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Effect of Lipopolysaccharide extracted from Escherichia coli on the growth and development of promastigote of *Leishmania tropica* (In vitro study)

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Abstract : In the present study, the inhibitory effect of lipopolysaccharide extracted from *Escherichia coli*, on growth of *in vitro Leishmania tropica* promastigote was investigated. The results showed that the lipopolysaccharide investigated had possesses a marked inhibitory effect, at different concentration on growth of the promastigotes of *L. tropica* .The concentration 100 µg\ml showed 41% inhibition ratio where as it is about 50% at both 25, 250 µg\ml , which representing L.D.50. The concentration 50 µg\ml showed 67% inhibition ratio. Whereas the 75 and 500 µg\ml showed the highest inhibition ratio as it was 78% and 85% , respectively. Lipopolysaccharide had the same effect damper on number of cells, number of generation and the generation time.
Key Word : *Leishmania tropica* , Lipopolysaccharide(LPS), *Escherichia coli*, *Leishmania* Media Culture , Inhibitory Effect of LPS.

Introduction

The species belonging to the genus *Leishmania* sp. to a group of flagellates parasites and biologically diverse that fall within the family Trypanosomatidae where leishmaniasis is the most types of pathogens for humans and some domestic animals , human infected with different types of leishmaniasis causing the occurrence leishmaniasis disease, which is clinically varies depending on the ability of a pathogen to spread in the of deep surface body tissue (Skin) has carried [1] which classification of leishmaniasis disease clinically to four types are: First Visceral form and second Muscocutaneous and third cutaneous form and fourth diffuse cutaneous form, one of the types of cutaneous form of the disease. Since the treatment chemical (Chemotherapy) for disease leishmaniasis include the use of toxic compounds high such as (Antimonials) and other which is given to the patient by intravenous or intramuscular where lead to incidence of side effects have with the patient's treatment in the hospital and stay under medical supervision, for example, when using the Pentamidine drug treatment cause side effects are low blood pressure and kidney failure and gastric disorders as well as severe headaches[2]. Leishmaniasis consider the vector-born parasites that lead to the occurrence of infectious diseases where it is one of the reasons the task of the courtyard in the world, particularly in developing countries. It is also parasite belonging to the genus leishmaniasis parasite obligate interacellular parasite causing disease incidence Alchammanah Leishmanial disease[3].

Morphology

The Leishmania parasites are generally two forms: -

1. Amastigote form is found in the final host.

2. Promastigote form is found in the form of insect carrier and artificial cultures. Amastigote feature being ovoid, small, inanimate, has a clear nucleus is located in the rear and on the nuclear body and have central karyosome, the kinetoplast a rod is located in front of the nucleus is composed of the parabasal body, and a length of about 3-5 µm, either width ranges between (1-3) µm. The promastigote being fusiform shape, moving, with a length of approximately (15-20) µm and width (1.5- 3.5) µm, with a whip longer than the length of the body where the (15-28) µm[4] and has cytoplasmic organelles the same as that found in the amastigote, and both forms are breeding by longitudinal fission. The features of cytoplasm clearly from the nucleus and mitochondria and vacuoles and base granular [5].

Life cycle:

The female sand fly consider the main carrier of the disease to humans, these infected while feeding on the blood containing the parasite from final host infected, and according to[6], the parasite completes its growth and development inside the insect through (4-25) days where passes to shift the main formal form of amastigote into promastigote which is produced in large numbers in the mid gut of the insect, then migrate to the pharynx or the front gut of the insect, where they are ready for injection into the final or vertebrates host [7] . It is noted in the period (6-9) days of eating insect meal infected blood it happen severe nasopharyngeal of the insect and the bite of the insect final host in this period lead to the spread of the disease where promastigote intervention under the skin and some of them are destroyed by phagocytic cells while entering others to the reticuloendothelial system, endothelial cells or phagocytic cells to other parts of the body such as the liver, skin, spleen, bone marrow, or some amastigote may absorb by sand flies during fed on definitive host blood infected where the circle is completed.[6,8,9].

