

## ARTICLE / INVESTIGACIÓN

## Effect of Synergism of Thalidomide and Liposomal Amphotericin-B on *Leishmania tropica* and *Leishmania donovani* Promastigote

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**Abstract:** This study aims to find safe and effective anti-leishmaniasis drugs; thus, the synergism between thalidomide and liposomal amphotericin-B was tested as antileishmanial on *L. tropica* and *L. donovani* promastigote *in vitro*. IC<sub>50</sub>, IC<sub>90</sub> were determined at the Log phase of thalidomide and were (10), (25) µg/ml for *L. tropica* and (12.5), (30) µg/ml for *L. donovani*. Moreover IC<sub>50</sub>, IC<sub>90</sub> were determined at Log phase of Liposomal amphotericin-B and were (5), (20) µg/ml for *L. tropica* and (5), (25) µg/ml for *L. donovani*. Additionally, synergistic effect IC<sub>50</sub> of the two drugs were determined when Liposomal amphotericin-B fixed it, and thalidomide concentrations changed was (2.5+0.5) µg/ml on *L. tropica* and (2.5+1) µg/ml on *L. donovani*. When thalidomide was fixed, and Liposomal amphotericin-B was changed, it was (2.5+2) µg/ml for both *L. tropica* and *L. donovani*. The synergistic effect on the morphology of both promastigotes forms was observed.

**Key words:** Leishmaniasis, thalidomide, liposomal amphotericin-B, synergistic.

### Introduction

Leishmaniasis is a zoonotic parasitic disease caused by the Leishmania parasite. Leishmaniasis occurring in most tropical and sub-tropical countries is classified as a neglected tropical disease<sup>1</sup>. Leishmaniasis is an intracellular parasite transmitted by the bit of sand fly species phlebotomist. Leishmaniasis considers one of the seven most crucial tropical diseases on all continents<sup>2</sup>. Approximately 98 species are suspected vectors of human leishmaniasis<sup>3</sup>. More than 20 species of obligate intracellular protozoa of Leishmania caused human infection<sup>4</sup>. Limiting access to the treatment caused infection of more than one billion persons<sup>5</sup>. According to the classification of WHO, there are three types of Leishmaniasis infected the human:

#### Visceral Leishmaniasis (VL)

It is a fatal disease; more than 95% of the case if untreated directly. Human infection occurs due to (*L. donovani*, *L. infantum*) known as *L. chagasi* in the new world. The protozoan infects the phagocytic cell (macrophage and monocytes), amastigote transformed by macrophages to the liver, spleen and bone marrow. It caused swelling in them<sup>6</sup>.

#### Cutaneous Leishmaniasis (CL)

It is a common form of Leishmaniasis and causes skin lesions. This typically evolves from papules to nodular plaques to ulcerative sores. In old-world include *L. major* and *L. tropica* while *L. mexicana*, *L. braziliensis*, *L. amazonensis*, *L. venezuelensis* and *L. ethiopia* found in the new world<sup>7</sup>.

#### Mucocutaneous Leishmaniasis (MCL)

This type of Leishmania causes disfiguring lesions of the nose, mouth and throat mucous membranes. Infection

can spread through the bloodstream or lymphatic system caused by *L. braziliensis* and *L. panamensis*<sup>8</sup>. The leishmaniasis treatment requires a very long time of therapy, is very limited, costly to the patients, and has high toxicity for most drugs<sup>9</sup>. Pentavalent antimonials are the most commonly used drugs for treating Leishmaniasis, despite their variable efficacy for both cutaneous and visceral leishmaniasis<sup>10</sup>. Due to its accumulation in the tissues, antimony can cause severe effects like vomiting, anorexia, nausea, myalgia, headache, abdominal pain, lethargy, and arthralgia; additionally, it rarely causes a severe reaction with cardiac arrhythmia<sup>11</sup>. Pentamidine and amphotericin B are the second-line drugs for Leishmaniasis. However, toxicity and emerging resistance has discontinued the pentamidine uses, whereas amphotericin-B is capable of causing severe toxicity, hospitalization requirements, and the high costs of therapy<sup>12</sup>. Miltefosine is registered as an anticancer agent; with regards to CL and VL therapy, the main advantages were oral efficacy and a short course of treatment; therefore, it has significant limitations in the leishmaniasis therapy due to its teratogenic effects and longevity, which may enhance the resistance to drugs<sup>13</sup>.

Thalidomide (Thalomid) is a synthetic of glutamic acid (alpha- N- phthalimido-glutarimide) is a glutamic acid<sup>14</sup>. The chemical structure is C<sub>13</sub>H<sub>10</sub>N<sub>2</sub>O<sub>4</sub> with a 258.23 g/mol molecular weight. It is very efficacious in treating patients with chronic erythema nodosum leprae. Thalidomide works as selectively inhibiting tumor necrosis factor-α gene expression, which is concerned with nerve damage's pathogenicity in leprosy<sup>15</sup>. Additionally, liposomal amphotericin B (AmBisome) is aliped-complex, colloidal and liposomal form; the composition consists of a mixture of phosphatidylcholine, cholesterol and distearoyl phosphatidylglycerol. Its structure

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re is C47H73NO17, with a molecular weight of 924.09 g/mol. Amphotericin B is an antifungal agent effective versus clinically pertinent molds and yeasts containing *Aspergillus*, *Candida spp.* and filamentous molds, approved to treat fungal infections worldwide<sup>16</sup>. The drug was developed to reduce adverse effects and improve pharmacokinetics, bioavailability and lower toxicity but still very expensive and unstable at higher temperatures<sup>11</sup>. The study tackled the inhibitory effect of thalidomide and liposomal amphotericin B separately and synergistically on *L. tropica* and *L. donovani* promastigote and tried to reduce the dose of amphotericin in the presence of thalidomide. That combined therapy may be promising for the treatment of Leishmaniasis.

