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In Vitro Assessment of Miltefosine Activity Against Promastigotes and Axenic Amastigotes of *Leishmania tropica*

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Abstract

Leishmaniasis is a worldwide disease still treated with expensive compounds that present severe side effects, and are frequently ineffective emphasizing the importance to search effective compounds against this disease. Miltefosine drug (HePC) that used as antitumor agent has been used against *Leishmania tropica* in two forms promastigote and axenic amastigote *in vitro* conditions. Different concentrations (5, 10, 15, 20, 25 and 30 µM) of HePC were performed and exposed to both parasite forms in comparison to sodium stibogluconate (Sb) drug. Parasites viability then was determined using MTT assay after 12, 24, and 48hr of exposure. DNA was extracted from treated and untreated parasites after 48hr of exposure and qualitative analysis of the total genomic DNA fragmentation was carried out through using agarose gel electrophoresis.

The IC50 of HePC and Sb were 17.07 and 25.72 µM against *L. tropica* promastigotes, respectively, while, the IC50 of them were 18.08 and 21.29µM against axenic amastigotes respectively. Fragmented DNA in agarose gel electrophoresis was revealed in both parasite forms exposed to all used concentrations of HePC. These results showed the significant activity of miltefosine against the viability of *L. tropica* promastigotes and axenic amastigotes.

Keywords: Cutaneous leishmaniasis, sodium stibogluconate, miltefosine, apoptosis

تقييم فعالية الملتيفوسين ضد الطور امامي السوط وعديم السوط الخارجي للشمانيا الاستوائيه داخل المختبر

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الخلاصه

داء اللشمانيا من الامراض العالمية الانتشار التي لا زالت تعالج بمركبات باهضة الثمن ولها اعراض جانبيه خطيره واحيانا تكون غير فعاله مما يؤكد على ضرورة البحث عن مركبات فعاله ضد هذا المرض. عقار الملتيفوسين المستخدم كعامل مضاد للاورام تم استخدامه ضد اللشمانيا الاستوائيه بشكليها الامامي السوط وعديم السوط الخارجي في المختبر. حضرت تراكيز مختلفه من عقار الملتيفوسين (5، 10، 15، 20، 25، و 30 مايكرومولر) وتمت معاملتها مع كلا الشكلين للطفيلي بالمقارنه مع عقار البنتوستام. ثم تم قياس حيوية الطفيليات بأستخدام فحص الحيويه (MTT) بعد حوالي 12، 24 و 48 ساعه من التعريض. تم استخلاص دنا الطفيليات المعامله بالعقاقير بعد 48 ساعه من التعريض وأجري تحليل نوعي لتكسير جينوم الدنا الكلي من خلال ترحيله الكهربائي على هلام الاكاروز. لقد كانت الجرعه المثبطه لنصف عدد الطفيليات (IC50) من عقاري الملتيفوسين والبنتوستام 17.07 و 25.72 مايكرومولر

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على التوالي ضد الطور الامامي السوط، بينما كانت قيمتها للطور اللاسوطي الخارجي 18.08 و 21.29 مايكرومولر على التوالي. لقد ظهر تكسير للدنا خلال ترحيله الكهربائي على هلام الاكاروز في كلا شكلي الطفيلي وبكافة التراكيز المستخدمه من عقار الملتيفوسين. أظهرت هذه النتائج فعاليه معنويه لعقار الملتيفوسين ضد حيوية الطور امامي السوط وعديم السوط الخارجي من اللشمانيا الاستوائيه.

Introduction

Leishmaniasis is a wide spectrum of diseases caused by trypanosomatida protozoan parasites of the genus *Leishmania*. In human, parasites withstand and reproduce within phagolysosomes of macrophages as intracellular amastigotes stage [1].

In the Old World (Europe, Africa, Central Asia and Middle East), the ulcerated skin lesions typical for Cutaneous Leishmaniasis (CL) are mainly caused by *Leishmania* major and *L. tropica*. Meanwhile, in the New World (Latin America), they are caused by *L. braziliensis*, *L. guyanensis* and *L. mexicana* species complexes, of which the former two species complexes can disseminated to the nasopharyngeal tissues and cause mucosal eradicative form (mucocutaneous leishmaniasis) [2].