Mode of transmission of parasite to the human

The way the prevailing and accepted to transmitted the parasite to the human is the bites of sand fly female that little insect carrier and it's a length about of 2-3 mm serves as a vital vector to leishmaniasis in various parts of the world known as convey genus *Phlebotomus*. The disease in the countries of the ancient world (Asia, Europe and Africa) while convey genus *Lutzomyia* convey the disease in the countries of the new world (Northern and Southern America)[3],[10] as reported[4] that sand flies of the relatively weak and be Soundless in flight and are found in wet and dark places, where activity is increasing in places The evening and during the night hours, with an estimated belonging to the genus *Phlebotomus* species, including approximately.

The (500) type in the world, but (30) species of which only promised as vectors of the disease[6, 11] have found that there are other modes uncommon to transporting the parasites, such as a congenital transmission where the parasite is transmitted through blood transfusions, the parasite is transmitted in occasional cases or through the shared use of injection among people rarely have to transport by vaccination in farms.[4,6]

Although there are a number of chemical compounds that are used to treat leishmaniasis but it is not devoid of toxic effects resulting from the long of the treatment period, as well as the high cost of treatment in a number of these compounds, so has the need to search for new compounds effective such as materials chemical and plant and bacterial extracts including compounds already used to treat bacteria, fungi and protozoa such as antifungal ketoconazole [12] and plant extracts *Caparis spinasa* and *Citrallus colocynthis* against leishmaniasis [13] . And water extracts for fruits *Melia tropica*, and leaves and fruits of *Nerium oleander* on the *L. tropica* [14] . The LPS on the gram negative bacteria considers as immune modulators that are still intensive research highlights it due to its chemical properties reflected on the vital effects because research indicated that LPS from natural materials that have superior capabilities in influencing the microorganisms, it is worth mentioning that some bacterial extracts toxic effect differ in their effects on different organisms, including protozoa[15,16] . So came the aims of the current study, to determine the effect of LPS derived from *E.coli* on the promastigote of *L. tropica In vitro.*

Materials and methods

Nutrient agar media

The media prepared by dissolving 23 grams of the agar in a liter of distilled water, adjust the pH at (7.2), then autoclave with temperature (121) c° and pressure (1.5) atmosphere for 15 minutes, and pouring in the petridishes.

Bacteria activate Initial medium

The media prepared by dissolving 20 gm. of glucose Glucose, (1) gm. of NH4Cl and (5.44) gm. of KH2PO4 in a liter of sterile distilled water, added to (6) ml of mineral salts solution[17].

The LPS production medium

The media prepared by dissolving 20 gm. of glucose, 0.5 gm. of NH4Cl, (2.72) gm. of KH2PO4 and (6) ml of mineral salts solution[17].

Bacterial sample

Pure isolation was obtained of the *Escherichia coli* from Bacteriological laboratory/Department of Biology / College of Science for women / Babylon university.

Bacterial growth

Bacterial isolate had grown at the nutrient agar media and the later put an incubator at a temperature of (28) c° for a period of 24 hours, then kept the temperature (4) c° until use.

- Bacteria has been actived on the Initial medium as inoculated with bacteria (3 ml) from the activation media and incubated at a temperature (28 - 32) c° for a period of 18 hours.
- Transport about (2%) from activation media to the production medium is the best media for the production of LPS[18]. And beakers incubated in shaker incubator (150) round / min at temperature (28) c° for a period of 72 hours.

Extraction of Lipopolysaccharide (LPS)

Followed the method of[19] modified by[18] as follows : -

- Added EDTA-Na2 to the production media with concentration(2) mM, beakers putted in shaker an incubator at a temperature of (37) c° for a period of 30 minutes.
- Bacterial cells were treated to ultra sonicator for 30 seconds at a frequency of 20 vibrations per second to break the cells, taking into account the use of crushed ice.
- Bacterial cells deposited by cooled centrifuge with speed (27420) g for half an hour at a temperature of (4) c°.
- Supernatant collect and centrifuged for one hours to get rid of all remnants of bacterial cells.
- Acetone was added to the supernatant at ratio 5: 1 (v / v) and leave at temperature (4) c° for 24hours to get LPS as white suspended minutes stuck.
- Use rotary evaporator temperature (40 - 50) c° for the purpose of obtaining LPS deposit is thick viscous liquid.
- Poured the substances in a petridishes and transferred to the incubator with degree (32) c° for a period of 48 hours for the purpose of drying, and preserved until use.

