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Evaluation of Silver Nanoparticles (Ag NPs) Activity Against the Viability of *Leishmania tropica* Promastigotes and Amastigotes *In Vitro*

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Abstract

Leishmaniasis is a disease caused by a protozoan parasite of the genus *Leishmania*. It is transmitted by the bite of sandfly (Subfamily Phlebotominae). Limited drugs are available for the treatment of leishmaniasis, and the general drug (pentostam) have many side effect on patients. Therefore, there is an urgent need for another drugs for the treatment of leishmaniasis.

This study aimed to develop new type of antileishmanial agents instead of classical drug (pentostam) and investigated the effectiveness of silver nanoparticles (Ag NPs) on *Leishmania tropica* parasites in both phases promastigote and amastigote in comparision to pentostam in *in vitro* condition.

This study showed the effects of Ag NPs in comparision to pentostam with different concentrations (0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 µg/ ml) on *L.tropica* promastigotes viability. The viability of promastigotes after 72 hr. recorded maximum cytotoxic effect of Ag NPs in highest concentration (2.1 µg/ml), it was 23.17 ± 0.45 % comparing with pentostam which was 69.33 ± 0.33 %, as well as IC_{50} was calculated for MTT assay and the result for Ag NPs was 1. 749 µg/ml after 72 hr., while pentostam drug did not show IC_{50} in all treatments.

On the other hand, the study also showed the effects of Ag NPs on *L.tropica* amastigote phase, and the viability was (3.10 ± 0.59 and 47.34 ± 0.87) % after 72 hr. in the highest concentration 2.1 µg/ml for Ag NPs and pentostam respectively, and IC_{50} was 1.148 µg/ ml for Ag NPs after 72 hr., but all results of pentostam stayed over than 50%.

Keywords: *Leishmania tropica*, pentostam, silver nanoparticles, promastigotes, amastigotes.

تقدير فعالية الفضة المتناهية الصغر على حيوية الطور امامي السوط و عديم السوط للشمانيا الأستوائية في المختبر

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الخلاصه

داء اللشمانيا هو مرض يسببه طفيلي من جنس اللشمانيا . ينتقل هذا المرض بواسطة لسعة حشرة ذبابة الرمل (من عائلة فليبوتومني).

ان العقاقير المتوفره لعلاج هذا المرض محدوده والعقار العام هو البنتوستام والذي له العديد من الاثار الجانبيه على المريض. لذلك فان هناك حاجه ماسه لايجاد عقارات اخرى لعلاج هذا المرض.

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تهدف هذه الدراسه الى تطوير نوع جديد من مضادات اللشمانيا بدلآ من العقار القديم (البنتوستام)، كما وتهدف الى استكشاف كفاءة دقائق الفضه النانونيه على حيوية طفيلي اللشمانيا الأستوائية في طوريه الأمامي السوط و عديم السوط ومقارنته مع البنتوستام في الاوساط الزرعيه.

لقد بحثت الدراسه تاثير دقائق الفضه النانويه مقارنة مع البنتوستام في تراكيز مختلفه (0.3 ، 0.6 ، 0.9 ، 1.2 ، 1.5 ، 1.8 و 2.1 مايكروغرام/مللتر) على حيوية الطفيلي في طور أمامي السوط. اظهرت النتائج التاثير التثبيطي الاقصى لدقائق الفضه على حيوية طور أمامي السوط بعد مرور 72 ساعه في التركيز الاعلى 2.1 مايكروغرام/مللتر هو 23.17 ± 0.45 % مقارنة مع البنتوستام الذي سجل 69.33± 0.33 % فقط . وكذلك تم تسجيل قيمة التركيز التثبيطي لنصف عدد الطفيليات بمقياس (ال ام تي تي) والتي كانت 1.749 مايكروغرام /مللتر بعد مرور 72 ساعه بينما لم يظهر البنتوستام اي تاثير في كافة التراكيز المستعمله.

