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Artemisinin Efficacy on iNOS Production in U937 Cell-line Infected with *Leishmania donovani*

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Abstract

Visceral leishmaniasis (VL), the second-most-serious parasitic illness after malaria, is currently endemic in more than 88 countries. Need for new anti-leishmanial compounds is currently being taken into consideration by researchers due to resistance and lack of effective vaccinations. This research was conducted to find out more about the effect of artemisinin (ART). ART was examined *in vitro* promastigotes stages and *ex vivo* amastigotes stages of the Iraqi strain of *Leishmania donovani* in U937 cell line after 24, 48 and 27 hours using MTT assay. In addition, the level of macrophage nitric oxide (NO) was measured using Griess assay in U937 cell line. The results of promastigotes viability percentage were less than 50 %, where the IC_{50} was 43.8, 37.9 and 36.6 µM respectively. Similar cytotoxic effect of ART was observed against amastigote forms, where the cell viability was 36.11, 40.2 and 37.4 µM respectively, after the three times of follow up. Furthermore, by using Griess assay the level of nitrite in infected macrophages was measured. The results after 48hr were 6.58 in high concentration and 1.22 in lower concentration. However, considering the absence of a significant difference, the observed concentrations are still higher than the control when compared to Griess assay. These findings demonstrated that ART has a cytotoxic effect on both forms of *L. donovani.* It also increased iNOS production in infected macrophages which can define artemisinin therapeutic efficacy against *L. donovani* infection.

Keywords: Visceral leishmaniasis, Artemisinin, MTT, Nitric oxide.

فعالية الأرتيميسينين على إنتاج iNOS في الخط الخلوي للبلاعم العملاقةU937المخمجة بالليشمانيا الاحشائية

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الخلاصة

داء الليشمانيا الحشوي هو ثاني أكثر الأمراض الطفيلية خطورة بعد الملاريا،حيثأنهمستوطن حاليًا فيأكثرمن 88 دولة. أخذ الباحثون في نظر الاعتبار الحاجة إلى مركبات جديدة مضادة لمرض الليشمانيا بسبب المقاومة ونقص التطعيمات الفعالة. في هذه الدراسة، تم فحص تأثير مادة الأرتيميسينين

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(Artemisinin) على طور أمامي السوط وطور عديم السوط المستقرفي المختبر خارج الجسم الحي من سلالة الليشمانياالاحشائيةالعراقية واستخدامالخطالخلوي937Uبعد 24 و48و27 ساعة بواسطة أختبار MTT. أضافة إلى ذلك ، تم قياس مستوى أوكسيد النيتريك للخلاية البلعميه (NO) بواسطة أختبار Griess في الخط الخلوي937U. كانت نتائج النسبة المئوية للبقاء الحيوي لطور أمامي السوط أقل من 50 ٪ ، حيث كانت الـ IC_{50} (43.8 ، 37.9 ، 36.6) مايكرومولر على التوالي. لوحظ وجود تأثير سام للخلايا مماثللأرتيميسينين ضد طور عديم السوط المستقر، حيث كان البقاء الحيوي لها (36.11، 40.2، 37.4) مايكرومولر بعد ثلاث أوقات من المتابعة، على التوالي. علاوة على ذلك ، تم قياس مستوى النتريت في الخلايا البلعمية المصابة بواسطة اختبار Griess. هذه النتائج وجدت أن مادةالارتيميسينين لها تأثيرمثبطعلى طفيلي الليشمانيا لكلا الطورين .

Introduction

Leishmaniasis is a neglected parasite disease spread by vectors and is caused by approximately 20 species of the genus *Leishmania*. Thus, causing an even wider range of diseases with various symptoms [1][2]. Despite many projects of disease control, Leishmaniasis is still a serious public health problem, especially in undeveloped countries [3]. According to WHO no inclusive vaccine has yet been approved for human use, despite the fact that numerous new vaccines have shown effectiveness in animal models [4]. Visceral type causes the most severe cases which can be fatal if not cured. The medications used to treat leishmaniasis have significant side effects, such as toxic consequences and the development of resistant forms [5]. Many researchers are trying to investigate naturally occurring substances against *Leishmania* parasites that have fewer adverse effects on people and are more accessible to the general public. Artemisinin (ART) is generated from *Artemisia annua L.* and is the best anti-malaria medication currently available [6]. ART, both in in *vitro* and in *vivo* studies, has demonstrated that it possesses anti-leishmanial properties [7].

