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Effect of silver nanoparticles on macrophage cytotoxicity upon exposure to *Leishmania tropica in vitro*

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Abstract

Cutaneous leishmaniasis (CL) is the most form of leishmaniasis disease prevalent in Iraq. CL remains a public health problem in numerous endemic countries because of the absence of safe, effective, and high-cost drugs for treatment. Macrophages are the main inhabitant cell for *Leishmania*; they phagocyte and allow parasite multiplying. Phagocytosis and anti-leishmanial activity of macrophage are the main factors in the elimination of *Leishmania* parasites. Phagosome-resident amastigotes also evade innate host defense mechanisms. Silver nanoparticles (Ag NPs) have an important effect in stimulating the production of oxygen species. The objective of this study was to examine macrophages cytotoxicity upon exposure to *L. tropica* and Ag NPs. Serially diluted concentrations (8, 4, 2, 1, 0.5 and 0.25 µg/ml) of Ag NPs were used following macrophages exposure to *L. tropica.* MTT assay was used for verification the viability of macrophages and *Leishmania* (promastigote and amastigote). The viability percentage of macrophages has increased 104.53 ± 4.62% compare with the control group. The results indicated the positive effectiveness of Ag NPs on proliferation of promastigote and amastigotes forms. The IC50 (50% inhibitory concentration) of Ag NPs on promastigotes was calculated 2.988 µg/ ml, and the IC50 of Ag NPs following infection of macrophages *in vitro* on amastigotes was measured (2.584µg/ml). The current results concluded that (Ag NPs) had an effect on macrophages stimulation to inhibit *L. tropica* growth *in vitro* following the infection with parasite.

Keywords: Silver nanoparticles, *Leishmania tropica*, Macrophages.

تأثير جزيئات الفضة المتناهية الصغر على سمية الخلايا البلعمية عند التعرض للشمانيا الجلدية في المختبر

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الخلاصه

داء اللشمانيا الجلدية هو أكثر أشكال داء الليشمانيا انتشارا في العراق. لا يزال هذا المرض مشكلة صحية عامة في العديد من البلدان المتوطنة عالميا بسبب عدم وجود أدوية مأمونة وفعالة بالاضافة الى غلاء ثمن هذه الادوية. الخلايا البلعمية هي الخلايا الرئيسية لاستيطان الليشمانيا; فهي تبتلع الطفيليات وتسمح لها بالتضاعف. تعتبر عملية البلعمة وتنشيط مضادات اللشمانيا في الخلايا البلعمية من العوامل الرئيسية في القضاء على طفيليات الليشمانيا. الطور عديم السوط المستقر في فجوات البلعمة أيضا له دور في الية الزيغ المناعي من دفاعات المضيف . جزيئات الفضة المتناهية الصغر (Ag NPs) لها تأثير هام في تحفيز إنتاج

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أنواع الأوكسجين. هدفت الدراسة إلى فحص سمية الخلايا البلعمية عند التعرض لليشمانيا الجلدية و (Ag NPs). استخدام تراكيز مخففة (8، 4، 2، 1، 0.5 و 0.25 ميكروغرام / مل) من (Ag NPs) بعد تعرض الخلايا البلعمية لللشمانيا الجلدية. استخدام اختبار MTT للتحقق من حيوية الخلايا البلعمية والليشمانيا (للطورين الامامي وعديم السوط). اظهرت النتائج ان حيوية الخلايا البلعمية زادت الى 104.53 ± 4.62 % مقارنة مع مجموعة السيطرة. أشارت النتائج إلى الفعالية الموجبة (Ag NPs) على نمو الطورين الامامي وعديم السوط. تم حساب IC50 (التركيز النصف مثبط) على الطور امامي السوط والذي بلغ 2.988 ميكروغرام / مل، و تم قياس IC50 من بعد اصابة الخلايا البلعمية مختبريا على الطور عديم السوط وقد كانت قيمتها (2.584 ميكروغرام / مل). وخلصت النتائج الحالية إلى أن (Ag NPs) كان له تأثير على تحفيز الخلايا البلعمية لتثبيط نمو الليشمانيا الجلدية في المختبر بعد الاصابة بالطفيلي.

