Leishmanicidal activity of Artemisinin against cutaneous Leishmaniasis, *in Vitro*

Ghuffran Muhammed Hassan   **Hayder Z. Ali**

Department of Biology / College of Science / University of Baghdad

E-mail : guffran_bio@yahoo.com , h.z.ali2010@gmail.com

Abstract

Background: Cutaneous leishmaniasis (CL) is a neglected disease in tropical countries, including Iraq. Several studies have sought to examine chemotherapies for leishmaniasis treatment but most of them are of toxic and/or undesirable side effect, therefore, the need for investigating new fewer toxic therapies is essential.

Aim of study: In this study, the cytotoxic effect of Artemisinin (ART), a novel herbal compound, was screened against the two forms, promastigotes and amastigotes, of the Iraqi isolate of *Leishmania tropica*, the causative agent of Baghdad boil.

Material and methods: Different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8) µM of Artemisinin were screened to investigate the leishmanicidal activity of the herbal compound against the two forms of the parasite along three times of follow up (24, 48, 72) hour using MTT cytotoxicity assay.

Results: The results showed that growth rate and cell viability were significantly decreased at all studied concentrations. The IC_{50} was measured after 72 hours of follow up and was 2.625 µM and 2.636 µM for promastigotes and amastigotes, respectively.

Conclusion: These findings approved the leishmanicidal efficacy of Artemisinin against the of *L. tropica* and can be further studied to screen its effectiveness *in vivo* for exploring a safer herbal drug for treatment of cutaneous leishmaniasis.

Key words: Artemisinin ART, Leishmniasis , *in vitro,* MTT.

Introduction

Leishmaniasis is one of the most serious epizootic diseases according to the World Health Organization (WHO) (1). Leishmaniasis is a crucial public health problem caused by *Leishmania* spp., class of Kinetoplastida, it affects 98 countries worldwide an estimated 1.3 million new cases and 20000 to 30000 deaths are reported annually around 310 million people are at risk of an infection (2). Cutaneous leishmaniasis, the skin form of the disease, is an endemic disease in Middle Eastern, such as Syria, Iraq, Kingdom Saudi Arabia, and Jordan and it is still considered as an essential health problem which requires international awareness (3). The drug favorite for therapy of CL. is Glucantime and Pentostam. Both of them possess toxic side effects. Therefore, scientists are evolving less toxic new therapies with minimal toxicity (4,5). Miltefosine and paromomycin are two medications that have been entered more newly for the therapy of leishmaniasis disease (6). However, long-term medication with miltefosine's long half-life (about 152 h) can enhance early starter of drug resistance, and potential teratogenic and abortifacient effects limit its prescription through gestation (7,8). *In vitro* tests have led to the emergence of paromomycin-resistant, miltefosine resistant (9) meglumine antimoniate-resistant (10) and pentamidine-resistant (11). Artemisinin and its derivatives represent a very important new class of antimalarials (12). Artemisinin and aqueous extract of *Artemisia sieberi* are of plant origin. Artemisinin is derived from a medicinal herb called qinghazo (sweet wormwood) or *Artemisia annua* and is still obtained from this plant. Artemisinin is relatively facilely purified after extraction from plants (13). Following their discovery and development of antimalarial drugs by Tu Youyou's group in the 1970s (14). Artemisinin and its derivatives have been investigated in treating parasitic diseases or parasitic infections caused by protozoan parasites including *Leishmania* spp., *Trypanosoma* spp., Toxoplasma *gondii*, Neospora *caninum*, *Eimeria* tenella, *Acanthamoeba castellanii*, *Naegleria fowleri*, *Cryptosporidium parvum*, *Giardia lamblia*, and *Babesia* spp. (15). They are efficient in inhibiting the parasite metabolism while showing limited adverse effects on the host, indicating a higher safety index of the drugs. A large number of *in-vitro* or *ex-vivo* studies have shown that ART and its derivatives have activities in controlling the parasites, and the herbal compound shown effective against the protozoan (16).

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guffran_bio@yahoo.com

Material and Methods

Artemisinin ($C_{15} H_{22} O_5$) was purchased from TOCRIC biotechne (England) and dissolved according to the manufacturer's protocol. In brief, 3 mg of artemisinin was dissolved in 500 μL of Dimethyl sulfoxide (DMSO). From the stock solution, different concentrations of ART were prepared as following (500, 400, 300, 200, 100, 50 and 10) μM.

Leishmania tropica isolate culture

L. tropica was isolated from a patient in AL-Karama hospital (Baghdad city), a patient diagnosed with Cutaneous Leishmaniasis and a sample was taken from a hand lesion (17). Procyclic promastigotes of *L. tropica* were cultured in M199 medium (Sigma Aldrich St. Louis, MO, USA). The medium was prepared according to manufacturer's procedure at pH 7.4 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin, culture was incubated at 26 °C For three days to enable proliferation of promastigotes into log phase (18). The production of axenic amastigotes was induced by transferring the procyclic promastigotes of 5×106 parasite/ml to a new RPMI140 medium supplemented with 20 % HIFBS, pH 5.5, and incubated at 35°C for two days to produce the infectious metacyclic promastigotes (19).