Leishmaniasis treatment includes four main options, the pentavalent antimony (sodium stibogluconate or meglumine antimoniate), the polyene amphotericin B (as deoxycholate salt or a liposomal formulation, AmBisome), the alkylphosphocholine miltefosine and the aminoglycoside paromomycin [1]. Despite recent advances, safety, resistance and cost issues necessitate the continued effort to identify an improved anti-leishmanial drug. Studies have also been undertaken to test the drug susceptibility of clinical isolates [3].

Oral miltefosine (Hexadecylphosphocholine: HePC), a drug that is used as antitumor agent, also has been used for treatment of visceral leishmaniasis in India [4]. A number of studies have been carrying out to elucidate the mechanism of action of HePC. Previously the antineoplastic activity of HePC has been attributed to its apoptosis-inducing potential [5]. Also apoptosis has been proposed as the mechanism of antiprotozoal activity of HePC [6].

The pathways and the mechanisms that lead to inhibition or induction of apoptosis in *Leishmania* spp. are of particular interest as they will be possible targets for development of anti-*Leishmania* drugs [7, 8].

This study aimed to clarify the assessment of the effectiveness of miltefosine against *L. tropica* comparing with Sodium stibogluconate *in vitro* conditions.

Materials and methods

Miltefosine drug (HePC) or C21H46NO4P, MW of 407.57 and purity of 99%, was manufactured by (Xian Wango Biopharm Co., Ltd. China). Serial of different concentrations (5, 10, 15, 20, 25and 30 µM) of HePC were performed and then used against promastigotes and axenic amastigotes of *Leishmania tropica* isolate which was commendable obtained from Biology Department, College of Science, University of Baghdad.

Culture of *Leishmania tropica*

Promastigotes were cultured in M199 media (pH 7.2, 10% Fetal Calf Serum (FCS) (Sigma, USA) with antibiotics at 25˚C and sub-cultured at cell densities of 2×10^7 to 2.5×10^7 cells/ml. then, promastigotes were seeded in 96-well culture plates at a density of 1×10^5 cells/ml and treated in triplicate with HePC in final concentrations ranging from 5-30 µM. The plates were incubated at 25˚C for 12, 24 and 48 hr before MTT assay.

Axenic amastigotes for *L. tropica* 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 cultured for 3days in NNN media (26ºC) then transferred to M199 medium at (pH 5.2, 5% of CO2, and 33ºC) to obtain this stage [9, 10].

Measurements of cell viability by MTT colorimetric assay

MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide](Sigma, USA) is a colorimetric assay which determine the reduction of tetrazolium by mitochondrial enzymes in viable cells into formazan, so, number of live parasites were determined based on the optical absorbance of HePC treated and untreated promastigotes and axenic amastigotes in triplicate wells using the following formula:

Viable parasites (%) = (AC-AB) × (AT-AB) /100

(AC), (AB), and (AT) is the optical density (OD) of untreated parasites, blank and HePC or Sb treated parasites, respectively.

Then the results were expressed as the concentration that inhibited parasite growth by 50% (IC_{50}) after 48 hr.

DNA fragmentation assay

Qualitative analysis of the total genomic DNA fragmentation was carried out through using agarose gel electrophoresis.

Each promastigotes and axenic amastigotes (1×10^5 cells/ml) was incubated separately with concentrations (5, 10, 15, 20, 25and 30µM) of HePC. They were harvested after 48hr of exposure. An apoptotic DNA ladder kit (Promega, USA) was used to extract DNA from treated and untreated parasites, following to the manufacturer's instructions. DNA (10 µg) of each sample was electrophoresed in (1.5%) agarose gel at (100 V) for 2 hr.

Statistical analysis

Results were expressed as the concentrations that prevent parasite growth by 50% (IC_{50}) after 48 hr. IC_{50} were calculated using Excel program [11, 12].

The statistical analysis system program was used to illustrate the effect of the different concentrations in the study [13]. Least significant difference (LSD) test was used to significant compare between means in this study.

Results and Discussion

Cytotoxic potential of miltefosine (HePC) on *L. tropica* promastigotes and axenic amastigotes was tested using the MTT assay in comparison to the cytotoxic potential of pentostam drug (Sb).

