ISSN: 0067-2904

The Effect of Zinc oxide Nanoparticles (ZnO NPs) on the Viability of *Leishmania tropic In Vitro*

Meaad A. Gharby*, Ban N. Al-Qadhi
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

Cutaneous leishmaniasis (CL) also known under local names like (tropical sore, oriental sore and Baghdad sore) is the most common form of leishmaniasis. It is a public health and a social problem in many developing countries. The Old World disease primarily is caused by *Leishmania major* in dry desert areas and *Leishmania tropica* in urban areas. Recently, metal oxide nanoparticales have been versatile platforms for biomedical applications and therapeutic interventions. There is an urgent need to develop new types of antileishmanial agents instead of classical drug (pentostam), especially, when its efficacy showed a decline towards some strains of *Leishmania*. Therefore, the present work was aimed to evaluating antileishmanial activity of zinc oxide nanoparticles (ZnO NPs) on metabolic activity (viability) of *Leishmania tropica* parasites in both phases (promastigote and amastigote) *in vitro* condition. This study revealed the effects of different concentrations (2, 2.5, 3, 3.5, 4, 4.5 and 5µg/ ml) of ZnO NPs and pentostam drugs on *L. tropica* promastigote viability, which was recorded the maximum cytotoxic effect (25.12 ±1.47 and 40.81± 1.47) % at high concentration (5 µg/ml) for ZnO and pentostam respectively after 72 hr. The IC_{50} was calculated depending on the results of MTT assay to determine the most effective concentrations of ZnO NPs on the viability of *L. tropica* promastigotes. The result was 4.318 µg/ ml after 72 hr., while pentostam drug recorded an IC_{50} value only after 72 hr. which was 4.897 µg/ ml. On the other hand, the study also showed the effects of ZnO on amastigote phase, and the viability decreased by increasing the concentrations and incubation time. So the highest concentration (5 µg/ ml) recorded lower percentage of viability (18.17 ± 0.60 and 36.07 ± 2.68) % for ZnO NPs and pentostam respectively after 72 hr., while the IC_{50} of the results of MTT assay for both ZnO NPs and pentostam drug was 3.84 µg/ ml and 4.734 µg/ ml respectively after 72 hr.

Keywords: *Leishmania tropica*, Zinc oxide nanoparticles, pentostam, promastigotes, amastigotes, MTT assay.

تاثير الدقائق المتناهية الصغر لأوكسيد الزنك على حيوية اللشمانيا الاستوائية في المختبر

ميعاد عبدالرزاق غربي* ، بان نوري القاضي
قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصه

داء اللشمانيا الجلدية يعرف ايضا باسماء عامية (الحبة الاستوائية، الحبة الشرقية وحبة بغداد) وهو من اكثر الانواع الشائعة من اللشمانيا. ويمثل مشكلة للصحة العامة في العديد من الدول النامية. مرض العالم

*Email: meaad_a_r@yahoo.com

القديم غالبا ما يتسبب بطفيلي اللشمانيا الرطبة في المناطق الصحراوية الجافة واللشمانيا الجافة في المناطق الحضرية. حاليا اوكسيدات المعادن المتناهية الصغر تعتبر دعامات متعددة التطبيقات في الطب الحيوي والتداخلات الجراحية وهناك حاجة ملحة لتطوير انواع جديدة من مضادات اللشمانيا بدلا من العلاج التقليدي (البنتوستام) خاصة عند بدء انخفاض كفائته ضد بعض السلالات من اللشمانيا. لذا هدفت الدراسة الحالية لتقييم الفعالية المضادة للشمانيا لدقائق اوكسيد الزنك على حيوية طفيلي اللشمانيا الاستوائية لكلا الطورين (امامي و عديم السوط) بظروف مختبرية. بينت الدراسة تأثير تراكيز مختلفة (2، 2.5، 3، 3.5، 4، 4.5، 5) مايكروغرام/ مل من اوكسيد الزنك والبنتوستام على الشكل الامامي السوط للشمانيا الجلدية ،حيث سجلت اعلى تأثير سمي على الخلايا بمقدار (25.12 ± 1.47) % و (40.81 ± 1.47)% عند اعلى تركيز (5) مايكروغرام/ مل لاوكسيد الزنك والبنتوستام على التعاقب بعد 72 ساعة. في حين أن التركيز القاتل لنصف العدد من الخلايا تم حسابه اعتمادا على نتائج اختبار ام تي تي لتحديد تركيز اوكسيد الزنك الاكثر تاثيرا على حيوية الشكل الامامي السوط للطفيلي. وكانت النتيجة 4.318 مايكروغرام/ مل بعد 72 ساعة بينما سجل البنتوستام 4.897 مايكروغرام/ مل. من ناحية أخرى، أظهرت الدراسة أيضا آثار اوكسيد الزنك على الطور عديم السوط، وانخفضت الحيوية من خلال زيادة التراكيز و وقت الحضانة. وبالتالي فإن أعلى تركيز (5 مايكروغرام/ مل) سجل نسبة أقل من الحيوية (18.17 ± 0.60 و 36.07 ± 2.68)٪ لأوكسيد الزنك والبنتوستام على التوالي بعد 72 ساعة، في حين أن التركيز القاتل لنصف العدد من الخلايا تم حسابه اعتمادا على نتائج اختبار ام تي تي لكل من اوكسيد الزنك والبنتوستام وكانت النتيجة 3.84 مايكروغرام/ مل و 4.734 مايكروغرام/ مل على التوالي بعد 72 ساعة.