The inhibitory effectiveness of the LPS testing

For the purpose of the test of promastigote to precipitate LPS sensitivity was prepared concentrated solutions of this extract in distilled water and then sterilized by filtration using membrane filters (0.22) and kept in the refrigerator as stock solution until used in the preparation of various concentrations of the extract, then add at least three repeated concentration of all the next day to add a promastigote. Then calculate the number of cells during different time stages 24.72, 96.120. Were compared with various concentrations of the extract with the control sample.

- Used high concentrations of LPS (100, 250, 500) µg\ml. with volume of 0.1, 0.3, 0.5 ml, respectively, as high dosages.

- Used low concentrations of LPS (25, 50,75) µ g\ml. with volume of 0.1, 0.3, 0.5 ml, respectively, as low dosages.

Leishmania samples

Samples were collected for skin leishmaniasis of patients admitted in Al-Hilla General Hospital and Public Health Laboratory in the Babylon province (Picture 1). So took the sample after sterilization of the skin with 70% ethyl alcohol and then withdraw 0.2 ml under the ulcer by using a clean medical syringe in order to preparation of direct smear and then culturing the parasite[20].

Direct smear preparation

The sample that was taken from under the skin on a clean glass slide and then dried and fixed by methyl alcohol for 1-2 minutes is placed and after being washed with distilled water and then dyed by slide with Geimsa stain (10-15%) for 30 minutes and then washed with distilled water and dried and examined under a compound microscope with oily immerssion 100 x (Picture 2).

Culturing of *Leishmania tropica*

L. tropica culture developed parasites in two phases media cultures (Tobie's medium) to get a good growth for promastigote in short time [21] .This media consists of two phases, the semisolid phase and liquid medium and components of these as the two mediums or phases as follows : -

Semisolid medium every liter contains as follows: -

NaCl	6.91gm	KCl	0.29gm	Peptone	1.0gm
$CaCl_2H_2O$	0.22gm	D-glucose	0.77gm	*Beef extract*	0.3gm
$NaHCO_3$	0.1gm	Agar	4.0gm	D.W	800ml
		pH=	7.4±0.1		

Dissolve all solids with distilled water except blood and antibiotic not dissolved and adjusts the pH to 7.2 .and then added to this medium 200 ml not coagulant blood rabbit is rennet and 50 gm of antibiotic Gentamycine then the media are placed in the autoclave at atmospheric pressure for 20 minutes and at a temperature of 121 c ° and taken from it 5 ml. stuck in the form of a bottle with a tight lid diagonally placed to get a larger surface area for the development of the parasite taking into account the non-pollution and kept refrigerated . the culture was used of the first to isolate the parasite and development [22] .

Liquid medium each liter contains as follows: -

NaCl	6.91gm	KCl	0.29gm	Beef extract	0.3gm
$CaCl_2$	0.22gm	D. Glucose	0.77gm	Brain heart infusion	10gm
$NaHCO_3$	0.1gm	Peptone	1.0gm	D.W	800ml
		pH=	7.4±0.1		

[23]descripted and modified for semi-solid special media[22], which is used to produce large numbers of the parasite. Each liter contains 200 ml of diluted non coagulant blood rabbit with ratio 2: 1 (water - Blood) and the antibiotic, which was added after media sterilization by autoclave at atmospheric one pressure for 20 minutes. all of these media are incubated at a temperature of 37 c° for 24 hours. then stocked under 4 c ° until use.

Add 2 ml of liquid medium to the semi-solid media and keep refrigerated until use. For the purpose of the growth of the parasites in the media convey inoculum about of 0.1 ml of liquid medium containing a good growth of promastigote to the culture by medical sterile syringe and kept in an incubator with at 26 c° and then follow the growth and reproduction of parasites of these media using an optical microscope for consecutive days and be careful not to contaminate circles until it reaches the number (1×10^7 to 1×10^7) parasites / ml

