In *Vitro* Study of Ag_2O, MgO Nanoparticles and Sb Drug Cytotoxic Effects on *Leishmania donovani*

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Abstract

Background: *Leishmania* is a parasitic protozoan that causes severe illness in humans. While pentavalent antimony compounds (Sb Drug) are used as antileishmanic drugs but are linked to limitations and many adverse complications.

Objective: Therefore, commitment is still needed to find a new and successful treatment.

Material & methods: In this study, the effect of Ag_2O, MgO nanoparticles with mean particles size of (12-28)nm and(7-12)nm respectively on *Leishmania* was evaluated. Viability percentage of promastigotes following the addition of different concentrations of Ag_2O, MgO nanoparticles and Sb drugs (25, 50, 100, 200 and 300 µg / ml) to the parasite culture, assessed using colorimetric ALAMAR stain in (24,48 72)hours.

Results: The concentration that the number of parasite by half (IC50) value was measured after 24, 48 and 72 hours for Sb, Ag_2O NPs and MgO NPs. For Ag_2O NPs the IC50 was 2.404 after 72 hours only, and doesn't reveal any value in 24 hour, 48 hour, in comparison to Sb drug and MgO NPs which doesn't reveal IC50 value in all used concentration and periods.

Conclusion: Our data determine the superiority of Ag_2O NPs and MgO NPs over standard in vitro pentostam that's it. Provides the possibility of using these Ag_2O NPs and MgO NPs as a drug target for candidates with better antileishmanial efficacy.

Key words: *Leishmania* , Ag_2O and MgO NPs, IC50, ALAMAR stain.

Introduction

Leishmaniasis, one of the most deserted tropical illnesses, is presently impacting 12 million of the world population (1). Leishmaniasis has lately received more public consideration because of the fact of morbidity and high infection rate(2). *Leishmania*-related diseases persist among the worldwide deadliest deserted tropical illnesses(3). The mortality percentage is significant, contributing to approximately dual million disability-adjusted life years (1).

Drugs for *Leishmania* treatments possess some drawbacks such as controlling difficulty, long-term treatment and low tolerability.(4) Pentavalent antimonial is an assembly of composites utilized for the leishmaniasis treatment**,** (5).(6)

Metal oxide nanoparticles attain unique chemical and physical features which are attributed to their nanometer structure, hence providing resourcefulness on different scales(7, 8). Metal oxide nanoparticles are considered to be a major factor in semiconductors, drug delivery, diagnosis, catalysis, sensing devices, etc. (9,10). Moreover, metal oxide nanoparticles are significantly used as an antimicrobial agent and thus recently have gained a considerable devotion (10-12). Metal oxides nanoparticles can be considered as toxicity medium by taking into account a number of actors such as exposure, dissolution and size(13). The poisonous quality for nanoparticles is subject to be assessed through several approaches such as *in vitro* and *in vivo*.

Materials and methods

Sb Drug and Nanoparticles concentrations

Metal oxide nanoparticles Ag_2O, MgO NPs powder was used with mean particles size of (12-28) nm and (7-12) nm respectively**,** and synthesized by using green methods, The stock solution of Ag_2O NPs and MgO NPs was serially diluted in phosphate buffered saline (PBS) (PH 7.4) additionally sonicated for 40 min for forming homogeneous suspensions, Prepare the resulting concentrations: 25, 50, 100, 200, and 300 µg/ml immediately before use.

Drug doses Pentostam or Sodium stibogluconate (Sb) in liquid form as an injectable ampoule (100 mg / ml), (Glaxo Activity UK Limited Barnard Castle, part of the Glaxo Smith Kline group of companies). The medication was processed below 25° C and it protects against light. The following concentrations were prepared by using a stock solution of Sb: 25, 50, 100, 200, and 300µg/ml immediately before use.

Parasite culture

Leishmania donovani strain (DUAA/ IQ/ 2005/MRU15) was collected from the Department of Biology, AL-Mustansiriya University. They were maintained and sub cultured every seven days, and cultivated with L-glutamine (Sigma, St Louis, MO) in RPMI-1640 medium.

Preparation of RPMI 1640 medium

Medium was easily washed by separating with a porosity of 0.22 micron through a sterile layer filter, using positive compared to vacuum pressure to reduce carbon dioxide loss, 90 ml of this RPMI-1640 filter medium was taken and 10 ml of fetal bovine serum (FCS) was applied. In the filtered mixture 100 µg/ml of penicillin was added. Mean liquid stored at 2-8° C. Cells (1×10^6 cells / mL) contained 5 mL of RPMI-1640 plus 10% FCS medium in addition to a flask and the flask was incubated at 27 ° C. Cells went around week after week(14).

Cytotoxicity assay (colorimetric ALAMAR stain)

Alamar assay has been widely used for biological and environmental applications of cell viability and cytoto xicity studies(15). Cell culture, in microtiter plate 96 well (200 µl cultures), was treat med with Sb drug, Ag_2O NPs and MgO NPs in different diluted concentration: 25, 50,100,200, and 300 µg/ml, and in different experimental incubations (24, 48 and 72) hours. After incubation the microtiter plate 96well was centerifuged and the suspension media was removed and 20 µl of ALAMAR blue stain was added incubated for 4 hours at 26° C and finally the absorbance by (ELISA) reader at 620 was added.

