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Miltefosine Efficacy on *Leishmania Donovani* Promastigote

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Abstract

In the current study, different concentrations of miltefosine drug, which is the first effective and safe oral treatment for visceral leishmaniasis, was evaluated against *L. donovani* promastigotes in comparison with pentosam drug. Direct counting microscopic assay was used to find 50% inhibitory concentration (IC50) of miltefosine and pentostam against *L. donovani* promastigotes. The IC50 of miltefosine drug was 45.42µg/ml, 46.76µg/ml and 36.68µg/ml after 24 hr, 48hr and 72hr respectively, In comparison with IC 50 of pentostam drug was 75.39 µg/ml after 72hr. There were significant differences ($P<0.05$) between IC50 values of miltefosine and pentostam drugs from first day to third day.

Keywords: *Leishmania donovani*, miltefosine, pentostam, promastigote**.**

فاعلية الملتيفوسين على الطور امامي السوط للشمانيا الاحشائيه

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الخلاصه

تهدف هذه الدراسة الى تقييم التراكيز المختلفة من عقار الملتيفوسين (وهو اول عقار فموي يثبت كفائته وسلامته في معالجة مرض اللشمانيا الاحشائية) بالمقارنة مع عقار البنتوستام ضد الطور الامامي للسوط لطفيلي اللشمانيا الاحشائية.تم استخدام العد المجهري المباشر لايجاد قيمة التراكيز المثبطة لنصف عدد الطفيليات IC50 لكل من عقار الملتيفوسين والبنتوستام ضد الطور الامامي السوط لطفيلي اللشمانيا الاحشائية.بينت الدراسة بأن قيمة التراكيز المثبطة لنصف عدد الطفيليات كانت45,42مايكروغرام/مل 46,76مايكروغرام/مل 36,68مايكروغرام/مل في اليوم الاول والثاني والثالث على التوالي بالمقارنة مع عقار البنتوستام التي بلغت قيمة التراكيز المثبطة لنصف عدد الطفيليات في اليوم الثالث 75,39مايكروغرام/مل. كما اوضحت الدراسة بأن هناك فروق معنوية ($P<0.05$) بين قيمة التراكيز المثبطة لنصف عدد الطفيليات لكل من عقار الملتيفوسين والبنتوستام من اليوم الاول الى اليوم الثالث.

Introduction

Leishmaniasis is caused by protozoan parasites of over 20 *Leishmania* species and is transmitted to humans via the bite of infected female sand flies. Visceral leihmaniasis is the most severe clinical manifestation, it is fatal if untreated. According to a recent report from the World Health Organization, [1] there are an estimated 1.3 million new cases of leishmaniasis worldwide and an estimated 20,000 to 30,000 deaths caused by these parasites annually.

Pentavalent antimony has been considered the mainstay of therapy in leishmaniasis for decade several limitations have decreased the use of antimonials: the variable efficacy against CL and VL, as well as the emergence of significant resistance has been increased [2].Other alternative agents are

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utilized in different clinical situations and guided by availability and effectiveness in different localities [3].

Miltefosine was the first oral drug approved for use for the treatment of visceral and cutaneous leishmaniasis and is currently registered for leishmaniasis treatment in India in 2002, as Impavido, Germany in 2004, and Colombia in 2005 [4]. This drug is also used clinically for the topical treatment of skin metastases associated with breast cancer and cutaneous lymphoma [5]. Miltefosine has shown high cure rates in the treatment of visceral cutaneous and mucocutaneous [6-8].

Materials and methods

Cultivation of *Leishmania dononvani promastigote*

Leishmania donovani isolate (DUAA/IQ/2005/MRU15) was obtained from The Medical Unit at College of Medicine, Al_Nahrain University. These parasites were routinely maintained through serial passage every two months in BALB/C mice. After isolation from animals, they were maintained on semisolid medium and sub-cultured every 15 days. To obtain large amount of parasites in promastigote stage *in vitro*, inoculums of one ml was transferred from culture contain growth to screw tube vials contain five ml of media (m 199) with 10% FCS, and then incubated at 26˚c. After three days the culture was examined under light microscope to ensure the growth of parasites and the absence of any other contamination, added amount of media to culture if need. By this way gain the active parasites in log phase [9].

Drug concentrations:

Pentostam (SbV)(Sodium Stibogluconate)

An injectable ampoules (100 mg/ml) manufactured by (Glaxo Operations UK Limited Castle, Member of the Glaxo Smith Kline Group companies). The drug stored below 25˚C and protect from light. It was gained from Al-Karama teaching hospital. A stock solution (100 mg/ml) of pentostam was used in this study. The following concentrations (5, 10, 20, 40, 45, 50, 60, 70, 80 µg/ml) were prepared for *L. dononvani* promastigotes.