## Materials and methods

### Biological activity of thalidomide and liposomal amphotericin B

#### Leishmania used

We used two local type of *L. tropica* and *L. donovani*. Stock culture gets from Biotechnology Center / Al- Nahreen University / Baghdad. The culture is cultivated in Tobie medium<sup>17</sup>.

#### Parasites cultivation

1.9 ml of liquid phase was added to McCantry vials containing 5 ml of slants solid phase, then 0.1 ml of parasites inoculums were taken from stock culture through the logarithmic phase, so the initial density was  $2 \times 10^7$  / ml, new promastigotes were incubated for 4 days at 26 °C, was counting the number of promastigote by using a hemocytometer chambers.

#### Thalidomide (Thalix-100)

Thalidomide was used as a capsule in a concentration of 100 mg made by Fresenius Kabi India get it from a local pharmacy. 1000 µg/ml was prepared as a stock solution, then prepared six concentrations (10, 12.5, 15, 20, 25, 30) µg/ml they were used to identify the IC50 and the IC90 for the cultured parasites.

#### Liposomal amphotericin B (Ambiome)

Liposomal amphotericin B also gets it from the local pharmacy as a 50mg injection concentration made by the

Ireland drugs company. The stock solution was prepared as 1000 g/ml then five concentrations (5, 10, 20, 25, 30) µg/ml were used to determine IC50 and IC90. The concentrations were kept in the refrigerator at 2-8 °C<sup>18</sup>. On the other hand, the Synergy between the two drugs was as follow: First, the liposomal amphotericin -B was fixed at a concentration of 2.5 µg/ml, while the concentrations of thalidomide were (0.25, 0.5, 1, 2) µg/ml. Secondly, the thalidomide was fixed at a 2.5 µg/ml concentration, whereas the concentrations of liposomal amphotericin-B were (1, 2, 3, and 4) µg/ml. All concentrations were used to determine the IC50. Promastigotes numbers were identified at different time intervals (24, 48, 72, 96) hrs.

### Effect of thalidomide and liposomal amphotericin -B on the morphology of *L. tropica* and *L. donovani* promastigotes

The effect of drugs on morphology was observed by using Giemsa stain for cutaneous and visceral promastigotes at IC<sub>50</sub> concentration; then, the parasites were examined and photographed using (OPTICA, ITALY) compound light microscope supported with a camera and compared with the control group (non-infection).

#### Statistical Analysis

Spss version 21 software was used to analyze the data<sup>19</sup>.

## Results and discussion

Table (1) shows the influence of various concentrations of thalidomide on the growth of *L. tropica* compared with the control group through different time intervals. 10 µg/ml represents 50% of the log phase (72) hrs. Inhibitory concentration. At the same time, the highest concentration that inhibits 90% of growth was 25µg/ml.

Table (2) display the effect of various concentrations of thalidomide on the growth of *L. donovani* compared to the control group through different times periods. 12.5 µg/ml appears to represent 50% the inhibitory concentration of the log phase (72) hrs. Whereas the highest concentration inhibits 90% of growth was 30 µg/ml.

This study appears highly effective of thalidomide it was 25, 30 µg/ml concentrations of it to get 90% inhibitory growth and 10, 12.5 µg/ml concentrations to get 50% inhibitory growth of *L. tropica* and *L. donovani*. These results which

| Exposure time (h)<br>Treatment (µg/ml) | 24                       |              | 48                       |              | 72                       |              | 96                       |              |
|--|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|  | Mean* ± SE               | Inhibition % | Mean*± SE                | Inhibition % | Mean* ± SE               | Inhibition % | Mean* ± SE               | Inhibition % |
| Cont.                                  | 1.13 ± 0.02 <sup>a</sup> | ---          | 22.9 ± 0.31 <sup>a</sup> | ---          | 46.5 ± 0.95 <sup>a</sup> | ---          | 33.2 ± 0.52 <sup>a</sup> | ---          |
| 10                                     | 0.63 ± 0.03 <sup>b</sup> | 44           | 16.5 ± 0.81 <sup>b</sup> | 28           | 37.1 ± 0.51 <sup>b</sup> | 20           | 29.5 ± 0.68 <sup>b</sup> | 11           |
| 12.5                                   | 0.53 ± 0.03 <sup>c</sup> | 53           | 11.2 ± 0.60 <sup>c</sup> | 51           | 22.9 ± 0.49 <sup>c</sup> | 50           | 16.7 ± 0.57 <sup>c</sup> | 49           |
| 15                                     | 0.43 ± 0.01 <sup>d</sup> | 61           | 8.73 ± 0.38 <sup>d</sup> | 61           | 17.8 ± 0.51 <sup>d</sup> | 61           | 11.8 ± 0.60 <sup>d</sup> | 64           |
| 20                                     | 0.30 ± 0.01 <sup>e</sup> | 73           | 4.53 ± 0.48 <sup>e</sup> | 80           | 15.0 ± 0.25 <sup>e</sup> | 67           | 9.20 ± 0.55 <sup>e</sup> | 72           |
| 25                                     | 0.25 ± 0.02 <sup>e</sup> | 77           | 3.50 ± 0.17 <sup>e</sup> | 84           | 7.66 ± 0.63 <sup>f</sup> | 83           | 6.50 ± 0.25 <sup>f</sup> | 80           |
| 30                                     | 0.14 ± 0.02 <sup>f</sup> | 87           | 3.10 ± 0.05 <sup>e</sup> | 86           | 3.93 ± 0.35 <sup>g</sup> | 91           | 3.50 ± 0.17 <sup>g</sup> | 89           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 1.** Effect of thalidomide on the growth of *L. tropica* promastigotes.

