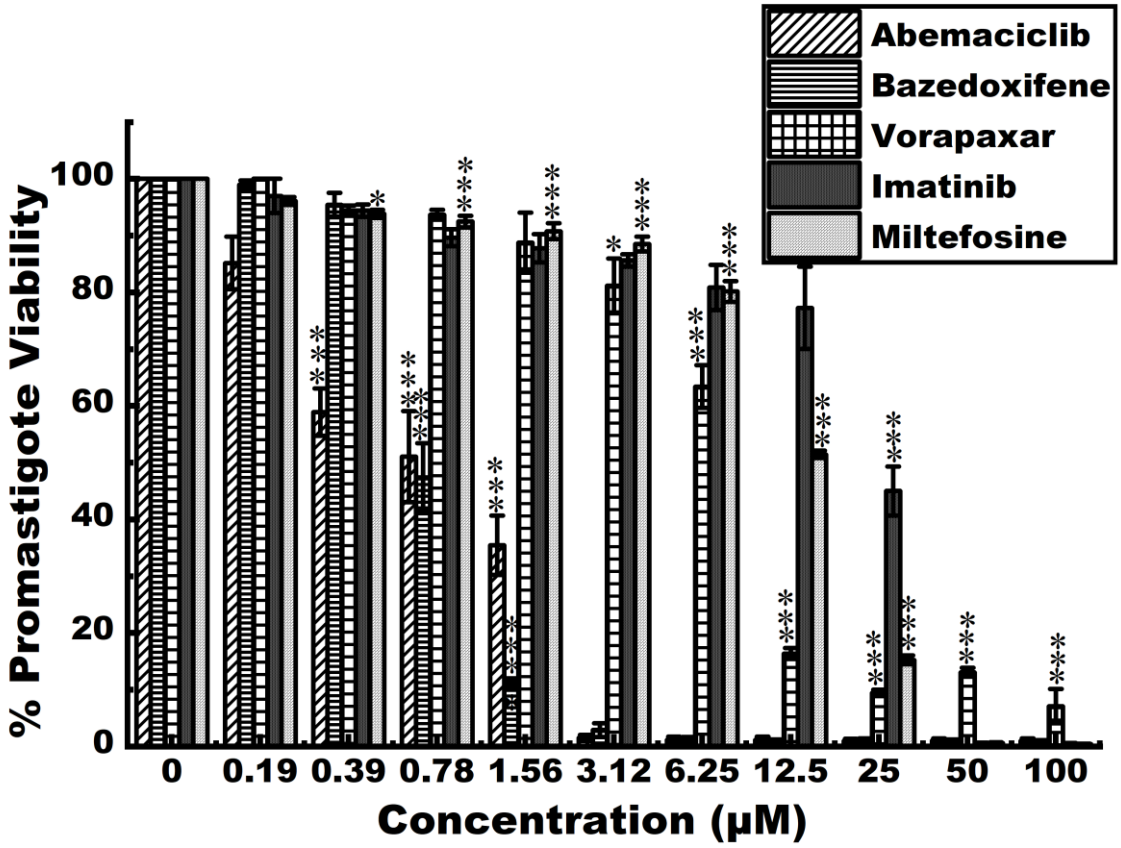

Figure 4.5: The dose-dependent antileishmanial effect of selected compounds such as Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib on the promastigote stage. The value represents the mean ± SEM of three independent experiments performed in triplicate. Significant difference compared with zero concentration (control) * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$).

For Abemaciclib, Bazedoxifene, Vorapaxar, and Miltefosine, the EC_{50} values were found to be 1.52 ± 0.37 µM, 2.11 ± 0.38 µM, 10.4 ± 1.27 µM, and 13.4 ± 0.53 µM, respectively. However, determining the EC_{50} for Imatinib was not feasible as macrophage cells were impacted by increasing concentrations of the compound, as shown in Table 4.3.

4.3.6 Cytotoxic effect of the selected compound on J774A.1 cell line.

In this study, vorapaxar demonstrated no adverse effects on macrophage viability up to 500 µM. The cytotoxic effects of all compounds on macrophages were depicted in Figure 4.7 (A), with increased concentrations of Vorapaxar and Miltefosine shown in Figure 4.7 (B). Abemaciclib, Imatinib, Bazedoxifene, and Miltefosine exhibited less cytotoxic impact on macrophage morphology and viability, with CC_{50} values of 83.35 ± 0.77 µM, 43.7 ± 0.42 µM,