Introduction

Leishmania is the eukaryotic parasite in charge of leishmaniases, a range of diseases that places at danger nearly 350 million of persons in 98 countries due to the Drugs for Neglected Diseases *initiative* (DND*i*). This organism has a multipart life cycle collected of two separate stages, the promastigote form establish in the female sandfly vector and the amastigote form that repeats in the mammalian host [1]. Cutaneous leishmaniasis (CL) is a main public health problematic and reasons a variety of diseases from self-healing infections to prolonged disfiguring disease [2]. Recent antileishmanial chemotherapy applications are far away from being effective [3] .While pentavalent antimonial drugs are the most frequently prescribed treatments for leishmaniasis, they yield severe side effects. Extra compounds, such as amphotericin B, miltefosine and pentamidine, are another antileishmanial but they furthermore produce side effects that can threaten the patient's life [4]. Nanotechnology has performed as an attractive alternative due to its enhanced bioavailability and lower toxicity, and extra characteristics that advantage to reduce the danger of this disease [5, 6]. Silver nanoparticles (Ag NPs) have attained a special focus [7]. Ag NPs has antibacterial effect and *Leishmania* parasites seem to be very sensitive to it also [8]. direct damaging impact of Ag NPs on different forms (promastigotes and amastigotes) of *Leishmania tropica* parasite, likewise, the destruction of parasites increases with concentrations of Ag NPs used [9]. The MTT assay is the most prevalent active for the evaluation of cytotoxicity or cell viability after exposure to toxic materials. The MTT assay was tested for its validity in various cell lines [10]. MTT pass in the cells through endocytosis and the reduction of MTT to a colored insoluble formazan take place on the mitochondria in viable cells. This assay has been used successfully to verify mitochondrial reduction capacity and viability [11].

Material and methods

Parasite culture

Leishmania tropica parasite was isolated from patients at Al Karama Hospital and was developed at the parasitology lab for post graduate studies in Biology Department, College of Science, University of Baghdad. The promastigotes were cultivated in 199 medium containing 50 µg/ml penicillin and 10% HI-FBS and incubate at 26 °C.

Culture of macrophage cells

Macrophage model was used to assess the antileishmanial activity of Ag NPs against intracellular amastigotes. The peritoneal macrophages were isolated from the peritoneum of BALB/c mice by injection the Thioglycollate. Isolated macrophage cells were counted by hemacytometer and completed the size by Dulbecco's modified Eagle's medium (DMEM), supplemented with10% fetal bovine serum. They were then seeded into flat- bottom 96 well microtiter plates for a period of 24 hrs. for attachment at 37°C in a humidified 5% CO_2 95% air incubator. Non-adherent cells were removed by one-step washing with medium.

Isolation of metacyclic stage parasites and raising *in vitro* infection

Metacyclic stage of *Leishmania* parasites were isolated after 6 day from the past parasite cultures that were centrifuged, washed two times with PBS (pH7.2) and resuspended in complete RPMI. FBS (5%) was added to the parasite solution and incubated for 30 min at 37˚C for opsonization to gain the virulent metacyclic stage parasites.

Preparation of Ag NPs concentrations

Nanoparticles of silver were imported from NANO pars SPADANA Technology, the final concentration of Ag NPs was 4000 mg/L [12]. The stock solution of Ag NPs was serially diluted two-fold in serum free DMEM media yielding concentrations (8, 4, 2, 1, 0.5 and 0.25 µg/ml). Sonication was used at 100W and 40 kHz for 40 min for forming homogeneous suspensions and small magnetic bars were placed in the suspensions for stirring during dilution to avoid aggregation and deposition of particles.

MTT (Thiozolyl Blue Tetrazolium bromide) assay

Determination non-toxic concentrations of Ag NPs on macrophages

Before starting experiments on *L. tropica* promastigotes and amastigotes in macrophage cultures, non-toxic concentrations of Ag NPs were determined on macrophage cell culture. Peritoneal macrophages cells were seeded at 2×10^3 cells/ well, 100 µl of cells in growth medium were added to each well of a sterile 96-well microtitration plate, incubated at 37°C in humidified atmosphere for 24 hrs. Following to incubation, (50µl) from different concentrations of Ag NPs (8, 4, 2, 1, 0.5 and 0.25 µg/ml) were put into each wells of the microplate. The wells that did not expose Ag NPs were determined as control. After 48 hrs incubation, MTT assay was conducted on macrophage cells. 28 µl of 2 mg/ml solution of MTT (Bio-World, USA) was added and incubating for 4 hrs. at 37°C. After removing the MTT solution, the crystals remaining in the wells was solubilized by the addition of 130 µl of Dimethyl sulfoxide (DMSO) followed by 37°C incubation for 15 min with shaking. The absorbency was determined on a microplate reader at 584 nm.