are in agreement with (20) The showed that uses of chlorpromazine as an antileishmanial agent in concentrations of 20, 25 µg/ml inhibit 90% of *L. major* and *L. donovani* growth in vitro at log Phase. Other researchers used zinc sulfate to test the effect on *L. major* and *L. tropica in vitro* and observed that parasites in the control group were atypical growth procedures, but in the experimental group revealed a much slower growth rate<sup>21</sup>. Another researcher indicates that synthesized pyrrolidine in different concentrations inhibits the growth of *L. infantum* compared with the control group<sup>22</sup>.

Thalidomide stimulates apoptotic-specific pathways via caspase 8-mediated cell death. At the mitochondrial level, they are in charge of c-junk terminal kinase (JNK) conditional release of cytochrome-c and S mac into the cytosol, where they organize the activity of the molecule that influence death apoptosis by stimulating T cells to produce IL-2, it alters natural killer cell (NK) function and numbers, thereby increasing NK-dependent cytotoxic activity<sup>23</sup>. Thalidomide bounded to factor (TNF)-alpha inhibiting the production of interleukin (IL), a growth factor of 50% of the living cell, and they activate apoptotic pathways, causing cell death of promastigotes and decrease the growth rate to kill the promastigote cell. Thalidomide uses for cutaneous lesions treatment. Thalidomide was hypothesized to inhibit the activity of CRBN-containing E3 ligases by directly binding CRBN<sup>24</sup>. This study agreement with (25) that the effect of some medicinal plants is used anti promastigote activity against *L. aethiopic* and *L. donovani*.

In the light of growth, the inhibitory effect of liposomal amphotericin B indicates that the lethal concentration of 50% of *L. tropica* was 5µg/ml, while the concentration of

20µg/ml was killed 90% of promastigotes at the log phase (72) hrs. compared with the control group, table (3)

Besides that, the inhibitory impact of liposomal amphotericin -B on *L. donovani* indicate the concentration of 5 µg/ml was killed at 50% of promastigotes, while the concentration of 25 µg/ml killed 90% of promastigotes at log phase (72) hrs. compared with the control group, Table (4).

There are many studies and research about leishmaniasis treatment. Most researchers used liposomal amphotericin -B to treat *L. tropica* and *L. donovani* promastigote in India; this drug is used because it is a safe and effective therapy for visceral and cutaneous leishmaniasis<sup>26</sup>. Additionally<sup>27</sup>, they proved that due to the failure of existing drugs, amphotericin-B has a cure rate of 100% at a dose of 0.75-1 mg/ kg for 1-20 intravenous infusions daily. It causes severe toxicity, e.g., hypokalemia, nephrotoxicity, myocarditis, and even death, also the high cost of therapy. However, doses of 5mg/kg/day of liposomal amphotericin B caused significantly lower toxicity rates than amphotericin B lipid complex or conventional amphotericin B deoxycholate. WHO recommended that 10 or 15 mg/kg of liposomal amphotericin B have high cure rates<sup>28</sup>. This study agreement with (29) reported that amphotericin-B acts by binding to ergosterol in the cell membranes; the binding might lead to modulation in cell membrane permeability, formation of pores, induction of metabolic shock, ion leakage and promotion of cell death.

Fluorescent and gold liposomes (loaded with amphotericin B or empty liposomes) were used in vitro and in vivo to elucidate the liposomes bounded with cell walls in pathogens responsible for the infection. Empty liposomes (without amphotericin) remain intact without causing cell disruption.

| Exposure time (h) | 24                       |              | 48                       |              | 72                       |              | 96                        |              |
|-------------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|---------------------------|--------------|
|                   | Mean* ± SE               | Inhibition % | Mean*± SE                | Inhibition % | Mean* ± SE               | Inhibition % | Mean* ± SE                | Inhibition % |
| Cont.             | 1.15 ± 0.01 <sup>a</sup> | ---          | 26.5 ± 0.32 <sup>a</sup> | ---          | 53.6 ± 1.28 <sup>a</sup> | ---          | 45.50 ± 0.55 <sup>a</sup> | ---          |
| 10                | 0.06 ± 0.00 <sup>b</sup> | 46           | 13.9 ± 0.49 <sup>b</sup> | 47           | 26.7 ± 2.80 <sup>b</sup> | 50           | 22.2 ± 0.60 <sup>b</sup>  | 51           |
| 12.5              | 0.55 ± 0.01 <sup>c</sup> | 52           | 12.7 ± 0.49 <sup>b</sup> | 52           | 20.1 ± 0.44 <sup>c</sup> | 62           | 19.7 ± 0.64 <sup>c</sup>  | 56           |
| 15                | 0.49 ± 0.01 <sup>d</sup> | 57           | 9.76 ± 0.53 <sup>c</sup> | 63           | 14.4 ± 0.20 <sup>d</sup> | 73           | 12.5 ± 0.20 <sup>d</sup>  | 72           |
| 20                | 0.42 ± 0.03 <sup>e</sup> | 63           | 7.50 ± 0.50 <sup>d</sup> | 71           | 12.3 ± 0.20 <sup>d</sup> | 77           | 10.20 ± 0.55 <sup>e</sup> | 77           |
| 25                | 0.31 ± 0.01 <sup>f</sup> | 73           | 4.26 ± 0.12 <sup>e</sup> | 83           | 5.85 ± 0.20 <sup>e</sup> | 89           | 4.16 ± 0.36 <sup>f</sup>  | 90           |
| 30                | 0.13 ± 0.00 <sup>g</sup> | 88           | 3.20 ± 0.05 <sup>e</sup> | 87           | 5.03 ± 0.12 <sup>e</sup> | 90           | 3.23 ± 0.08 <sup>f</sup>  | 92           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 2.** Effect of thalidomide on the growth of *L. donovani* promastigotes.