Introduction

Leishmaniasis is a disease caused by a protozoan parasite of the genus *Leishmania*, a unicellular kinetoplastid flagellate. The main forms of the disease are cutaneous leishmaniasis (CL) caused by *Leishmania tropica, mexicana* and *major* etc., visceral leishmaniasis (VL) caused by *Leishmania donovani* and *Leishmania infantum* and mucocutaneous leishmaniasis (MCL) caused by *Leishmania braziliensis* [1, 2]. During the life cycle of the genus *Leishmania* assume various morphologic and functional stages. In mammalian hosts, the parasites occur as amastigote forms and are found within the phagolysosomes of macrophages, while in sandfly hosts the parasites occur as promastigotes within the gut [3- 5].

The first line of treatment for all types of leishmaniasis is the pentavalent antimonials- meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam), it is remained the standard therapy for more than 60 years [6]. This agent has multiple toxicities and is increasing unsuccessful due to expansion of parasite resistance, and there are many defects such as painful of administration and long period of treatment [7- 9]. Due to acute forthright effects, lots of patients who are infected with leishmaniasis refuse treatment, so there is serious need to new alternative treatments. Nano medicine developments are storming the disease racetrack, and they are being introduced into the clinic [10].

Zinc oxide nanoparticles (ZnO NPs) is one of the five zinc components that are actuality registered as generally recognized as safe by the United State Food and Drug Administration [11, 12]. This nanoparticle has effective on reluctant microorganisms, and an antibacterial effect on gram- negative and gram- positive bacteria such as *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* [13, 14].

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

Promastigote cultivation

To reap large amount of parasites in promastigote stage *in vitro*, inoculum of one ml was relocated from NNN culture contain growth to universal tube vials contain five ml of media (m 199) at pH 7 with 10% FCS, and then incubated at 26 ˚C. After three days the culture was examined under light microscope to ensure the growth of parasites and the absence of any other contamination, a small amount of media added to culture if needed. By this way gain the active parasites in log phase [15].

Axenic amastigote cultivation

The productions of axenic amastigotes were induced by some modification in pH of media and temperature of incubation. The promastigotes were inoculated in NNN medium at 26 ℃ for three days, then replaced by liquid phase (M199 medium) at pH 5 supplemented by 10% FCS , and incubated at 33℃ for at least five days [15, 16].

Pentostam (SbV)

An injectable ampoules (100 mg/ml) of pentostam manufactured by (Glaxo Operations UK Limited Castle, Member of the Glaxo Smith Kline Group companies) were used in this study. They were gained from Medical City Hospital in Baghdad.The drug was stored below 25˚C and protect from light. A stock solution of pentostam was used to prepare the following concentrations (2, 2.5, 3, 3.5, 4, 4.5 and 5 µg/ ml) before used as anti-promastigote and anti-amastigote assay, as a control group for zinc oxide nanoparticales (ZnO NPs).

Zinc Oxide Nanoparticles (ZnO NPs) concentrations

Nanoparticles ZnO colloid was imported from NANO pars SPADANA Technology. The original concentration was 400 mg/ml. The stock was dispersed in ultrapure water by sonication at 100W and 40 kHz for 40 minute to form homogeneous suspensions, then serially diluted in sterile ultrapure water and additionally sonicated for 40 min. Small magnetic bars were placed in the suspensions for stirring during dilution to avoid aggregation and deposition of particles.This NPs stored at the room temperature and protect from light. A stock of ZnO NPs was used to prepare the following concentrations (2, 2.5, 3, 3.5, 4, 4.5 and 5 µg/ ml) immediately before used as anti-promastigote, and the same concentrations were prepared immediately before used as anti-amastigote.

The viability of parasites by MTT assay

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 formazanby cleavage of the tetrazolium ring by dehydrogenase enzymes [17]. 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 [18]. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 490 nm using a microplate reader [19]. Relative numbers of live cells 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 [20].

MTT assay protocol

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

Statistical Analysis

The Statistical Analysis System- SAS [21] 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. On the other hand, IC_{50} values were calculated at different concentrations of pentostam and ZnO NPs at both phases promastigotes and amastigotes. To determine the

concentration at which the parsite is inhibited by 50%. Using excel program to calculate the values of IC_{50} [22, 23].

Results and Discussion:

Cytotoxic effect of ZnO NPs on *L. tropica* promastigotes by colorimetric assay (MTT):

The results showed that the highest concentration (5µg/ml) of ZnO NPs after 72hr. gave maximum cytotoxic effect (25.12 ± 1.47), while the viability of promastigotes to the same concentration and the same time of pentostam was 40.81 ± 1.47, it was higher than ZnO NPs, this disclosed that the best concentrations of the ZnO NPs led to destroy and kill many of parasites. On the other hand, the lowest concentration of ZnO NPs and Pentostam (2 µg/ ml) after 72 hr. recorded higher viability (69.12 ± 0.53) and (87.86 ± 1.44) respectively (Table-1), this indicated to the lowest killing effect on parasites in this concentration.