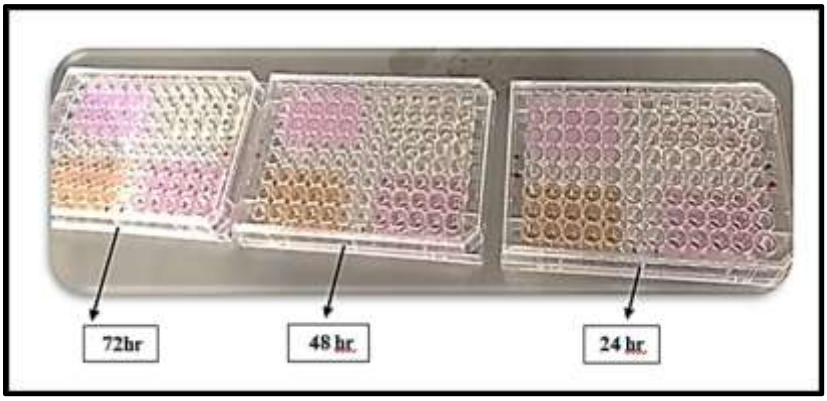

Figure (1): Microtiter Plate of 96 wells, cytotoxicity of Ag_2O NPs, MgO NPs and Sb drug against *L.donovanai* promastigotes after (24, 48, 72) hours, before adding ALAMAR dye.

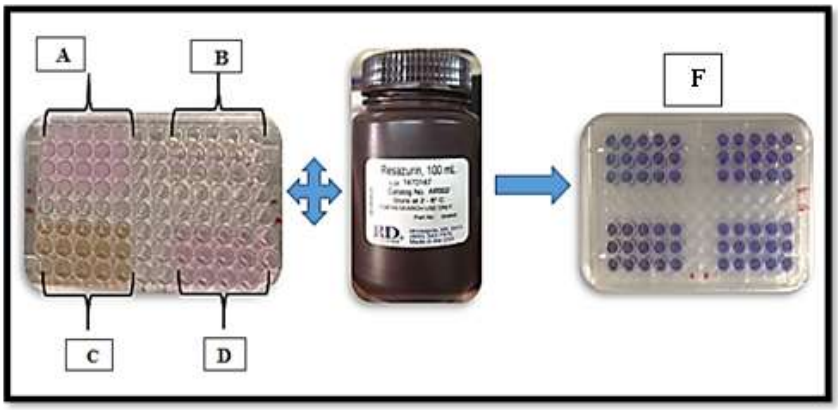

Figure (2): A Microtiter Plate of 96 wells showing the cytotoxicity against *L. donovanai* promastigotes. A: cytotoxicity of MgO NPs, B: Control, C: cytotoxicity of Ag_2O NPs D: cytotoxicity of Sb drug, F: after adding ALAMAR dye and incubation 4 hours

Result

In vitro procyclic promastigotes of *L. donovani* were screened with Ag_2O NPs, MgO NPs and Sb drugs following three times of follow up (24, 48, 72) hours. The results of cytotoxicity revealed the activity of Ag_2O NPs, MgO NPs to inhibit the parasite's growth and limit the proliferation, difference of colorimetric absorption was detected using Alamar Blue.

Alamar Blue is a suitable colorimetric suitability pointer for tranquilize screening measures with promastigotes of *L. major*. Because of the connection of promastigote number with the absorbance of Alamar Blue, the impacts of medications can be evaluated photometrically (16).

Statistically, there was a significant ($P \geq 0.05$) difference in absorption of micro titer plate reader data between the test and the control group for promastigotes at all concentrations (25, 50, 100, 200 and 300) µg/ml after 24, 48 and 72 hours of follow-up in Figure (1) . Moreover, the effect was most apparent after 72 hours of treatment.

After 24 hours the Ag_2O NPs showed a significant ($P<0.05$) cytotoxicity in the percentage of viable promastigotes compared with the promastigotes treated with MgO NPs, penostam and the untreated promastigotes. At lowest concentration of Ag_2O NPs was (25µg/ml), the percentage of viable cells was (0.469 ± 0.08), while the highest concentration (300µg/ml) displayed (0.390 ± 0.04) percentage of viable cells. The percentage of viable promastigotes treated by MgO NPs in lowest concentration was (25µg/ml) while with highest concentration (300µg/ml) was (0.550 ± 0.06) and (0.492 ± 0.06) respectively. The percentage of viable promastigotes treated by pentostam in lowest and highest concentration was (0.600 ± 0.11) and (0.50 ± 0.07) respectively and untreated parasites was (0.645 ± 0.07).