HePC showed a dose-dependent cytotoxic effect on both stages of parasite. It was reduced the viable cells to 3.1% for *L. tropica* promastigotes at a concentration of 30µM after 48hr in comparison to 30.9% viable cells exposed to Sb at the same concentration and duration with a significant ($P< 0.05$) difference between them and between the lowest and highest concentrations, as shown in Tables -1 and 2.

Table 1-The percentage of viable cells of *L. tropica* promastigotes exposed to HePC.

Drug concentrations	**Percentages of promastigotes viability after exposed to HePC**			**mean ± SD**	**LSD P ≤ 0.05**
	12 hr	**24hr**	**48hr**		
5 µM	95.3	90.1	87.9	91.1 **a** ± 3.8	41.05
10 µM	92.8	88.4	71.2	84.1 **a** ± 11.4	
20 µM	78.6	54.3	32.2	55.0 **abc** ± 23.2	
25 µM	71.4	29.2	12.7	37.8 **bc** ± 30.3	
30 µM	69.1	12.8	3.1	28.3 **c** ± 35.7	
mean ± SD	82.75 **a** ± 11.29	56.98 **ab** ± 331.36	43.45 **b** ± 33.3		
LSD P ≤ 0.05	33.47				

Table 2-The percentage of viable cells of *L. tropica* promastigotes exposed to Sb.

Drug concentrations	**Percentages of promastigotes viability after exposed to Sb**			**mean ± SD**	**LSD P ≤ 0.05**
	12 hr	**24hr**	**48hr**		
5 µM	97.7	93.1	90.1	93.6 **a** ± 3.8	28.22
10 µM	95.1	90.9	77.9	88.0 **ab** ± 9.0	
15 µM	92.4	87.3	61.4	80.4 **ab** ± 16.6	
20 µM	86.4	79.1	55.9	73.8 **abc** ± 16.0	
25 µM	80.07	67.7	47.7	65.2 **bc** ± 16.3	
30 µM	79.9	48.2	30.9	53.0 **c** ± 24.9	
mean ± SD	88.6 **a** ± 7.7	77.7 **ab** ± 17.2	60.7 **b** ± 21.2		
LSD P ≤ 0.05	20.12				

However, for *L. tropica* axenic amastigotes the cytotoxic effect of HePC showed almost 100% death at 30µM after 48hr in comparison to 14.7% of viable cells exposed to Sb at the same concentration and duration with a significant (P< 0.05) difference between them and other lower concentrations, as exhipited in Tables-3 and 4.

Table 3- The percentage of viable cells of *L. tropica* amastigotes exposed to HePC.

Drug concentrations	**Percentages of amastigotes viability after exposed to HePC**			**mean ± SD**	**LSD P ≤ 0.05**
	12 hr	**24hr**	**48hr**		
5 µM	87.7	81.3	67.3	78.8 **a** ± 10.4	35.39
10 µM	79.9	70.9	52.1	67.6 **a** ± 14.2	
15µM	71.3	66.3	43.7	60.4 **ab** ± 14.7	
20µM	60.2	42.4	28.2	43.6 **abc** ± 16.0	
25µM	58.02	21.9	5.04	28.4 **bc** ± 27.1	
30µM	56.1	13.4	0	23.2 **c** ± 29.3	
mean ± SD	68.9 **a** ± 13.0	49.4 **ab** ± 27.8	32.7 **b** ± 26.6		
LSD P ≤ 0.05	28.87				

Table 4-The percentage of viable cells of *L. tropica* amastigotes exposed to Sb.

Drug concentrations	**Percentages of amastigotes viability after exposed to Sb**			**mean ± SD**	**LSD P ≤ 0.05**
	12 hr	**24hr**	**48hr**		
5 µM	94.7	92.4	90.9	92.7 **a** ± 2.0	29.9
10 µM	90.04	79.3	77.4	82.2 **ab** ± 6.9	
15 µM	88.2	71.7	63.7	74.5 **ab** ± 12.5	
20 µM	81.8	61.2	50.1	64.4 **abc** ± 16.1	
25 µM	76.6	55.3	33.6	55.2 **bc** ± 21.5	
30 µM	70.1	42.7	14.7	42.5 **c** ± 27.7	
mean ± SD	83.6 **a** ± 9.2	67.1 **ab** ± 17.8	55.1 **b** ± 28.2		
LSD P ≤ 0.05	24.55				

From the viability results the IC_{50} of HePC and Sb were determined against *L. tropica* promastigotes and axenic amastigotes after 48 hr of exposure.