| Exposure time (h) | 24                       |              | 48                       |              | 72                       |              | 96                       |              |
|-------------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|                   | Mean* ± SE               | Inhibition % | Mean*± SE                | Inhibition % | Mean* ± SE               | Inhibition % | Mean* ± SE               | Inhibition % |
| Cont.             | 2.30 ± 0.23 <sup>a</sup> | ---          | 22.0 ± 1.15 <sup>a</sup> | ---          | 44.1 ± 0.49 <sup>a</sup> | ---          | 32.8 ± 0.95 <sup>a</sup> | ---          |
| 5                 | 1.13 ± 0.03 <sup>b</sup> | 50           | 10.7 ± 0.60 <sup>b</sup> | 51           | 22.4 ± 0.05 <sup>b</sup> | 49           | 16.1 ± 0.63 <sup>b</sup> | 50           |
| 10                | 0.51 ± 0.04 <sup>c</sup> | 77           | 6.03 ± 0.17 <sup>c</sup> | 72           | 7.93 ± 0.08 <sup>c</sup> | 82           | 7.20 ± 0.63 <sup>c</sup> | 78           |
| 20                | 0.12 ± 0.01 <sup>d</sup> | 94           | 0.43 ± 0.15 <sup>d</sup> | 98           | 4.13 ± 0.60 <sup>d</sup> | 90           | 3.9 ± 0.45 <sup>d</sup>  | 88           |
| 25                | 0.10 ± 0.01 <sup>d</sup> | 95           | 0.27 ± 0.07 <sup>d</sup> | 99           | 1.07 ± 0.04 <sup>e</sup> | 97           | 1.86 ± 0.20 <sup>e</sup> | 94           |
| 30                | 0.06 ± 0.00 <sup>d</sup> | 97           | 0.10 ± 0.01 <sup>d</sup> | 100          | 1.02 ± 0.15 <sup>e</sup> | 98           | 0.50 ± 0.05 <sup>e</sup> | 98           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 3.** Effect of liposomal amphotericin -B on the growth of *L. tropica* promastigotes.

However, the binding of amphotericin-B-loaded liposomes resulted in cell death. This may be due to the inactivation of liposomes and the secretion of amphotericin B to bind the cell membrane of ergosterol and exhibit antiparasitic activity<sup>30</sup>. Amphotericin B can translocate from the liposome to the membranes of parasite or fungal cells and bind to parasitic or fungal ergosterol higher than cholesterol<sup>29</sup>.

The inhibitory mechanism of leishmanial action is thought to be drug-binding to parasite ergosterol precursors such as lanosterol, disrupting parasite membrane, producing and changing membrane permeability, then killing parasite<sup>27</sup>. The specialized formulation has properties that increase activity, whereas toxicity reduces: penetration and constant levels into tissues, such as the spleen and liver; high transformation temperature leading to blood, tissues and macrophages stabilization; the presence of cholesterol in the liposome, that reduces the interaction of drugs with the membranes of mammalian cells and decrease their toxicity; It is highly related to ergosterol and its precursors to ensure anti-microbial efficacy. Initial doses higher than 5mg/kg provide better penetration and more prolonged tissue continuation than repeated lower doses<sup>31</sup>.

This study shows that *L. donovani* required a higher dose of liposomal amphotericin-B than *L. tropica* promastigote. It is 20mg/kg. However, the minimum effective dose remains unknown<sup>30</sup>. The researchers appear liposomal-loaded with amphotericin-B led to the death of cells. This possibly led to liposome disruption and amphotericin-B secretion to bind ergosterol in the cell membrane showing antiparasitic efficacy.

It is worth noting that other experiments were conducted on the effect of the two drugs together. It showed an inhibitory effect on *L. tropica*, when amphotericin was fixed at

a concentration of 2.5 µg/ml, and thalidomide concentration changed, the IC50 was 2.5 + 0.5 µg/ml at log phase (72) hrs. Table (5).

Additionally, the results demonstrated the inhibitory effect on *L. donovani*; when amphotericin was fixed at a concentration of 2.5 µg/ml, and thalidomide concentration changed, the IC50 was 2.5 + 1 µg/ml at log phase (72) hrs. Table (6).

Furthermore, regarding the synergism of the two drugs, the result clarified the effectiveness of *L. tropica* and *L. donovani* promastigotes after fixation of thalidomide concentration at 2.5 µg/ml and liposomal amphotericin -B changed. The IC50 was 2.5 + 2 µg/ml at log phase (72) hrs. for the two types of parasites (table 7 and 8).