Miltefosine (Miltex) (an alkyl phospholipid)

This drug present in powder form (10 g) with molecular weight 407.57 and purity 99 % manufactured by (Xian Wango Biopharm Co., Ltd. China). The following concentrations (5, 10, 20, 40, 45, 50, 60, 70, 80µg/ml) were prepared for *L. donovani* promastigotes.

Testing the effect of each drug on the parasite promastigote:

Large amount of parasites stage was harvest in log phase. Drug concentrations (SbV, Miltex) were estimated and prepared in triplicate using the equation [C1 V1= C2 V2] immediately before each experiments.The required number of sterile screw tube vials containing 5ml of M199 media was prepared. The parasite stage was added (1 x 10^6parasite /ml) to each vial medium then it was counted daily for 3 days using hemocytometer.

Statistical analysis:

The results of each experiment were analyzed by the method described by both Hills *et al.*, [10] and Huber and Koella [11]. The Statistical Analysis System- SAS (2012) [12] was used to effect of different factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

Results and Discussion

Table-1 and -2 (Figure-1 and -2) showed the effect of different concentrations (5, 10, 20, 40, 45, 50, 60, 70, 80) µg/ml of pentostam and miltefosine drugs on *L. donovani* promastigotes. *L. donovani* promastigotes treated with all concentrations of miltefosine drug showed significant ($P<0.05$) decreases in the parasite numbers in comparison with pentostam drug and untreated parasites. The number of promastigotes treated with high concentrations of miltefosine (50,60,70,80) µg/ml after 24 hr was (80,35,28,16 $\times10^5$ cell/ml) while the number of promastigotes treated with same concentrations of pentostam was (752,720,650,592$\times10^5$ cell/ml) and untreated parasites which was1000$\times10^6$ cell/ml.

After 48 and 72 hr number of promastigotes treated with miltefosine decreased clearly reaching to (20,20,17,8 $\times10^5$ cell/ml) and (10,10,7,3$\times10^5$ cell /ml) respectively, compared with promatigotes treated with pentostam (200,177,130,150$\times10^5$ cell/ml) and (90,82,70,52 $\times10^5$ cell/ml) respectively and untreated parasites recorded 1400$\times10^6$ and 1730$\times10^6$ respectively.

The present study showed that the *L. donovani* promastigotes are less susceptible to pentostam than miltefosine. Thus, pentavalent antimonials are prodrugs that require biological reduction to the trivalent form [Sb(III)] for antileishmanial activity. The site (amastigote or macrophage) and mechanism of reduction (enzymatic or non-enzymatic) remain controversial. However, several studies have reported that axenic amastigotes (i.e., cultured in the absence of macrophages) are susceptible to pentostam Sb (V), whereas promastigotes are not, suggesting that some stage specific reduction occurs in this life cycle stage[13,14]. Unfortunately, the standard drugs administered parenterally, including pentavalent antimonials, amphotericin B, and paromomycin, are toxic and expensive. Moreover, the emergence of resistance to antimonials is now a widespread phenomenon, and almost all these standard treatments are relatively inefficient against leishmaniasis-human immunodeficiency virus coinfection [15]. pentavalent antimony (SbV) enters the host cells, crosses the phagolysosomal membrane and is converted into trivalent antimony (SbIII). Then, SbIII acts against amastigotes by compromising the cells thiol redox potential by inducing efflux of intracellular thiols and consequently inhibiting trypanothione reductase (TR) [16]. SbV reduction can be non-enzymatic, under acidic conditions such as those found in the phagolysosome, by glutathione (GSH), glycylcysteine and trypanothione, or enzymaticby thiol-dependent reductase (TDR1) [17]and antimonite reductase (ACR2) [18].ACR2 also increases the sensitivity of Leishmania to SbV[19]. SbV may also kill parasites by indirect mechanisms, such as increasingcytokine levels [20].

The leishmaniacidal activities of miltefosine have been associated with perturbation of the alkyl-phospholipid metabolism and the biosynthesis of alkyl-anchored glycolipids and glycoproteins [21, 22].Miltefosine has been considered to inhibit the translocation of CTP: phosphocholine-cytidyltransferase, the key enzyme of phosphocholine biosynthesis, from its inactive cytosolic form to its active membrane-bound form[23]. In addition, sphingomyelin biosynthesis has been shown to be inhibited by miltefosine, leading to increased levels of cellular ceramide [24].Studies on *L. mexicana* suggested that miltefosine might cause perturbation of ether-lipid metabolism, GPI anchor biosynthesis, and leishmanial signal transduction [22].