Determination of *L. tropica* promastigotes metabolic activities

MTT test was used in order to determine metabolic activities of *L. tropica* promastigotes after their exposure to (50 µl) from different concentrations of Ag NPs (8, 4, 2, 1, 0.5 and 0.25 µg/ml). For this purpose, 2×10^4 cells/well stationary phase *L. tropica* promastigotes were seeded 100 µl into each wells of a 96-well-microplate. Promastigotes that were exposed to Ag NPs was evaluated as control. After 48 hrs incubation at 26°C, metabolic activities of parasites were detected with MTT assay that was described in detail at above.

Post-phagocytosis study

Determination viability percentage of parasites and macrophages when treatment of macrophages with Ag NPs *in vitro* following infection with *L. tropica*

The antileishmanial efficacies of Ag NPs were studied following the macrophages exposure to *L. tropica* promastigotes. For the following treatment, cultures were infected with 100 µl of promastigotes (2×10^4 cells/ well) at a ratio of 1:10 (macrophage/parasites) and incubated at 37°C for 4 hrs. After their incubation, 50 µl serially diluted Ag NPs (8, 4, 2, 1, and 0.5 µg/ml) were introduced to each culture and were incubated for 24 and 48 hrs (Figure- 1). Untreated macrophages and macrophages exposed to *L. tropica* promastigotes only in the absence of Ag NPs were used as controls. Viability percentage of parasites and macrophages following infection were detected with MTT assay that was described in detail at above.

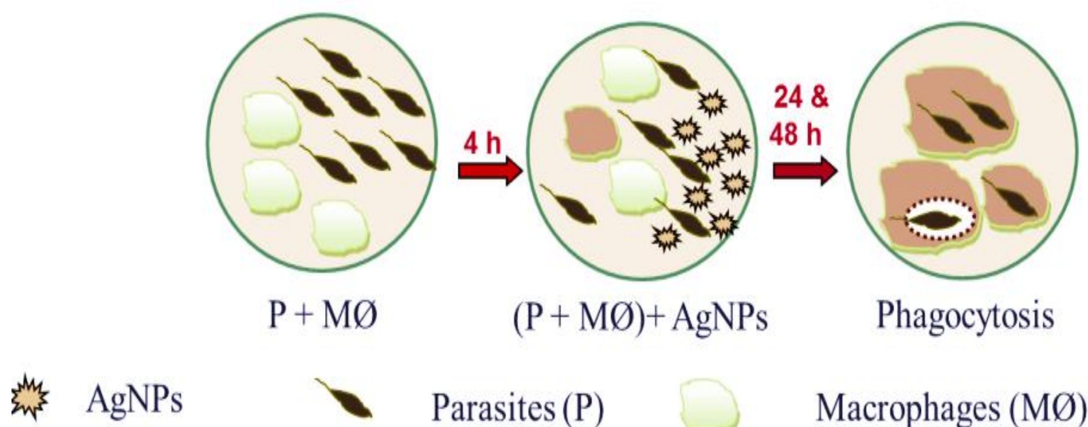

Figure 1-Effects of Ag NPs following macrophages exposure to *L. tropica* parasite [13].

Data Analysis

The Statistical Analysis System program was used to study the effect of difference factors in study parameters. Least significant difference –LSD test was used to test the significant values compared between means and Chi-square test was used to test the significant compared between percentages in this study.

