Miltefosine or Antimonials: An *in vitro* Evaluation of Sensitivity against *Leishmania tropica*

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Abstract

Antimony containing agents, meglumine antimoniate and sodium stibogluconate, are the first line of chemotherapy against cutaneous leishmaniasis in Pakistan. A comparison of the efficacy of these antimonials and miltefosine, the only oral therapy for leishmanisis was assessed in vitro. Eight different isolates of L. tropica were exposed to 4 different concentrations of the drugs for 48 hours under the standard conditions for L. tropica cultivation. At the end of exposure time, viability of the parasites was checked through XTT colorimetric assay. The efficacy of miltefosine stood matchless as compared to pentostam and glucantime; standard antimonial drugs. The average activity of miltefosine differed Significantly than the other standard drugs (miltefosine vs Glucantime p=0.003; miltefosine vs pentostam p=0.0045; glucantime vs pentostam p=0.006). The mean IC_{50} values of mltefosine, glucantime and pentostam were respectively 1.476 ± 1.088µg/mL, 6.746 ± 2.438µg/mL and 85.970 ± 82.201µg/mL. Significant differences were noted in IC_{50} of the drugs (miltefosine vs glucantime p=0.0003; miltefosine vs pentostam p=0.0242; glucantime vs pentostam p=0.0501). This study showed miltefosine as a more suitable option for treating L. tropica. This might be of practical importance in areas with higher prevalence of resistance to antimonials.

Keywords: Miltefosine, Antmonials, *in vitro* Sensitivity, *Leishmania tropica*.

Introduction

Leishmaniasis is a vector-borne disease caused by protozoan parasites belonging to the genus *Leishmania* (Cecílio et al., 2022). Humans contract the disease when bitten by the female *Phelobotomus* or *Lutzomyia* sand flies infected with the parasite (Torres-Guerrero et al., 2017). The disease manifests in three main clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Mann et al., 2021). Out of these cutaneous leishmaniasis usually caused by *Leishmania*

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tropica in endemic regions is of significant concern to public health. The skin lesions caused by cutaneous leishmaniasis can leave permanent scars and may cause lasting effects on psychological, psychosocial and physical well-being of the patients, specifically in areas where there is limited access to healthcare facilities (Bilgic-Temel et al., 2019). Leishmaniasis causes potential risk to about 350 million people across 88 tropical, subtropical and temperate countries of the world. Every year, the disease leads to 1.5-2 million new cases and about 70,000 deaths attributed to VL worldwide (Torres-Guerrero et al., 2017). Leishmaniasis is cosmopolitan in distribution spanning from the deserts of West Asia to the central and South American rainforests. More than 90% of all VL cases that occur globally are reported from Sudan, Bangladesh, Nepal, Brazil and India (CDC, 2024). Substandard housing conditions, lack of sanitation, open sewage systems, improper waste disposal and poverty are the determinant factors for leishmaniasis (WHO, 2023). The disease is more frequent in rural than in urban settings and transmission typically occurs through sandflies which are most active during early morning and at nighttime (CDC, 2024).

The antimony containing agents including megulmine antimoniate and sodium stibogluconate that cause senile changes in *Leishmania* have been in use for almost 6-7 decades (Kumar et al., 2018; Buchelt et al., 2025). Nevertheless, various drawbacks like lengthy therapeutic regimens, cytotoxicity, differential cure rates, and acquisition of resistance have compromised their efficacy, therefore necessitate the development of new alternatives (Alcântara et al., 2018). In recent years several alternative chemotherapeutic agents have been implied in the treatment of various forms of leishmaniasis. Miltefosine is one of such candidates and have been the one and only treatment given through oral route. This drug has shown very good results in treating several different species of *Leishmania*. Miltefosine induces changes in the lipid metabolism and consequently leads to mitochondrial and membrane damages. Varied and species-specific activity of the drug, however, restrict the use of the drug (Khan et al., 2024; Masne et al., 2024). Although investigations done in vitro do not consert with the ones done in vivo but they still bring forth valuable insights about the effects of a drug on a parasite (Gupta and Nishi, 2011; Bhushal et al., 2025).

The present endeavored aimed at assessing the anti-leishmanial potential of miltefosine against the flagellate forms of *L.tropica* in comparison with the antimonial drugs. The outcome of this study may have substantial impacts on lessening adverse effects, improving patient's wellbeing and combating issues of drug resistance in leishmaniasis treatment.

Methodology

Ethical Approval

The study was endorsed by the ethical committee of the institution vide letter no. (213/EC-FLES-UOP/2023).