Miltefosine proved its effectiveness on *L. donovani* promastigotes especially when used in high concentrations (more than 40 mg/ml). Previous studies proved its effectiveness as a treatment for human visceral leishmaniasis [25, 27].It has been hailed as potentially the first oral treatment of human leishmaniasis[28,30]. Miltefosine is effective *in vitro* and *in vivo* against *Leishmania* species [31] demonstrated efficacy in animals via the oral route [32].

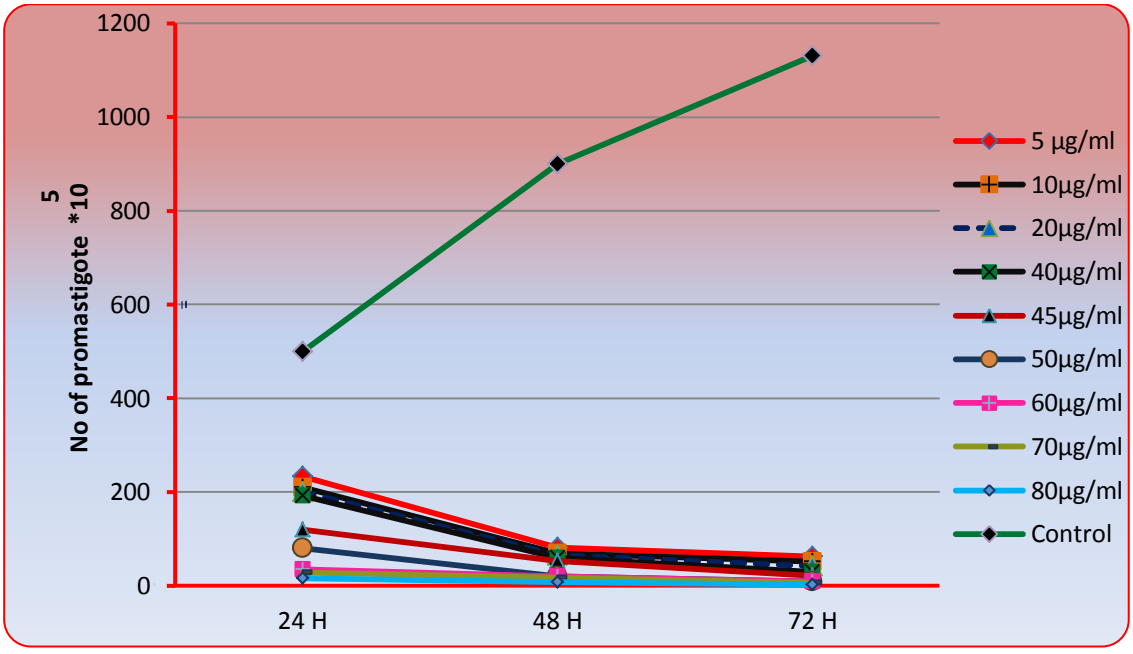


Figure 1-The effect of different concentration of miltefosine drug on *L.donovani* promastigote

Table1-The effect of different concentration of miltefosine drug on *L.donovani* promastigote

Time (hr)	Miltefosine concentration (µg/ml)										LSD
	control	5	10	20	40	45	50	60	70	80	
24	1000	233	210	200	192	120	80	35	28	16	125.74 *
48	1400	82	70	66	60	53	20	20	17	8	192.55 *
72	1730	63	52	42	30	22	20	10	7	3	172.03 *
LSD	139.6 *	42.85 *	39.68 *	56.22 *	41.68 *	38.50 *	21.09 *	19.68 *	19.33 *	11.71 *	---
* (P<0.05)											