Producing activated oxygen radicals and nitric Oxide (NO) are the two main methods for eradicating intracellular parasites [8]. NO generation by iNOS can be regulated at the transcriptional level. Most cells have extremely low levels of iNOS protein, the cause is unclear. However, activating these cells with cytokines or growth factors causes increased iNOS gene transcriptions and consequent synthesis of high quantities of NO [9]. ART has been demonstrated to serve a dual role in the control of leishmaniasis in recent studies. It enhances NO and iNOS production in uninfected macrophages and effectively regulates the immune system by elevating Thl cytokine expression [10] [7]. The goal of this investigation was to see if ART had any cytotoxic effects on promastigotes and amastigotes. In addition the role of ART in macrophages NO production under *ex vivo* condition was also investigated.

Materials and Methods

Parasite and Macrophages Cell Line U937 Used in This Study

Iraqi isolate (MHOM/IQ/2005/MRU15) of *L. donovani* was kindly provided by the laboratory of Parasitology, Graduate Studies, Department of Biology, College of Science, University of Baghdad. It was previously diagnosed by PCR [11]. Macrophage U937 cell line was purchased from Rawafid-Aleloom laboratories, Hillah, Iraq.

Promastigote Culture:

Axenic procyclic promastigotes were cultivated in RPMI1640 cell culture media. This medium was supplemented with 10% HIFCS, 100 IU/ml pen-strep [12]. The parasites were concentrated to $1x10^6$ parasites/ml and put into 5 ml universal tubes to be stored at 26°C. Later on promastigote forms were counted using a Neubauer chamber enhanced bright-line hemocytometer from Germany.

Amastigotes Differentiation from Promastigotes, *in vitro*

Amastigotes differentiation was performed by transferring $1x10^6$ promastigotes/ml to RPMI1640 medium supplemented with 20% HIFBS, pH 5.5, and incubating at 35°C for two days to create infectious metacyclic promastigotes. This was used to promote the formation of axenic amastigotes [13].

Macrophage Host U937 Cell Line Culture

The cell line U937 was grown in RPMI1640 medium with 10% HIFBS and 1% pen-strep at 37°C in an incubator that had been humidified with CO2 levels of 5%.

Artemisinin Preparation:

Artemisinin was made by dissolving 5 mg of ART powder in 3.5 ml of dimethyl sulfoxide (DMSO), as directed by the manufacturer (TOCRIS bioscience/UK).

Cytotoxicity Assay (Colorimetric MTT Assay)

MTT assay was used to screen *L. donovani* promastigotes and amastigotes for ART, as described by [14], with some modification to the doses of artemisinin. Three of 96well microtiter plates with *L. donovani* procyclic promastigotes cell suspension and three axenic differentiated amastigotes suspended in M199 medium, were utilized. Artemisinin concentrations were used to incubate the test groups: 1000, 500, 250, 125, 62.5, 31.25 and 15.6 µM. Instead of artemisinin, a triplicate of parasite culture without artemisinin was also placed on each plate as a control with DMSO. Plates were incubated for three different time periods: 24, 48 and 72 hours. Each well received 10 µl of MTT solution to the manufacturer's (iNtRON®). ,The plates were then incubated for another four hours. Later on 20 µL of DMSO was added to each well. The ELISA reader was used to read the results.