تم تكرار التجربه المذكوره على الطفيلي في طور عديم السوط وكانت نتائج حيوية الطفيلي هي 3.1± 0.59 و 47.34± 0.87 % بعد مرور 72 ساعه في اعلى التراكيز لجزيئات الفضه والبنتوستام على التوالي. وقد كان التاثير التثبيطي لنصف عدد الطفيليات هو 1.148 مايكروغرام/مللتر لدقائق الفضه النانونيه بينما ثبت البنتوستام فوق النصف (اكثر من 50%) لجميع التراكيز العلاجيه.

Introduction

Leishmaniasis has been known for many hundreds of years, as one of the first clinical descriptions made in 1756 by Alexander Russell and called Aleppo boil. It is also known as (tropical sore, oriental sore, chiclero's ulcer or chiclero ulcer), and it is the most common form of leishmaniasis affecting humans [1].

Pentavalent antimonial is the gold standard for treatment of leishmaniasis, but it is suffer from one or other limitations like high toxicity, much expensive and there are several cases of *Leishmania* being resistant to antimonials [2].

Nanoparticles promise an alternative approach to current antibiotics in treatment of infectious diseases [3]. Silver nanoparticles (Ag NPs) confer many advantages over other drugs. In the same concentrations which are low enough to be susceptible to human cells, it is lethal for many pathogenic microorganisms without any side effects on humans [4]. Different studies have reported certain antileishmanial effects of silver due to their high level of germicidal capacity, Ag NPs have unique physicochemical properties such as tiny size (in the range of 10-1000 nm), great surface area, electrical charge and shape [5].

This study aimed to develop new type of antileishmanial agents instead of classical drug, and investigated the effectiveness of Ag NPs on *Leishmania tropica* parasites in both phases (promastigote and amastigote) in comparision to pentostam in *in vitro* condition.

Materials and methods

Leishmania tropica isolate

Leishmania tropica parasite was obtained from parasitology lab for post graduate students, College of Science, University of Baghdad.

These parasites were maintained and sub-cultured every 1-2 weeks in Novy- MacNeal-Nicolle (NNN) medium till used.

Cultivation of *L. tropica in vitro*

To reap large amount of parasites in promastigote stage, inoculum of one ml was relocated from NNN culture contain growth to screw tube vials contain five ml of (m 199) media at pH 7 with 10% FCS, and then incubated at 26 ˚C [6].

On other hand, the productions of axenic amastigotes were induced by some modification. The promastigotes were inoculated in NNN medium at 26° C for three days, then replaced by liquid phase (M199 medium) at pH 5 supplemented by 10% FCS , and incubated at 33°C for at least five days [6-7].

Pentostam concentrations

An injectable ampoules (100 mg/ml) of pentostam manufactured by (Glaxo Operations UK Limited Castle, Member of the Glaxo Smith Kline Group companies). A stock solution (100 mg/ml)

of pentostam were used in this study to prepare the following concentrations (0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 µg/ ml) before used in anti-promastigote and anti-amastigote assay as a positive control group for silver nanoparticales (Ag NPs).

Silver nanoparticales (Ag NPs) concentrations:

Ag NPs was imported from NANO pars SPADANA Technology. The original concentration was 4000 mg/l, the stock was dispersed in distilled water (D.W) by sonication at 100 W and 40 kHz for 40 minute to form homogeneous suspensions and sonicated for 40 min, it was stored at the room temperature and protect from light. A stock of Ag NPs was used to prepare the following concentrations (0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 µg/ ml) immediately before used in anti-promastigote assay, and the same concentrations were prepared again before used as anti-amastigote.

The viability of *Leishmania tropica in vitro*

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide; thiazolyl blue] is a water soluble tetrazolium salt yielding a yellowish solution. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes [8]. This water insoluble formazan can be solubilized using Dimethyl sulfoxide (DMSO), and the dissolved material is measured spectrophoto-metrically yielding absorbance as a function of concentration of converted dye [9].

Relative numbers of living parasites were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula:

Viable cells (%) = (AT-AB) / (AC-AB) ×100

Where **AT, AC** and **AB** is the absorbance of the treated samples, untreated samples and blank respectively [10].

