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Original paper

Artemisinin efficacy against old world *Leishmania donovani*: *in vitro* and *ex vivo* study

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ABSTRACT. Visceral leishmaniosis is one of the most fatal old-world neglected disease with estimated 90 thousand worldwide cases emerge each year. In Iraq, the cutaneous and visceral form are endemic but available chemotherapies are either toxic with diverse side effects, expensive available drugs or parasite resistant is arising. Artemisinin (ART) is a semi-synthetic compound which proved its effectiveness against protozoan parasites, such as malaria and *Leishmania*. In this study, the efficacy of different concentrations of pure artemisinin was screened *in vitro* against promastigotes and axenic amastigotes by MTT assay after 24, 48 and 27 hours follow up. In addition, the infectivity ability and number was investigated of intra-cellular Leishman bodies in treated murine peritoneal macrophages after 24 and 48 hours ART treatment. The results verified ART efficacy against the promastigotes and axenic amastigotes viability with IC_{50} measured after 24, 48- and 72-hours treatment. Infectivity percentage of murine macrophages and parasite burden were significantly reduced in treated cells. These findings indicate the leishmanicidal activity of ART against the Iraqi isolate of *L. donovani* and further in vivo study is recommended for assigning ART as a natural anti visceral leishmaniosis compound.

Keywords: artemisinin, old world visceral leishmaniosis, cytotoxicity, promastigotes, amastigotes

Introduction

Leishmaniosis is a sand-fly-borne disease, which is caused by the intracellular protozoan eukaryotic parasite of the trypanosomatid genus *Leishmania*; it is endemic in large areas of tropical and subtropical regions with different forms of cutaneous leishmaniosis (CL) and visceral (VL) are localized [1]. The visceral type of leishmaniosis is one of the most neglected diseases in poverty populations and considered the second-most incurable parasitic infection after malaria, worldwide [2,3]. Most chemotherapies used for VL treatment, such as pentavalent antimonials, have concerns in terms of side effect, high cost or parasite-resistant reports were described, especially in endemic areas [4–7]. Furthermore, *Leishmania donovani* developed a significant intransigence to combination of anti-leishmaniosis drugs, such as miltefosine-paromomycin and stibogluconate-paromomycin through experimentally step-wise process [8]. Similar studies indicated the minor impact of stibogluconate on the amastigotes form of *L. donovani*, which is the residing form of vertebrate host [9]. Moreover, there is no efficient vaccination against leishmaniosis and most understandings are based on experimental models with unsuccessful consequences on human trials [10,11]. Therefore, ongoing research are attempting to explore natural compounds against *Leishmania* parasites, with less side effect on humans and more affordable for public. One of the promising anti-parasitic plant-extracted semi-

synthetic compounds is artemisinin (ART) and its derivatives, the aerial part extract of *Artemisia annua* [12–14]. Artemisinin was first explored as anti-malarial agent, which demonstrated its noxious action against the most deadly species of *Malaria falciparum* [15–17]. Further investigation verified ART anti-leishmanial activity via inducing parasite programmed cell death and facilitating the pro-inflammatory cytokines of infected macrophages [18–20]. Here, we have introduced the investigation of anti-parasitic efficacy of artemisinin on the old world Iraqi strain of *L. donovani* procyclic promastigotes and intra-cellular *ex vivo* amastigotes.

Materials and Methods

***Leishmania* isolate.** *L. donovani* isolate (MHOM/IQ/2005/MRU15) was kindly provided at the Dept. of Biology, College of Science, University of Baghdad and was previously identified by PCR [21].

Chemicals. Artemisinin ($C_{15}H_{22}O_5$) was purchased from TOCRIS biotechne, UK and prepared according to manufacturer's, in which 3 mg was dissolved in 500 l of dimethylsulfoxide (99.9%). Giemsa stain was purchased from Jouri-Labs, Ethiopia.