***Ex-vivo* Infection of U937 Macrophages with *L. donovani* Amastigotes:**

Cells were scraped from the bottom of a healthy culture flask and counted using a haemocytometer before being cultured on a 6 well-plate (Falcon/USA). Phosphate buffered saline (PBS) was used to wash the cells twice. The cells were then re-suspended at a final concentration of 1 x 10^5 cell/ml in RPMI media supplemented with 10% HIFBS, 1% pen-strep in a humidified incubator at 37°C. *L. donovani* amastigotes were added in a ratio of 1:10. The following concentrations of artemisinin were added to the wells: 1000, 750, 500, 250, and 125 µM. Triplicate plates were prepared and incubated for three times for follow-up: 24, 48 and 72 hours [15]. Plates were fixed and stained with DAPI stain after the specified incubation time. A separate plate was prepared as control where no ART was added.

Griess Reagent System Assay for Nitric oxide (NO) Measurement [16]

U937 macrophages 1 $x10^6$cell/ml of complete RPMI 1640 seeded in 6-well plates were infected for 5 hours at 37°C in 5% CO_2 with *L. donovani* promastigotes at a macrophage/parasite ratio of 1:10. Infected cells were incubated with Artemisinin 1000, 750, 500, 250 and 125 µM at 37°C. Supernatants were collected at 24, 48 and 72 hrs. NO was measured using Griess reagents (Promega, USA) and $NaNo_2$ (0-100 µM) was used to create a standard curve.

Statistical Analysis

SPSS software 2010 was used for t test analysis of cytotoxicity and Griess assay screening, and IC_{50} calculation, where $p\ value \leq 0.5$ was considered significant.

Result and Discussion

Cytotoxicity of Artemisinin against *L. donovani in vitro*

The drug cytotoxicity was tested against the *L. donovani* Iraqi strain on axenic culture of procyclic promastigotes and amastigotes, as well as the U937 cell line, in order to determine its cytotoxicity before *ex-vivo* infection and its effects on the viability of the *Leishmania* parasite which was used to examine cytotoxicity and cell viability. For all artemisinin concentrations, the results were plotted and compared to the control group. Cytotoxicity was measured using data of microtiter-plate reader absorbance and computed as mean± standard deviation SD [17]. The cytotoxicity result showed artemisinin ability to inhibit parasite development and proliferation, as well as a difference in colorimetric absorption. After 24, 48 and 72 hours follow-up was performed for promastigotes at all concentrations: 1000, 500, 250, 125.5, 62.5, 31.25 and 15.6 µM), and was found out that there was a statistically significant ($p \geq 0.05$) difference in microtiter plate reader absorption between test and control. The toxicity result for the highest concentration of 1000 µM for three times was 0.095, 0.063 and 0.123 respectively, and the toxicity result for the lowest concentrations of 15.6µM was 0.264, 0.137 and 2.347 for three times respectively, (Figure 1).

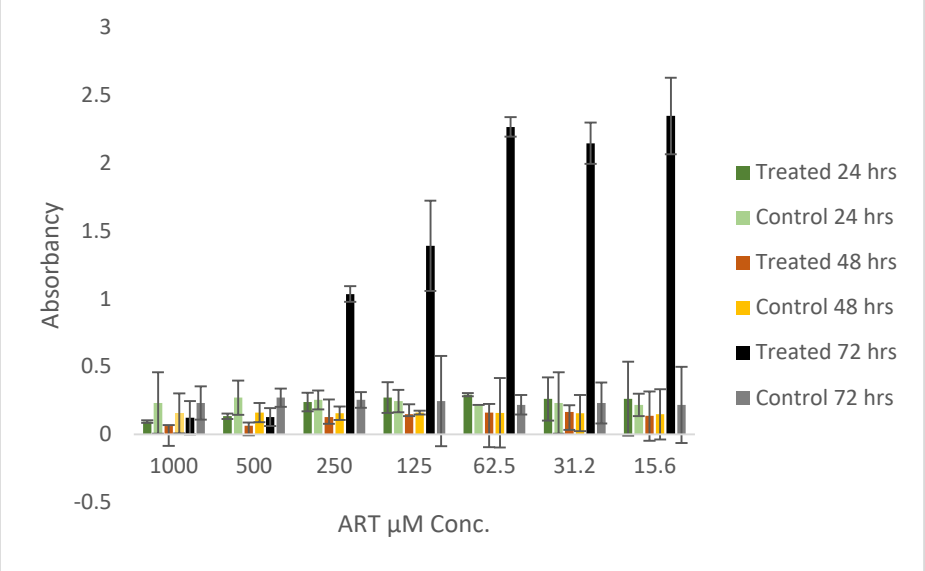

Figure 1: Artemisinin cytotoxicity against *L. donovani* promastigotes after 24, 48 and 72 hours of incubation.