The IC_{50}s of HePC and Sb were 17.07µM and 25.72 µM against *L. tropica* promastigotes after 48hr, respectively, as shown in Figure-1.

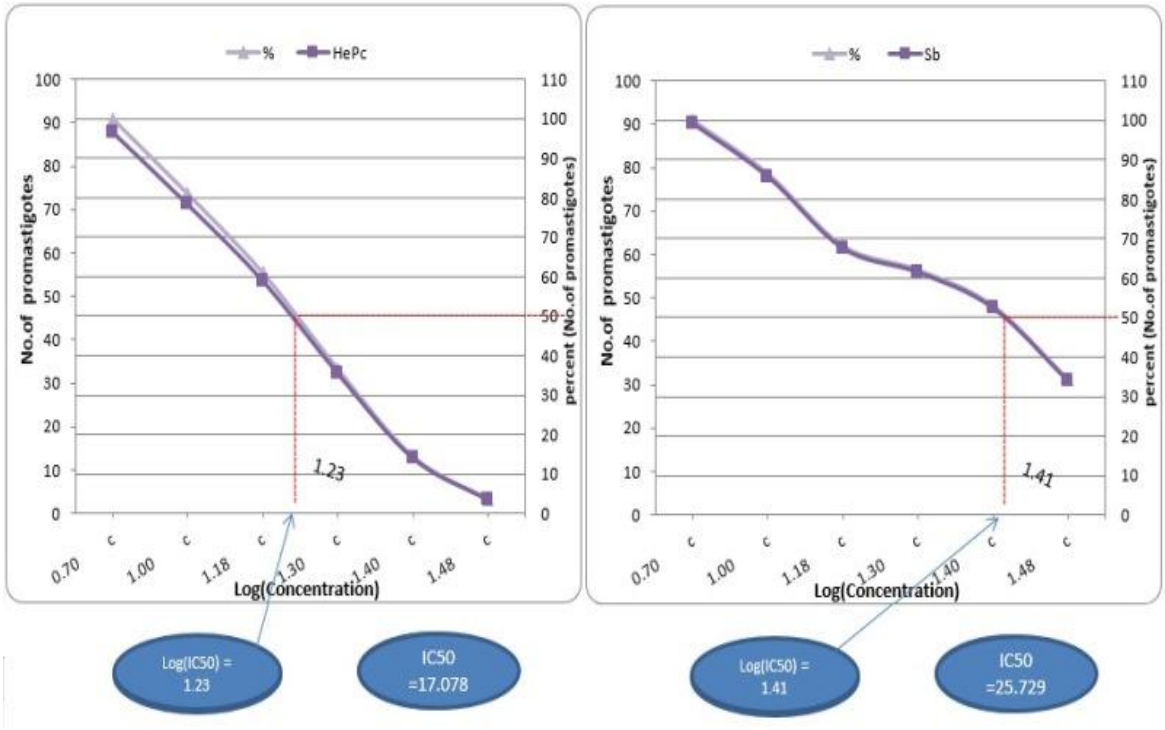

Figure 1-The IC50 of HePC and Sb against *L. tropica* promastigotes after 48hr.

While the IC_{50}s for *L. tropica* axenic amastigotes were 18.08µM and 21.29µM after the same periods, respectively, as shown in Figure -2.