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

Drug concentration (µg)	**Percentage of viable cells**		**LSD value**
	ZnO NPs	**Pentostam**	
2	69.12 ± 0.53	87.86 ± 1.44	7.405 *
2.5	62.30 ± 1.45	86.99 ± 1.71	7.612 *
3	57.07 ± 1.48	86.02 ± 1.52	8.932 *
3.5	53.75 ± 1.91	83.45 ± 1.83	7.336 *
4	42.01 ± 1.52	80.13 ± 1.57	7.025 *
4.5	30.53 ± 0.99	56.67 ± 1.84	7.842 *
5	25.12 ± 1.47	40.81 ± 1.47	7.935 *
LSD value	6.239 *	7.479 *	----
* ($P<0.05$).			

The results of this study showed that ZnO NPs have dose-dependent anti-leishmanial activities because the cell viability and proliferation of promastigotes are decreased after treatment with nanoparticles, which indicates that nanoparticles damage mitochondrial enzymes and cell cycle proteins.

These results agree with other researches such as studies of Jebali and Kazemi (2013) [24], that searched in cytotoxicity assessment of different nanoparticles including ZnO NPs on cutaneous leishmaniasis using both MTT assay and formazan crystals observation. There was no formazan observed in parasites which were treated with high concentrations of nanoparticles. On the other hand, formazan was seen in negative control cells that were not treated with any nanoparticles.

The results also agreed with study of Delavari *et al.* (2014) [25], that searched in evaluating the cytotoxic effect of ZnO NPs on *L major* promastigotes *in vitro*. In addition, other studies carried out on the effectiveness of zinc sulphate on *L. tropica* and *L. major* were found an inhibitory action on utmost virulence enzymes and enzymes of carbohydrate, also the most paramount regulatory enzyme in the glycolytic pathway was dampened [26, 27].

It is noticeable that pentostam is usually not very effective *in vitro* since it is processed *in vivo* before acting against *Leishmania* parasites [28]. Furthermore, promastigote of cutaneous leishmaniasis was found to be reluctant to some drugs like pentamidine and pentostam [29].

The MTT assay is based on the cellular metabolic activity and the ability of the mitochondrial enzyme, succinate-dehydrogenase of viable cells to convert the MTT tetrazolium salt into a purple colored formazan product, thus color formation serves as a useful and convenient marker of only the viable cells. MTT formazan is commensurate to the number of living cells present, cells with rapidly dividing cells exhibit high rates of MTT reduction. In contrast cells with a low metabolism reduce very little MTT [30, 31], as happened in rustles of this study.

The IC_{50} is the concentration which inhibits growth and numbers of cells to the half number (50 %) compared with control group,in this study it was calculated for each ZnO NPs and pentostam for MTT assay.

IC_{50} of ZnO NPs and pentostam after 72 hr. was 4.318 µg/ ml and 4.897µg/ ml as shown in Figures -1 and 2 respectively.

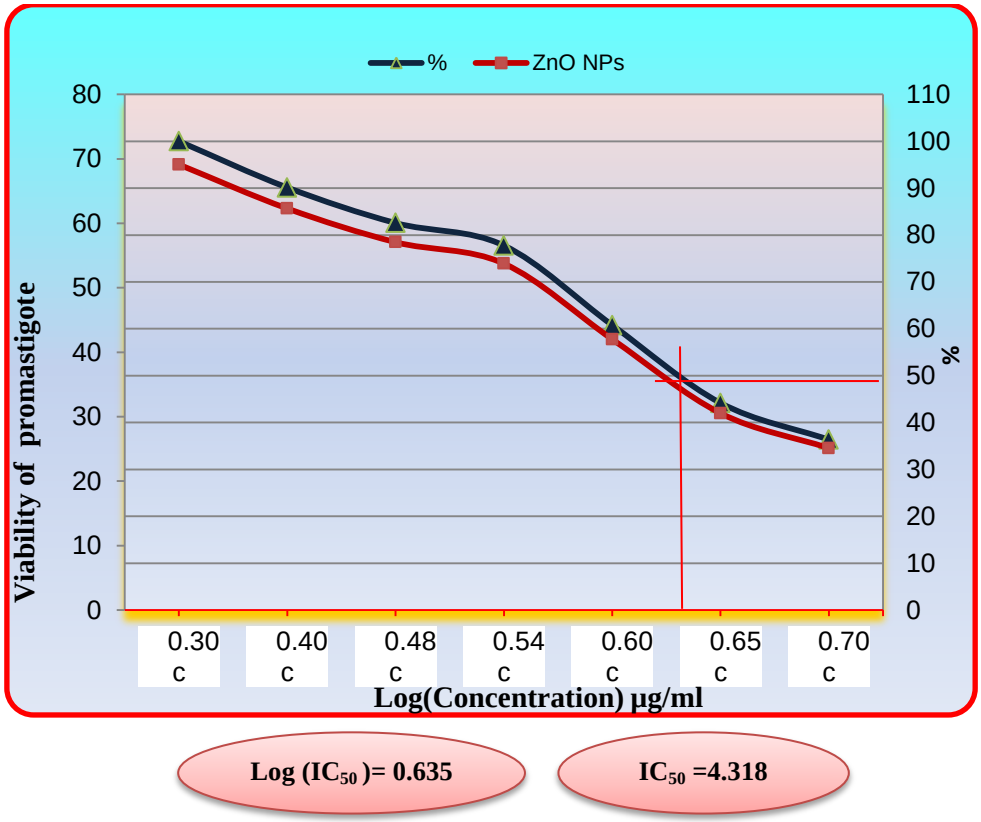

Figure 1- IC_{50} of ZnO NPs against *L. tropica* promastigotes by MTT assay after 72 hr.