Results and Discussion

Cytotoxicity of Ag NPs against macrophages and *L. tropica* promastigote *in vitro*

In current study, the effect of Ag NPs on macrophages and *L. tropica* promastigotes *in vitro* at different concentrations (0.25, 0.5, 1, 2, 4 and 8 µg/mL) was studied for 48 hrs. The viability percentage of macrophage for 48 hrs of Ag NPs exposure showed significant (P<0.05) differences. The lowest used concentration of Ag NPs (0.25 µg/ml) listed (95.6± 3.61%) of viable macrophage percentage and the highest used concentration of Ag NPs (8 µg/ml) displayed (104.53±4.62%) of viable macrophages percentage. Ag NPs did not record IC50 value because macrophages stayed over than 50%. *L. tropica* promastigotes viability after 48 hrs of Ag NPs exposure showed significant (P<0.01) differences among all concentrations of Ag NPs. The viability percentage was (71.36±2.64%) for promastigotes treated with 0.25 while it was (18.74 ± 1.04%) for promastigotes treated with 8 µg/ml of Ag NPs (Table- 1). Thus, by increasing the Ag NPs concentrations, the viability of *L. tropica* promastigotes will decrease compared with the control group. IC_{50} value of Ag NPs on *L. tropica* promastigotes was 2.988 µg/ml (Figure- 2) while Ag NPs did not record any value of IC50 on macrophages because macrophages stayed over than 50% (Figure- 3).

Table 1- Viability percentages of macrophages and *L. tropica* upon exposure to Ag NPs after 48 hrs of treatment.

Ag NPs (µg/ml)	MØ viability %	*L. tropica* viability %
0.25	95.6 ± 3.61	71.36 ± 2.64
0.5	96.59 ± 4.08	60.28 ± 3.05
1	99.39 ± 4.52	54.77 ± 2.71
2	101.37 ± 3.97	46.26 ± 2.26
4	104.35 ± 4.09	27.99 ± 1.17
8	104.53 ± 4.62	18.74 ± 1.04
LSD value	7.431 *	8.941 **

* (P<0.05).

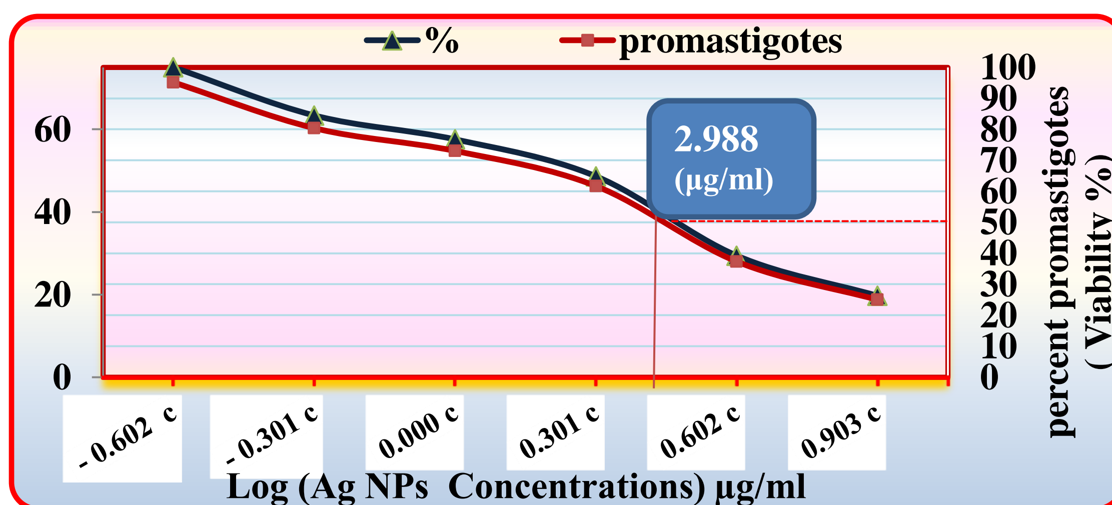

Figure 2- IC_{50} of Ag NPs on *L. tropica* promastigotes after 48 hrs. of treatment.