The percentages of parasite viability were shown against logarithm artemisinin concentrations to calculate cell viability. All Artemisinin log concentrations were compared between test and control as means ± standard deviation (SD) (Quinn and Keough, 2002). At the greatest concentration of 1000 µM, the mean of promastigote cell viability was observed to be 41.0, 40.7 and 4.48% after 24, 48 and 72 hours, respectively (SD± 1.95). Furthermore, at the lowest concentrations of 15.6 µM, the mean of cell viability was determined 121.3, 92.4 and 86% after 24, 48 and 72 hours, respectively (SD± 4.3), and the IC_{50} of ART was 43.8, 37.9 and 36.6 µM for three times respectively (Figure 2).

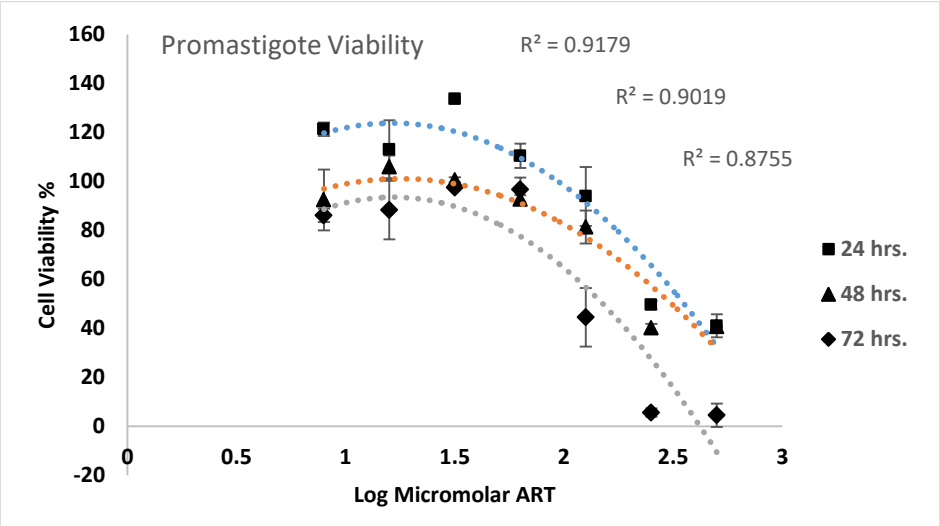

Figure 2: Cell viability of *L. donovani* promastigotes treated with Artemisinin, after 24, 48 and 72 hours of incubation.

Following three periods of follow-up, 24, 48 and 72 hours, axenic amastigotes of *L. donovani* were screened with artemisinin. In RPMI medium, promastigotes were differentiated into amastigotes and incubated at 35°C. The findings revealed that ART has an anti-leishmanial proliferative impact of the amastigote during all incubation periods, with colorimetric absorption differences between the test and control groups. The toxicity result for the highest concentration of 1000 µM for three times was 0.167, 0.118 and 0.155 respectively, and for the lowest concentration of 15.6 µM, the toxicity result was 1.522, 1.269 and 2.099 for three different timings respectively. The mean of cell viability of amastigote at a concentration of 1000 µM was detected 8.53, 10.60 and 9.30 respectively (SD ± 0.49) and cell viability average of amastigote 15.6 µM was the lowest concentration that could be measured as 83. 84, 103.81 and 90.01 respectively (SD ± 9.5) after 24, 48 and 72 hours of the follow-up. The IC_{50} of ART was 36.11, 40.2 and 37.4 µM for three different timings respectively. There were statistically significant ($p \geq 0.05$) differences in microtiter plate reader data absorption between test and control for amastigotes (Figures 3, 4) at examined concentrations of 1000, 500, 250, 125.5, 62.5, 31.25 and 15.6 µM.

