Efficacy of Amphotericine B Ddrug Against Promastigote and Axenic Amastigote of *Leishmania Dononvani in Vitro*

Zhor M. Abbas[1]*, Khawla H. Zghair[1] and Nada M. Al-Bashir[2]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
[2]Medical Research Unit, College of Medicine, Al-Nahrain University, Baghdad ,Iraq.

Abstract

Leishmania are protozoan parasites belonging to the family *Trypanosomatidae* that cause high morbidity and mortality levels with a wide spectrum of clinical syndrome. This study aimed to investigate the effect of liposomal amphotericine B (AmBisome) drug on promastigote and axenic amastigote stages of *Leishmania donovani* isolate (MHOM/IQ/2005/MRU15)in comparison with pentostam SbV drug. Different concentrations of AmBisome and SbV drugs were investigated against *Leishmania donovani* promastigote and axenic amastigote. The IC_{50} values of SbV and AmBisome drugs on promastigote were10.12 mg/ml and 2.21µg/ml, respectively, while they were 0.77µg/ml for axenic amastigote for both drugs. The present study concluded that axenic amastigote was more sensitive than promastigote against both drugs, and AmBisome drug showed high effectiveness against both stages with low concentrations in comparison with pentostam.

Keywords: AmBisome, pentostam , visceral leishmanasis , axenic amastigote.

تاثير عقار الامفوترسين ب ضد الطور الامامي السوط وعديم السوط لليشمانيا الاحشائيه خارج جسم الكائن الحي

زهور محسن عباس*[1]، خوله حوري زغير [1] ، ندى محمد طه البشير [2]

[1]قسم علوم الحياة ،كلية العلوم ،جامعة بغداد، بغداد ،العراق

[2]وحدة البحوث الطبيه، كلية الطب ، جامعة النهرين، بغداد، العراق.

الخلاصه

الليشمانيا طفيليات ابتدائيه تعود الى عائلة المثقبيات التي تسبب معدلات عاليه من الامراضيه والوفيات مع مدى واسع من الاعراض السريريه. هدفت الدراسه الى اختبار فاعلية عقار الامفوترسين الشحمي على الطور امامي السوط وعديم السوط خارج الخلوي لليشمانيا الاحشائيه. تم الحصول على العزله الاحشائيه من وحدة البحوث الطبيه في كلية الطب جامعة النهرين .اختبرت تراكيز مختلفه من عقار البنتوستام والامفوتيرسين الشحمي ضد الطورين الامامي السوط وعديم السوط خارج الخلوي من اللشمانبا الاحشائيه و كانت قيم IC₅₀ لهذين العقارين 10.12ملغم /مل و 2.21 مايكروغرام /مل للطور الامامي السوط على التوالي بينما كانت قيمها 0.77 مايكرو غرام /مل للطور عديم السوط لكلا العقارين. اظهرت النتائج ان الطور عديم السوط خارج الخلوي اكثر حساسيه تجاه العقارين من الطورامامي السوط، وأن الامفوتيرسين الشحمي فعال ضد الطورين وبتراكيز اقل منها للبنتوستام.

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Email: zhorbio@yahoo.com

Introduction

The Leishmaniasis are a group of diseases with wide epidemiological and clinical diversity caused by the protozoan parasite *Leishmania*. *Leishmania* parasites live a dual-form life cycle (digenetic life cycle), as either a promastigote flagellar or an amastigote form. The promastigotes are found in the insect vector and are injected into the mammalian host during the vector's blood meal. Then, they are phagocytised by macrophages, dendritic cells and/or neutrophils attracted to the biting site in the skin. Once inside the phagosome, promastigotes differentiate into amastigotes, multiply by simple division until bursting the host cell. In the mammalian host, these protozoa are obligate intracellular parasites of macrophage dendritic cell lineages. This complex life cycle includes several facets that might be exploited for drug design optimization and development [1]. Visceral leishmaniasis (VL) is a systemic disease that is fatal in the absence of treatment [2]. It is estimated that 88 countries are leishmaniasis-endemic. There are 500,000 new cases of VL and more than 50,000 deaths from the disease every year [3].