Parasite culture. Promastigotes was cultured in M199 media (Sigma-Aldrich) supplemented with 10% heated inactivated foetal bovine serum (HIFBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich), pH 7.4 and incubated at 26°C [22]. Axenic amastigotes was differentiated by shifting temperature to 35°C, pH was adjusted to 5.5 in M199 media according to [23].

Murine macrophages. Primary peritoneal macrophages were isolated by peritoneal lavage of BALB/c mice post 72 hours thioglycollate intraperitoneal injection, according to [24]. Isolated macrophages were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% HIFBS, 100 U/ml – 100 µg/ml Pen-Strep and incubated at 37°C, 5% CO_2.

***In vitro* assessment of anti-promastigotes and anti-amastigotes activity.** ART was screened against procyclic promastigotes and axenic amastigotes in 96 well-plates (Falcon, USA). Briefly, log phase promastigote or amastigote, 1×10^6 parasite/ml, was added to each well of 96 plate containing 100 µl of M199 and was incubated with artemisinin starting with 1000 µM. Serial dilution was made to end with minimum 7.8 µM ART, wells were set in triplicates and plates were incubated at 26°C and 35°C for promastigotes and amastigotes, respectively, for 24, 48 and 72 hours follow-up before MTT assay handling [25]. Cell viability was analysed by plotting absorbency against ART micromolar concentration *via* OD values of microplate reader (Biotek; 570 nm); IC_{50} was calculated after 24, 48 and 72 hours for both forms by plotting cell viability against log ART concentrations.

***Ex vivo* assessment of anti-intracellular amastigotes activity.** Murine macrophages were harvested and washed in DMEM, cells were concentrated and maintained in 6 well-plates (Falcon, USA) as 10^6 cell/well and incubated for 24 hours in 37°C, 5% CO_2 as previously described [26]. Next day, growing macrophages were experimentally infected with axenic amastigotes in a ratio of 1:10; Artemisinin was added at a final concentration of (100, 200, 300, 400, 500) µM, plates were prepared in triplicates and incubated for three courses of follow-up. After the desired incubation, plates were fixed and stained with Giemsa stain according to [27].

Parasite load calculation. All plates were examined under microscope (100×), random fields were scanned to count at least 100 cells/well. Number of infected or uninfected macrophages and number of intracellular amastigotes per cell were counted, for treated and untreated plates [28].

Statistical analysis. Cell viability was analyzed by GraphPad Prism v7.0, significant differences was calculated by t-test at p value ≤ 0.05, also, IC_{50} was calculated by non-linear regression.

Results

Promastigotes and amastigotes *in vitro* screening

The *in vitro* screening approved the anti-leishmanial growth effect of ART on both forms. Significant difference of absorbency was observed between treated and untreated parasite growth for the two forms after 24, 48 and 72 hours incubation, this was noted for all studied concentrations, however, the promastigotes showed less susceptibility to ART along the three times of follow-up (OD up to 0.7) in comparison with amastigotes screening (OD up to 3.5) (Fig. 1 and 2). Cell viability was determined by plotting OD values of promastigotes or axenic amastigotes, after 3 incubation periods, against ART micromolar concentrations, and the results demonstrated a