Parasite Isolation and Characterization

In this study the strains of *Leishmania tropica* used were acquired from patients in Khyber Pakhtunkhwa province, Pakistan attending district headquarter hospital (DHQ), Jamrud (District Khyber), and Kuwait teaching hospital, Peshawar. The species was characterized by using k-DNA and ITS-1 PCR.

Compounds

The compounds tested in the present work were Miltefosine; hexadecyl phosphocholine (Profounda, USA), Glucantime; meglumine antimoniate (Sanofi-Aventis), and Pentostam; sodium stibogluconate (GlaxoSmithKline, UK). Stock solutions were prepared from the crystalline solid form of the drugs and stored at -20ºC.

Drug Exposure of Promastigotes

For drug sensitivity assays the parasites were cultivated in fresh RPMI-1640 (Sigma-Aldrich, R8758) culture medium added with 10% FBS (Sigma-Aldrich, F9665) and 100µg/mL of each streptomycin and penicillin in 25mL culture flask (Thermo scientific, 156340) at $1x10^6$ cell/mL initial density. Cultures were maintained at 24°C–26°C in cooled incubator. Promastigotes in the late log to stationary phase were counted using a Neubauer hemocytometer and viability was assessed by trypan blue exclusion. Only cultures with viability equal to or exceeding 98% were used for drug sensitivity assays.

Prior to drug application, promastigotes were seeded into 96-well flat-bottom culture plates at a density of 1×10^6 cells/well. Using serial dilution, four concentrations of each test drug (miltefosine = 407.568µg/mL, 40.7568µg/mL,4.07568µg/mL and 0.407568µg/mL; glucantime=365.98µg/mL, 36.598µg/mL, 3.6598µg/mL and 0.36598µg/mL; pentostam= 910.9µg/mL, 91.09µg/mL, 9.109µg/mL and 0.9109µg/mL) were added in triplicate for each concentration. Negative control wells contained only growth medium and *L. tropica* promastigotes without any drug. The plates were incubated at 26°C for 48 hours.

Post Exposure XTT Viability Assay

After 48 hours of continuous exposure to the drugs, relative viability of the parasites was assessed through XTT colorimetric viability assay. Briefly, 20µL of 4 mg/mL XTT was added to each test and control wells. The plate was incubated for 6 hours at 26°C, and optical density was measured at 490 nm and 630 nm using a Biotek ELx-800 ELISA reader (Srisuton et al., 2019).

Data Analysis

The percentage inhibition of each replicated well was calculated using the standard formula:

$$\% \text{ Inhibition} = Ab_{control} - Ab_{test} / Ab_{control} \times 100$$

The IC_{50} values were calculated by non-linear regression using GraphPad prism, and the significance was determined using t-test.

Results

Inhibitory effects of the drugs against *Leishmania tropica* promastigotes were found to be strictly concentration dependent. A steep increase was observed in the leishmanicidal activity of the drugs with the increasing dose. The average percentage inhibitions of miltefosine at 407.568µg/mL, 40.7568µg/mL, 4.07568µg/mL and 0.407568µg/mL were respectively 68.22%, 62.70%, 51.01% and 16.34%. Strain specific variability in the inhibitory effects was, however, evident (Figure 1A). Glucantime gave next higher inhibition of the promastigotes. Its mean inhibitory activity at 365.98µg/mL, 36.598µg/mL, 3.6598µg/mL and 0.36598µg/mL was 45.51%, 39.43%, 19.38% and 5.76% respectively. Strain specific difference in the antileishmanial activity was there (Figure 1B). Pentostam revealed the lowest activity against *L. tropica* promastigotes. Its four different concentrations 910.9µg/mL, 91.09µg/mL, 9.109µg/mL and 0.9109µg/mL gave mean percentage inhibition values of 38.43%, 24.71%, 15.79% and 6.39% respectively. Again, the strain specific variations in the activity were evident (Figure 1C). Furthermore, when statistically compared with each other, the drugs showed significant difference in the efficacy against *L. tropica* promasigtes (miltefosine vs Glucantime p=0.003; miltefosine vs pentostm p=0.0045; glucantime vs pentostam p=0.006).