There are two types of VL, which differ in their transmission characteristics: zoonotic (ZVL) is transmitted from animal to vector to human and anthroponotic (AVL) is transmitted from human to vector to human. In the former, humans are occasional hosts and animals, mainly dogs, are the reservoir of the parasite [4]. ZVL is found in areas of *L. infantum* transmission whereas AVL is found in areas of *L. donovani* transmission. Following an incubation period that generally lasts between 2 and 6 months, VL patients present symptoms and signs of persistent systemic infection including fever, fatigue, weakness, loss of appetite and weight loss and parasitic invasion of the blood and reticulo-endothelial system, lead to enlarged lymph nodes, spleen and liver. Fever is usually associated with rigor and chills and can be intermittent. Fatigue and weakness are worsened by anemia, which is caused by the persistent inflammatory state [2]. VL is widely spread in different parts of the world. Iraq regarded as an endemic place, especially in the middle and south parts, the main reasons, is due to adaptation of the vector sand fly in these areas [5]. The pentavalent antimonials sodium stibogluconate and meglumine antimoniate have been the first-line treatment for VL in many areas for more than 70 years. Cheaper generic forms of these drugs are available that have been shown to be equivalent to the branded products [6]. Antimonials are toxic drugs with frequent, sometimes life-threatening, adverse side effects, including cardiac arrhythmia and acute pancreatitis. Patients under the age of 2 or aged 45 or over with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, VL complications or a combination of these factors[7]. Conventional Amphotericin B has replaced antimonials as the first-line treatment for VL in some areas of the Bihar State of India where treatment failure rates for antimonials reached >60%.AmB and its formulations are increasingly being used and are considered as the best existing drugs against VL and have a 97% cure rate with no reported resistance [2,8]. Lipid formulations of amphotericin B have been developed in order to improve its bioavailability and pharmacokinetic (PK) properties, considerably reducing side effects [9- 11]. The larger lipid particles are rapidly assimilated by the mononuclear phagocyte system (hepatic macrophages), where *Leishmania donovani* parasites accumulate and VL develops. Another advantage is that smaller liposomes stay in the blood stream longer than the free drug [11]. The main limitations are its high cost, administration route and lack of stability at high temperature .This study aimed to demonstrate the effectiveness of different concentrations of liposomal formulation (AmBisome) of amphotericin B drug against *L. donovani* promastigote and axenic amastigote comparing with sodium stibogluconate (Sb) *in vitro* conditions.

Materials and methods

Leishmania donovani promastigotes isolate (MHOM/IQ/2005/MRU15) were obtained respectively, from The Medical Research Unit at College of Medicine, University of Al-Nahrain. These parasites were routinely maintained through serial passages every two months in BALB/c mice. After isolation from animals, they were maintained on semisolid medium and sub- cultured every 15-days.

Promastigote cultivation

To obtain a large amount of parasites in promastigote stage *in vitro*, inoculums of 1ml was transferred from culture contain growth to screw tube vials contain 5ml of media (M199) with 10% fetal calf serum (FCS), and then incubated at 26°C. After three days the culture was examined under compound microscope to ensure the growth of parasites and the absence of any other contamination,

added amount of media to the culture if need, by this way gain the active parasites in log phase (3-4 days after cultivation) [12].

Axenic amastigote cultivation

Axenic amastigotes for *L.donovani* have been generated and cultured *in vitro* by mimicking those environmental signals, temperature and pH, that parasites encounter in the macrophage phagolysosomal vacuole. Promastigotes were transferred in M199 medium at (ph 5.2, 5% of CO_2, 37°C) to obtain this stage [12].

Drug concentrations

A stock solution (100 mg/ml) of sodium stibogluconate (SbV) was used in this study. The following concentrations (2, 4, 8, 16, 32 and 64 mg/ml) were prepared for promastigote and (0.2, 0.4, 0.6, 0.8, 1 and 1.2 µg / ml) for axenic amastigote, while for AmBisome drug the following concentrations (0.6, 1.2, 2.4, 4.8, 8.16, 16.32 and 32.64 µ g /ml) were prepared for promastigote and (0.2, 0.4, 0.6, 0.8, 1 and 1.2 µg / ml) for axenic amastigote.

