

Cytotoxic Effect of Methotrexate on Leishmaniadonovani Promastigotes

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Abstract: *Leishmania* is auxotroph to folic acid, antifolates drug inhibit the synthesis and conversion of folate derivatives. In this study, cytotoxic effect of methotrexate was investigated on the procyclic promastigotes proliferation of *L. donovani*. The results showed a significant ($p \geq 0.05$) difference in growth of treated groups at high concentrations (1000, 500, 250, 125.5) μM after 24, 48 hrs., while at 72 hrs. significant difference was observed at all concentration. The IC_{50} values was measurable after 24, 48 and 72 hrs. and it was 174.238, 52.283 and 109.175 μM , respectively. The present study showed the cytotoxic effect of methotrexate on the proliferation of promastigotes of the visceral type of *Leishmania*.

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I. Introduction

Leishmaniasis, a complex disease caused by protozoa of the genus *Leishmania* spp., which can infect humans and several animals through the bite of phlebotomine insect vector (1), it was distributed worldwide and is responsible for a wide spectrum of diseases, including visceral, mucocutaneous or espundia and cutaneous leishmaniasis (1,2). *Leishmania* distinct two morphologically forms during its life cycle which required promastigotes, flagellated insect stage and amastigote, non-flagellated form to be completed (3, 4). *Leishmania* is transmitted through bite sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the new world (5). Leishmaniasis is prevalent in 88 countries, affecting an estimated 12 million people with approximately 2 million new cases per year. Leishmaniasis has been deemed a tropical affliction that composes one of the seven entities on the list of most important diseases of World Health Organization/Tropical Disease Research (WHO/TDR) in addition to malaria, African Trypanosomiasis, Schistosomiasis, Chagas disease, Filariasis, Leprosy and Tuberculosis, Among these, 5,00,000 are visceral leishmaniasis and 15,00,000 are cutaneous leishmaniasis (6).

Leishmania usually reside within the macrophage of the vertebrate host; to enter the macrophage, *Leishmania* utilizes a variety of cellular receptors to mediate endocytosis. Once inside the macrophage, *Leishmania* is protected from phagolysosome degradation by a variety of adaptations to inhibit cellular defense mechanisms (7). No vaccines are presently available against *Leishmania* infection and treatments rely primarily on chemotherapy (8).

The chemotherapeutic arsenal is limited and includes pentavalent antimonials sodium stibogluconate (Sb) or (Pentostam) as first-line drugs exhibited some problems such as prolonged systemic therapy, less effectivity against various forms and high toxicity and the second-line drugs amphotericin B and pentamidine have limitations for use because of, prolonged length of therapy, high cost and adverse reactions. *Leishmania* is sensitive to MTX, the drug is not used clinically to treat leishmaniasis (9-11). Methotrexate, folate anti-metabolite used since more than 40 years as a potent anticancer agent in cases of leukemia, sarcoma and rheumatic disorders, it was less toxic than the then-current treatments (4, 12). MTX has been found to be toxic on some parasites, such as malaria spp. and thus, more investigation are currently on process for screening of this drug on other parasites, such as *Leishmania* (13). In this study, different concentrations of MTX were screened on the procyclic forms of *Leishmania donovani* and follow-up was made for 72 hours.

II. Material And Methods

2.1. Parasite culture:

Leishmania donovani isolate was kindly provided by Biotechnology Research Centre/ Al-Nahreen University and it was previously diagnosed as *L. donovani* by PCR (14). Parasite culture was routinely maintained in vitro in cell-culture media (RPMI) and incubated at 26°C with continuous passages, three times a week.

2.2. Drug concentrations:

The following concentrations (15.6, 31.25, 62.5, 125, 250, 500, 1000) μM of methotrexate was investigated for cytotoxicity screening against *L. donovani* promastigotes, follow up was made for three times, 24, 48 and 72 hours. Control was made by following the same procedure above but pentostam was added instead of MTX. Triplicates was made for each concentration.

2.3 Cytotoxicity screening (Colorimetric assay):

Alamar Blue is widely used as a metabolic indicator for living cells because it is nontoxic. The biochemical mechanism to resazurin (Blue) is reduced to resorufin (pink). Resazurin was added to the culture in micro-plate of 96 flat bottom wells in a ratio of 1:10 and incubated for 4 hours at 26°C prior reading the plates by ELISA reader (Avusturya®) at 570/600 nm wavelength(15).