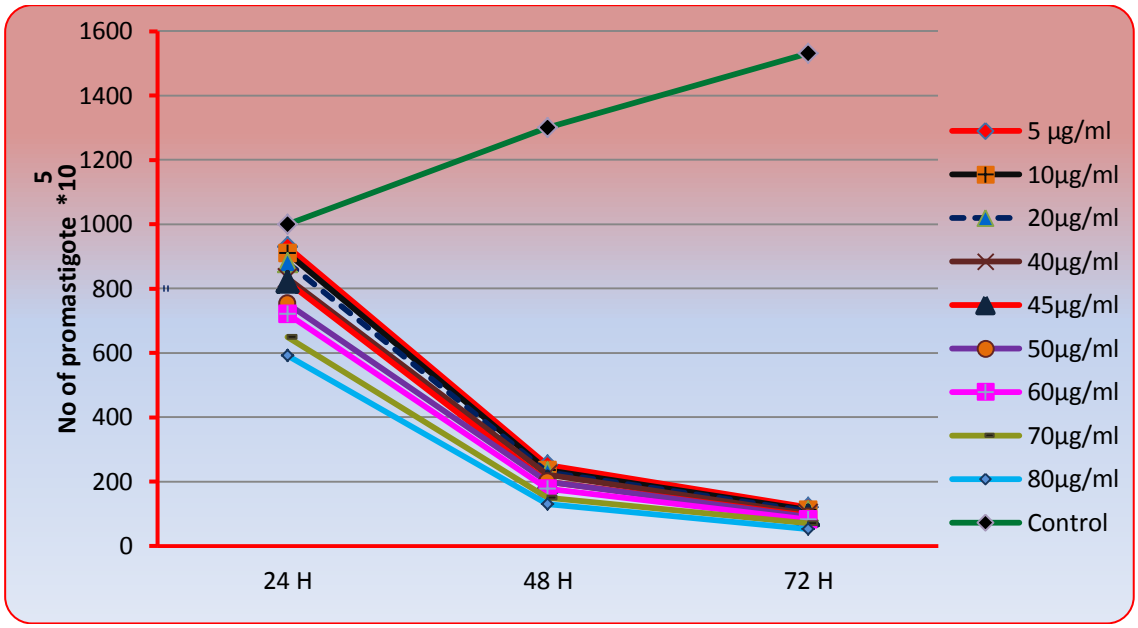


Figure 2-The effect of different concentration of pentostam drug on *L. donovani* promastigote

Table 2-The effect of different concentration of pentostam drug on *L. donovani* promastigote

Time (hr.)	Pentostam concentration (µg/ml)										LSD
	control	5	10	20	40	45	50	60	70	80	
24	1000	930	909	880	833	820	752	720	650	592	107.43 *
48	1400	252	235	230	221	200	200	177	150	130	139.58 *
72	1730	120	112	108	100	93	90	82	70	52	166.74 *
LSD	139.6 *	94.59 *	87.14 *	91.28 *	84.57 *	77.61 *	72.35 *	77.05 *	69.42 *	51.66 *	---
* (P<0.05)											

Table 3- Comparison between the effect of different concentrations of miltefosine and pentostam drugs against *L.donovani* promatigotes after 24 hr.

Concentration (µg/ml)	No.of promastigotes treated with miltefosine	No.of promastigotes treated with pentostam	L.S.D value
5	233±19.14	930±41.06	87.35*
10	210±16.55	909±33.96	103.49*
20	200±21.06	880±27.63	73.42*
40	192±13.57	833±31.29	85.33*
45	120±7.61	820±37.62	94.61*
50	80±4.06	752±41.05	69.57*
60	35±2.79	720±33.94	81.20*
70	28±2.06	650±37.81	64.48*
80	16±1.77	592±22.39	71.29*
L.S.D value	64.92*	124.85*	---
*(P<0.05),NS: Non-significant			

Table-3 showed significant differences between *L.donovani* promastigotes treated different concentrations of miltefosine and pentostam drugs after 24 hr (P<0.05), At 5, 45, 80 concentrations was 87.35, 94.61 and 71.29 respectively.

Table 4- Comparison between the effect of different concentrations of miltefosine and pentostam drugs against *L.donovani* promatigotes after 48 hr.

Concentration (µg/ml)	No.of promastigotes treated with miltefosine	No.of promastigotes treated with pentostam	L.S.D value
5	82±3.69	252±25.72	42.19*
10	70±3.05	235±21.95	39.55*
20	66±2.84	230±14.51	39.06*
40	60±2.19	221±18.43	34.68*
45	53±1.66	200±14.22	46.74*
50	20±1.72	200±14.60	41.39*
60	20±1.44	177±13.52	33.96*
70	17±1.09	150±16.85	26.58*
80	8±0.73	130±12.39	19.66*
L.S.D	46.73*	74.82*	---
*(P<0.05),NS: Non-significant			

Table-4 showed significant differences between *L.donovani* promastigotes treated with different concentrations of miltefosine and pentostam drugs after 48 hr (P<0.05), At 5, 45, 80 concentrations was 42.19 ,46.74 and 19.66 respectively.

Table 5- Comparison between the effect of different concentrations of miltefosine and pentostam drugs against *L.donovani* promatigotes after 72 hr.