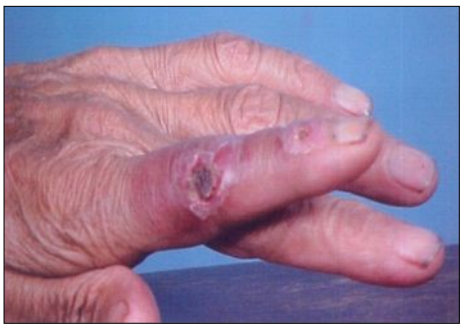
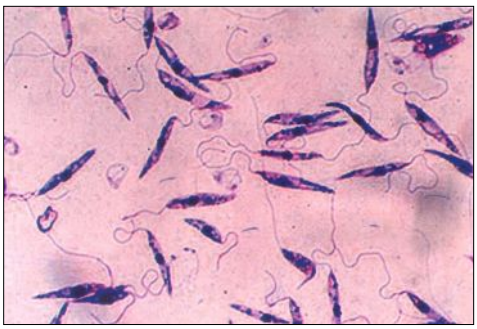


Figure (1): Type wet skin ulcer *L.tropica*



Figure(2) : Promastigote *L.tropica* , Geimsa stain by[24]

Results:

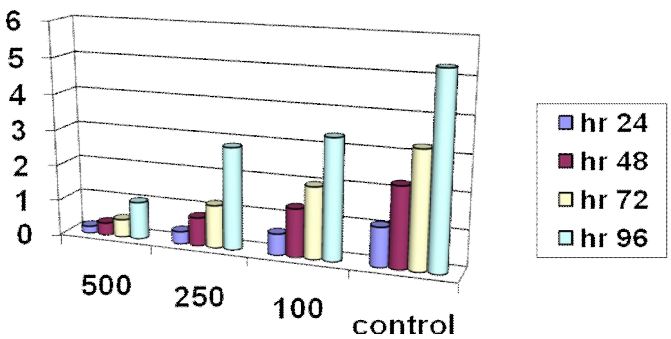
Determine the logarithmic phase of the promastigote *L.tropica*.

Table(1) and (2) shows the effect of high and low concentrations lipopolysaccharides on the number of promastigote of *L.tropica* (control group) developing or growth in the Tobies media and select which developed the logarithmic phase by red blood cells count (Haematocytometer). Since the linear growth increasingly limit the period of 96 hours and zero linear increase after this period, so this period consider the period of logarithmic growth-phase of the promastigote (Figure 3). The number of generations 5.61 generation and generation time its 16.65.

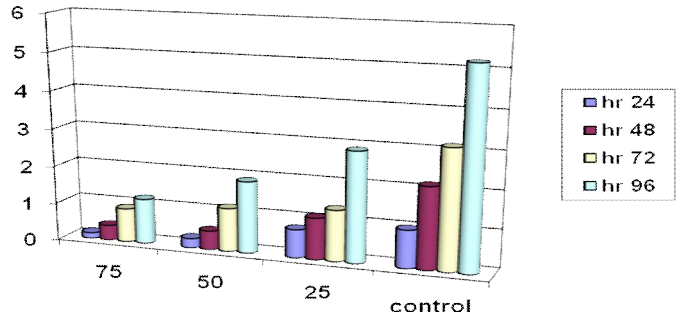
The influence of LPS of *E.coli* on the growth of promastigote for *L.tropica*.

In the current study was to determine the LPS effectiveness of different concentrations added to the growth medium against the promastigote, where the average number of measurement promastigote number capable of movement in comparison with the control group, was to determine the effect of the extract on the growth of leishmaniasis by observing the growth curve (Fig. 3, 4). The results of the current study showed that the LPS extract effective against promastigote to parasite, since the inhibition rate showed a 78% .85% for two concentration(75, 500) micrograms / mL, respectively, and has led to the reduction of the number of generations to 3.28 generation at the concentration 500 µg / ml , after 96 hours of treatment compared to the control group which was 5.61, as for the concentration of 75 µg / ml decline was 3.6 generation compared to the control group (5.61) .Also two concentration (75, 500) micrograms / ml, record generation time of 25.83 and 28.2 hours , respectively , compared to the control group , which were

16.65 hours (Table 1, 2) .



Figure(3) : Effect of high concentrations of lipopolysaccharides on the growth of promastigote for *L.tropica*



Figure(4) : The effect of low cocentration of lipopolysaccharides on growth of promastigote for *L.tropica.*

Table (1) : The effect of highest concentrations of lipopolysaccharides on the number of promastigote *L .tropica* and the number of generations and generation time and the percentage of growth inhibition.