In a series for the eight different strains MHOM/PK/2023/Jam12, MHOM/PK/2023/Jam17,MHOM/PK/2023/Jam43,MHOM/PK/2023/Jam 46,MHOM/PK/2023/Jam54,MHOM/PK/2022/KWH1,MHOM/PK/2022/ KWH3, MHOM/PK/2022/KWH4 miltefosine gave IC_{50} of 3.15 µg/mL, 2.21 µg/mL, 2.815µg/mL, 0.754µg/mL, 0.521µg/mL, 0.887µg/mL, 0.361µg/mL, 1.109µg/mL, respectively. Mean IC_{50} of miltefosine was

1.476 ± 1.088µg/mL (Table 1). Similarly, the IC_{50} values of glucantime for the eight different strains in the preceding series were 6.548µg/mL, 10.16 µg/mL, 5.645µg/mL, 5.811µg/mL, 4.570µg/mL, 5.837µg/mL, 4.502µg/mL, 10.895µg/mL respectively with mean value being 6.746 ± 2.438µg/mL (Table 1). For pentostam, the IC_{50} values were 9.953µg/mL, 58.910µg/mL, 259.6µg/mL, 143.4µg/mL,27.5µg/mL, 25.7µg/mL, 70.2µg/mL and 92.5µg/mL, respectively, with mean value of 85.970 ± 82.201µg/mL (Table 1).

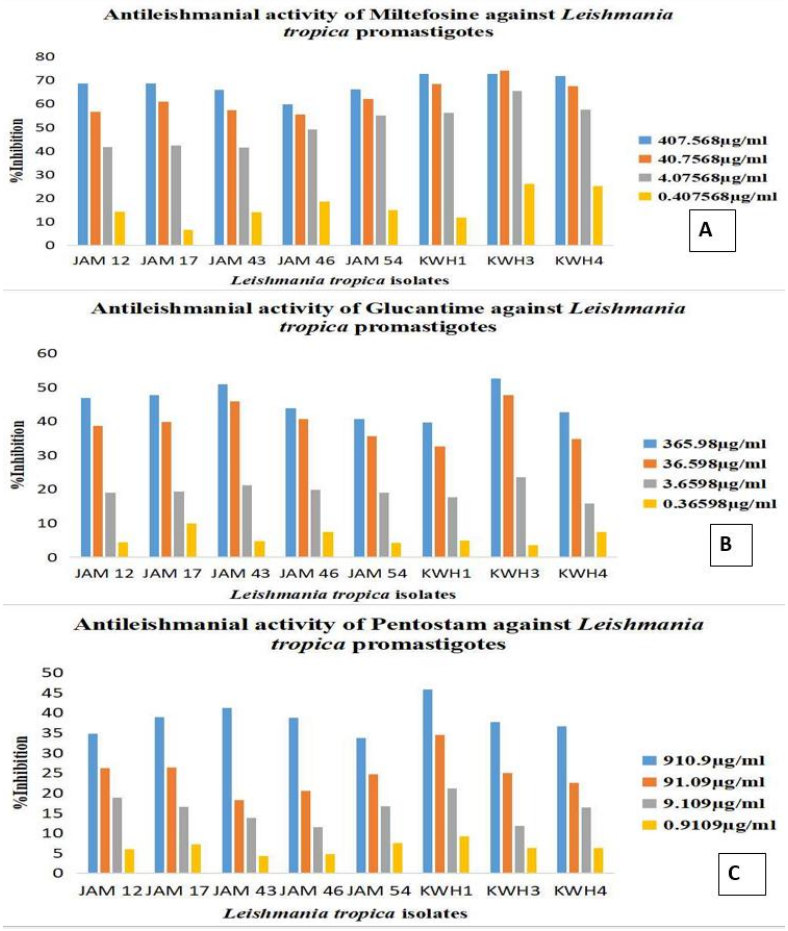

Figure 1: Dose-dependent inhibition responses of eight different strains at varying concentrations of Miltefosine, Glucantime, and Pentostam. Each bar represents the mean inhibition percentage at a given concentration. The graph highlights the variability in response among the strains across the tested concentrations.

When analyzed statistically, the IC_{50} values showed significant difference Miltefosine vs Glucantime p=0.0003; Miltefosine vs Pentostam p=0.0242; Glucantime vs Pentostam p=0.0501).

Table 1: IC_{50} values of Miltefosine, Glucantime, and pentostam against L. tropica Promastigotes.