Cytotoxicity assay (Colorimetric   MTT assay)

Promastigotes and amastigotes were cultured in plates of 96 wells flat bottom, with the different concentrations: (1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8) μM, each concentration was made in triplicates and each plate was incubated at 26°C and 35°C for promastigotes and amastigotes, respectively. After 24, 48 and 72 hours of incubation, 20 μL of MTT solution was added to each well and the plates was incubated at 26°C and 35°C. The medium was aspirated and the formed formazan crystals were solubilized by adding 50 μL DMSO per well for 10 min. Finally, the intensity of the dissolved formazan crystals was quantified by ELISA reader at 620 nm (20).

Statistical Analysis

To determine the significant differences between means of control and test values for each concentration after time (24, 48, and 72) h, using t-test and Different between means have analyzed at ($p \leq 0.05$) and expressed as (Mean ± SE) (21).

Results and Discussion

The results of cytotoxicity revealed the activity of Artemisinin to inhibit parasite's growth and limit the proliferation, difference of colorimetric absorption was detected. Statistically, there was a significant ($p \leq 0.05$) difference in absorption of microtiter- plate reader data, between test and control for promastigotes at all concentrations (1000, 500, 250, 125.5, 62.5, 31.25, 15.6 and 7.8) μM after 24, 48 and 72 hours of follow-up. Moreover, the effect was most apparent after 72 hours of treatment. According to the cytotoxicity results above, the IC_{50} was calculated along the three times of follow up and demonstrated a time-dependent inhibition of the parasite growth in which the IC_{50} value was only measurable after 72 hours and it was 421.743 μM.

Cell viability was calculated by plotting the percentages of parasite viability against logarithm Artemisinin concentrations. Results were compared between test and control for all Artemisinin log concentrations as means ± standard deviation (SE) (21). The percentage of promastigotes viability for the treated groups for all concentration was more than 50 % after 24- and 48-hours treatment, therefore no IC_{50} could be calculated; while after 72 hours treatment, the cell viability of test group dropped below 50 %. The mean of cell viability measured at the highest concentration of 1000 μM was (76.61, 70.03, 41.71) % after 24, 48 and 72 hours, respectively figure 1, 2 and 3. Furthermore, the mean of cell viability measured at the lowest concentration of 7.8 μM was (94.76, 92.67, 92.05) % after 24, 48 and 72 hours.
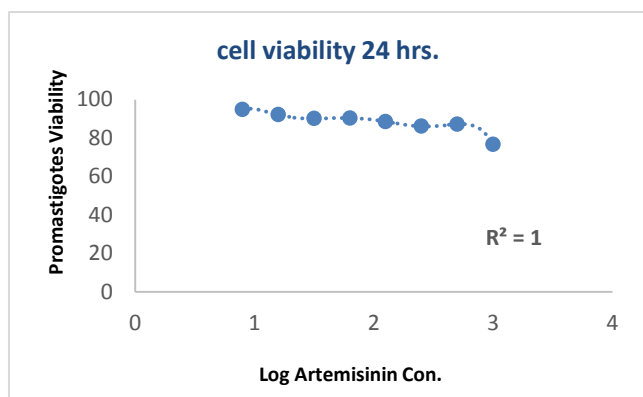
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Figure (1): Cell viability of *L. tropica* promastigotes treated with Artemisinin, after 24 hours of incubation
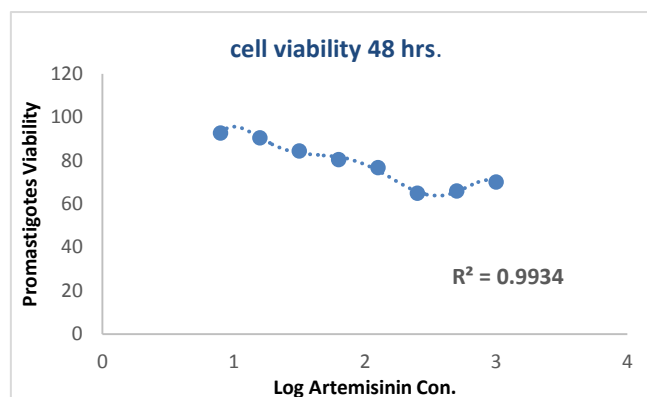