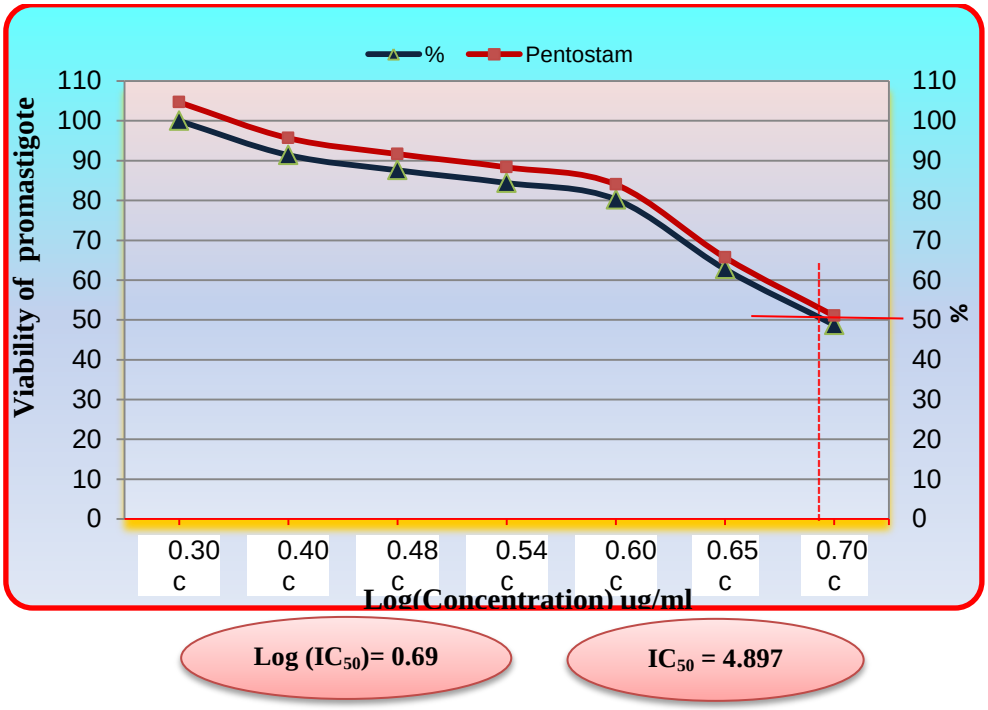

Figure 2-The IC_{50} of pentostam on *L. tropica* promastigotes by MTT assay after 72hr**.**

The results of this study denoted that nanoparticles have dose dependent anti-leishmanial activities because of susceptibility of *L. tropica* to ZnO NPs at low concentrations used in this study, unlike the low effect of pentostam drug against *L. tropica* in the same concentrations.

There are some evidences prove that nanoparticles have anti-leishmanial activities on *L. tropica* and *L. major* [32]. Jebali and Kazemi (2013) [24] postulated that nanoparticles bind to different parts

of promastigote membrane, inactivate membrane proteins, and then lead to inhibition of access to macrophages, so that inactivated and impaired of parasites. Other researchers looked at the IC_{50} value on *leishmania* parasites, Delavari *et al.* (2014) [25] indicated that IC_{50} of ZnO NPs on Iranian strain of *L. major* promastigotes was measured 37.8 µg/ml after 24 hr. This value is higher than values in this study; this may be due to the difference between strains of parasite, where *L. major* is more resistant than *L. tropica*, difference of the producing company of nanoparticles and difference of environmental and *in vitro* conditions.

Bondarenko *et al*. (2013) [33] studied L(E)C_{50} or minimal inhibitory concentrations (MIC) values of some nanoparticles against bacteria. MIC values for bacteria (*Vibrio fischeri*) were 7.1, 200 and 500 mg/L for Ag, CuO and ZnO NPs sequentially. Another study of Najim *et al*., (1998) [27] on *L. tropica* and *L. major* searched the effect of zinc sulphate, and there results showed that the ED_{50} for *L. tropica* promastigote was 161.8 µg/ml while for *L. major* was 221.9 µg/ml. These results pointing to the fact that *L. tropica* is more sensitive to zinc sulphate than *L. major*. The same study showed that the ED_{50} for sodium stibogloconate for *L. tropica* promastigote was 2.55 g/ ml and 334.7 µg/ ml for *L. major*. These values were higher than that of zinc sulphate.

On the other hand, the studies on pentostam drug revealed that IC_{50} values were high compared to those of NPs. A study done by Callahan *et al*., (1997) [34] showed that IC_{50} of Pentostam for *L. mexicana* promastigote was 10 µg/ ml. While another study recorded that IC_{50} of Pentostam for *L. major* promastigote was 139.31 µg/ ml, and it was 28.41 µg/ ml for *L.donovani* [35]. However, results of Vermeesch *et al*., (2009) [36] study recorded that IC_{50} of pentostam against *L. donovani* promastigote was > 64µg/ ml. This means it was not active because it cannot kill at least the half number of parasites.