Statistical analysis

The results of each experiment were analyzed by the method described by both Hills *et al.,* [13] and, Huber and Koella [14]. Briefly, Hills proposed finding two concentrations, $x1$ and $x2$, such that the parasite density, $y1$, at concentration $x1$ (and all lower concentrations) was more than half of the density found in the control, $y0$, and that the parasite density, $y2$, at concentration $x2$ (and all higher concentrations) was less than half of $y0$. The IC_{50} value for each drug was then found by linear extrapolation between $x1$ and $x2$:

Log (IC50) = log($x1$) + [($y1$ - $y0$/2) / ($y1$ - $y2$)] [log($x2$) - log($x1$)].

Least significant difference –LSD test was used to significant compare between means in this study.

Results and discussion

Drug susceptibility of *Leishmania donovani* to liposomal amphotericin B (AmBisome) and pentostam Sb (V) drugs was determined on both stages: the promastigote and the axenic amastigote.

The effect of (2, 4, 8, 16, 32 and 64 mg/ml) concentrations of Sb (V) on *L. donovani* promastigotes shown in figure 1. This figure revealed highly decreased in the number of the parasites from the 2nd day of exposure in all drug concentrations especially the high doses in comparison with the control. The effect of low concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2 µg/ml) of Sb (V) drug was investigated on the axenic amastigotes of *L. donovani* which showed rapid decreased in the number of *L. donovani* parasites (figure- 2).
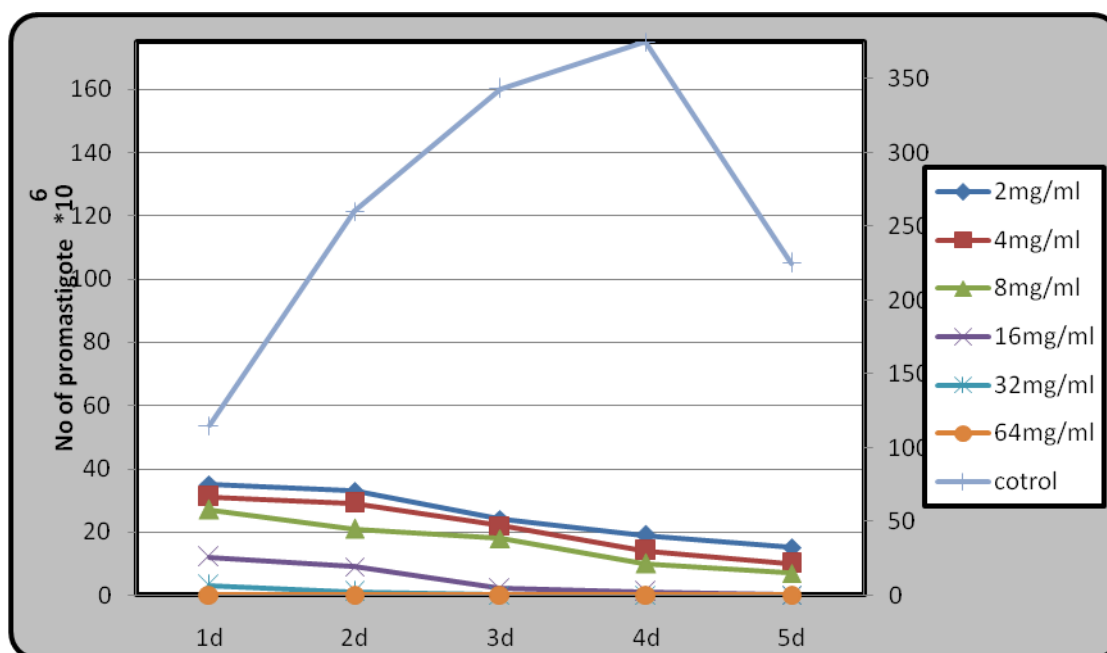