2.4 Statistical analysis

The t test was used to determine the significance of methotrexate effect and IC50 was calculated as previously described by (16)

III. Result and discussion

Procyclic insect stage promastigotes of *L. donovani* was treated with different concentrations of methotrexate to detect the cytotoxicity of this drug on the parasite viability. Results showed that there was a significance difference in absorption between test and control for the high concentration (1000, 500, 250, 125.5) μM after 24, 48 hrs., while at 72 hrs. significance ($p \geq 0.05$) was detected at all concentrations shown in figure [1, 2, 3 and 4].

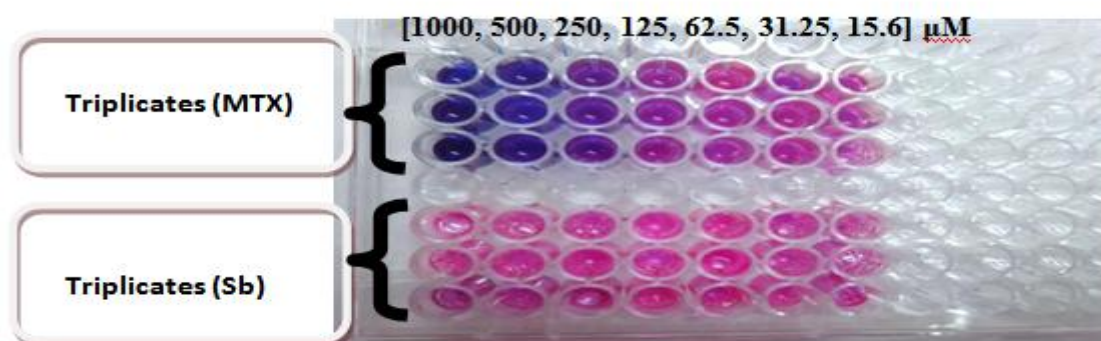


Fig.1 Microtiter Plate of 96 wells, cytotoxicity of Methotrexate against *L. donovani* promastigotes after 48 hrs. treatment.

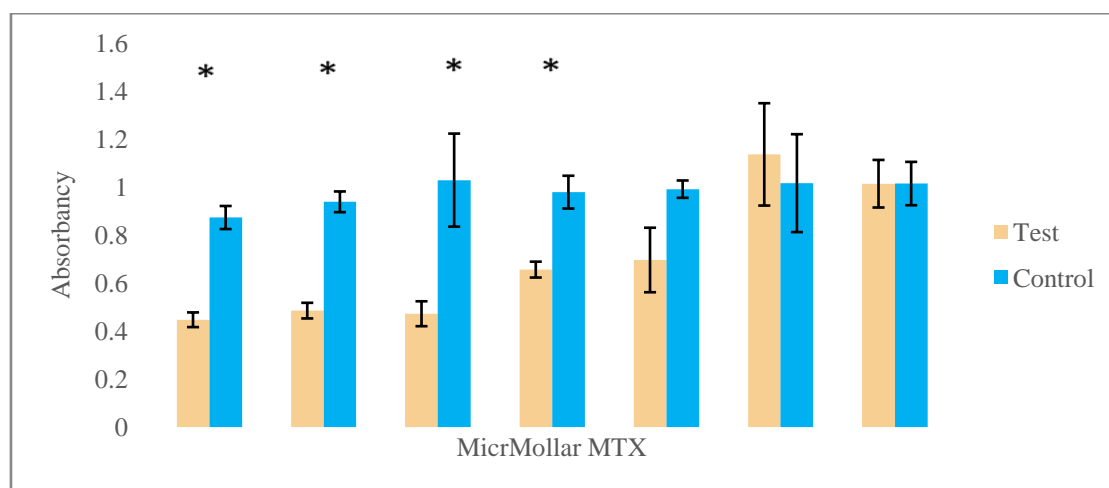


Fig. 2 Methotrexate cytotoxicity, 24 hrs. incubation, (*) significant difference.