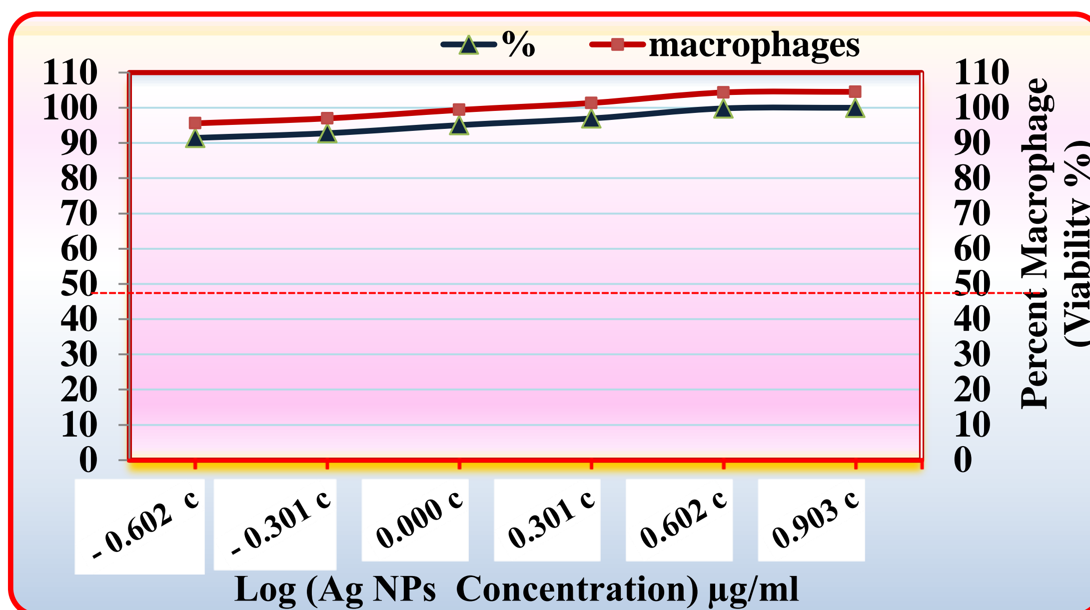

Figure 3- IC_{50} of Ag NPs on macrophages after 48 hrs. of treatment.

Macrophages can produce high amounts of ROS in order to kill microbial agents [14]. However, this production is prevented by *Leishmania* through the inhibition of the enzymatic mechanism responsible for producing reactive oxygen species (ROS), and *Leishmania* can live inside macrophages without exposed to any damage [15]. Therefore, it may be suggested that in order to prevent *Leishmania* parasites with a ROS-based treatment, these oxygen derivatives must be produced in a physical way such as through Ag NPs, instead of in an enzymatic way that can be blocked by parasites. Ag NPs presented significant anti-leishmanial effects by inhibiting the promastigotes proliferation and metabolic activity [8]. A study about cytotoxicity of Ag NPs on mammalian cells especially macrophages by Baker *et al*. (2005) revealed that Ag NPs are attractive because they are non-toxic to the human body at low concentrations and have wide spectrum antibacterial actions[16]. The concentrations used of Ag NPs in current study have no cytotoxic effect against macrophages. Also, the result showed that Ag NPs have antileishmanial effect following infection of macrophages. The results of Gharby *et al*. (2017) showed the effectiveness of Ag NPs in the decrease viability of *L. tropica* promastigotes to 31.38% in the concentration 2.1 µg/ ml. The IC_{50} of promastigotes was 1.908 µg/ml [9]. Studied on Ag NPs indicated induced a strong dose-dependent antiproliferative influence on *L. infantum* promastigotes with a dose of 50 µM of Ag prompted 100% promastigotes death and the IC_{50} were 2.18 ± 0.33 µM [17].

Post-phagocytosis study

Cytotoxicity of Ag NPs against macrophages following infection with *L. tropica in vitro*

The viability percentage of macrophages treated with Ag NPs following infection with *L. tropica* showed significant ($P<0.05$) differences among all concentrations of Ag NPs. The lowest used concentration of Ag NPs (0.25 µg/ml) listed (91.23 ± 3.86%) of viable cells, while the highest one (8 µg/ml) listed (101.98 ± 4.78%) of viable cells. There was a significant difference ($P<0.01$) when this condition compared with the control group of macrophages infected with *L. tropica* and not treated with Ag NPs (67.461 ± 2.83%) (Table 2). Ag NPs did not recorde IC_{50} value on macrophages treated following infection with *L. tropica* Figure- 4.

Table 2-The macrophages viability percentage after 48 hrs. of Ag NPs treatment following infection.

AgNPs (μg/ml)	MØ viability %
0.25	91.23 ± 3.55
0.5	91.96 ± 3.27
1	93.9 ± 3.97
2	96.53 ± 3.81
4	98.78 ± 4.15
8	101.98 ± 4.85
Control	67.461 ± 2.83
LSD value	8.368 **
** (P<0.01), NS: Non-significant.	