All of the results mentioned above showed high effectiveness of ZnO NPs on parasites which was higher than effectiveness of pentostam, and explained that high effectiveness of nanoparticles is due to the ability of NPs to produce reactive oxygen species (ROS) that are capable of causing alteration in macromolecules, like proteins, lipids and nucleic acids, which is known as oxidative stress. These oxygen derived free radicals are short-lived, unstable, and influence prokaryotic or eukaryotic cell viability, righteousness, and health. Thus leading to cell death [37, 38]. ROS induces protein oxidation and lipid peroxidation that damage the rigidity of cell membrane by affecting fluidity and permeability, inflicting changes in the ion transport, and inhibiting the metabolic processes [39].

Cytotoxic effect of ZnO NPs on *L. tropica* amastigote by colorimetric assay (MTT):

Leishmania tropica amastigotes treated with ZnO NPs showed low viability compared to those treated with pentostam in all used concentrations, and these results indicated to the positive effectiveness of ZnO NPs on amastigotes proliferation.

The results in this study showed significant differences ($p< 0.05$) between different concentrations of the drugs, and the viability decreased by increasing the concentrations, as shown in Table-2.

After 72 hr., the results showed that amastigotes viability have recorded maximum cytotoxic effect at high concentration (5µg/ ml) of ZnO NPs, it was 18.17 ± 0.60 and 36.07 ± 2.68 for ZnO NPs and pentostam respectively.

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

Drug concentration (µg)	**Percentage of viable cells**		**LSD value**
	ZnO NPs	**Pentostam**	
2	60.13 ± 2.62	87.97 ± 1.04	6.552 *
2.5	58.32 ± 0.56	77.52 ± 1.07	8.396 *
3	50.12 ± 1.41	75.93 ± 0.63	8.405 *
3.5	39.02 ± 2.06	72.46 ± 1.49	8.912 *
4	26.01 ± 1.01	71.07 ± 1.67	7.553 *
4.5	22.83 ± 1.67	51.33 ± 0.68	7.409 *
5	18.17 ± 0.60	36.07 ± 2.68	7.966 *
LSD value	8.225 *	7.986 *	----
* (P<0.05).			

All of the results showed that the percentages of viable amastigotes in ZnO NPs were less than Pentostam drug at all doses after incubation.

A perfect drug delivery system must possess two important elements: controlled and targeted delivery. In this consideration, ZnO NPs have outcropped as the potential and effective drug delivery systems. Drugs have an optimum concentration within which they are salutary. Therefore, in designing the ZnO NPs, the major goal is to control the particle size and surface properties to fulfill the controlled emission of the pharmacologically active agent at a specific site at the therapeutically optimal rate within the dose regime. Due to their ultra-small and controllable size, NPs can facilely penetrate body cells, and more importantly, they show high reactivity with biological systems of microbes [40].

Xie *et al*., (2011) [41] reported that ZnO NPs induce generation of ROS can lead to cell death when the antioxidative capacities of the cells are surpassed. Moreover, the death of parasites by ZnO NPs may be related to the mechanism of disruption of the cell membrane lipids and proteins that resulted in the leakage of intracellular contents eventually.

In the case of ZnO NPs, generation of ROS has been imputed to their nano level and semiconductor characteristics that lead to generation of ROS even in the absence of light. Oxidative stress reflects an imponderable between the systemic semblance of reactive oxygen species and a biological system's capacity to readily reform the resulting damage [42].

On the other hand, the IC_{50} of amastigotes was calculated for the results of MTT assay for ZnO NPs and Pentostam drug. It was 3.84 µg/ ml and 4.734 µg/ ml for ZnO NPs and pentostam respectively after 72 hr. as shown in Figures- 3 and 4.