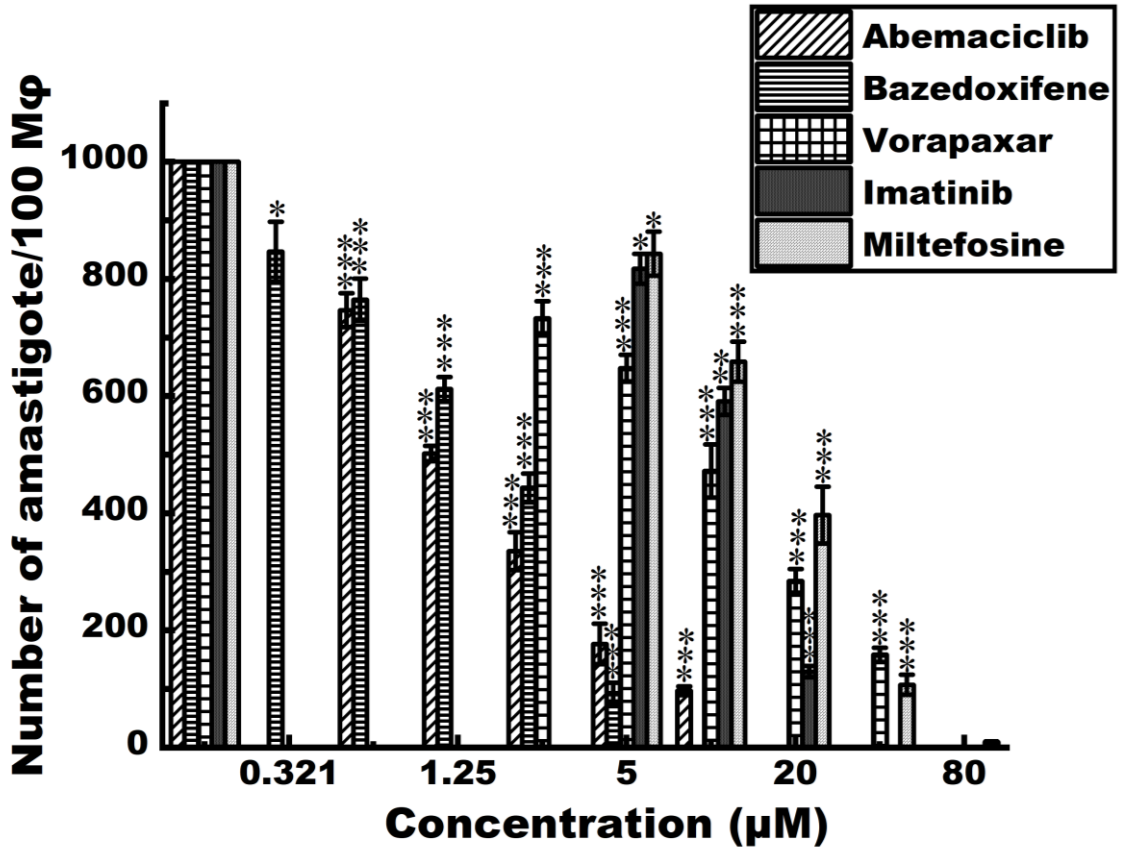

Figure 4.6: The dose-dependent antileishmanial effect of selected compounds such as Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib on Intra-macrophagic amastigote stage of *Leishmania donovani*. The value represents the mean ± SEM of three independent experiments performed in triplicate. Significant difference compared with zero concentration (control) * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$).

11.8 ± 0.56 µM and 153.6 µM, respectively, as detailed in Table 4.3. The selectivity index (SI), calculated as the ratio of macrophage CC_{50} to parasite IC_{50}, was employed to compare macrophage toxicity with parasite activity against *L. donovani*. Since Vorapaxar displayed no toxicity up to 500 µM, its CC_{50} was not computed. As shown in Table 4.3, the SI of Abemaciclib for promastigotes was 90.50, while for amastigotes, it was 54.83. For Vorapaxar, the SI for promastigotes was >81.97, and for amastigotes, it was >48.08. Bazedoxifene and Miltefosine exhibited SI values of 18.15 and 5.59 for promastigotes and 11.29 and 11.72 for amastigotes, respectively. However, Imatinib demonstrated an SI value of 1.82 for promastigotes, and due to its toxicity to cells, an SI for amastigotes was not determined. These findings indicate that Abemaciclib and Vorapaxar selectively target parasites without adverse