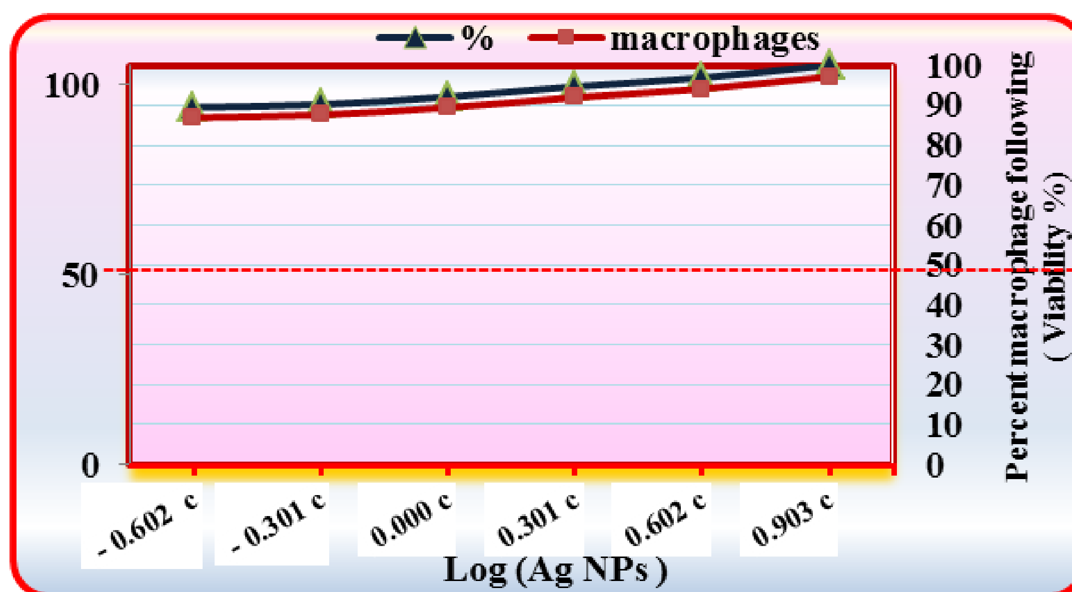

Figure 4- IC_{50} of Ag NPs on macrophages following infection after 48 hrs. of treatment.

The Ag NPs have been revealed to be cytotoxic at higher concentration more than 6 μg/ml [18]. Also in this study, the concentration 8 μg/ml of Ag NPs was non-toxic. It was found that low concentrations such as 1, 5, and 10 μg/ml of Ag NPs had no toxic effects on macrophages in the dark or under UV light [8]. [19] described that smaller Ag NPs (3 nm) are more cytotoxic than larger particles (25 nm) at a concentration of 10 μg/ml suggesting importance of particle size. The toxicity of Ag NPs was studied by its localization in Hep G2 cell line. It was found that Ag NPs get localized in the mitochondria and have an IC_{50} value of 251 μg/ml [20]. The current study demonstrated that the Ag NPs cannot recorded any value of IC_{50} on macrophages in all experiments because the used of low concentrations from Ag NPs to avoid toxicity on macrophages and in the same time induce them to kill the parasite.

Cytotoxicity of Ag NPs against *L. tropica* amastigotes following infection of macrophages *in vitro*

The viability percentage following the infection of macrophage by *L. tropica* treated with Ag NPs showed significant (P<0.01) differences among all concentrations of the Ag NPs. The lowest used

concentration of Ag NPs (0.25 µg/ml) listed (47.00 ± 2.81%) of viable cells, however the highest one (8 µg/ml) listed (20.58 ± 1.41%) of viable cells. There was a significant difference ($P<0.01$) when this condition compared with the control group of *L. tropica* amastigotes in the infected macrophages and not treated with Ag NPs (89.371 ± 3.75%) (Table 3). The IC_{50} value of Ag NPs on *L. tropica* amastigotes following infection of macrophages for 48 hrs. was 2.584µg/ml (Figure-5).

Table 3- *L. tropica* viability percentage following infection after 48 hours of Ag NPs treatment.