MTT assay protocol

Both phases (promastigote and amastigote) of *Leishmania tropica* at cocentration of 1×10^4 parasite/ ml and test compounds (Ag NPs and pentostam drugs) were prepared and dispensed in a flat-bottom 96-well microtiter plate containing a final volume of 100µl/ well, and the microtiter plate was incubated at 25˚C for three days. 10 µl of MTT solution was added per well to achieve final concentration of 0.5 mg/ml, then the microtiter plate was incubated for 4 hr at 25˚C. After that, the media was removed and 100µl of DMSO solution was added in order to solubilize the formazan crystals. On the other hand the microtiter plate was stirred gently then, left for 15 minutes, and absorbance was read at 490 nm by ELISA reader.

Statistical 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 significant compare between means in this study [11].

On the other hand, IC_{50} values were calculated at different concentrations of pentostam and Ag NPs, at both phases promastigotes and amastigotes. To determine the concentration at which the parasite is inhibited by 50%. Using excel program to calculate the values of IC_{50} [12-13].

To optain IC_{50} using excel application, x_ axis represent logarithm of drug concentration and y_ axis reresent parasite density relative to the density of cotrol, by interpolation method between two concentrations (x_1, morthan y_0, x_2 less than y_0) find IC_{50}.

Results and Discussion

The present study investigated to determine the effectiveness of Ag NPs on the viability of *L. tropica* promastigotes and amastigotes *in vitro* in comparison to standard drug (Pentostam), using a pertinent viability test (MTT assay) to evaluate the cytotoxic effect of Ag NPs on parasite.

Cytotoxicity effect of Ag NPs on *L. tropica* promastigotes

The results showed that promastigotes vibility after 72 hr. of drug exposure characterized by a significant differences ($p < 0.05$) between all concentrations and the maximum percentage (2.1 µg/ml) of Ag NPs was recorded at 72 hr., it was 23.17 ± 0.45 comparing with pentostam which was 69.33 ± 0.33, this mean that the high concentration of the Ag NPs led to destroy high percentage of parasites.

Results showed that Ag NPs had dose-dependent anti-leishmanial effects, because cell viability and proliferation of promastigotes were decreased after treatment with this nanoparticles with increasing doses, Table -1.

Table 1- The viability percentage of *L. tropica* promastigots which exposed to Ag NPs and Pentostam drugs by MTT assay after 72 hr.

Drug concentration (µg)	Percentage of viable cells		LSD value
	Ag NPs	Pentostam	
0.3	72.92 ± 1.94	90.23 ± 1.40	8.469 *
0.6	70.60 ± 1.42	90.07 ± 1.14	8.603 *
0.9	66.01 ± 2.07	88.52 ± 1.30	7.924 *
1.2	62.32 ± 1.14	84.61 ± 1.96	8.779 *
1.5	53.97 ± 2.31	82.59 ± 1.66	8.053 *
1.8	34.37 ± 1.72	76.55 ± 0.92	8.304 *
2.1	23.17 ± 0.45	69.33 ± 0.33	8.912 *
LSD value	8.971 *	7.257 *	----
* ($P<0.05$).			

Previous study showed that silver polypyridyl complexes are biologically efficient against *Leishmania mexicana*, where they impact with DNA [14]. As well as silver has been shown to have antibacterial activity at very low concentrations, since it reacts easily with protein carboxylates, hydroxyls, and thiols, thus silver compounds were used as antibacterial agents [15]. An article by Kwan *et al*. (2011) [16] studied the biological effects of Ag NPs, it was shown that in wounds treated with Ag NPs, there was preferable collagen alignment after healing when compared to controls, which resulted in better mechanical strength.

On the other hand, the IC_{50} was computed for each Ag NPs and pentostam, it was 1.749 µg/ ml for Ag NPs after 72 hr. as shown in Figure -1 . But all results of pentostam remained over than 50%, thus no IC_{50} could calculated, Figure -2.