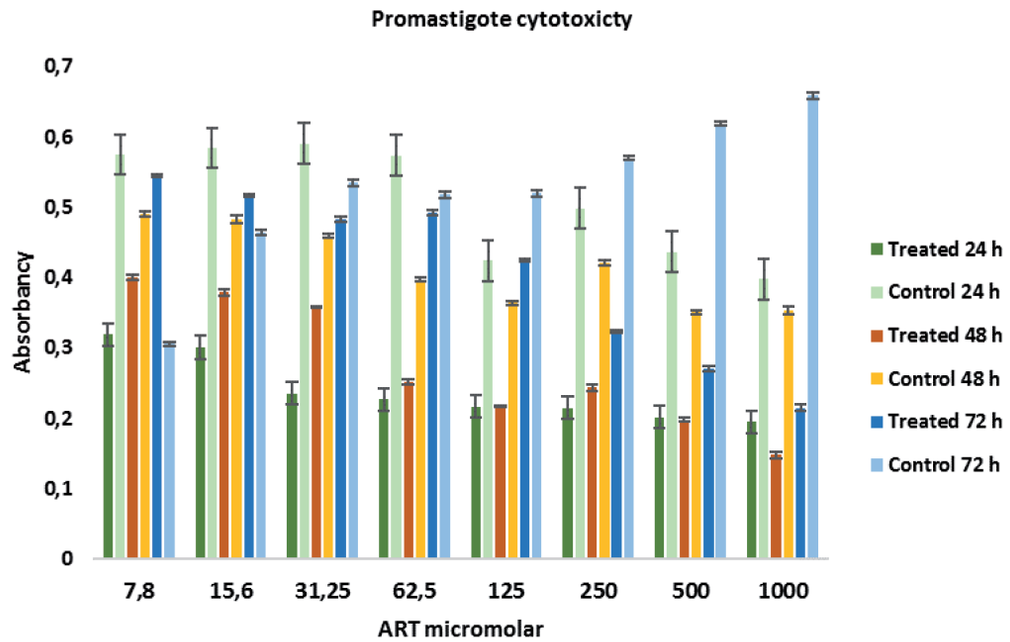

Figure 1. Absorbency screening against promastigotes 24, 48, and 72 hours

Table 1. IC_{50} values of promastigotes and amastigotes, 24, 48, 72 hours post treatment

IC_{50} (μM)	24 hours	48 hours	72 hours
Promastigotes	88.4	25.28	204.45
Amastigotes	50.24	48.33	180.6

gradual decline in parasite viability during follow-up in which the maximum inhibition was detected after 72 hours for both forms (Fig. 3 and 4).

IC_{50} was determined by plotting the cell viability of treated promastigotes or axenic amastigotes divided by percent of cell viability of untreated parasite against the logarithm of log artemisinin concentration. The minimum IC_{50} values for amastigotes and promastigotes were reported after 48 hours (Table 1). Notably, the IC_{50} values for axenic amastigotes were less than their counterparts of promastigotes, this may confirm the results of screening in Figure 2.

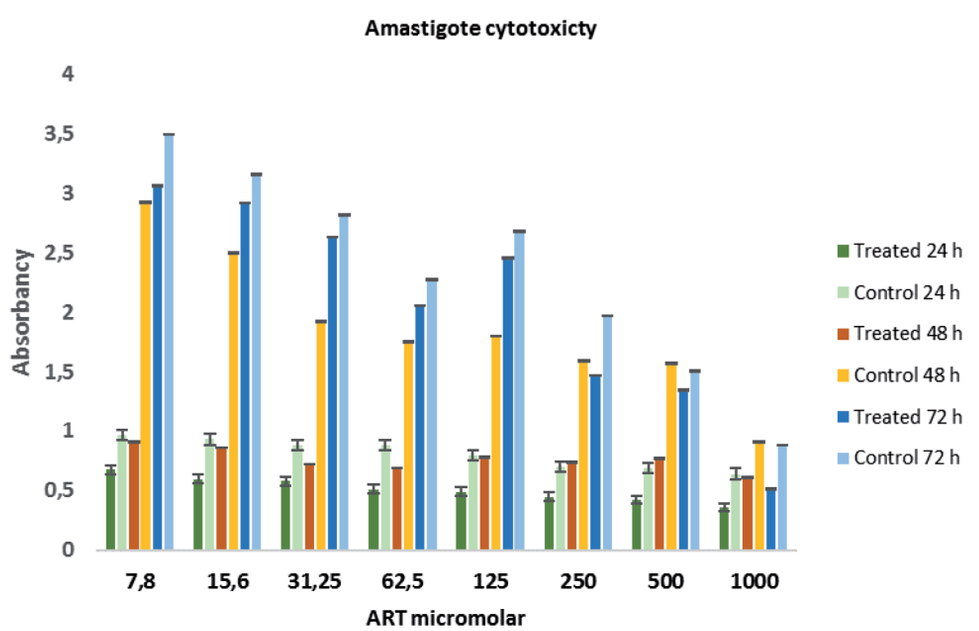

Figure 2. Absorbency screening against axentic amastigotes with artemisinin 24, 48, and 72 hours