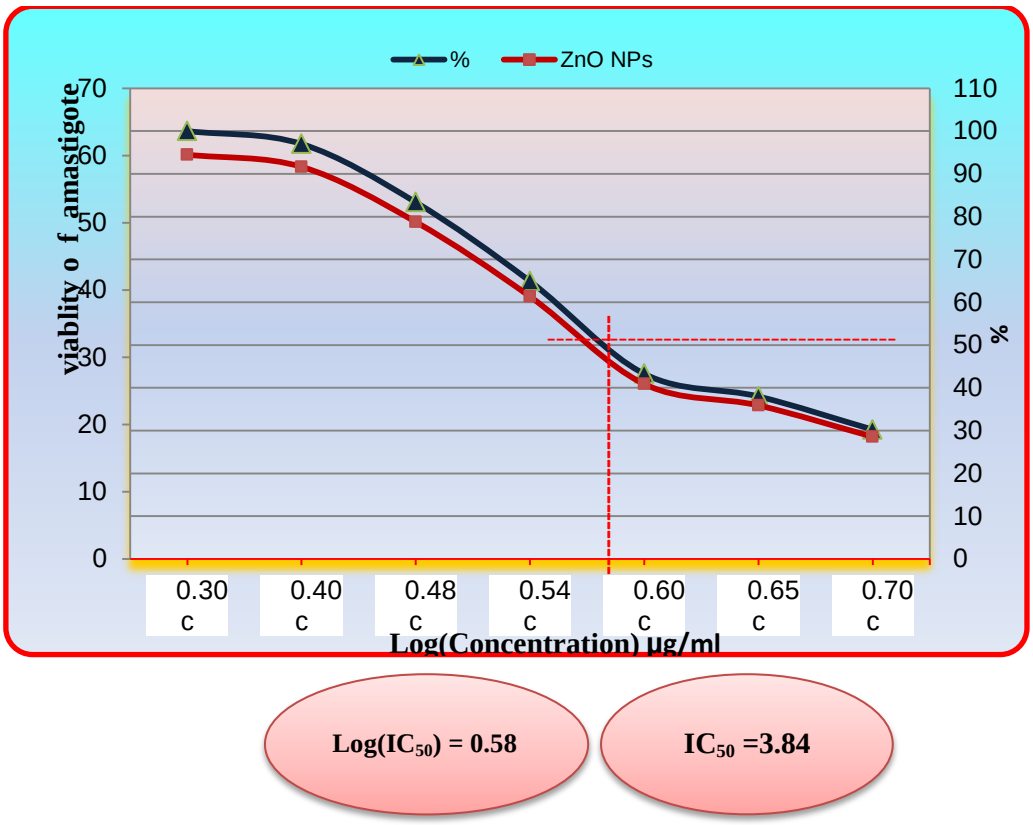

Figure 3- The IC_{50} of ZnO NPs against *L. tropica* amastigotes by MTT assay after 72 hr.

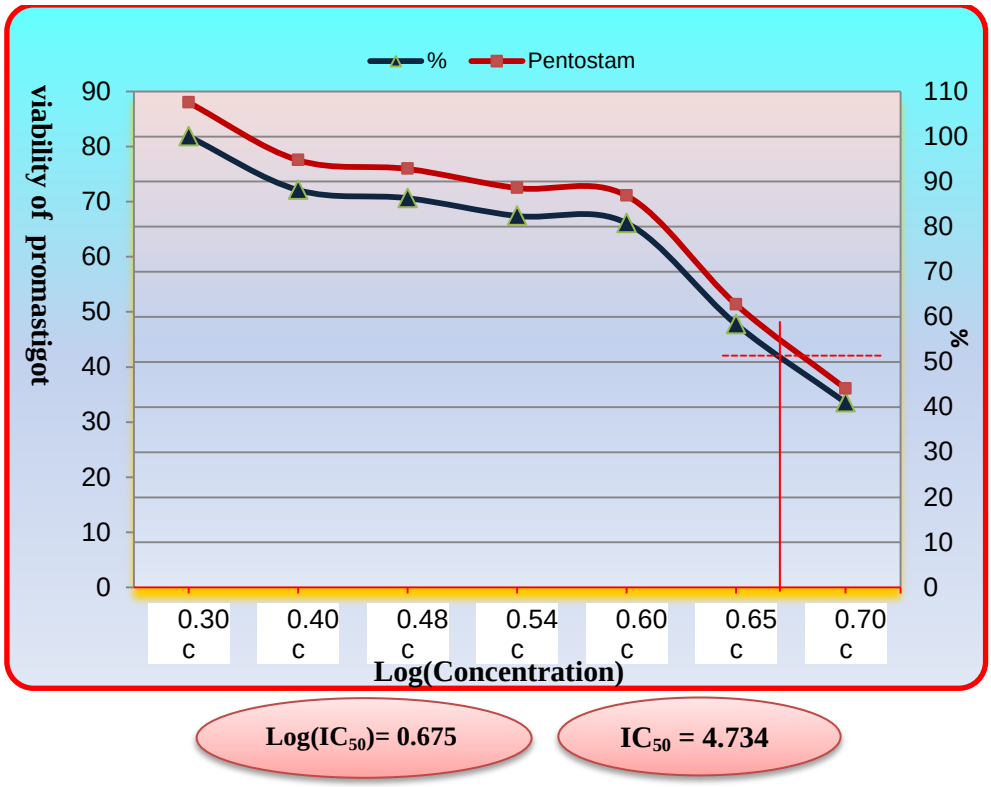

Figure 4-The IC_{50} of pentostam against*L. tropica* amastigotes by MTT assay after 72 hr.

The results of IC_{50} in this study showed high effectiveness of ZnO NPs on amastigote numbers with low concentrations, in comparison to the growth of amastigotes treated with pentostam. This may be related to the low concentrations of pentostam that didn't have the ability to destroy the parasites at these used concentrations, unlike nanoparticles and their ability to kill the microorganisms at low concentration.

Some evidence suggests that nanoparticles have anti-leishmanial activities, and there are researches of metal compounds with potential applications in medicine and the reactivity of metal ions and their interaction with a wide range of biomolecules like proteins and DNA. The effect was mainly associated with generation of ROS and nitrogen species that could kill intracellular parasites [43, 44]. Zinc oxide increases fat oxidation in prokaryotic and eukaryotic cell membranes and disruption of the cell membrane proteins and lipids that resulted in the infiltration of intracellular contents and finally the death of cells [41].

There is a study done by Najim *et al*., (1998) [27] on *L. tropica* and *L. major* amastigote form, searched about the effect of zinc sulphate. Their results showed that the ED_{50} for *L. tropica* axenic amastigote was 137.9 µg/ ml while for *L. major* was 126.87 µg/ ml. The same study showed that the ED_{50} for sodium stibogloconate for *L. tropica* axenic amastigote was 163.3µg/ ml, and 139.2 µg/ ml for *L. major*. These values of sodium stibogloconate are higher than that of zinc sulphate.