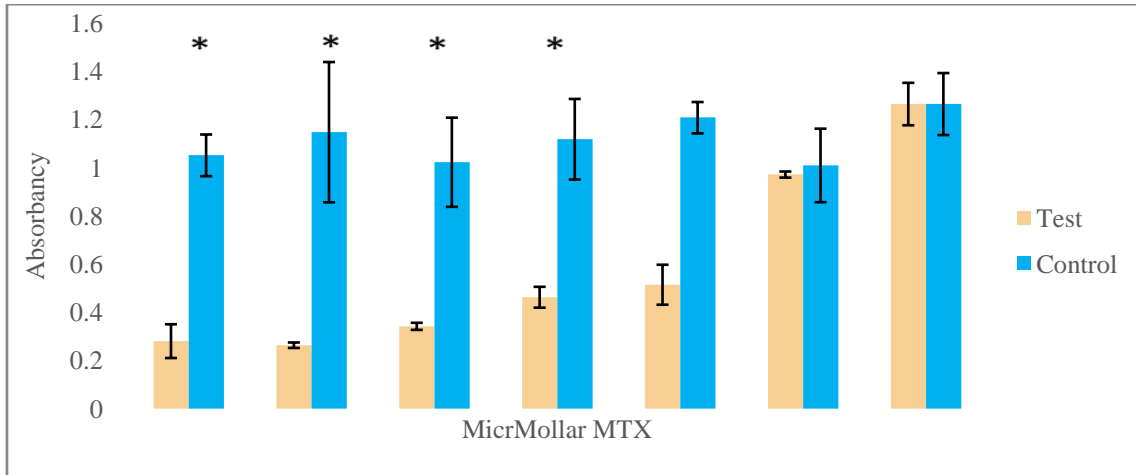


Fig.3 Methotrexate cytotoxicity, 48 hrs. incubation, (*) significant difference.

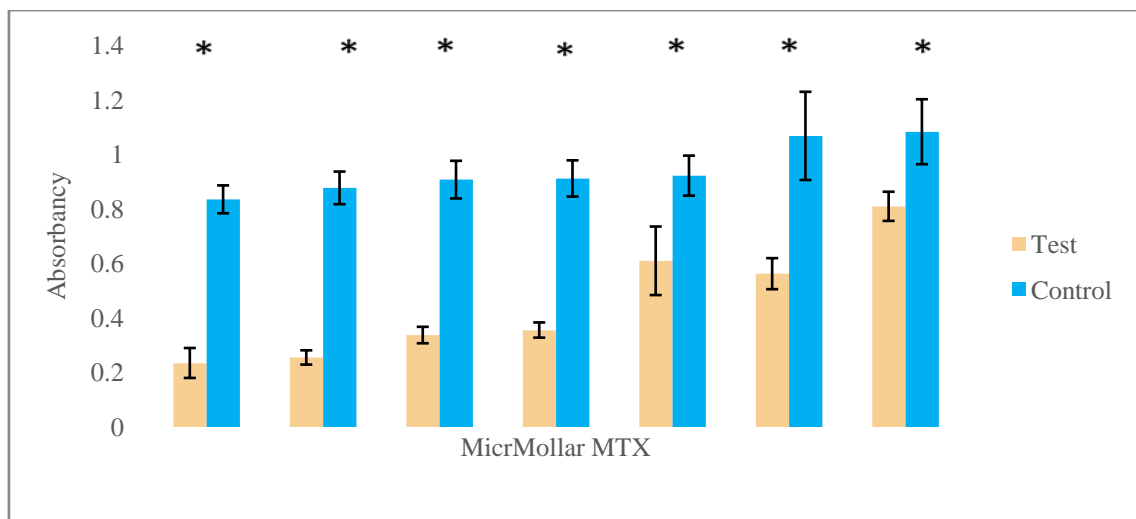


Fig.4 Methotrexate cytotoxicity, 72 hrs. incubation, (*) significant difference.

The mean of cell viability measured at the highest concentration of 1000 μM was (51.22, 26.65, 28.02) % after 24, 48 and 72 hours, respectively. Furthermore, the mean of cell viability measured at the lowest concentration of 15.6 μM was (99.93, 100, 74.70) % after 24, 48 and 72 hours respectively, Figures (5, 6 and 7). According to the cytotoxicity and cell viability results, the IC_{50} was calculated along the three times of follow up and demonstrated a time-dependent inhibition of the parasite growth to 50 % in which the IC_{50} value was measurable after 24, 48 and 72 hrs. and it was 174.238, 52.283 and 109.175 μM .