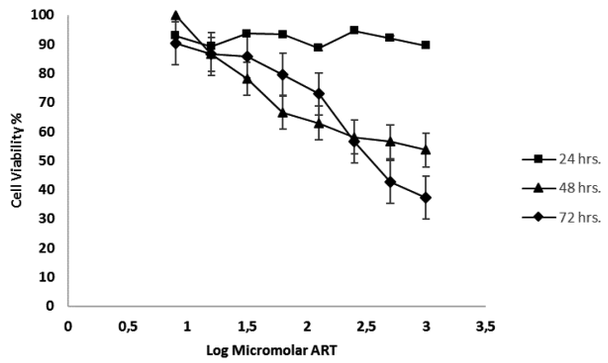

Figure 3. Cell viability of promastigotes after 24, 48 and 72 hours treatment with ART

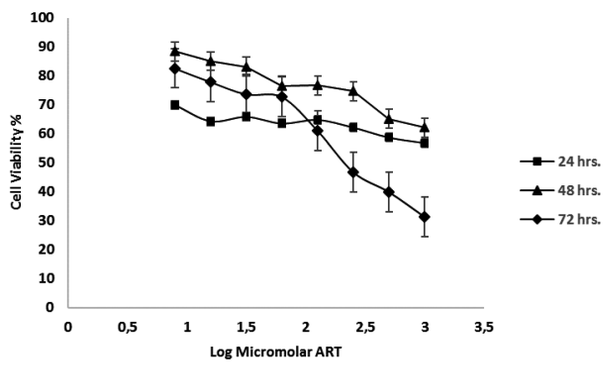

Figure 4. Cell viability of axenic amastigotes after 24, 48 and 72 hours treatment with ART

ART-Murine macrophage experimental *ex vivo* infection

Artemisinin anti-amastigotes potential was examined against intra-cellular amastigotes in mice peritoneal macrophages to test its capability to reduce the macrophage infection. Results demonstrated the ability of ART to inhibit amastigotes infectivity. The inhibition effect was observed after 24 hours in which percentage of infected macrophages was decreased into 36% and was further decreased into 20% at 500 µM after 48 hours; however, significant decline of infectivity was noticed in all studied ART concentrations (100–500) µM. Furthermore, there was a major reduction in the percent of amastigotes per 100 macrophages, between treated and untreated groups; although none of the concentrations showed a difference in the percent of amastigote per infected cell. This is due to the significant decline in the number of infected macrophages, in comparison to the high proportion of infected ones in control group (Table 2, Fig. 5).

Discussion

In order to investigate the inhibitory effect of artemisinin on procyclic promastigotes and axenic amastigotes of the old world *L. donovani,* different concentrations of the semi-synthetic artemisinin were examined against the two *in vitro* forms of the parasite. Statistically, the ART concentrations used in this study (7.8–500) µM exhibited a strong effect on the parasite growth, under optimum growing condition, compared with untreated parasite forms. Such results are in parallel with ART anti-parasitic activity on malaria and other protozoan and helminth parasites and continuing researches are investigating the prospective effect on cutaneous and visceral forms of *Leishmania* [29]. Additionally, ethanol extracts of eleven species of *Artemisia* spp. proved the efficacy to inhibit or kill the in vitro promastigote form of *L. major* with IC_{50} between 100–200 µg. [30]. Previous studies

Table 2. *Ex-vivo* infection of macrophage with *L. donovani* amastigotes after 24 and 48 hours ART treatment (cell counting under fluorescent microscope)