Zinc sulphate was orally administered to Iraqi patients suffering from cutaneous leishmaniasis that caused by *L. tropica* and *L. major*. This salt showed very favorable healing rates (96.9%) against cutaneous leishmanaisis in a 45-days treatment with oral daily doses of 10 mg/kg. After a comparative study between oral zinc sulfate and meglumine antimoniate in the treatment of cutaneous leishmaniasis, zinc sulphate was nearly close cure percentage to systemic meglumine antimoniate injections without any side effects [45].

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 ZnO NPs on different forms (promastigotes and amastigotes) of *Leishmania tropica* parasite, also, the destruction of parasites increases with concentrations of ZnO NPs used, and the best concentration was 5 µg/ ml after 72 hr.

References

1. Herwaldt, B. L. **1999**. Leishmaniasis. *Lancet*, **354** (9185): 1191- 1199.
2. Igbineweka, O., Aghedo, F., Idusuyi, O. and Hussain, N. **2012**. Evaluating the efficacy of topical silver nitrate and intramuscular antimonial drugs in the treatment of cutaneous leishmaniasis in sokoto, Nigeria. *African Journal Of Clinical And Experimental Microbiology*, **13**: 90- 97.
3. Sacks, D.L. **1989**. Metacyclogenesis in *Leishmania* promastigotes. *Experimental Parasitology*, **69** (1): 100- 103.
4. Killick-Kendrick, R. **1990**. The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. *Annales De Parasitologie Humaine Et Comparee*, **65** (1): 37- 42.
5. Bates, P. A. and Tetley, L. **1993**. *Leishmania mexicana*: induction ofmetacyclogenesis by cultivation of promastigotes at acidic pH. *Experimental Parasitology*, **76**: 412- 423.
6. Fr´ezard, F., Demicheli, C. and Ribeiro, R. R. **2009**. Pentavalent antimonials: new perspectives for old drugs. *Molecules*, **14**(7): 2317- 2336.
7. Croft, S. L. and Coombs, G. H. **2003**. Leishmaniasis-current chemotherapy and recent advances in the search for novel drugs. *Trends In Parasitology*, **19**(11): 502- 508.
8. Desjeux, P. **2004**. Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases*, **27** (5): 305- 318.
9. World Health Organization (WHO). **2010**. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva. World Health Organ. *Technical Report Series*, **949**: 1- 186.
10. Ferrari, M. **2005**. Cancer nanotechnology: opportunities and challenges. *Nature Reviews Cancer*, **5**: 161- 171.
11. Liu, Y., He, L., Mustapha, A., Li, H., Hu, Z. Q. and Lin, M. **2009**. Antibacterial activities of zinc oxide nanoparticles against Escherichia coli. *Journal of Applied Microbiology*, **107**: 1193- 1201.
12. Premanathan, M., Karthikeyan, K., Jeyasubramanian, K. and Manivannan, G. **2011**. Selective toxicity of ZnO nanoparticles toward Gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. *Nanomedicine*, **7**: 184- 192.
13. Hirota, K., Sugimoto, M., Kato, M., Tsukagoshi, K., Tanigawa, T. and Sugimoto, H. **2010**. Preparation of zinc oxide ceramics with a sustainable antibacterial activity under dark conditions. *Ceramics International*, **36**: 497- 506.
14. Emami-Karvani, Z. and Chehrazi, P. **2011**. Antibacterial activity of ZnO nanoparticle on gram-positive and gram-negative bacteria. *African Journal of Microbiology Research*, **5**: 1368- 1373.
15. Al-Bashir, N. M. T., Rassam, M. B. and Al-Rawi, M. **1992**. Axenic cultivation of amastigotes of *Leishmania donovani* and *Leishmania major* and their infectivity. *Annals of Tropical Medicine and Parasitology*, **86**(5): 487- 502.
16. Bates, P. A. **1994**. Complete development cycle of *Leishmania mexicana* in axenic culture. *Parasitology*, **108**: 1- 9.
17. Terry, L. R., Richard, A. M., Andrew, L., Helene, A. B., Tracy, J. W., Lisa, M., Douglas, S. and Yvonne, R. **2004**. Cell viability assay, In: Assay Guidance Manual, Sittampalam, G. S., *et al*.,(Eds.), National Library of Medicine, USA.
18. Mosmann, T. **1983**. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**: 55- 63.
19. Tanaka, A. K., Valero, V. B., Takahashi, H. K. and Straus, A. H. **2007**. Inhibition of *Leishmania (Leishmania) amazonensis* growth and infectivity by aurebasidin A. *Journal of Antimicrobial Chemotherapy*, **59**(3): 487- 492.
20. Verma, N. K. and Dey, C. S. **2004**. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy*, **48**: 3010- 3015.
21. Statistical Analysis System (SAS). **2012**. User's Guide. Statistical.Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.
22. Werner, H. and Jaccob, C. K. **1993**. A comparaision of three methods of estimation EC_{50} in studies of drug resistance of malania parasites. *Acta Tropica*, **55**(4): 257- 261.