Concentration (µg/ml)	No.of promastigotes treated with miltefosine	No.of promastigotes treated with pentostam	L.S.D Value
5	63±2.57	120±7.82	18.74*
10	52±2.08	112±7.64	23.68*
20	42±2.14	108±6.49	21.04*
40	30±1.97	100±4.91	16.41*
45	22±1.49	93±5.06	14.39*
50	10±0.74	90±4.33	17.95*
60	10±0.79	82±3.62	14.66*
70	7±0.52	70±2.97	12.07*
80	3±0.17	52±2.46	14.48*
L.S.D	41.39*	68.22*	---
*(P<0.05),NS: Non-significant			

Table-5 showed significant differences between *L.donovani* promastigotes treated different concentrations of miltefosine and pentostam drugs after 72 hr (P<0.05), At 5, 45, 80 concentrations was 18.74,14.39 and 14.48 respectively.

The 50% inhibitory concentration (IC_{50}) of the drugs

IC_{50} the drug concentration that decreases the rate of cell growth by 50%. In this study the IC_{50} of the two drugs was calculated by direct microscopic counting for three days. IC_{50} of miltefosine drug was 45.4µg /ml, 46.76 µg/ml and 36.68µg/ml after 24, 48 and 72 hr respectively Figure-3,-4,-5. The number of promastigotes treated with pentostam drug remained high even after 24 and 48 hr (above 50%). IC_{50} of pentostam drug was 75.39µg/ml for 72 hr , Figure-6,-7,-8.

The present results revealed high values of miltefosine IC_{50} against *L. donovani* promastigote in comparison to the results obtained in previous studies on different *Leishmania* species, these differences may be due to the different strains and other laboratory conditions.

Previous reports showed that there is a significant difference between the IC_{50} values after 72 h of miltefosine treatment of promastigotes from the species *L.donovani, L.major, L.tropica, L.aethiopica, L.mexicana, L.panamensis, L.braziliensis, L.amazonensis* and *L.infantum.* The IC_{50} values ranged from 0.12-1.32 µM in *L. donovani* to 4.8-13.1 µM in *L. major* [33-35]. IC_{50} for *L.donovani* promastigotes ranging from 1.5-7.1 µM, IC_{50} for *L.guyanensis* promastigotes ranging from 5-10 µM and 13.5-25 µM for *L.braziliensis* promastigotes [36].

IC_{50} for L. *(L.) amazonensis* amastigotes was 3.21µM, IC50 for *L.(L.) braziliensis, L.(L.) guyanensis*, L.(L.) chagasi and *L.(L.) donovani* amastigotes was 5.40µM , 4.02µM, 4.46µM and 0.22µM respectively[37]. IC_{50} on T. cruzi at a concentration of 55.4µM [38].