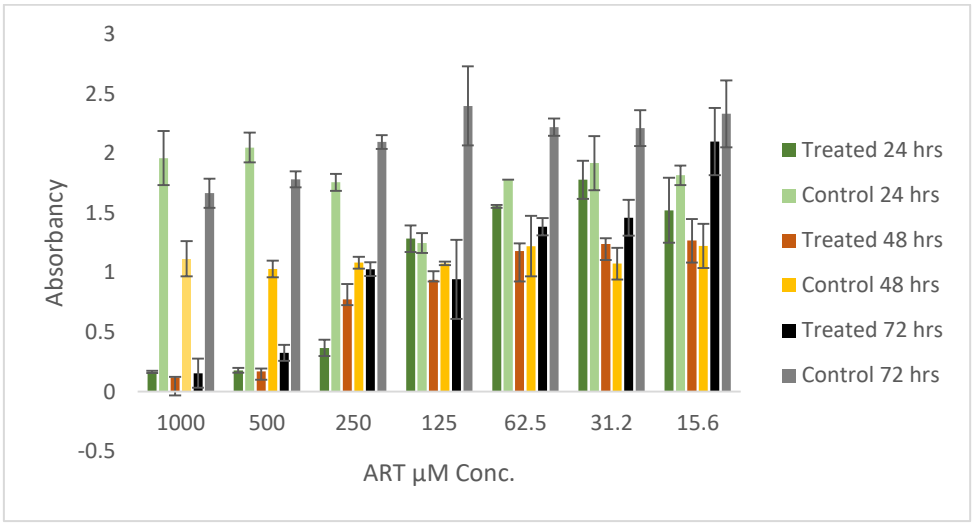

Figure 3: Artemisinin cytotoxicity against *L. donovani* amastigotes, after 24, 48 and 72 hours of incubation.

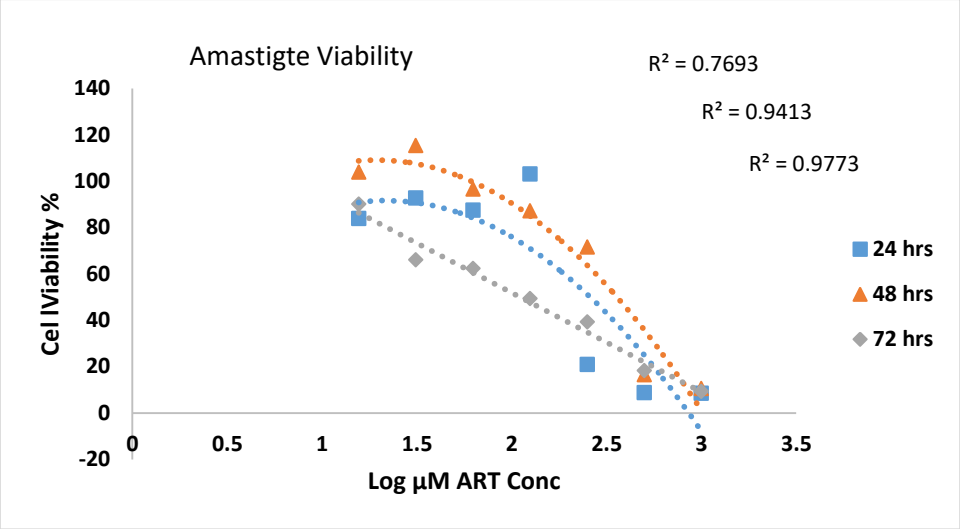

Figure 4: Cell viability of *L. donovani* amastigotes a treatment with Artemisinin after 24, 48 and 72 hours of incubation.

MTT assays demonstrated that drug concentration had an impact on the percentage of parasites that survived after 72 hours with higher concentrations resulting in lower survival rates ($p < 0.05$).