Figure 1- The effect of different concentrations of Sb (V) drug on promastigote of *L. donovani*
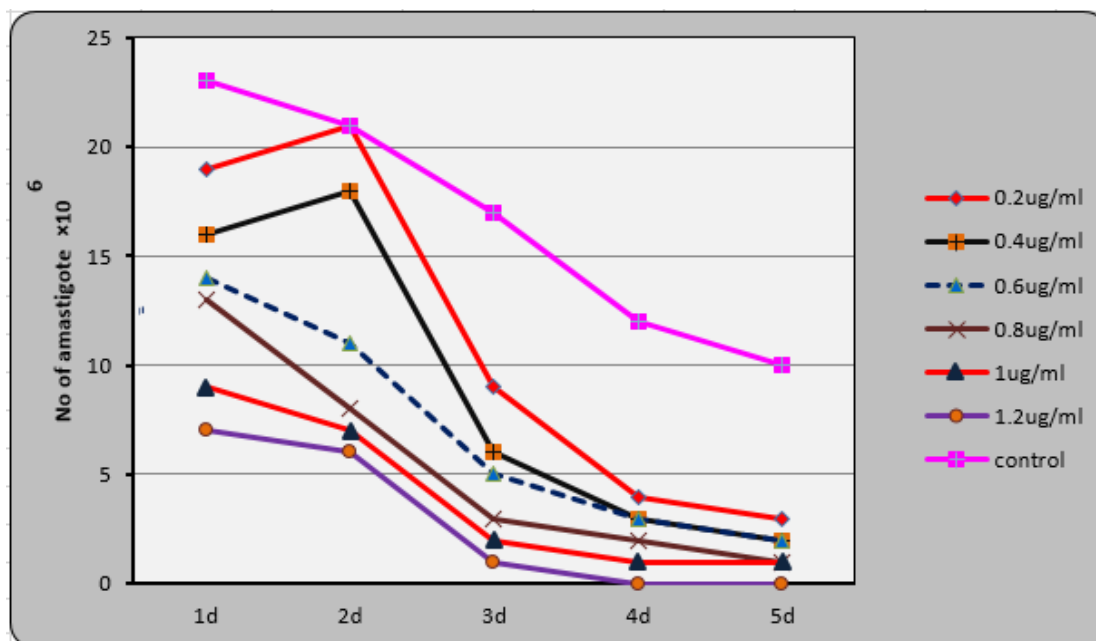
Figure 2- The effect of different concentrations of Sb (V) drug on axenic amastigote of *L. donovani*

Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades with little evidence of resistance.The growing resistance to Sb (V) is in India while it's still remained sensitive all over the world could be due to the fact that leishmaniasis usually has zoonotic transmission except in the Indian subcontinent and East Africa where the transmission is largely anthroponotic. In an anthroponotic cycle, once Sb (V) resistance gets established, it spreads exponentially and organisms sensitive to the drug get eliminated quickly, whereas the drug-resistant parasites continue to circulate in the community [15].

Pentavalent antimony Sb (V) undergoes biological reduction to much more active/toxic trivalent form of antimony Sb (III) that exhibits antileishmanial activity. However, the site of (amastigote or macrophage) and the mechanism of reduction (enzymatic or nonenzymatic) remain controversial. For instance, amastigotes but not promastigotes can reduce Sb (V) to Sb (III). This explains why amastigotes are more susceptible to Sb (V) but promastigotes some times are not [16]. Conversion of Sb (V) to Sb (III) may occur at both sites, that is, macrophage and parasite, and the parasite plays a major role in the generation of higher, lethal concentrations of Sb (III) within the parasite [17].

Different concentrations (0.6, 1.2, 2.4, 4.8, 8.16, 16.32 and 32.64 µg/ml) of liposomal amphotericin B (AmBisome) drug were investigated against the promastigote form of *L. donovani* parasites. The high concentrations (8.16, 16.32 and 32.64 µg/ml) of AmBisome drug clearly decreased the number of *L. donovani* promastigotes (figure -3).