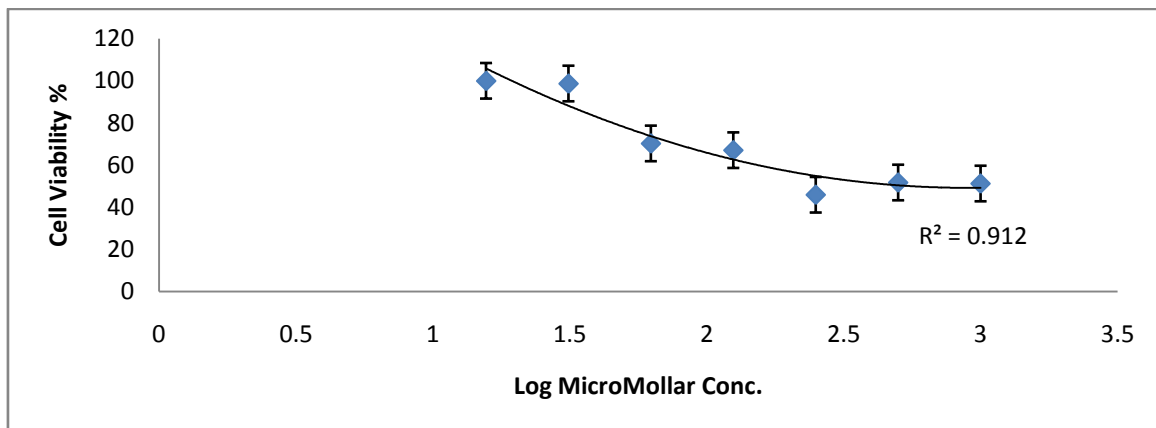


Fig.5 Cell viability of *L. donovani* promastigotes treated with Methotrexate, after 24 hours of incubation.

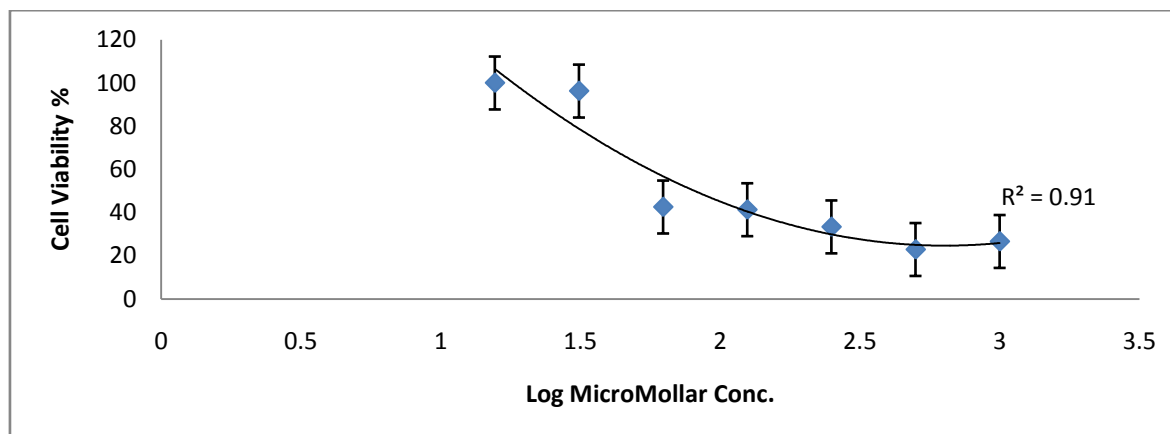


Fig.6 Cell viability of *L. donovani* promastigotes treated with Methotrexate, after 48 hours of incubation.

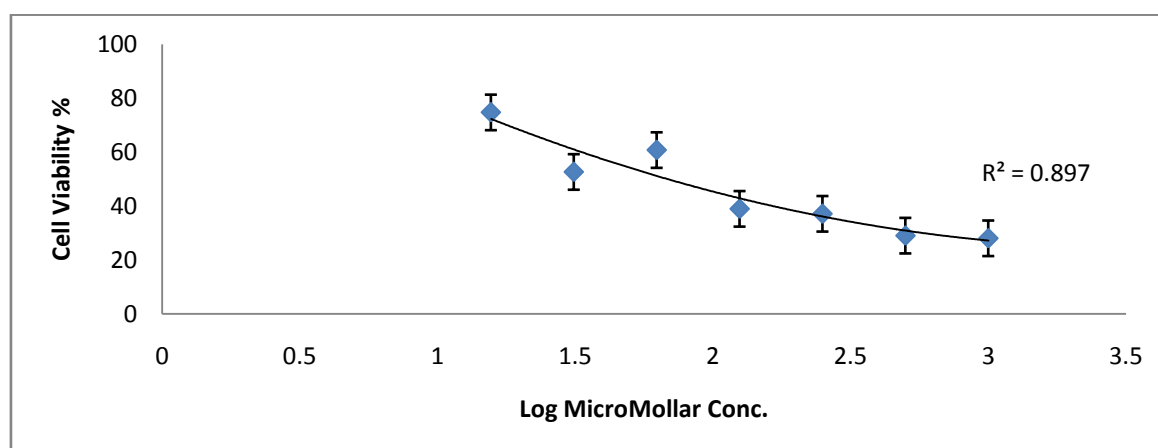


Fig.7 Cell viability of *L. donovani* promastigotes treated with Methotrexate, after 72 hours of incubation.