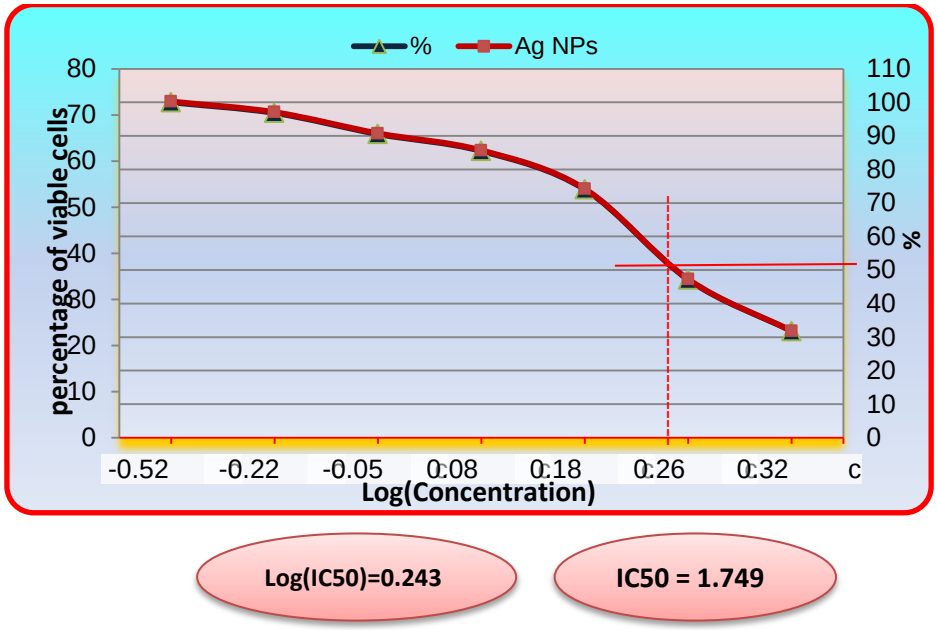

Figure 1- The IC_{50} of Ag NPs on *L.tropica* promastigotes by (MTT assay) after 72 hr.

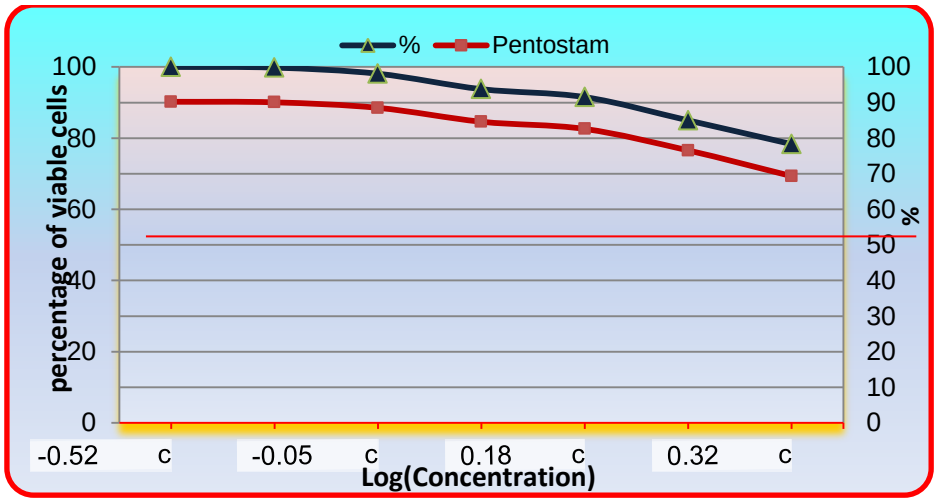

Figure 2- The IC_{50} of pentostam on *L.tropica* promastigotes by (MTT ass ay) after 72 hr.

The results of this study showed antileishmanial effect of Ag NPs more than the effect of pentostam on the parasites promastigotes, this may related to the pentostam was unsatisfactory because of their limited efficiency, recurrent side effectiveness and increasing drug resistance as explained by Croft and Seifert (2005) [17].

Other researchers studied the effect of different metal nanoparticles such as silver colloids, and showed the higher antibacterial activity, it is closely affined to their small size. Furthermore, the catalytic activity of these nanoparticles is also follow on their size, size distribution, structure, shape and chemical–physical environment [18].