(%) Inhibition	(%) Growth	Generation time (hours)	Generation No.	No. of promastigote 10^6 \ ml.×	Time (hours)	Concentration Microgram\ ml.
82	18	24	0.9	0.1	24	**500**
75	15	26.4	1.7	0.25	48	
76*	15	30.9	2.22	0.4	72	
85*	19	28.2	3.28	0.95	96	
69*	31	13.17	1.7	0.25	24	**250**
65*	35	15.9	2.9	0.7	48	
64	36	20.01	3.47	1.1	72	
53	53	19.75	4.72	2.75	96	
46*	54	9.17	2.47	0.5	24	**100**
40	60	12.7	3.64	1.25	48	
39	61	16.5	4.21	1.9	72	
41	63	18.8	5.01	3.25	96	
0	100	6.8	3.34	1.0	24	**Control**
0	100	10.5	4.38	2.15	48	
0	100	14.23	5.01	3.15	72	
0	100	16.65	5.61	5.2	96	
F calculated= 25.9 * F tabulated=6.36						

Significant differences($P \leq 0.05$).*

Table (2) : The effect of lowest concentrations of lipopolysaccharides on the number of promastigote *L .tropica* and the number of generations and generation time and the percentage of growth inhibition.

(%) Inhibition	(%) Growth	Generation time (hours)	Generation No.	No. of promastigote 10^6 \ ml.×	Time (hours)	Concentration Microgram\ ml.
88*	22	18.05	1.21	0.15	24	**75**
88*	22	20.05	2.21	0.4	48	
70	30	21.56	3.22	0.9	72	
78*	24	25.83	3.6	1.2	96	
69*	31	13.2	1.9	0.25	24	**50**
64	26	18.45	3.47	0.5	48	
62	38	19.67	3.53	1.15	72	
67*	37	22.11	4.12	1.9	96	
33	77	7.68	3.07	0.75	24	**25**
47	53	13.3	3.47	1.1	48	
66*	44	18.55	3.74	1.35	72	
51*	56	19.45	7.80	2.9	96	
0	100	6.8	3.34	1.0	24	**Control**
0	100	10.5	4.38	2.15	48	
0	100	14.23	5.01	3.15	72	
0	100	16.65	5.61	5.2	96	
F calculated= 50.6 * F tabulated=6.36						

Significant differences($P \leq 0.05$).*

Discussion

It became clear that the leishmaniasis is globally epidemic spread, so it is necessary to find the right treatment for its , especially the medical and pharmaceutical materials provide an effective and without side effects to eradicate the disease is very limited.

The use of lipopolysaccharides (LPS) extracted from the *E.coli* as influential on the vitality and effectiveness of parasites skin leishmaniasis and studies on the use of this substances is not available, but using only of this material against protoscolices of the *Echinococcus granulosus* as immune modulators, which gave good results in stimulating the immune system and thus in the killing of these protoscolices and limit the spread [25].

Most of the people living in endemic disease with leishmaniasis heavily depends on processing traditional medicine to relieve the symptoms of the disease , has current studies headed towards the investigation of plant and bacterial extracts , which have effective against *Leishmania* , and this is done by using the form of promastigote so as to ease keep it under appropriate conditions *In vitro* as well as the promastigote study could indicate the possibility of the existence of effectiveness against *Leishmania* of material to be tested [26].

Where notes effectiveness through influence on the preparation of promastigote the and the generation time and the number of generation have and this is what we have to study it in this research were to prove the existence of the influence of so-called against *Leishmania* LPS *In vitro* through the effect of these extracts on the growth of promastigote in the of Tobies media as well as their impact on the number of time and generations .

And generally it has been to determine the number of promastigote in the Tobies media that growth during the period (96) hours of any growth during the logarithmic phase, where it was noted that the numbers of promastigote multiply several times to increase the period of growth until it reaches the phase logarithmic reaching number (5.2×10^6) parasite / cm^3 Table (1) and shape (3 and 4) and this is similar to the result to some

extent brought by Hayali (2000), where the number of promastigotr that growth in the Tobies media during the phase logarithmic to (3 - 5 × 10^7) parasite / cm^3 for parasites *L. donovani* and *L. major*, while the numbers of promastigote to parasites *L. major* about (3.45 × 10^7) parasite / cm^3 (Khan , 2001) and approximately

(2 - 4 × 10^7) parasite / cm^3 for parasites *L. tropica* and *L. donovan* [27].