On the other hand lower concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2 µg/ml) of AmBisome drug were investigated against axenic amastigote of *L. donovani*. This stage was affected by all AmBisome concentrations as shown in figure 4, although the high concentrations (0.6 to 1.2 µg/ml) of AmBisome drug were the most influential on its growth.
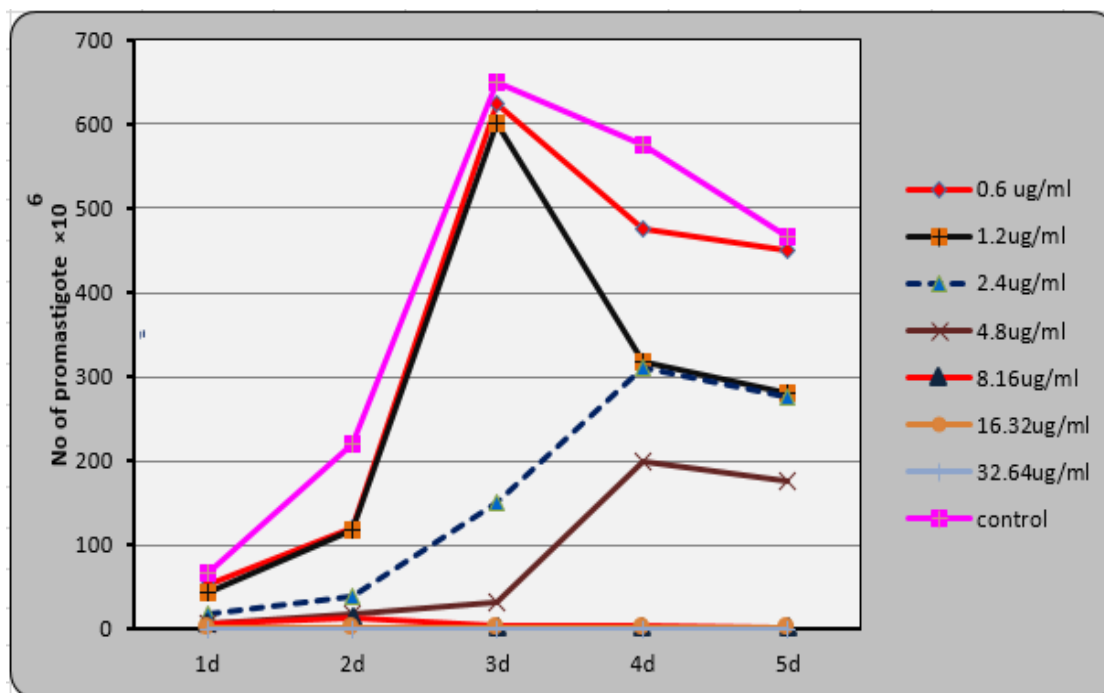
Figure 3- The effect of different concentrations of AmBisome drug on promastigote *L.donovani*
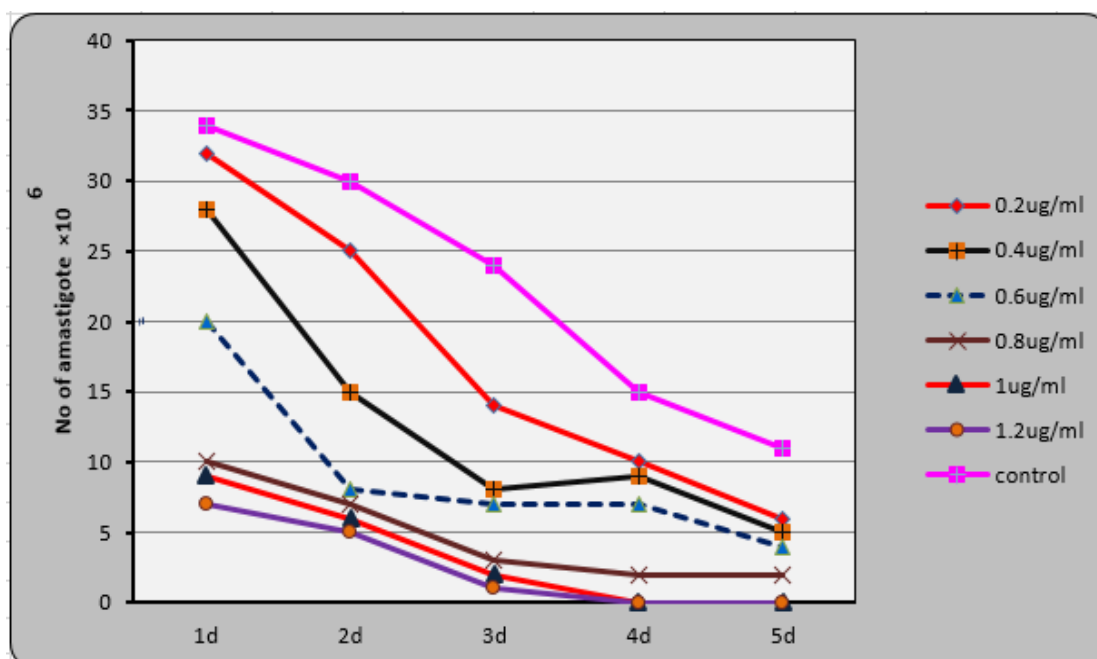

Figure 4- The effect of different concentrations of AmBisome drug on axenic amastigote of *L. donovani*.