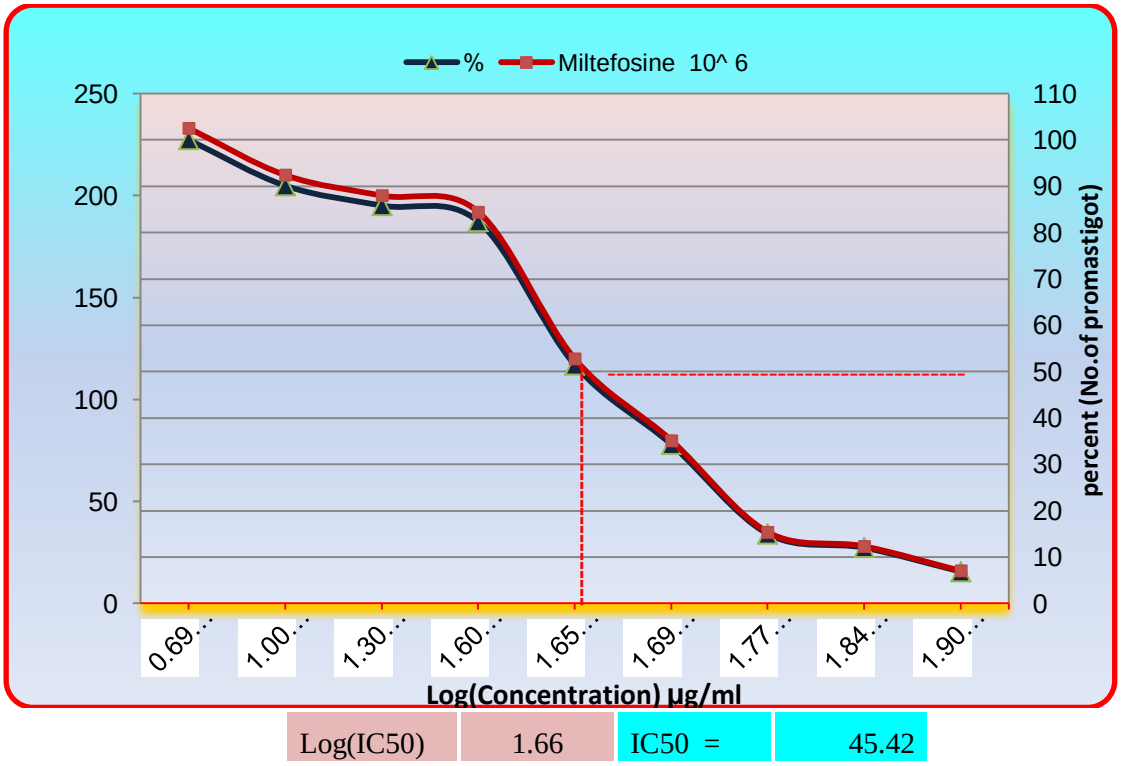


Figure 3- Effect of miltefosine on *L.donovani* promastigote (24hr)

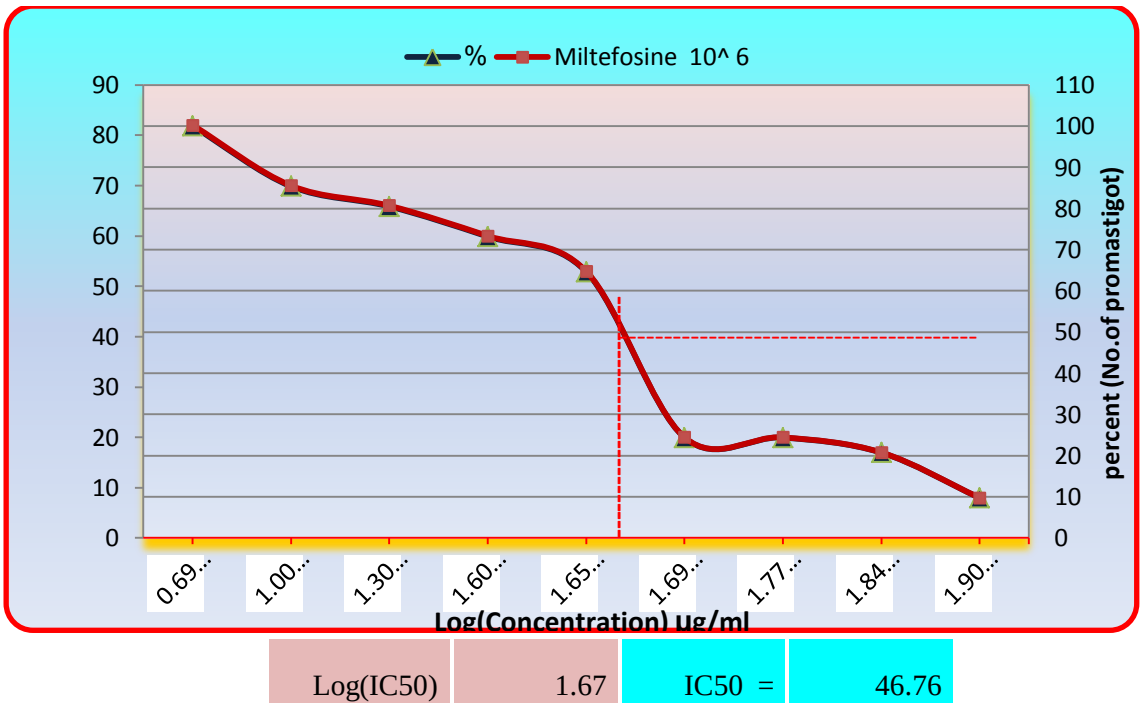


Figure 4-Effect of miltefosine on *L.donovani* promastigote (48hr)