The number generation and generation time account (Control group) of promastigote of *L. tropica* where was (5.61) and generation (16.65) an hour, respectively, when the logarithmic phase Table(1 and 2). In light of these results show that there is a toxic effect and a clear LPS on the growth of parasites *L. tropica*, through Note the gradual decrease of promastigote with increasing concentrations of extracts used, and this is in line with what it says[13].in the course of his studies on the effect of extracts of *Caparis spinasa* on the growth of the promastigote for *L. major* as well as his studies on the effect of the same extracts on *Trichomonas vaginalis* parasite growth[28] .

As well as what brought[29,].Where the promastigote exposing *L. major* to various concentrations of anhydrous copper sulfate to a gradual decline in the numbers of promastigote, which increases with concentration and duration of exposure . The results showed that the focus half lethal dose LD50 for LPS extract multi sugar fatty value is 25 micrograms when the phase logarithmic growth is 51% (Table 2 and Figure 4) while the value of the LD50 for the concentration of 250 micrograms is 53% (Table 1).

The concentrations LPS extract 500 and 75 micrograms , which were the best in their effects and to a percentage of inhibition of the growth has led up to (85%) and (78%), respectively, where the number of promastigote 0.95 × 10^6 and 3.28 generation of concentration of 500 micrograms and number of promastigote 1.2 × 10^6 and 3.6 generation of the concentration of 75 micrograms during logarithmic phase , and this is in line with what it says [29] .Where the of promastigote exposing *L. major* for the concentration (12.5) µg / ml of anhydrous copper sulfate to lower growth rate (64%) ,

These results are also consistent or agree with the[20], in his study of the effect of drug Chloropromazine on the growth and metabolism of *L. donovani* and *L. major* where he found that the use of high concentrations (20) and (25) µg / ml led to the occurrence of the inhibition of the growth rate (90%) of promastigote for *L. donovani* and *L. major* respectively.

Inhibitory effect of these extracts may be due to contain the effective compounds affect the forward growth of promastigote *In vitro* where is LPS extracts of gram negative bacteria of immune modulatores that are still intensive research highlights it due to its properties of chemical reflected on the effects vital as research indicated that the LPS from natural materials that have the ability to increase the functional and metabolic efficiency of phagocytosis in macrophages operations and kill germs including LPS extracted from the *Escherichia coli* [30, 31, 32, 33] , and that the LPS when associated with isolated liver cells leads to the production of tumor necrosis factor (TNF) and released , it also stimulates a monocytes to build mediator agents itself and excreted [34] , It is clear that there are similarities between two concentrations(75 and 500)micrograms in effect inhibitory possibly due to differences in molecular weight between them because the LPS concluded in stages and this is agree with [35] , that the LPS a high weights of molecular and affect concentrations of low-lying and this explains why the effect of low-lying concentrations in the current study

On the other hand, the reason for the effect of LPS may be due to adhesion receptors on the outer wall of *Leishmania*, which led to prevent contact with the outside environment, affecting vitality. studied[36], Effect of LPS extracted from the bacteria *Rhizobium* sp. on the vitality and effectiveness of the protoscolices of hydatid cysts noted that this materials may have affected the vitality and effectiveness of the protoscolices by (100%) after the tenth day and when using two concentrations (125, 250) µg / ml . It may be the effect of LPS against cutaneous leishmaniasis lesions in the current study may have interfered with or cleared the important fatty acid inside the cell as it guarantees to some extent on the construction of other compounds such as proteins, carbohydrates and nucleic acids

Where [13] explained that there are changes in the quality of fatty acids of promastigote of *L.major* that treated by LD50 of bitter melon extracts and compared to control group after 96 hours and varied quality of fatty acid for any experiments . Perhaps this change return to the lipid to promastigote -treated and non –treated to the fatty acids in the culture media[37,38, 39] as the lipid A presence in multi sugar fatty extracted from the bacteria *Pseudomonas aeruginosa* has the effect of endotoxins to *Leishmania* [38 -42].

Conclusion

So conclude from the present study that the LPS effective against cutaneous leishmaniasis lesions so we recommend expanding the study in this area to determine the best concentrations can be used in such studies *In vitro* and *In vivo* to open new horizons in the treatment of leishmaniasis .

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