The mechanisms of antimicrobial effect of nanoparticles are not well known, but ion release, binding to protein and cell components and catalytic oxidation are some proposed mechanisms. Some nanoparticles are able to produce reactive oxygen species (ROSs) under ultra violet (UV) light, and can destroyed microorganisms [19-20].

Cytotoxicity effect of Ag NPs on *L.tropica* amastigotes

A amastigotes of *Leishmania tropica* treated with Ag NPs showed low viability compared to those treated with pentostam in all concentrations, and these results due to the effectiveness of Ag NPs on dampen amastigotes proliferation.

The results in this study showed significant differences ($p< 0.05$) between different concentrations of the drugs. The viability decreased by increasing the concentrations, as shown in Table -2 which recorded lower viability in the highest concentration (2.1 µg/ml), it was 3.10 ± 0.59 and 47.34 ± 0.87 for Ag NPs and pentostam respectively. This mean it is the best between used concentration to destroy the parasites.

Table 2- The viability percentage of *L. tropica* amastigots which exposed to Ag NPs and Pentostam drugs by MTT assay after 72 hr.

Drug concentration (µg)	Percentage of viable cells		LSD value
	Ag NPs	Pentostam	
0.3	35.68 ± 1.56	83.24 ± 1.46	9.412 *
0.6	30.77 ± 2.13	80.73 ± 1.48	8.958 *
0.9	23 .56 ± 1.81	80.12 ± 1.62	9.074 *
1.2	16.81 ± 1.03	73.86 ± 1.15	7.220 *
1.5	11.23 ± 1.49	69.32 ± 1.59	8.508 *
1.8	5.50 ± 0.70	55.51 ± 1.84	8.341 *
2.1	3.10 ± 0.59	47.34 ± 0.87	8.943 *
LSD value	6.773 *	7.369 *	----
* ($P<0.05$).			

The results showed that the lowest cell viability was observed in groups of parasites which treated with Ag NPs, while the highest viability observed in groups treated with pentostam.

This result was agreed with previous result by Jebali and Kazemi, (2013) [21] who studied on Ag, Au, Ti O_2, ZnO, and MgO nanoparticles and there activities against cutaneous leishmaniasis. They referred that all nanoparticles dampened formazan crystal formation in MTT assay. The lowest formazan formation was recognized in parasites treated with Ag NPs, and the highest formation was observed in parasites treated with MgO NPs. Moreover, their study showed that cell viability was dose dependent, and declined progressively with increase of nanoparticle concentration.

On other hand, IC_{50} was also computed for each Ag NPs and pentostam, it was 1.148 µg/ ml for Ag NPs after 72 hr. as shown in Figure -3 , the results of pentostam showed no values of IC_{50}, Figure -4 .

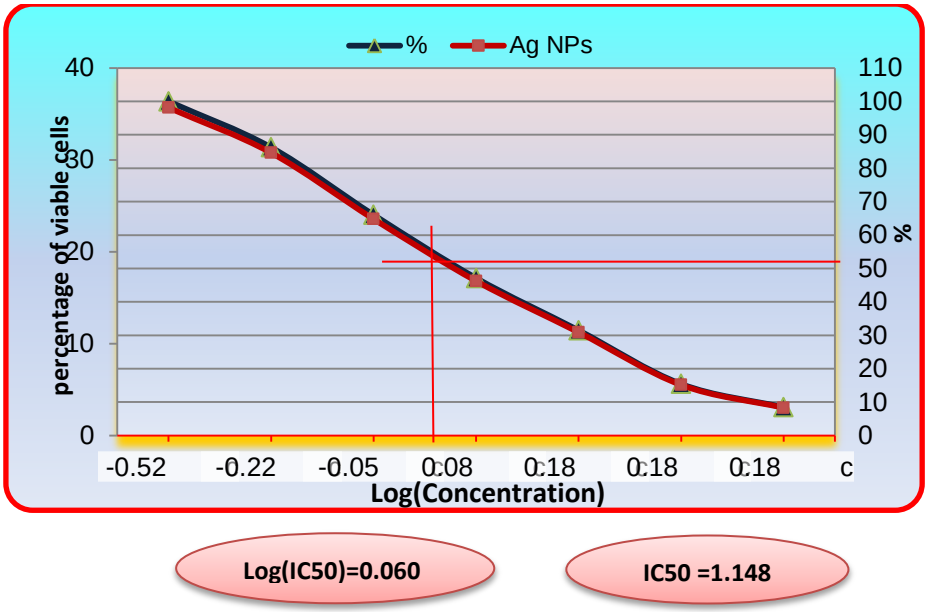

Figure 3- The IC_{50} of Ag NPs on *L.tropica* amastigotes by MTT assay after 72 hr.