A study by Scott et al. (17) indicated leishmaniacidal activity of *L. donovani* at 100 μM methotrexate, it was high enough to kill wild type cells in medium not supplemented with folate. MTX at a concentration of 4 μM did not significantly decrease the rate of cell division of MTXA5 cell (multiply in folate-deficient medium) to *L. donovani*, whereas the EC_{50} value of the drug was approximately 130 μM . Previous results indicated that MTX are potent effect against *P. falciparum*, with $\text{IC}_{50} < 50 \text{ nM}$. The concentration of methotrexate required to inhibit the growth of wild type *L. donovani* in Dulbecco's modified Eagle's medium was 2 orders of magnitude higher than that reported to inhibit the growth of wild type *L. major* in M199 medium (17).

In previous study by (18) proved that MTX has more potent parasitological effect on both resistant and susceptible *P. falciparum* in vitro than miltefosine dose on *L. donovani* promastigotes and the IC_{50} was 25 μM . The Differential toxicity of methotrexate, due to the different of folate concentration contain media between the DME-L culture medium 9.1 μM and the M199 medium 23 nM folate. Another study (19) detected the IC_{50} of Amphotericin (nM), Miltefosine (μM) and Pentamidine (μM) against *L. donovani* was (8.88, 17.5 and 1.3) μM . *L. infantum* (42, 15.5, and 2.87) respectively. *Leishmania donovani* and *L. m. mexicana* grew in very high (500 $\mu\text{g ml}^{-1}$, 1.1 mM) concentrations of the drug. By contrast, *L. major* could not sustain growth in concentrations of methotrexate greater than 5 $\mu\text{g ml}^{-1}$ (17).

The traditional therapy of cutaneous leishmaniasis is usually carried out by the glucantime drug but it is known for its toxicity and leading to broad side effects (20). Miltefosine and Paromomycin are two drugs that have been introduced in the last decade for the therapy of leishmaniasis disease (21). While the IC_{50} to relative drug such miltefosine for amastigotes *L. donovani* was 52.0 μM . Methotrexate is a potent inhibitor of the enzyme dihydrofolate reductase and causes a depletion of the cellular tetrahydrofolate pools. Despite the insensitivity of *L. mexicana* promastigotes to methotrexate, the dihydrofolate reductase from this organism was inhibited 50% by MTX at a concentration of only $2 \times 10^{-9} \text{ M}$ (17). Tetrahydrofolates are necessary for both purine and thymidylate nucleotide biosynthesis in animal cells, and mammalian cells (4). Another study (22) demonstrated the effects of antimonials and NO donors resistance of amastigotes *L. infantum* 4.7 μM and 37.7 μM , respectively. Furthermore, one MTX conjugate that is known to accumulate selectively in the spleen reduces *L. donovani* infection in vitro and in experimental animals MTX elicited an inhibitory chemotactic response 30–40%, even at concentrations far lower its IC_{50} . Another study done by Scott et al. (17) indicated the

M&B 35769 (May & Baker compounds used, 2,4-diamino-5-(3-[4-chlorophenylphenoxy]-propyl)-1H-imidazo[4,5-b]pyridine) was similarly active against promastigotes of *L. donovani* and *L. major*. The M&B 35769-resistant line of *L. mexicana* could grow in 120 μM (50 μg/ml) however, revealed that there had been no change in the specific activity of the enzyme. The IC₅₀ of *L. mexicana* dihydrofolate reductase to inhibition by methotrexate 2×10^{-3} , was very similar to that reported for *L. major*. The effectiveness of several 2,4-diaminopyrimidines in killing *L. mexicana* promastigote demonstrates that antifolates do have potential as agents against this parasite (17).

IV. Conclusion

This study is considered one of the very first trials of screening MTX cytotoxicity on promastigotes of the Iraqi isolates of visceral leishmaniasis, *L. donovani*.

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