	Intra-macrophage amastigote ART treatment – 24 hours					
Parameter	500 µM	400 µM	300 µM	200 µM	100 µM	Control
% of infected MØ	36*	30*	40*	50*	60*	90
% of non-infected MØ	64	70	60	50	40	10
# of amastigote /100 MØ	56*	40*	48*	68*	66*	120
% of amastigote / MØ	1.5	1.3	1.2	1.4	1.1	1.3
	Intra-macrophage amastigote ART treatment – 48 hours					
% of infected MØ	20*	30*	36*	38*	46*	67
% of non-infected MØ	80	70	64	62	44	33
# of amastigote /100 MØ	22*	32*	36*	38*	10*	54
% of amastigote / MØ	1.1	1.1	1	1	0.8	1.1

Explanations: MØ = macrophage; * = $p \leq 0.05$

demonstrated the apoptotic effect of artemether (*Artemisinin derivatives*) on both promastigote and amastigote forms of old world cutaneous *L. major* and *L. tropica* with low cytotoxicity on parasite host cell [29,31]. Furthermore, experimental human application exhibited the anti-malarial effectiveness of ART with IC_{50} of 1.2–16.2 nanomolar [32]; notably, other protozoan parasites, such as *Trypanosoma* spp. have shown to demand higher ART concentrations in micromolar [33].

A very recent study on *L. donovani* showed that IC_{50} value for amastigotes after 72 hours of ART treatment was equal to 21.62 micromolar [34] while another study examined the apoptotic ability of ART alone or in combination with glucantime or shark cartilage extract (as an immunomodulatory agent) demonstrated the necrotic and apoptotic impact on the promastigotes form of *L. infantum,* after 72 hours incubation with 25 µM/ml [35]. Moreover, fraction of ART leaves and seeds confirmed the apoptotic mechanism of ART treated promastigotes via externalization of phosphatidylserine serine from constitutional to outward cell membrane [20]. In addition, phase-contrast microscopy verified the shrinkage and cytoplasm condensation of ART-treated *L. donovani* promastigotes, which lead to inhibition of in vitro proliferation [36]. Similar study evidenced that ART depleted parasite mitochondria via induction of transmembrane depolarization in *L. donovani* promastigotes mitochondria at IC_{50} equal to 62 µM [37]. Another studies showed the effective *in vitro* inhibitory cytotoxic effect of ART on many parasitic protozoa metabolism with limited side effect on the host, which puts this compound to the front of drugs with high safety index [29,38]. Similar in vitro studies suggested that the apoptotic action of ART and the production of haem-ART products may generate free radicals, this can eliminate the promastigote by alkylation via impairing parasite's proteins [18,39].

The results of *ex vivo* experiments are in line with correlated studies that proved the susceptibility of intra-amastigotes of visceral *L. infantum* to artemisinin metabolite, dihydroartemisinin, although the IC_{50} was higher than its counterpart of control drug Amphotericin B (AMB) but the cytotoxic effect of ART on macrophages was very low in comparison with AMB; besides, AMB high cost limits its medical application [40–42]. Another study presented successful treatment of patients suffering from cutaneous leishmaniosis ulcer by *Artemisia* leaf powder with no cytotoxic or genotoxic effect on