23. Yuta, A. and Hirotaka, S. **2012**. Rapid and a ccurate IC_{50} determination using logarithmic concentration generator. 16th international conference on miniaturizyed system of chemistry and life sciences. Okinaws, Japan.
24. Jebali, A. and Kazemi, B. **2013**. Nano-based antileishmanial agents: A toxicological study on nanoparticles for future treatment of cutaneous leishmaniasis. *Toxicology in Vitro*, **27**: 1896- 1904.
25. Delavari, M., Dalimi, A., Ghaffarifar, F. and Sadraei, J. **2014**. *In Vitro* Study on Cytotoxic Effects of ZnO Nanoparticles on Promastigote and Amastigote Forms of *Leishmania major* (MRHO/IR/75/ER). *Iran Journal of Parasitology*, **9**(1): 6- 13.
26. Berman, J. D. **1991**. Biochemical mechanisms of clinical antileishmanial agents: a review. *Journal of Cell Pharmacology*, **2**: 75- 82.
27. Najim, R. A., Sharquie, K. and Farjou, I. B. **1998**. Zinc sulfate in the treatment of cutaneous leishmaniasis an *in vitro* and animal study. *Memórias do Instituto Oswaldo Cruz Rio de Janeiro*, **93**(6): 831- 837.
28. Ngure, P. K., Tonui, W.K., Ingonga, J., Mutai, C., Kigondu, E., Ng'ang'a, Z., Rukunga, G. and Kimutai, A. **2009**. *In vitro* antileishmanial activity of extracts of Warburgia ugandensis (Canellaceae), a Kenyan medicinal plant. *Journal of Medicinal Plants Research,* **3** (2): 061- 066.
29. Berman, J. D. and Wyler, D. J. **1980**. An *in vitro* model for investigation of chemotherapeutic agents in leishmaniasis. *Journal of Infectious Diseases*, **142**: 83- 86.
30. Berridge, M.V. and Tan, A.S. **1993**. Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Archives of Biochemistry and Biophysics*, **303**: 474- 482.
31. Berridge, M.V., Herst, P. M. and Tan, A. S. **2005**. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Review*, **11**: 127- 152.
32. Torabi, N., Mohebali, M., Shahverdi, A. R., Rezayat, S. M., Edrissian, G. H., Esmaeili, J. and Charehdar, S. **2011**. Nanogold for the treatment of zoonotic cutaneous leishmaniasis caused by *Leishmaniamajor* (MRHO/IR/75/ER): an animal trial with methanol extract of Eucalyptus camaldulensis. *Journal of Pharmaceutical Sciences*, : 113- 116.
33. Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M. and Kahru, A. **2013**. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells *invitro*: a critical review. *Archives of Toxicology*, **87**: 1181- 1200.
34. Callahan, H. L., Roberts, W. L., Rainey, P. M. and Beverley, S. M. **1997**. The PGPA gene of *Leishmania major* mediates antimony (Sb III) resistance by decreasing influx and not by increasing efflux. *Molecular and Biochemical Parasitology*, **68**(1): 145-149.
35. Kamau Ngure, P., Willy, K. T., Johnstone, I., Charles, M., Elizabeth, K., Zipporah, N., Geoffrey, R. and Albert, K. **2009**. *In vitro* antileishmanial activity of extracts of *Warburgia ugandensis* (Canellaceae), a Kenyan medicinal plant. *Journal of Medicinal Plants Research,* **3**(2): 061- 066.
36. Vermeersch, M., Raquel, I., Kim, T., Jean-Pierre, T., Paul, C. and Louis, M. 2009. *In Vitro* Suseptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: Partical relevance of stage-specific differences. *Antimicrobial Agents Chemotherapy*, : 3855- 3859.
37. Simon, H. U., Haj-Yehia, A. and Levi-Schaffer, F. **2000**. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, **5**(5): 415- 418.
38. Palmieri, B. and Sblendorio, V. **2007**. Oxidative stress tests: overview on reliability and use. *European Review for Medical and Pharmacological Sciences,* **11**(6): 308- 342.
39. Catala, A. **2009**. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemical and Physical Lipids*, **157** (1): 1- 11.
40. Zhang, L., Gu, F. X., Chan, J. M., Wang, A. Z., Langer, R. S. and Farokhzao, O. C. **2007**. Nanoparticles in Medicine: Therapeutic Applications and Developments. *Clinical Pharmacology and Therapeutics*, **83**: 761- 769.
41. Xie, Y., Shi, X., He, Y., Irwin, P. L. and Jin, T. **2011**. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. *Applied and Environmental Microbiology*, **77**: 2325- 2331.

42. Chang, Y. N., Zhang, M., Xia, L., Zhang, J. and Xing, G. **2012**. The Toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials*, **5**: 2850- 2871.

43. Sánchez-Delgado, R. A., Anzellotti, A. and Suárez, L. **2004**. Metal complexes as chemotherapeutic agents against tropical diseases: malaria, trypanosomiasis and leishmaniasis. In: Sigel A and Sigel H (Eds). Metal Ions in Biological Systems: Metal Ions and Their Complexes in Medication. FontisMedia and Marcel Dekker, **41**, pp: 379- 420.

44. Bruijnincx, P. C. and Sadler, P. J. **2008**. New Trends for Metal Complexes with Anticancer Activity. *Current Opinion in Chemical Biology*, **12**(2): 197- 206.

45. Yazdanpanah, M. J., Banihashemi, M., Pezeshkpoor, F., Khajedaluee, M., Famili, S., Rodi, I. T. and Yousefzadeh, H. **2011**. Comparison of oral zinc sulfate with systemic meglumine antimoniate in the treatment of cutaneous leishmaniasis. *Dermatology Research and Practice*, , Article ID 269515, p: 4.