		IC_{50} values		
S.No.	*L.tropica* strains	Miltefosine	Glucantime	Pentostam
1.	MHOM/PK/2023/Jam12	3.15 µg/mL	6.548µg/mL	9.953µg/mL
2.	MHOM/PK/2023/Jam17	2.21 µg/mL	10.16 µg/mL	58.910µg/mL
3.	MHOM/PK/2023/Jam43	2.815µg/mL	5.645µg/mL	259.6µg/mL
4.	MHOM/PK/2023/Jam46	0.754µg/mL	5.811µg/mL	143.4µg/mL
5.	MHOM/PK/2023/Jam54	0.521µg/mL	4.570µg/mL	27.5µg/mL
6.	MHOM/PK/2022/KWH1	0.887µg/mL	5.837µg/mL	25.7µg/mL
7.	MHOM/PK/2022/KWH3	0.361µg/mL	4.502µg/mL	70.2µg/mL
8.	MHOM/PK/2022/KWH4	1.109µg/mL	10.895µg/mL	92.5µg/mL
	Mean ±SD	1.476 ± 1.088µg/mL	6.746 ± 2.438µg/mL	85.970± 82.201µg/mL

Discussion

In vitro studies provide a platform for fast and preliminary sorting of drug sensitivity in both infectious disease and anticancer therapeutic development. Any candidate compound shall pass through *in vitro* assessment to make its way to any further trial. This study is one such effort to test the effectiveness of miltefosine against 08 different local strains and to compare it with standard antimonial therapy. Results of the present study (IC_{50} =0.361µg/mL to 3.15 µg/mL) suggested a consistent efficacy of miltefosine for treating cutaneous leishmaniasis and has been quite in line with the earlier findings with IC_{50} of 5.89µM and 23.7µM for isolates of *L. infantum* from different geographic areas in Brazi (Espada et al., 2021). Miltefosine has been investigated and found to cause apoptotic cell death of *L. major* and *L. tropica* with fifty percent inhibitory concentrations of 11µM and 22µM respectively (Khademvatan et al., 2011). Several previous studies have ascertained greater efficacy of miltefosine to the standard antimonial therapy. Studies on cutaneous *Leishmania* species in French Guiana has confirmed the IC_{50}value from 1.55 to 11.7µg/mL for miltefosine and 1,597 (< 937.5) ug/mL to 18,699 (> 30,000) µg/mL for meglumine antimoniate (Ginouvès et al., 2017). When compared, miltefosine and pentostam gave IC_{50} values of 25.72 µM and 17.07µM respectively against *L. tropica* promastigotes (Zghair, 2017). Antimonials being standard and first line of therapy against different species of *Leishmania* have been extensively investigated for their resistance in the parasite. Resistant strains usually present higher IC_{50} values in vitro. Sensitive and resistant strains of *L. tropica* has been previously found to exhibit much different IC_{50} values i.e, 52.2µg/mL and 170µg/mL (Mahmoudvand et al., 2017). Various studies that have

employed different extracts in relation to antimonials to check the efficacy against *Leishmania* promastigotes have determined different inhibitory concentrations i.e, 32.62 ± 0.66µg/mL (Madah et al., 2020) and 340µg/ml (Jafari et al., 2013).

As is evident, the effectiveness of glucantime antimonials against *L. tropica* varies greatly depending on the specific isolate, still they are essential therapeutic option, particularly in areas with well-established treatment procedures. Their inconsistent efficacy *in vitro*, however, raises the possibility that resistance and strain-specific variables could affect their effectiveness (Hadighi et al., 2006).

Compared to standard antimonials, miltefosine has proven to be significantly more effective against *Leishmania tropica* and typically exhibits broad-spectrum activity. This study reiterates the importance of reevaluating the existing therapeutic protocols especially in areas where resistance to antimonials is widely observed. Owing to its high potency, Miltefosine might be used as a primary treatment option or as a subcomponent of combination therapy to optimize treatment outcomes and to reduce the risk of resistance (Goswami et al., 2020). Erstwhile lab dish findings of miltefosine efficacy and medication through oral route make miltefosine a better choice over antimonials. A myriad of determinants affect the activity of a medication against a disease agent (Ware et al., 2021).

A myriad of determinants affect the activity of a medication against a disease agent. Miltefosine's potency over a range of *Leishmania* species and serotypes has made it the first choice of therapy against the disease (Sundar and Olliaro, 2007).

Conclusion

The present work validated miltefosine as preferable candidate over antimonials. The present results are enough in line with the requirement of the physicians and practitioners to cope with the treatment failures of antimonial therapy due to acquired resistance. However further studies are needed to verify its activity against intracellular amastigotes, determine its safety profile and evaluate its clinical applications for the treatment of cutaneous leishmaniasis.

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Conflict of interests

The authors confirm no conflicts of interest.

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