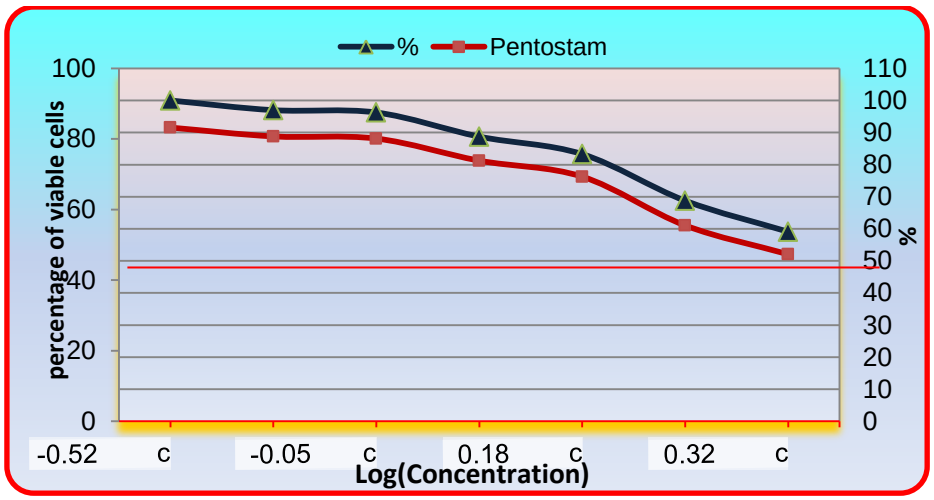

Figure 4- The IC_{50} of pentostam on *L.tropica* amastigotes by MTT assay after 72 hr.

Several studies have showed that Ag NPs declared high efficacy in inactivating viruses and bacteria. The explanation of antileishmanicidal activity of Ag NPs in the present results may related to the catalytic oxidation caused by metalic Ag and reaction with dissolved single valent Ag ions as previously showed by Shrivastava *et al.*, (2007) [22], Lu *et al.*, (2008) [23] and by Lara *et al.*, (2010) [24] on its activity on bacteria.

Chatterjee *et al*., (2011) [25] elucidated that some nanoparticles absorbed Near-infrared (NIR) energy, increase the temperature, and damage the target cells. Treatment of cutaneous leishmaniasis (CL) using infra red (IR) has been scrutinized by other researchers, it was shown that heat produced by IR is effect in CL, as good as 10 days of intravenous administration of pentostam [26].

The basic role of the antimicrobial effect of NPs is their ability to produce reactive oxygen species (ROS) [27]. *Leishmania* parasite is also known to be sensitive to ROS [28]. The production of ROS from macrophage is prevented by *Leishmania* through the inhibition of the enzymatic mechanism. Therefore, it may be posited that in order to inhibit *Leishmania* parasites with ROS, it must be produced in a physical way like Ag-NPs, instead of in an enzymatic way that can be blocked by parasites [29]. The recent studies showed that Ag-NPs, with their ability to produce high amounts of ROS, can be used as an effectiveness agent in the treatment of leishmaniasis.

Conclusions

Based on the present results, it has been concluded that pentostam drug had little effect on *L. tropica* at the low concentrations *in vitro*. On the other hand there is a direct destructive effect of Ag NPs on different forms (promastigotes and amastigotes) of *Leishmania tropica* parasite, also, the destruction of parasites increases with concentrations of Ag NPs used, and the best concentration of used Ag NPs which destroyed the parasites was 2.1 µg/ ml after 72 hr.

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