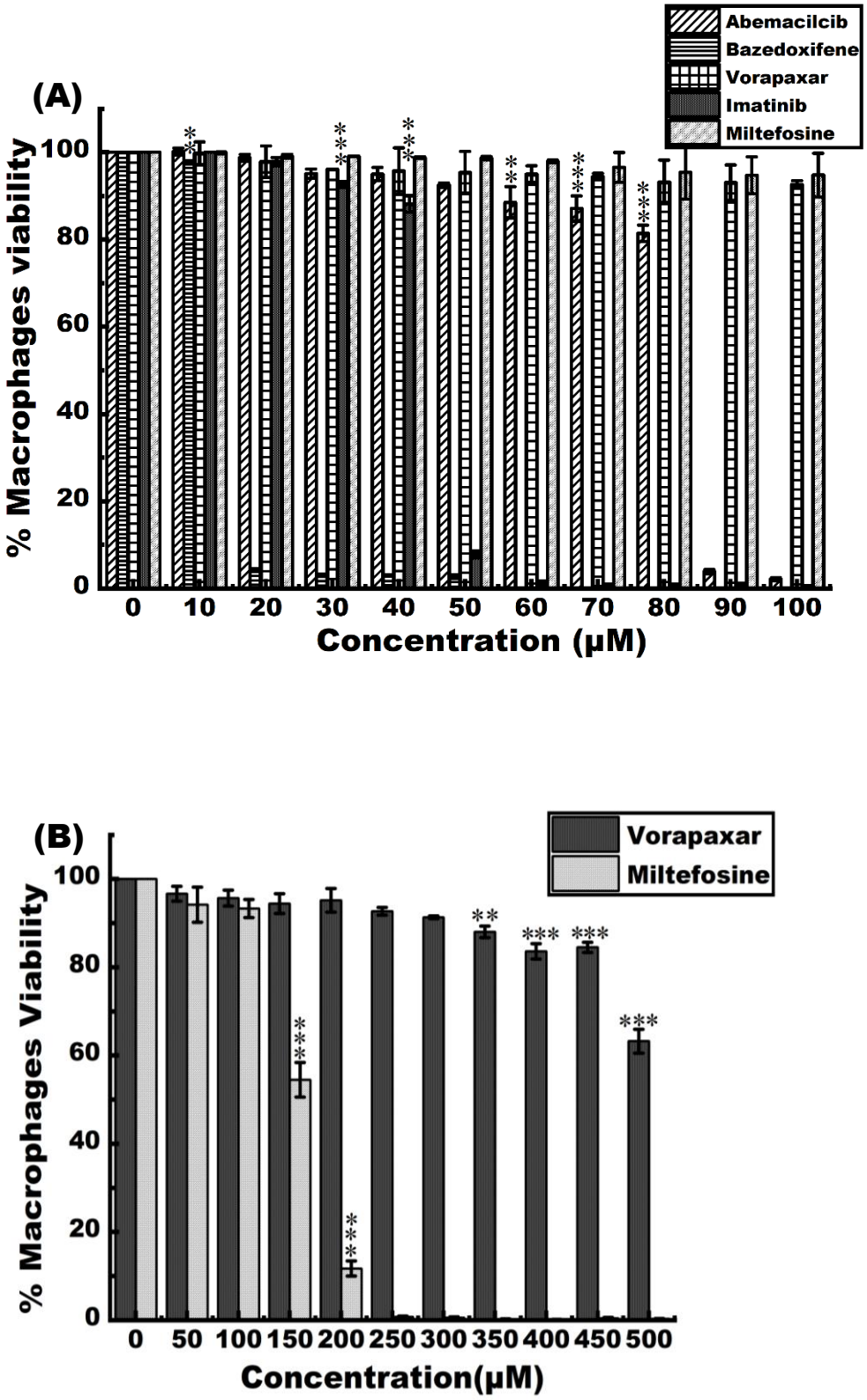

Figure 4.7: The Cytotoxic effect of selected compounds on murine macrophages J774 A.1. (A) The dose-dependent Cytotoxic effect of selected compounds such as Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib up to 100 µM on murine macrophage cell lines J774A.1 and compared with a Miltefosine (Standard drug). B) The Cytotoxic effect of Vorapaxar up to 500 µM and compared with Miltefosine (Standard drug). The value represents the mean ± SEM of three independent experiments performed in triplicate. Significant difference compared with zero concentration (control) * ($p < 0.01$), ** ($p < 0.001$), *** ($p < 0.0001$).

effects on macrophages. Bazedoxifene also shows promise. Generally, lead compounds with SI values above 10 were considered promising [Tabrez et al., 2021].

Table 4.3: Antileishmanial activity and selectivity index of Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib on promastigote & Intra-macrophagic amastigotes as well as their Cytotoxicity effect on the murine macrophages cell line J774A.1.