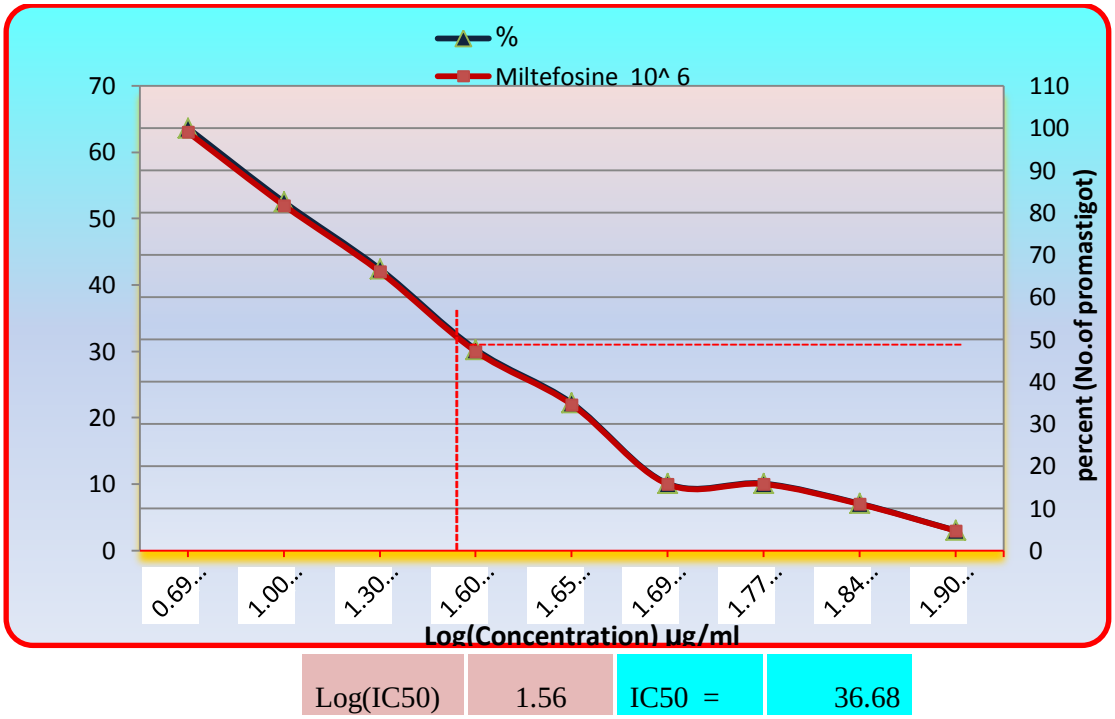


Figure 5- Effect of miltefosine on *L.dodnovani* promastigote (72hr)

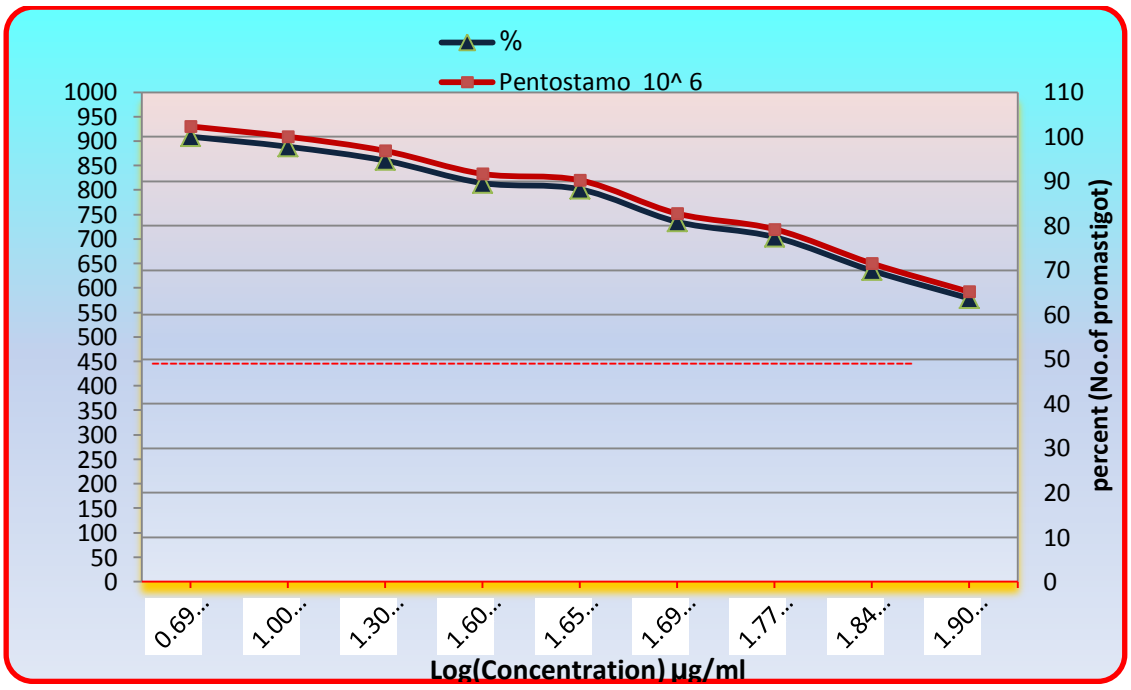


Figure 6- Effect of pentostam on *L.donovani* promastigote (24hr)