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. The major limiting factor about the use of this drug is due to their toxicity. Currently, toxic effects of amphotericin B have been largely ameliorated with the advent of lipid formulations. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and facilitating its preferential uptake by reticuloendothelial cells. Thus, this drug delivery result in increasing efficacy and reduced toxicity [18]

AmBisome have been considered as a high effective, non-toxic form of treatment for VL when administered in a short course [19]. The optimal regimen have been recommended with a total dose of 20 mg/Kg, given in 5 doses of 3-4 mg/Kg over 10 days [20]

Most patients are now treated with AmBisome, which is safe and very effective in a short time. Reduction of the toxic effects by using lipid formulations allows the infusion of higher doses of amphotericin B [15].

The mechanism of action of amphotericin B might be based on the peculiar metabolism of sterols of *Leishmania* species. In contrast to mammalian host, 24-ergosterol is the main sterol synthesized and existing in fungal and *Leishmania* membranes. Polyene macrolides bind to these molecules, creating pores that leak ions [21].

Interestingly, this drug, which was used to treat unresponsiveness to pentostam, was highly potent against extracellular amastigotes at concentrations below the maximal concentration achieved in serum. These data showed that amphotericin B was directly toxic to the parasite at the amastigote stage and did not seem to depend upon macrophage activation for its antileishmanial activity [22]

AmBisome is the most effective clinically prescribed therapeutic drug to treat VL. Although the development of AmBisome as a therapy against leishmaniasis has its origin in the discovery that it is a potent leishmanicidal agent [2, 9]. Amphotericin B, especially its liposomal form has become the reference treatment of human visceral leishmaniosis in southern Europe [23, 24].

A study on HIV/*L. infantum* co-infect patients receiving a long term secondary prophylaxis with AmBisome showed that there was no *in vitro* resistance to AmBisome of the strains isolated in course of disease relapses from these cases [25]

The main observation for all reference drugs was that log phase promastigotes tended to be less sensitive than the other extracellular or intracellular stages and were fully refractory to Sb (V). On the other hand, susceptibility to amphotericin B, which are known to affect membrane integrity directly or indirectly and which are able to exert antileishmanial action independently of cell-mediated parasiticidal mechanisms, has been clearly demonstrated [26]. Amphotericin B showed slightly higher potency in the cellular model than in the axenic models, a difference that is likely related to accumulation in the phagolysosome.

The IC_{50} is the concentration of drug which decreases cell numbers by 50% compared to numbers of control cells grown in the absence of drug. The current study showed clear differences between the IC_{50} values for the drugs on promastigote and axenic amastigote form of *L. donovani*. The axenic amastigote was more susceptible for both drugs than promastigote. As clarified in table 1 the number of the parasites was inhibited at concentrations less than the inhibited concentrations for promastigote for Sb (V) and AmBisome, this due to the susceptibility of this stage in comparison to promastigote.

Table 1-The total values SbV and AmBisome drugs IC_{50} against *L. donovani* promastigote and axenic amastigote

Drugs \ stages	Promastigote	Axenic amastigote
SbV	10.12 mg/ml	0.77 µg/ml
AmBisome	2.21 µg/ml	0.77 µg/ml

The IC_{50} of AmBisome drug obtained by Mutiso *et al.*, [27] was (0.16 ± 0.32µg/ml) against *L. donovani* promastigote. While another study done by Kamau Ngure *et al.*, [28] recorded the IC_{50} of pentostam against *L. donovani* and *L. major* promastigote which was (28.41 and 139.31 µg/ml) and it was for AmBisome (49.90 and 2.38 µg/ml) respectively.

The variations in the previous results of these studies and the present study are due to many effected factors, such as the used strain, used drug (type, concentrations and the origin of the drug), and other condition of the test used.

The IC_{50} for AmBisome drug is approximately the same against axenic amastigote with the IC_{50} value for Sb (V) drug against the same stage for both strains. So, due to the less toxicity and more safety of AmBisome, it could be the best alternative drug for Sb (V) leishmanial resistant cases.

The overcome of the present study indicated that AmBisome drug is effective against promastigote and axenic amastigote forms for L.donvani. It was highly effective upon axenic amastigote from promastigote when it's used concentrations were too low and effectiveness on inhibition the axenic amastigote parasite growth.

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