The interaction between the drugs provides a successful effect; it appears to have high toxicity on both types of leishmania promastigotes, especially when amphotericin was fixed. The synergism caused a high inhibitory effect, decay and degeneration in the living cell organelles finally, cell death and disappearance. These results are in agreement with (32) they concluded that combination treatment with thalidomide and glucantim reduced significantly the disease course than single treatment with each one of the drugs in mice infected with visceral Leishmania, and this effect was dependent on the production of 1L-10 and 1FN-Y, also illustrate that thalidomide is capable of downregulating disease advancement nevertheless it didn't completely discourage it. Others prove that the synergy between liposomal amphotericin -B and an antibacterial or antifungal agent reduces the viability of fungal cells, increases the permeability of the cell membrane, and increases cellular metabolic activity shortly after one hour of exposure<sup>33</sup>.

Thalidomide could hypothetically be included among

| Exposure time (h)<br>Treatment (µg/ml) | 24                        |              | 48                       |              | 72                       |              | 96                        |              |
|--|---------------------------|--------------|--------------------------|--------------|--------------------------|--------------|---------------------------|--------------|
|  | Mean* ± SE                | Inhibition % | Mean*± SE                | Inhibition % | Mean* ± SE               | Inhibition % | Mean* ± SE                | Inhibition % |
| Cont.                                  | 3.70 ± 0.05 <sup>a</sup>  | ----         | 22.1 ± 0.66 <sup>a</sup> | ----         | 44.8 ± 0.58 <sup>a</sup> | ----         | 37.2 ± 0.51 <sup>a</sup>  | ----         |
| 5                                      | 1.90 ± 0.11 <sup>b</sup>  | 48           | 12.6 ± 0.63 <sup>b</sup> | 43           | 22.3 ± 0.41 <sup>b</sup> | 50           | 18.96 ± 0.84 <sup>b</sup> | 49           |
| 10                                     | 0.90 ± 0.02 <sup>c</sup>  | 75           | 4.36 ± 0.26 <sup>c</sup> | 80           | 15.8 ± 0.54 <sup>c</sup> | 64           | 14.8 ± 0.20 <sup>c</sup>  | 60           |
| 20                                     | 0.42 ± 0.04 <sup>d</sup>  | 88           | 0.70 ± 0.14 <sup>d</sup> | 96           | 4.30 ± 0.05 <sup>d</sup> | 88           | 6.13 ± 0.40 <sup>d</sup>  | 83           |
| 25                                     | 0.35 ± 0.01 <sup>de</sup> | 90           | 0.44 ± 0.08 <sup>d</sup> | 98           | 2.48 ± 0.45 <sup>e</sup> | 92           | 3.20 ± 0.25 <sup>e</sup>  | 91           |
| 30                                     | 0.20 ± 0.02 <sup>e</sup>  | 94           | 0.20 ± 0.01 <sup>d</sup> | 99           | 0.66 ± 0.10 <sup>f</sup> | 98           | 1.86 ± 0.23 <sup>e</sup>  | 95           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 4.** Effect of liposomal amphotericin-B on the growth of *L. donovani* promastigotes

| Exposure time (h)<br>Treatment (µg/ml) | 24                        |              | 48                       |              | 72                       |              | 96                       |              |
|--|---------------------------|--------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|  | Mean* ± SE                | Inhibition % | Mean*± SE                | Inhibition % | Mean* ± SE               | Inhibition % | Mean* ± SE               | Inhibition % |
| Cont.                                  | 2.30 ± 0.41 <sup>a</sup>  | ----         | 21.2 ± 0.99 <sup>a</sup> | ----         | 42.4 ± 0.31 <sup>a</sup> | ----         | 43.1 ± 1.18 <sup>a</sup> | ----         |
| 2.5 + 0.25                             | 2.06 ± 0.30 <sup>a</sup>  | 10           | 20.2 ± 1.33 <sup>a</sup> | 5            | 27.6 ± 0.40 <sup>b</sup> | 35           | 21.5 ± 0.60 <sup>b</sup> | 37           |
| 2.5 + 0.5                              | 1.60 ± 0.20 <sup>ab</sup> | 30           | 17.4 ± 0.80 <sup>b</sup> | 18           | 21.3 ± 0.64 <sup>c</sup> | 50           | 17.0 ± 0.28 <sup>c</sup> | 50           |
| 2.5 + 1                                | 1.15 ± 0.07 <sup>bc</sup> | 50           | 15.9 ± 0.20 <sup>b</sup> | 27           | 16.9 ± 0.49 <sup>d</sup> | 60           | 13.4 ± 0.12 <sup>d</sup> | 60           |
| 2.5 + 2                                | 0.77 ± 0.01 <sup>c</sup>  | 66           | 14.9 ± 0.82 <sup>b</sup> | 30           | 8.93 ± 0.47 <sup>e</sup> | 79           | 10.6 ± 0.68 <sup>e</sup> | 69           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 5.** Effect of synergism between liposomal amphotericin-B and thalidomide on *L. tropica* (Note: liposomal amphotericin B fixed at 2.5 µg/ml).