AgNPs µg/ml	***L.tropica* "following"%**
0.25	47± 2.81
0.5	34.14 ± 2.05
1	29.60 ± 1.77
2	24.38± 1.52
4	22.± 0.74
8	20.58 ± 0.69
control	89.371 ± 3.75
LSD value	6.831 **

** ($P<0.01$), NS: Non-significant.

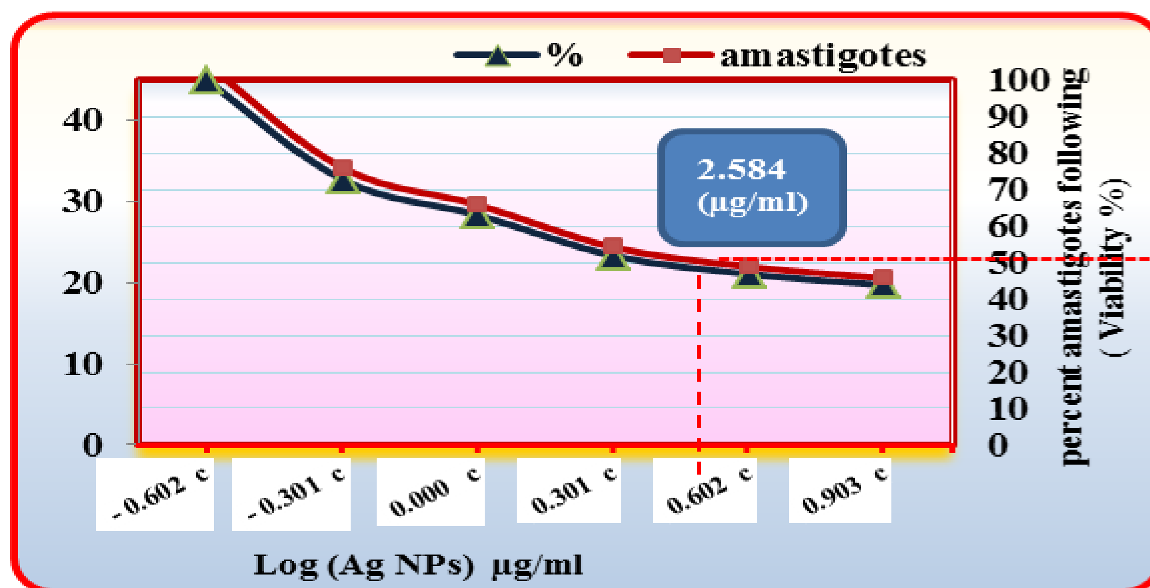

Figure 5- IC_{50} of Ag NPs on *L. tropica* promastigotes following infection after 48 hrs of treatment.

A study revealed that Ag NPs had inhibitory effects on amastigote forms of *Leishmania* parasites even in low concentrations that had non- toxic effects on macrophages. Remarkably, Ag NPs concentrations that had no antileishmanial effects on promastigotes significantly inhibited the existence of amastigotes inside macrophages [8]. The results of Gharby *et al*. (2017) showed decreases in the viability of *L. tropica* axenic amastigotes to 10.58% in the concentration 2.1 µg/ml. The IC_{50} of amastigotes was 1.298 µg/ml [9]. Also, Baiocco *et al*. (2010) studied the efficiency of Ag NPs on *L.*

infantum amastigotes and it was evaluated in murine macrophages from Balb/c mice. The groups of Ag NPs which involving 8-10 µM Ag were found to be highly toxic for the macrophages cells and stimulate cell lysis. Nevertheless, the toxic effect of Ag NPs on macrophages was revealed to be linked with dose dependent and a leishmanicidal activity. In the concentration range 0.2-4 µM, Ag NPs create a decrease in intracellular amastigotes number ranging from 10.8 to 65.0% and IC_{50} was 1.76 ± 0.24 µM [17].

Conclusion

Consequently, this study determined that non-toxic concentrations of Ag NPs was (8µg/ml) has the ability to activat the macrophages and enhanced inhibitory effects on *L. tropica* promastigotes and amastigotes *in vitro* following the infection after 48 hrs. of treatments. These results may then participate in the development of safe, non-toxic and effective anti-leishmanial drugs based on nanotechnology access against Leishmaniasis. Investigation these effects with *in vivo* studies would be a very important for use of these nanoparticles in the treatment of leishmaniasis.

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