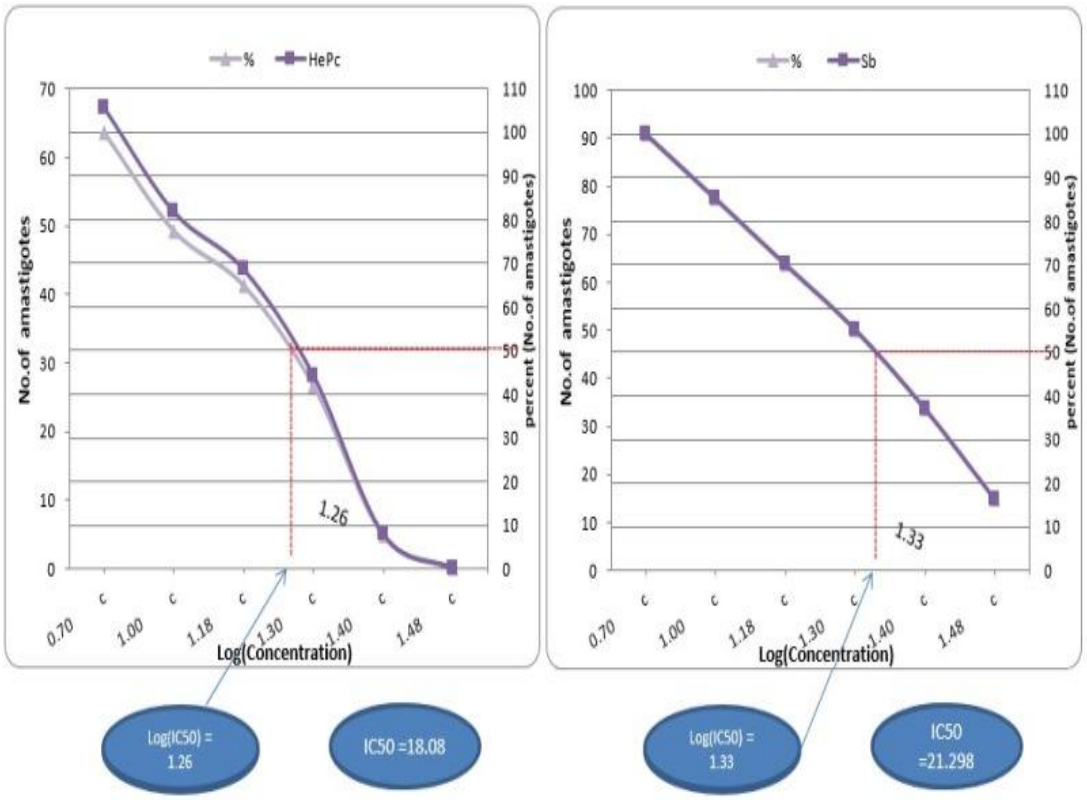

Figure 2-The IC50 of HePC and Sb against *L. tropica* amastigotes after 48hr.

However, HePC is one of the oral drugs that recently have been developed for treatment of leishmaniasis, which is undergoing clinical trials in several countries [4]. The IC_{50} is commonly used as a measure of drug potency in pharmacological research. In the current study, IC_{50} of HePC on *L. tropica* promastigotes and axenic amastigotes was determined using the results of viability test (MTT assay).

The present results showed that both *L. tropica* promastigotes and axenic amastigotes were more sensitive to HePC than Sb (17.07 µM vs. 25.72µM) and (18.08µM vs. 21.29µM) respectively. The observed IC_{50} of HePC on *L. tropica* promastigotes was higher than the IC_{50} against *L. infantum* promastigotes obtained by Khademvatan *et al.* [14], which was 7µM and the IC_{50} against *L. donovani* promastigotes after 48 hr was 11.73mM obtained by Al-shakir and Zghair, [15], Also it was higher than the IC_{50} of HePC achieved at 22 µM and 11µM for *L. major* and *L. tropica* promastigotes respectively, and the ED_{50} of *L. major* and *L. tropica* amastigotes were 5.7 µM and 4.2µM respectively [16], while it was lower than the IC_{50} of *L. donovani* promastigotes treated with HePC obtained by Verma and Dey [6], which was 25µM.

The reported IC_{50} for different strains of *Leishmania* was different from that of the current study. Different IC_{50} among *Leishmania* species showed that each organism responds to various drug concentrations.