| Exposure time (h) \ Treatment (µg/ml) | 24                       |              | 48                        |              | 72                       |              | 96                       |              |
|---------------------------------------|--------------------------|--------------|---------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|                                       | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE      | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % |
| Cont.                                 | 2.63 ± 0.72 <sup>a</sup> | ---          | 22.5 ± 0.05 <sup>a</sup>  | ---          | 44.8 ± 0.55 <sup>a</sup> | ---          | 31.5 ± 0.87 <sup>a</sup> | ---          |
| 2.5 + 0.25                            | 1.65 ± 0.09 <sup>b</sup> | 37           | 21.8 ± 0.23 <sup>ab</sup> | 3            | 27.6 ± 0.28 <sup>b</sup> | 38           | 24.8 ± 0.60 <sup>b</sup> | 21           |
| 2.5 + 0.5                             | 1.30 ± 0.07 <sup>c</sup> | 50           | 21.1 ± 0.37 <sup>b</sup>  | 6            | 25.1 ± 0.95 <sup>c</sup> | 44           | 21.6 ± 0.80 <sup>c</sup> | 31           |
| 2.5 + 1                               | 1.05 ± 0.02 <sup>c</sup> | 60           | 18.3 ± 0.44 <sup>c</sup>  | 19           | 22.1 ± 1.03 <sup>d</sup> | 50           | 16.4 ± 0.62 <sup>d</sup> | 48           |
| 2.5 + 2                               | 0.45 ± 1.89 <sup>d</sup> | 82           | 11.1 ± 0.52 <sup>b</sup>  | 49           | 19.1 ± 0.31 <sup>e</sup> | 57           | 11.4 ± 0.47 <sup>e</sup> | 64           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 6.** Effect of synergism between liposomal amphotericin B and thalidomide on *L. donovani* (Note: liposomal amphotericin B fixed at 2.5µg/ml)

| Exposure time (h) \ Treatment (µg/ml) | 24                       |              | 48                       |              | 72                       |              | 96                       |              |
|---------------------------------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|                                       | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % |
| Cont.                                 | 2.40 ± 0.05 <sup>a</sup> | ---          | 20.5 ± 0.56 <sup>a</sup> | ---          | 41.3 ± 0.52 <sup>a</sup> | ---          | 36.7 ± 0.92 <sup>a</sup> | ---          |
| 2.5 + 1                               | 1.60 ± 0.05 <sup>b</sup> | 33           | 17.1 ± 0.57 <sup>b</sup> | 17           | 31.5 ± 1.15 <sup>b</sup> | 24           | 22.8 ± 1.11 <sup>b</sup> | 38           |
| 2.5 + 2                               | 1.03 ± 0.01 <sup>c</sup> | 57           | 12.6 ± 0.36 <sup>c</sup> | 38           | 21.0 ± 1.00 <sup>c</sup> | 49           | 17.8 ± 0.62 <sup>c</sup> | 51           |
| 2.5 + 3                               | 0.95 ± 0.08 <sup>c</sup> | 60           | 10.7 ± 0.82 <sup>d</sup> | 48           | 13.6 ± 0.33 <sup>d</sup> | 67           | 13.3 ± 0.65 <sup>d</sup> | 64           |
| 2.5 + 4                               | 0.77 ± 0.01 <sup>d</sup> | 68           | 9.02 ± 0.29 <sup>e</sup> | 56           | 12.0 ± 0.40 <sup>d</sup> | 71           | 8.85 ± 0.55 <sup>e</sup> | 76           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 7.** Effect of synergism between thalidomide and liposomal amphotericin B on *L. tropica* (Note: thalidomide fixed at 2.5µg/ml)

| Exposure time (h) \ Treatment (µg/ml) | 24                       |              | 48                       |              | 72                       |              | 96                       |              |
|---------------------------------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|--------------------------|--------------|
|                                       | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % | Mean <sup>±</sup> SE     | Inhibition % |
| Cont.                                 | 2.45 ± 0.11 <sup>a</sup> | ---          | 21.9 ± 0.17 <sup>a</sup> | ---          | 43.2 ± 0.05 <sup>a</sup> | ---          | 32.5 ± 0.94 <sup>a</sup> | ---          |
| 2.5 + 1                               | 1.75 ± 0.14 <sup>b</sup> | 29           | 20.3 ± 0.10 <sup>b</sup> | 7            | 23.5 ± 1.14 <sup>b</sup> | 45           | 17.7 ± 0.14 <sup>b</sup> | 45           |
| 2.5 + 2                               | 1.25 ± 0.25 <sup>c</sup> | 49           | 18.1 ± 0.03 <sup>c</sup> | 17           | 21.6 ± 1.12 <sup>b</sup> | 50           | 15.1 ± 0.57 <sup>c</sup> | 54           |
| 2.5 + 3                               | 1.05 ± 0.00 <sup>c</sup> | 57           | 13.2 ± 1.01 <sup>d</sup> | 40           | 18.2 ± 0.59 <sup>c</sup> | 58           | 9.28 ± 0.43 <sup>d</sup> | 71           |
| 2.5 + 4                               | 0.85 ± 0.02 <sup>c</sup> | 65           | 10.6 ± 0.05 <sup>e</sup> | 51           | 15.7 ± 0.24 <sup>d</sup> | 64           | 7.33 ± 0.08 <sup>e</sup> | 77           |

\* Three replicators used in each treatment. Mean were multiplied x10<sup>6</sup>

\*\* Different letter indicates significant differences among values according to one-way analysis test at p ≤ 0.05

**Table 8.** Effect of synergism between thalidomide and liposomal amphotericin -B on *L. donovani* (Note: thalidomide fixed at 2.5µg/ml)