Leishmaniasis is one of the most significant illnesses in the world, and the World Health Organization (WHO) requires it to be eradicated. The disease is controlled by removing vectors and reservoirs, as well as supporting health treatment. In the absence of effective immunizations, researching novel anti-leishmanial drugs that were efficacious, safe and inexpensive is also a must [18].

Plant compounds have long been proven to be beneficial and have a wide range of antibacterial action against a range of infections. ART is one of these plant component chemicals. Combination therapy, which employs currently accessible medications, strives to reduce treatment costs, toxicity and durations [19]. Researchers recently revealed that Artemisia chemicals such as artemisinin and its derivatives have antiparasitic, antioxidant, antibacterial, anti-inflammatory and anticancer properties [20]. ART and its derivatives have been established to be effective in inhibiting *Leishmania* species in a variety of *in vitro* and *in vivo* studies. The medications were found to be effective against a variety of protozoa, including *Trypanosoma spp*., *Acanthamoeba* spp., *Babesia* spp. and schistosomiasis [21]. Artemisinin (Chinese-qinghaosu), a sesquiterpene lactone, and its derivatives are anti-malaria drugs that have shown efficacy in a variety of experimental leishmaniasis models [12]. Miltefosine and paromomycin are two medications that have recently been approved for the treatment of leishmaniasis [22]. *In vitro* tests were performed on extracts (methanol and dichloromethane extracts) from the leaves and aerial portions of four Artemisia species (*Artemisia absinthium*, *A. abyssinica*, *A. afra*, and *A. annua*) grown in Ethiopia. The dichloromethane extract from *A. abyssinica* was the most active (IC_{50} = 19.13 µg/mL), but ART from *A. annua* also has anti-trypanosomal and cytotoxic properties (IC_{50} = 35.91 µg/mL), which could be attributable to lipophilic sesquiterpene lactones in the extract [23]. Long-term treatment with miltefosine, which possesses a lengthy half-life (approximately 152 hours), can increase the development of resistance to drugs, and its potential teratogenic and abortifacient effects limit its use during pregnancy [24][17], assessed the potential use of ART as an antileishmanial medication in 2019, finding that ART alone inhibits the growth of *Leishmania infantum* promastigotes. Previous research found that the survival of *L. major* promastigotes declined progressively when the concentration of Artemisinin was increased,

with an IC_{50} of 750 µM for extracellular amastigotes and less than 30 µM for intracellular amastigotes [25].

Leishmania donovani, Leishmania infantum, Leishmania tropica, Leishmania braziliensis, Leishmania mexicana, and *Leishmania amazonensis* were used to study the anti-promastigote activity of ART on old and new world leishmaniasis, including *Leishmania donovani, Leishmania infantum, Leishmania tropica, Leishmania braziliensis, Leishmania mexicana,* and *Leishmania amazonensis,* respectively. In all of the above-mentioned species, artemisinin caused a comparable group of leishmanicidal efficacy, with IC_{50} values of 115, 120, 100, 100, 120 and 120 M respectively. ART has a broad spectrum of leishmanicidal activity, as evidenced by the comparability of IC_{50} values [26]. Treatment of *Leishmania tropica, Leishmania major,Leishmaniainfantum, Leishmania mexicana, Leishmania braziliensis* and *Leishmania amazonensis* showed similar results [26] [24].

Furthermore, [27]demonstrated that ART and its equivalents are efficient against *Leishmania donovani* promastigotes in 2003. As a result, they indicated that ART analogues could be evaluated as suitable leishmaniasis drug candidates[28]. Another research by [29] in 2007 reported that Artemisinin had anti-leishmania activity on both promastigotes and amastigotes of *Leishmania donovani*, with IC_{50} values of 160 and 22 PIM, respectively. In 2003 [30] also showed that Artemisinin has a high safety index (>22fold) against macrophage cells. The same author and coworkers reported antileishmanial activity of Artemisinin against Leishmania *donovani* and *Leishmania infantum* in 2010.