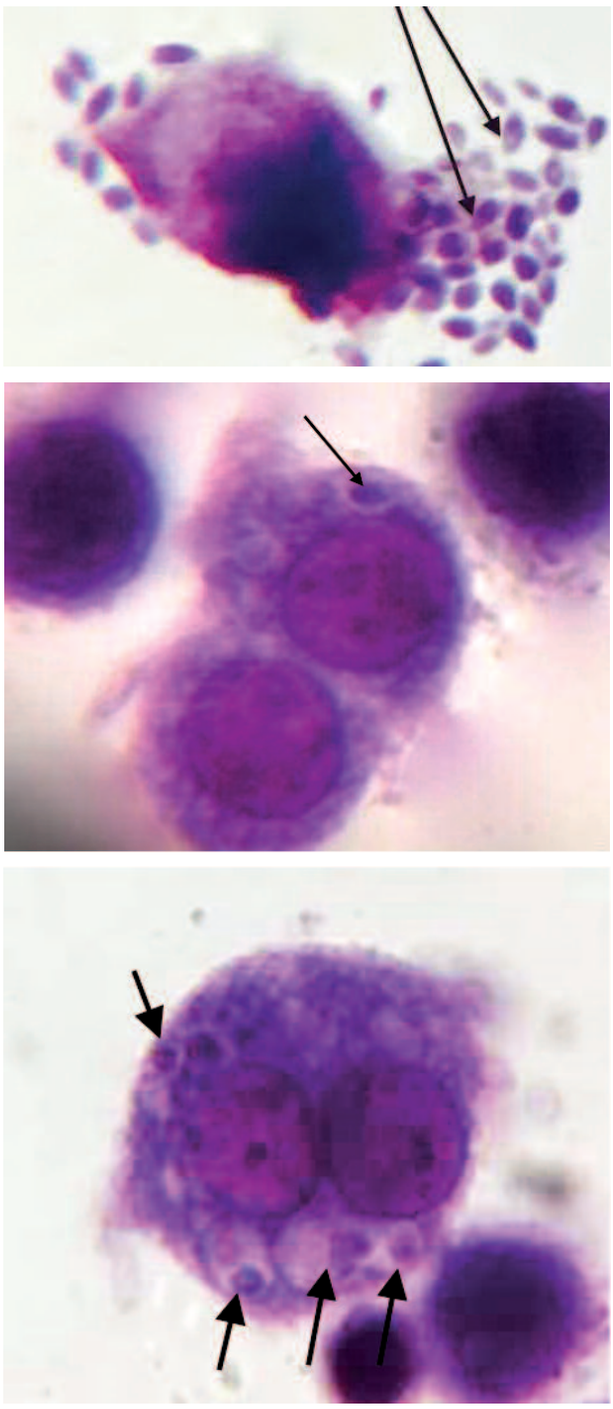

Figure 5. Mice macrophage infected with *L. donovani* amastigotes stained with Giemsa:
A: treated macrophage with 500 µM ART showing aggregation of amastigotes out of the cell; B: treated macrophages with 300 µM ART showing a single intra-amastigote; C: untreated macrophages showing multiple intra-amastigote infection; 48 hours post-infection.

macrophages [43]. Moreover, the *in vivo* mechanism of ART was elucidated by increasing the production of host macrophage Th1, IFNɣ and IL-4, which promote programmed cell death of the parasite and

control amastigote burden in macrophages [38]. An experimental *L. donovani* research on mice, verified the survival of animals after treatment with ART alone or in combination with diminazene with IC_{50} of 4.64 µg/ml and 2.28 µg/ml, respectively, concluding safety, curative and low-cost advantage of the herbal extract [19].

Comparable study on RAW macrophage cell line showed the ability of ART to restore NO production in infected macrophages after impairment of lethal NO production by *L. donovani* intra-amastigotes [39]. Similar ex vivo study exhibited the effect of ART to reduce the number of infected macrophages with *L. donovani* by recovering the NO production of infected cells [41]. Recent researchers have developed to analyse nanoparticles of nanoliposomal artemisinin to overcome the lipophilic characteristic of free-ART and the incorporated ART-liposomes had improved the anti-leishmanial activity, for *in vivo* and *ex vivo* trials, furthermore, a very recent study verified ART strong linkage to haeme compounds in its degradation activity against *L. tarentoale* [44,45].

This study is the first trial of screening artemisinin on the old world Iraqi isolate of *L. donovani*. This research proved the cytotoxic activity of ART against *in vitro* promastigotes, axenic amastigotes and *ex vivo* intra-amastigotes of this Iraqi visceral strain; although the mechanism of compound action did not be studied, many relevant researches have been reviewed and more studies are recommended for *in vivo* experimental investigations.

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