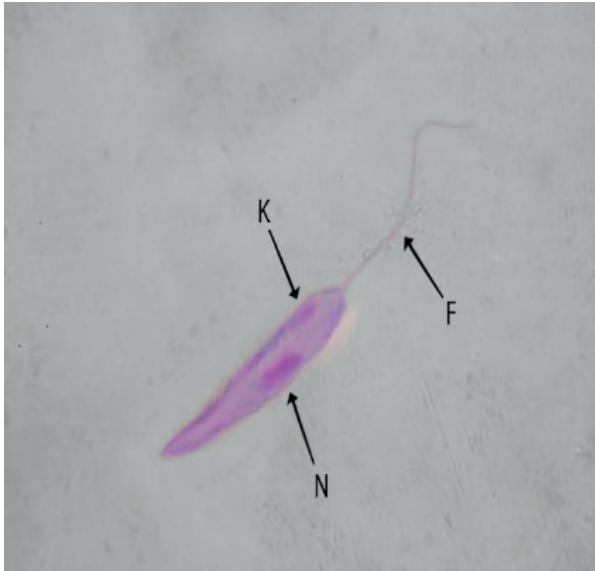
potential drugs tested for its association with COVID-19<sup>34</sup> based on its potent anti-inflammatory properties and activity in alleviating excessive inflammation and cytokine storms. (35) confirmed the agreement to this work it was significant of the in vitro anti-leishmanial effect of the combination of allicin and AmB to reduce the required doses of the antibiotic and thus the toxicity associated with its use. Using a constant ratio analysis with the extra-cellular phase and a checkerboard analysis with amastigotes, the combination of the 2 drugs was moderate to synergistic at minimum concentrations versus both amastigotes and promastigotes; this represented an approximately two-fold decrease in the IC<sub>50</sub> of the antibiotic added alone. The conclusion from (33) appears that the synergism between liposomal AmB and other drugs causes inhibition of DNA-dependent RNA polymerase leading to the repression of RNA synthesis and cell death. This results in agreement with (36) that is proved the liposomal amphotericin-B and selenium and the combination between them showed superior efficacy in the treat-

ment of CL also noticed that no toxicity was reported.

The synergism between thalidomide and liposomal amphotericin- B appears to be more efficient in inhibiting the growth of promastigotes than when treated with each drug alone. This means that the synergism effectively inhibited both types of Leishmaniasis at lower concentrations.

It is evident from the figures (2), (3) for *L. tropica* and (7), (8) for *L. donovani* that using the treatment leads to changes in the morphological appearance from the spindle shape to the oval, circular or even irregular shape as well the occurrence of bulges and cracks in these parasites, figures (4), (5) for *L. tropica* and (9), (10) for *L. donovani* when treated with IC<sub>50</sub> in comparing with untreated parasites, figures (2) and (6).

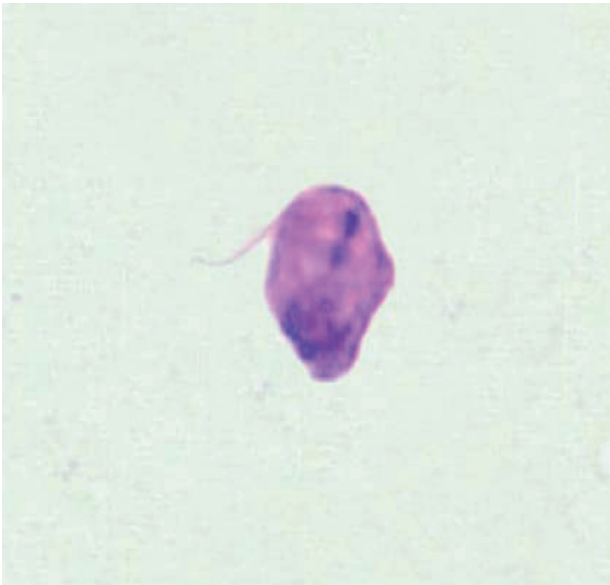
The result is consistent with those manifested by (37) to show that shape converted from spindle to rounded or irregular shape and became swelling; this indicates that the cell membrane permeability was affected, the osmotic cell was changed and allowed to liquid pass across the cell membra-



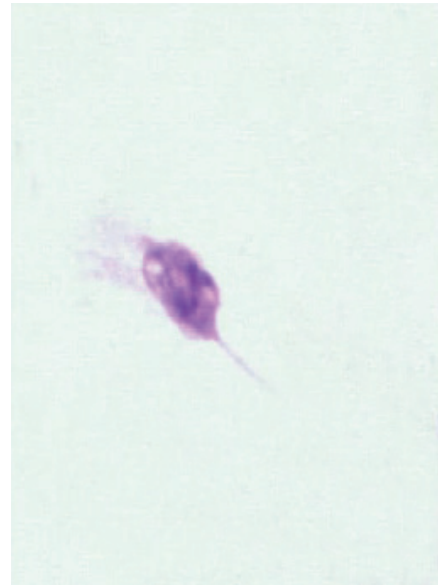
**Figure 1.** Untreated *L. tropica* N- nucleus, K-kinetoplast, F-flagellum.



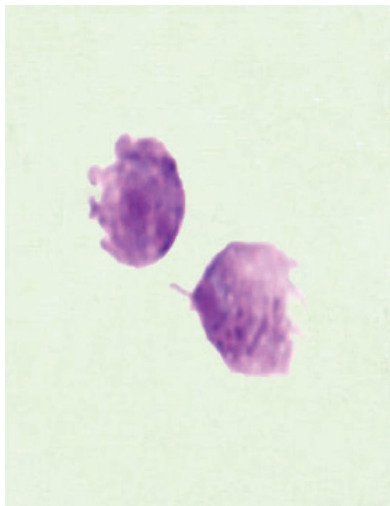
**Figure 2.** *L. tropica* promastigote treated  $IC_{50}$  of the thalidomide.