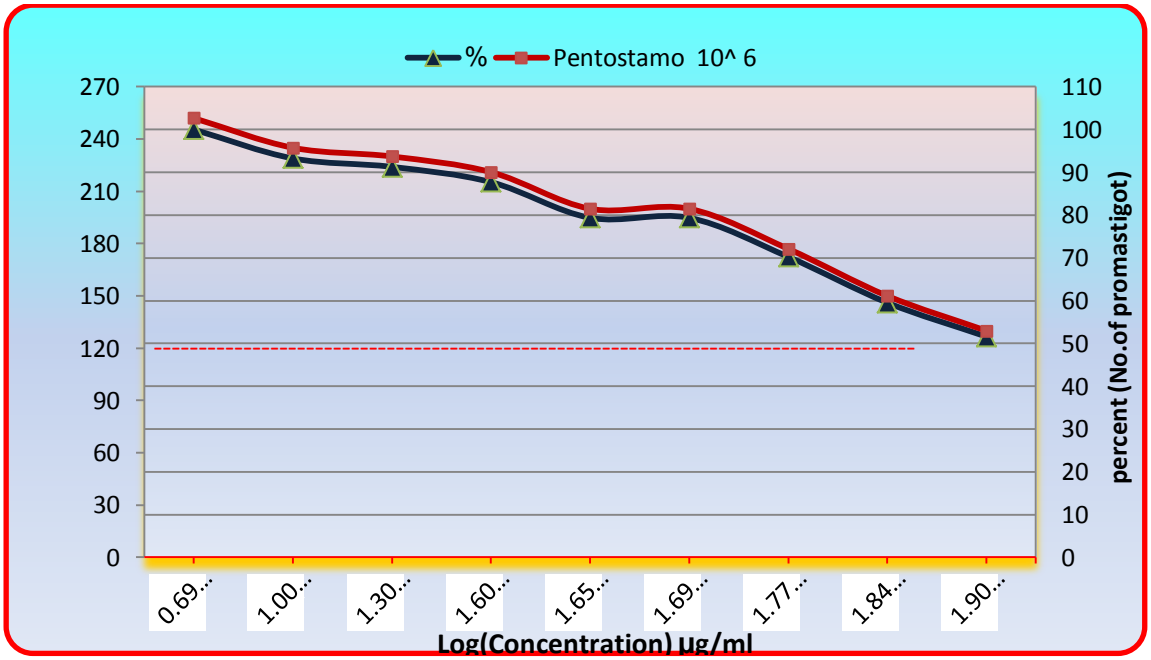
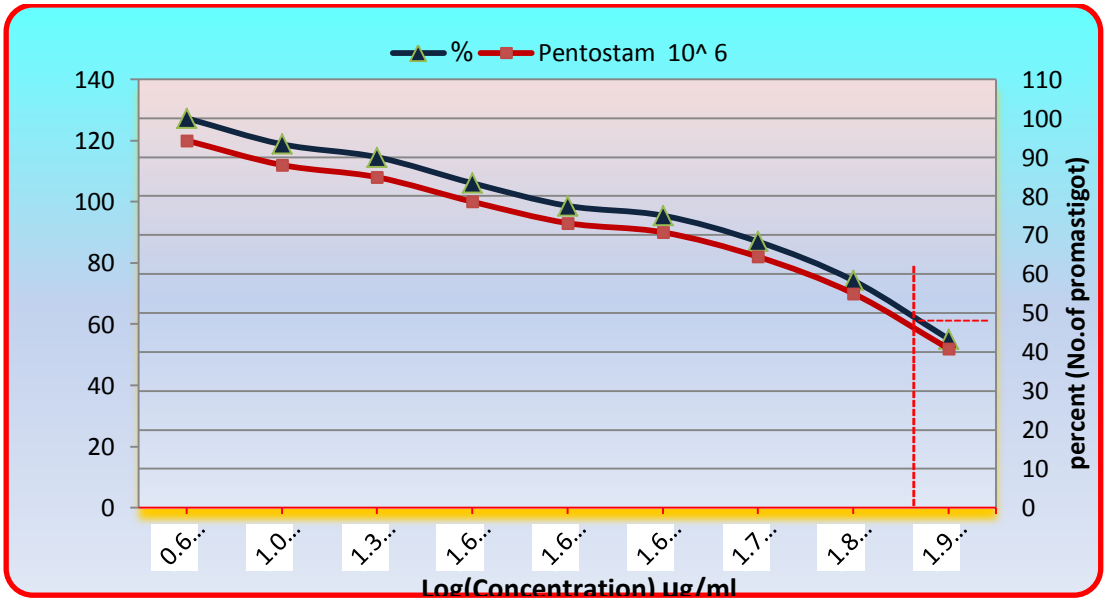


Figure 7- Effect of pentostam on *L.donovani* promastigote (48hr)



Log(IC50)	1.88	IC50 =	75.39

Figure 8- Effect of pentostam on *L.donovani* promastigote (72hr)

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