Figure (2): Cell viability of *L. tropica* promastigotes treated with Artemisinin, after 48 hours of incubation
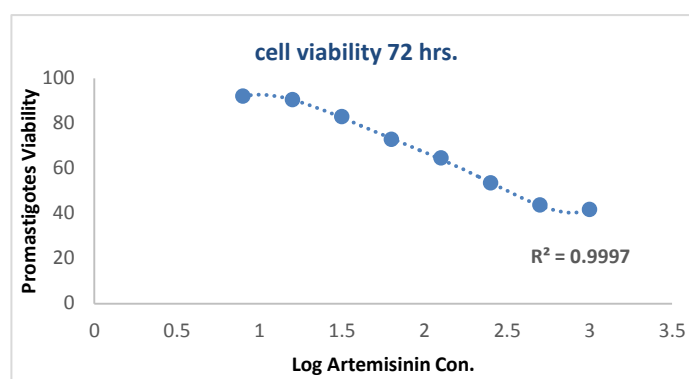

Figure (3): Cell viability of *L. tropica* promastigotes treated with Artemisinin, after 72 hours of incubation

For amastigotes screening, results have shown that the Artemisinin had inhibited amastigotes proliferation. Statistically, there was a significant ($p \leq 0.05$) differences of absorption of microtiter- plate reader data between treated groups and control for amastigotes at all studied concentrations (1000, 500, 250, 125.5, 62.5, 31.25, 15.6 and 7.8) µM after 24, 48 and 72 hours of follow-up.

The percentage of cell viability of amastigotes for test group was more than 50 % after 24 and 48hours incubation, therefore no IC_{50} could be calculated. While after 72 hours treatment, cell viability of test group dropped below 50 %. IC_{50} was detectable after 72 hours and calculated as 432.661 µM. The mean of cell viability measured at the highest concentration of 1000 µM was (68.83, 62.09, 31.26) % after 24, 48 and 72 hours, respectively. Furthermore, the mean of cell viability measured at the lowest concentration of 7.8 µM was (88.24, 84.47, 82.62) % after 24, 48 and 72 hours, respectively. Figure (5,6 and 7), Figure(4,5) and(6).
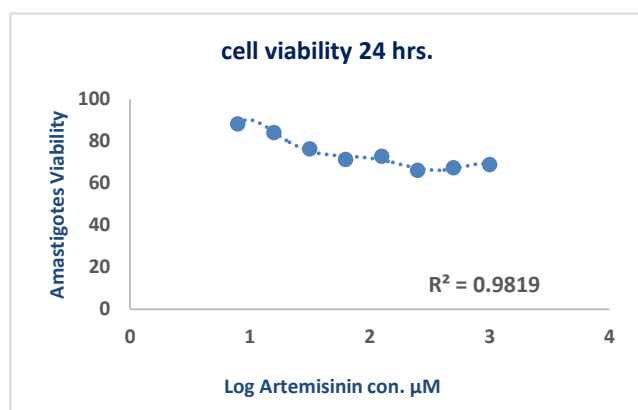
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Figure (4): Cell viability of *L. tropica* amastigotes treated with Artemisinin, after 24 hours of incubation
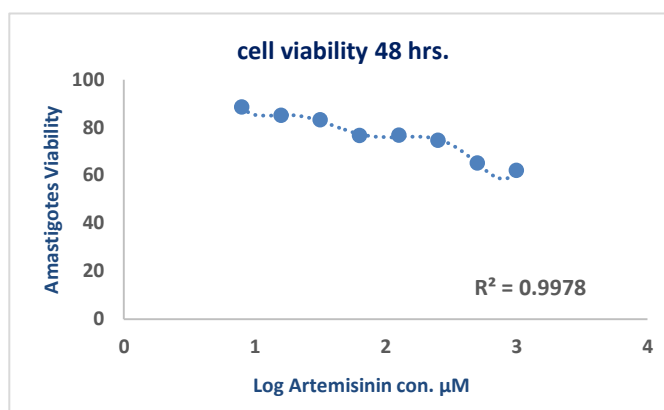

Figure (5): Cell viability of *L. tropica* amastigotes treated with Artemisinin, after48 hours of incubation
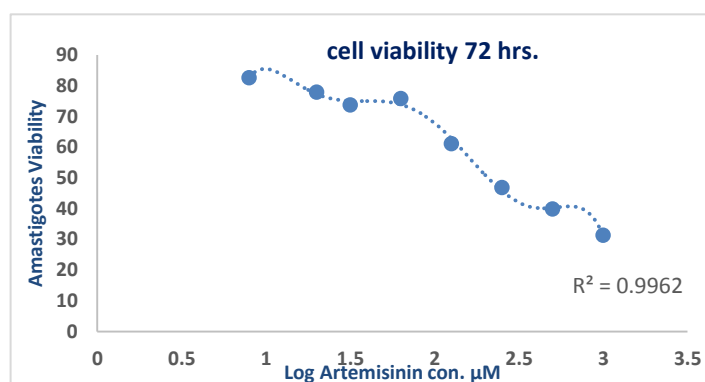