DNA fragmentation in promastigotes of *L. tropica* was verified by the presence of fragmented DNA in agarose gel electrophoresis after 48hr for promastigotes and axenic amastigotes treated with 5-30 μM HePC for 48 hr in comparison to Sb treated and untreated cells which didn't show DNA fragmentation , Figures -3a, and 3b

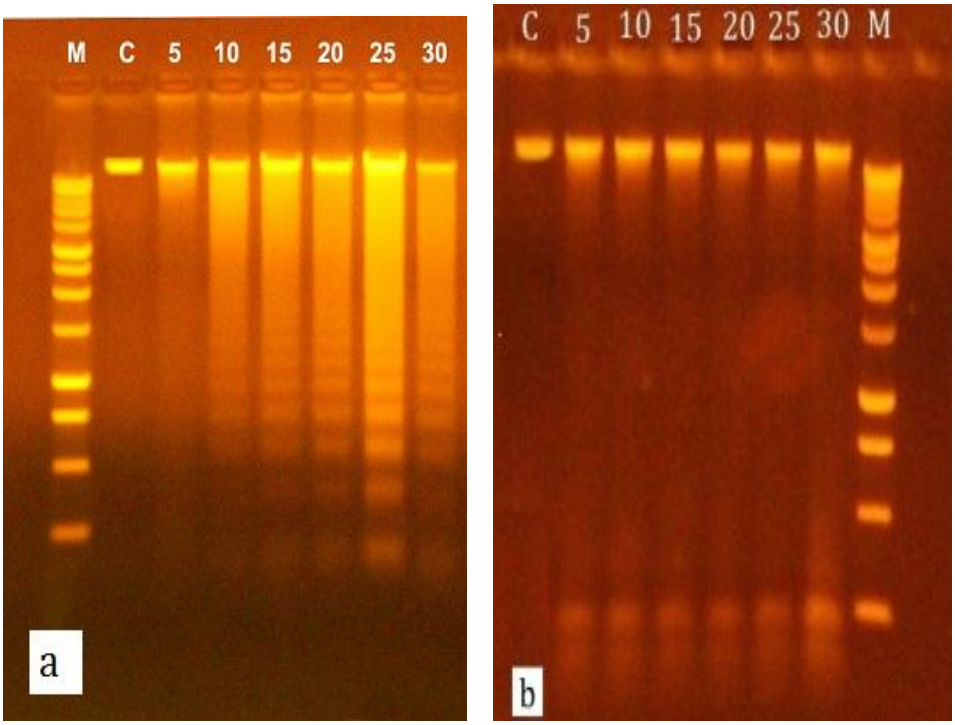

Figure 3- DNA fragmentation detected by agarose gel electrophoresis of (a) *L. tropica* promastigotes, (b) amastigotes, treated with HePC after 48hr. Lanes 5-30 exposed to HePC concentrations, C: untreated control, M: marker (1Kbp).

In the present study, both forms of *L. tropica* (promastigotes and axenic amastigotes) treated with HePC showed programmed cell death features; include cell shrinkage, DNA fragmentation. Similarly to a study conducted previously by Khademvatan *et al.* [14], that *L. infantum* promastigotes exposed to HePC, reported a cell death that shares most features associated with metazoan apoptosis. Also, HePC is able to induce this programmed apoptosis-like cell death in *L. donovani* promastigotes [15], *L. amazonensis* [17], and intra-/extracellular amastigotes of *L. donovani* [6].

HePC has demonstrated activity against *Leishmania* parasites and neoplastic cells. Remarkably, very similar molecular modes of action of HePC were identified against both *Leishmania* parasites and human cancer cells, linking its activity mainly to (i) apoptosis and (ii) disturbance of lipid-dependent cell signaling pathways [18].

Apoptosis-like cell death comparable to metazoan apoptosis has been demonstrated in *Leishmania* promastigotes following exposure to reactive oxygen species, resulting in e.g. nuclear condensation, DNA fragmentation and loss of cell volume [19, 20]. This is confirming by previous finding that tolerance of programmed cell death in *Leishmania in vitro* is linked to emerging multidrug resistance within the parasite [21].

An outstanding advantage of HePC is, its significant activity after oral administration, since few other antileishmanial drugs that are effective by oral administration are known. Ketoconazole, allopurinol, and allopurinol riboside are effective *in vitro*; however, clinical trials showed that cures could be achieved in only a few patients [22].

In conclusion, use of HePC can be a promising treatment. Better understanding of the mechanisms of HePc action may help in finding new targets for the treatment of *Leishmania* parasites.

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