Compounds	Cytotoxicity against J774A.1 Cell lines CC_{50} (µM)	Antileishmanial activity against *Leishmania donovani*			
		Promastigotes		Intracellular amastigotes	
		IC_{50} (µM)	Selectivity index (SI)	EC_{50} (µM)	Selectivity index (SI)
Abemaciclib	83.35 ± 0.77	0.92 ± 0.02	90.59	1.52 ± 0.37	54.83
Bazedoxifene	11.8 ± 0.56	0.65 ± 0.09	18.15	2.11 ± 0.38	5.59
Vorapaxar	>500	6.1 ± 0.91	>81.97	10.4 ± 1.27	>48.08
Imatinib	43.7 ± 0.42	23.9 ± 1.27	1.82	ND	ND
Miltefosine	153.6	13.6	11.29	13.4 ± 0.53	11.72

4.4 Discussion

To assess the potential of LdCS as a drug target and identify specific inhibitors to overcome the challenge of current antileishmanial drug resistance, the four compounds: Abemacilcib, Bazedoxifene, Vorapaxar, and Imatinib were selected from *in-silico* studies, and its binding affinities were evaluated through intrinsic fluorescence spectroscopy as tyrosine residues were present in the active site of LdCS. Enzyme activity was also evaluated based on the formation of a yellow TNB complex at 412 nm, indicating the amount of coenzyme A reacting with DTNB. Kinetic characterization studies were conducted to screen inhibitors and determine

their modalities. The Km and Vmax values for acetyl-CoA were 57.03±0.94 μM and 0.48 ± 0.13 μM/min, respectively, while for oxaloacetic acid, they were 1.14 ± 0.07 μM and 0.37 ± 0.01 μM/min, suggesting lower affinity towards acetyl-CoA. Similar features of citrate synthase have been reported in other organisms. To assess LdCS's potential as a drug target, the selected top hits from *in-silico* studies—Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib were used to examine their inhibition properties against LdCS. These compounds exhibited competitive inhibition towards acetyl-CoA and uncompetitive inhibition towards oxaloacetic acid, with Ki values in the range of 2 to 3 μM. Further validation was performed through antileishmanial and cytotoxicity studies against promastigote and amastigote forms of Leishmania and murine macrophages J774. MTT assays showed significant dose-dependent inhibition of promastigote viability, with IC_{50} values of 0.92 ± 0.02 μM, 0.65 ± 0.09 μM, 6.1 ± 0.91 μM, and 23.9 ± 1.27 μM for Abemaciclib, Bazedoxifene, Vorapaxar, and Imatinib, respectively. Parasitic burden within macrophages was also reduced in a dose-dependent manner, with EC_{50} values of 1.52 ± 0.37 μM, 2.11 ± 0.38 μM, and 10.4 ± 1.27 μM, respectively. These values were lower than those of Miltefosine (13.4 ± 0.53 μM). Vorapaxar showed no cytotoxic effects on macrophage viability up to 500 μM. Furthermore, Abemaciclib, Imatinib, and Bazedoxifene exhibited less cytotoxicity to macrophages (CC_{50} values: 83.35 ± 0.77 μM, 43.7 ± 0.42 μM, and 11.8 ± 0.56 μM, respectively) compared to Miltefosine (CC_{50} values: 153.6 μM). The selectivity index of Abemaciclib, Bazedoxifene, and Vorapaxar was superior to Miltefosine. These compounds demonstrated significant inhibition against LdCS and selectivity towards the parasite without detrimental effects on macrophages. *In-vivo* assays are necessary to validate the potency of these compounds as potential drug candidates against leishmaniasis.

4.5 Conclusion

In this investigation, enzyme kinetics studies were performed to elucidate its functional role.

Interestingly, in kinetic analyses, LdCS displayed a lower affinity for acetyl-CoA compared to oxaloacetic acid. However, it exhibited a notable binding affinity towards inhibitors—Imatinib, Bazedoxifene, Vorapaxar, and Abemacilcib—identified through *in-silico* research, as demonstrated in fluorescence studies. Moreover, these inhibitors displayed a competitive mode of inhibition towards acetyl-CoA, with inhibition constants falling within the range of 2 to 3 μM.

Remarkably, three of these selected compounds—Abemacilcib, Bazedoxifene, and Vorapaxar—effectively hindered the growth and proliferation of *L. donovani* promastigotes in a dose-dependent manner. Furthermore, they demonstrated the ability to reduce the intracellular parasitic load within infected macrophages without causing harm to the host macrophages. Collectively, our findings highlight the potential of FDA-approved compounds as inhibitors of LdCS, suggesting their candidacy for therapeutic interventions against leishmaniasis in the future.