Figure (6): Cell viability of *L. tropica* amastigotes treated with Artemisinin, after 72 hours of incubation

Artemisinin (Chinese-qinghaosu), a sesquiterpene lactone and its derivatives are anti-malaria drug, have demonstrated effectiveness in many experimental research forms of leishmaniasis (22,23). The first study of investigating the leishmanicidal activity of ART (24) and its efficacy against experimental cutaneous leishmaniasis was demonstrated. Although Artemisinin is widely utilized as an anti-malarial agent, it has also shown its anti-promastigote activities and its inhibitory effect on *Leishmania* was increased(25). Furthermore, Artemisinin proved efficacy on different helminthes in the experimental method including schistosomiasis (25), Trichinellosis (26), clonorchiasis (27) and other helminths (28).

A major number of *in-vitro* and *in-vivo* studies have shown that Artemisinin and its derivatives have efficacy in controlling *Leishmania* species. The drugs showed effectiveness against many protozoan such as *Trypanosomae spp.*, *Acanthamoeba spp.* and *Babesia spp.* (29). Previous studies showed that viability of the *L. major* promastigotes decreased gradually with increasing concentrations of Artemisinin with an IC_{50} value of 750 nM and 3–30 μM for intracellular amastigotes (25).

The anti-promastigote activity of ART was studied on old and new world leishmaniasis, including *L. donovani, L. infantum, L. tropica, L. braziliensis, L. mexicana, L. amazonensis* respectively. Artemisinin induced a similar group of leishmanicidal efficacy in all species aforementioned, with the IC_{50} values being 115, 120, 100, 100, 120 and 120 μM, respectively. The similarity of IC_{50} values is an evidence of Artemisinin has a broad spectrum of leishmanicidal activity (30). Comparable effects have been observed for the treatment of *L. tropica, L. major, L. infantum, L. mexicana, L. braziliensis* and *L. amazonensis* (31,32).

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Artemisinin exhibited high toxicity and apoptotic effect on promastigotes to *L. major* and lowering development of this disease in BALB/c mice (33). Another study by (38) reinforced all studies former *in-vitro* and *in-vivo* about effects of Artemisinin, they showed that Artemisinin inhibited the growth of *L. major* promastigotes. Its inhibitory activity toward promastigotes determined the IC_{50} values of 283 μM. While in another study on *L. donovani*, the IC_{50} of Artemisinin was 160 μM for promastigotes, which further decreased to 22 μM in amastigotes (34).They are effective in inhibiting the parasite metabolism while exhibiting limited adverse effects on the host, indicating a higher safety index of the Artemisinin (35,36).

Another study showed a significant ($P \leq 0.05$) decline in the proliferation of amastigotes inside the lesion's macrophages in the Artemisinin treated mice compared with the other macrophages (37). When exposed to microorganisms, (39) demonstrated a blast in oxygen consumption coincident with the production of nitric oxide in large amounts with H_2O_2, exhibited more effective leishmanicidal effects (38).

A study by (32) showed that Artemether (derivatives) had an apoptotic effect on *L. major* promastigotes, and could inhibit the growth of both of promastigotes and amastigotes *in-vitro*. Artemether is considered a derivative of Artemisinin, had exhibited an anti-leishmanial effect due to the methyl ether derivative of Artemisinin and including high efficacy and low toxicity that could become patented for the therapy of leishmaniasis (39).

The IC_{50} value of Artemisinin as an anti-malarial drug is in the nanomolar range (4.2–16.2 nM) whereas, for antileishmanial activity, micromolar concentrations are required, However, the safety index of the drug as an anti-leishmanial is high and, as oral, parenteral and rectal dosages can achieve micromolar concentrations (40), Artemisinin could well be considered a potential anti-leishmanial drug. ART compounds have also been shown to inhibit *in vitro* growth of *Trypanosoma cruzi* and *Trypanosoma brucei* subsp. rhodesiense at micromolar concentrations, providing evidence of its expanding therapeutic potential (41).

ART and its derivatives share a common structural feature called endoperoxide linkage (45). Miltefosine is the last new drug which is active in the oral administration. However, miltefosine-resistant parasites have been obtained in laboratory indicating that the miltefosine-resistance is at risk in the field (42). For this reason, the search for new antileishmanial drugs should continue to investigate the antileishmanial activity of compounds having lethal properties on other parasites. Thus, Artemisinin derivatives, dihydroartemisinin(DHA), artesunate, artemether, and Arteether, are currently used for the treatment of malaria in artemisinin combination therapies(43).

Conclusion

This work is first experiment to investigate the Artemisinin antileishmanial activity on Iraqi strain of *L. tropica* and could be suggested as a promising Therapy for Cutaneous Leishmaniasis, *in vivo* studies are required to complete this concept.

Acknowledgments

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