Nitric oxide production in culture media

Griess assay was used to assess levels of NO, a stabilized oxidized NO product that accumulated in the medium of culture and thus served as an indication of NO production. The results demonstrated that U937 cells were *L. donovani* infection with ART treatment in different concentrations, 1000,750, 500, 250 and 125 µM. produced the highest amount of NO when compared with control (infected U937 cells without ART) groups. In most of the concentrations, significant differences appeared after 48 and 72 hours. There is no significant difference between the 24 hour concentration and the remainder of the concentrations. However, considering the absence of a significant difference, the observed concentrations are still higher than the control when compared. The most stimulatory effect was observed in infected cells that were exposed to different concentrations of ART as in Figures 5, 6 and 7.

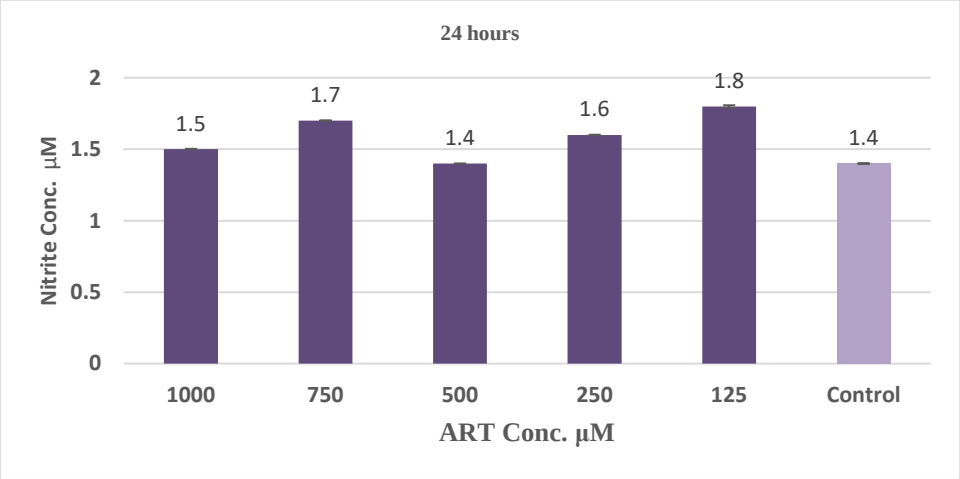

Figure 5: Nitric Oxide productions from U937 cells infection by *L. donovani* and treated with ART in culture media after 24 hours.

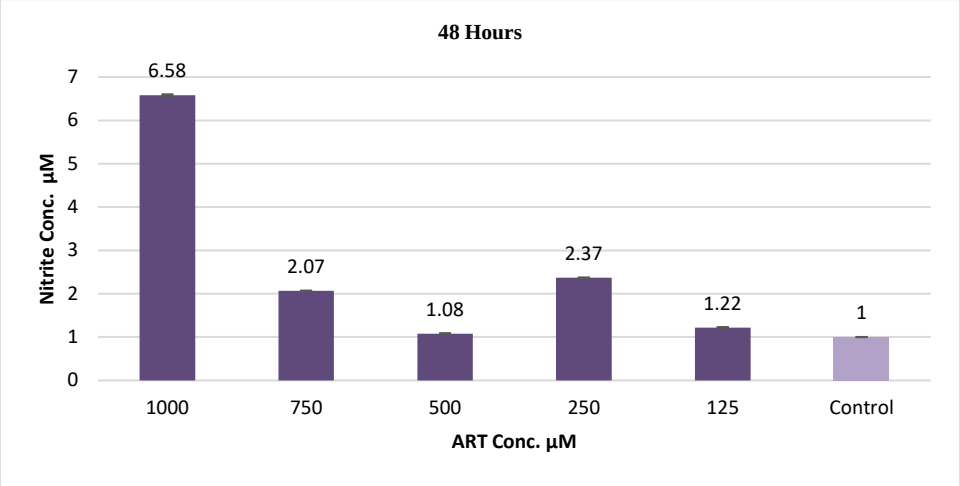

Figure 6: Nitric Oxide productions from U937 cells infection by *L. donovani* and treated with ART in culture media after 48 hours.