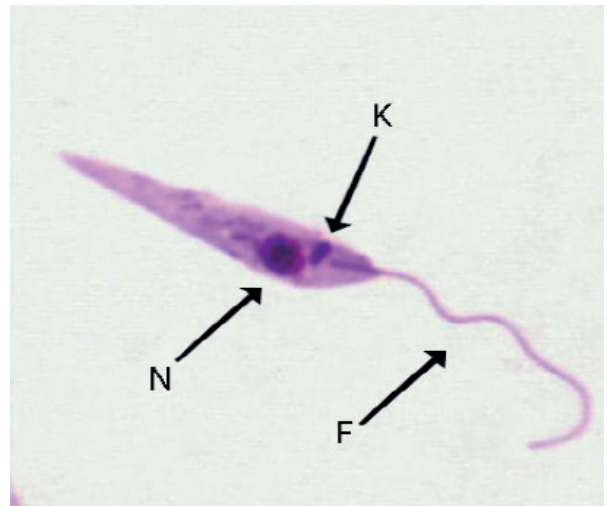
**Figure 3.** *L. tropica* promastigote treated  $IC_{50}$  of liposomal amphotericin-B.



**Figure 4.** *L. tropica* promastigote treated  $IC_{50}$  of synergism (liposomal amphotericin-B fixed).



**Figure 5.** *L. tropica* promastigote treated  $IC_{50}$  of synergism (thalidomide fixed).



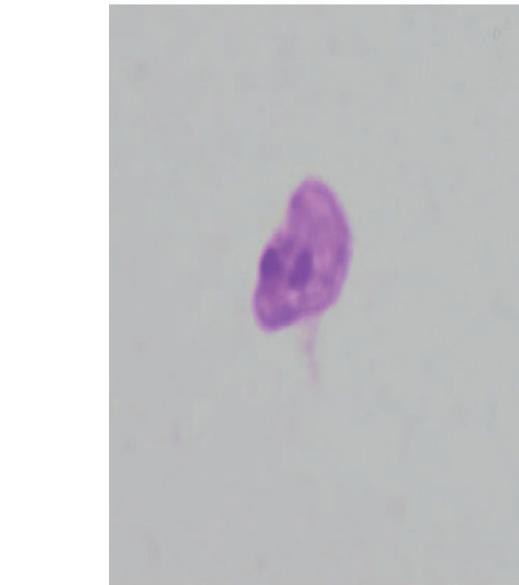
**Figure 6.** Untreated *L. donovani* promastigote N- nucleus, K- kintoplast, F- flagellum



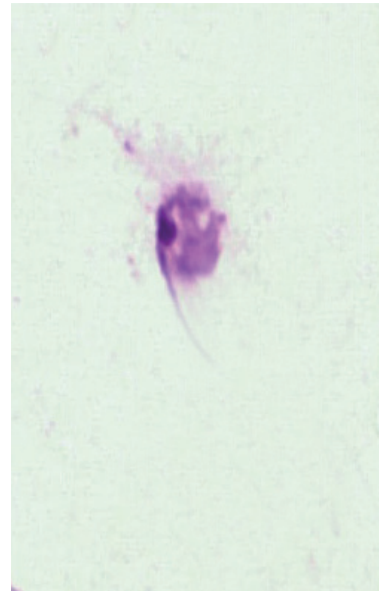
**Figure 7.** *L. donovani* promastigote treated  $IC_{50}$  of the thalidomide note: swollen promastigotes.



**Figure 8.** *L. donovani* promastigote treated  $IC_{50}$  of liposomal amphotericin -B- note, circular shape.



**Figure 9.** *L. donovani* promastigote treated  $IC_{50}$  of synergism (liposomal amphotericin-B fixed).



**Figure 10.** *L. donovani* promastigote treated  $IC_{50}$  of synergism (thalidomide fixed).

ne. The inhibitory influence of the two types caused shrinkage and degeneration in the living cell organelles, finally death the cell and disappearing it in low mixed concentrations. The mechanism of liposomal amphotericin-B, considered anti Leishmaniasis, has been associated with cholesterol and distearyl phosphatidyl glycerol within phospholipid bilayer synthetic and bound with protein in the medium to improve drug<sup>38</sup>. Other researchers like (39) concluded the mechanism of action of cloquinol has been examined at *L. infantum* and *L. amazonensis*; results showed morphological and biochemical changes in cloquinol treated parasites, including reduction in cell size, absence of mitochondrial membrane potency, rupture of the plasma membrane, increasing the ROS production and antileishmanial activity of cloquinol versus both related species of *Leishmania* and suggests that the parasites' mitochondria probably a potential biological target leading to parasite necrosis.

Change the particular enzyme or physiological activities<sup>40</sup>; therefore, the promastigote became smallest and shortened flagellate, lost the motility and became vibrating

motion then cell crash. Another research clear the effect of drugs on leishmania morphology as (41) treated parasites showed significant changes in cell morphology and fluorescence emission pattern in comparison with control cells, including the appearance of abnormally round-shaped cells, lack of cellular motility, cytoplasmic enlargement and an increase in the numerical of acidophilic vesicular organelles, indicating serious physiological alterations, also showed that the cell death by apoptosis and autophagy, the parasites showing found of multi nuclei and this might affect the microtubule dynamics in species of *Leishmania*.

## Conclusions

We conclude from the above that the use of drugs had high toxicity in cutaneous and visceral Leishmaniasis in vitro at low concentrations, whether for each drug alone or in the case of synergism. additionally, the treatment led to morphological changes

In these parasites, from spindle shape to the oval, circular, and irregular also, the occurrence the bulges and rupture of the plasma membrane indicate the effect on the permeability of the parasite's cell membrane.

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### Conflicts of Interest

Authors declare that they have no conflict of interest for this study.

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