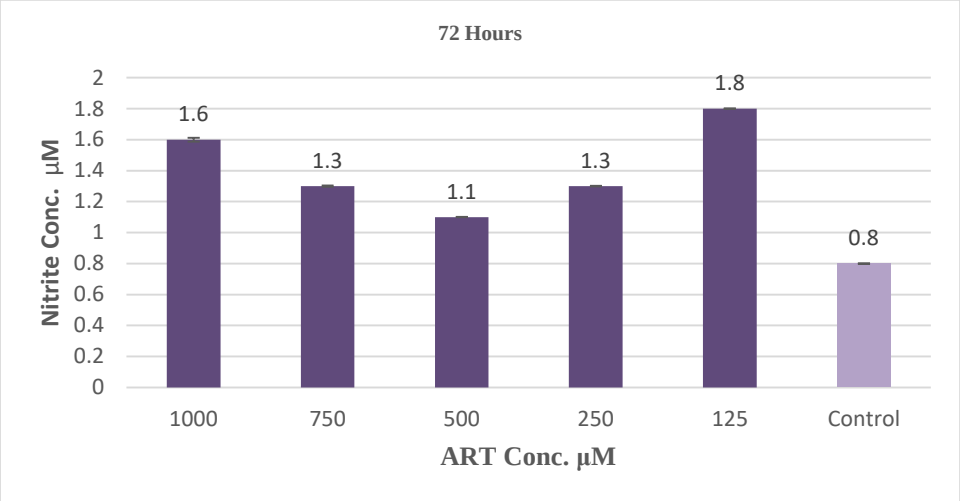

Figure 7: Nitric Oxide productions from U937 cells infection by *L. donovani* and treated with ART in culture media after 72 hours.

The process of producing (NO), mediated by nitric oxide synthases (NOS), is one of the important *Leishmania* eradication mechanisms. Inducible nitric oxide synthase (iNOS or NOS2) produces a lot of nitric oxide which helps in antimicrobial defense [31][32]. Several investigations have shown that iNOS may plays a role in leishmaniasis. However these findings are based primarily on *in vitro* cultures and animal experimentation [33]. [34] reported that *Leishmania* infection resulted in a decline in NO generation, which was followed by a large rise with the addition of ART. Only to the levels comparable to uninfected macrophages, infection resulted in a decrease in iNOS mRNA expression, which was recovered by ART and observed 50% reduction in parasite load, is mostly due to a direct parasiticidal impact rather than a NO-dependent mechanism, according to the results. And [35] show NO generation was higher in C3H/He cells in the absence of a stimulus and these cells also had a reduced infection rate and parasite load. NO generation increased and infection rate reduced when C3H/He and BALB/c macrophages were activated with LPS and IFN-Y.IL-4 and IL-10, both cytokines known for their macrophage deactivation characteristics, inhibited this leishmanicidal activity. These cytokines inhibited parasite killing by decreasing NO productions which was reversed by adding exogenous NO, indicating that they plays a roles in the control of *L. major* [36][37]. Since mice missing iNOS are unable to

regulate infection, NO is essential for parasite elimination [36]. Furthermore, LPG and other membrane compounds in *Leishmania*, such as glycoinositolphospholipids, have been shown to block macrophages iNOS and, as a result, NO generation [38]. A previous study by [39] discovered that tissues with high levels of iNOS are resistant to infection by *L. major*. This is also true *in vivo* as evidenced by the presence of iNOS expression in patient skin biopsies [40]. In fact [41]has shown that the creation of local and systemic NO early in the course of *L. mexicana* infection resolution is essential. Related study by [42] found adaptable technique for using host signaling during *L. amazonensis* infection, which has been linked to iNOS modulation. *L. amazonensis*-infected macrophages have been shown to lower LPS-induced mRNA iNOS levels, protein production and NO production. According to the present findings, ART has demonstrated its antileishmanial activity and enhanced NO in macrophage in *ex-vivo* condition.

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