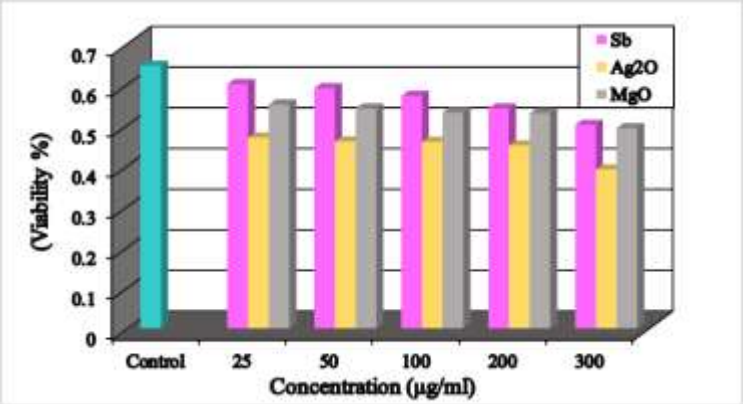

Figure (3): Viability of *L. donovani* exposed to Sb drug, Ag_2O NPs and MgO NPs by ALAMER stain assay after 24 hours.

After 48 hours in the lowest concentration of Ag_2O NPs (25µg/ml), the percentage of viable cells was (0.506 ± 0.08), while the highest concentration (300µg/ml) displayed (0.334 ± 0.04) percentage of viable cells. The percentage of viable promastigotes treated by MgO NPs in lowest concentration (25µg/ml) and highest concentration (300µg/ml) was (0.598 ± 0.14) and (0.450 ± 0.07) respectively.

The percentage of viable promastigotes treated by pentostam in lowest and highest concentration was (0.629 ± 0.12) and (0.550 ± 0.09) respectively and untreated parasites was (0.640 ± 0.06).

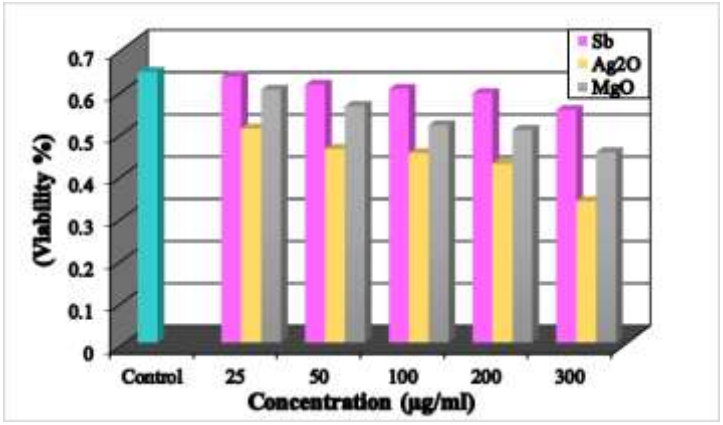

Figure (4): Viability of *L. donovani* exposed to Sb drug, Ag_2O NPs and MgO NPs by ALAMER stain assay after 48 hours.

After 72 hours at lowest concentration of Ag_2O NPs (25µg/ml) the percentage of viable cells was (0.413 ± 0.07), while the highest concentration (300µg/ml) displayed (0.155 ± 0.04) percentage of viable cells. The percentage of viable promastigotes treated by MgO NPs in lowest concentration (25µg/ml) and highest concentration (300µg/ml) was (0.474 ± 0.08) and (0.349 ± 0.05) respectively.

The percentage of viable promastigotes treated by pentostam in lowest and highest concentration was (0.628 ± 0.09) and (0.530 ± 0.11) respectively and untreated parasites which was (0.637 ± 0.08).

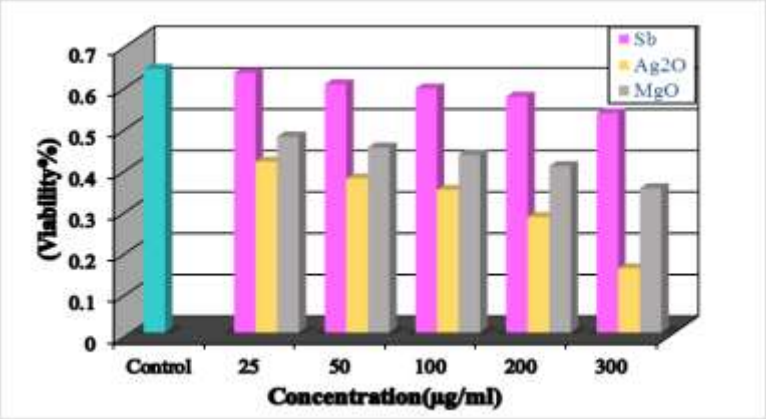

Figure (5): Viability of *L. donovani* exposed to Sb drug, Ag_2O NPs and MgO NPs by ALAMER stain assay after 72 hours.

The results of this study have shown lower efficacy of pentostam on *L. donovani* promastigotes in all using concentrations while Ag_2O NPs and MgO NPs have shown higher efficacy through all days of treatment with concentrations (25, 50, 100, 200 and 300 µg/ml) . The viability decreased by increasing the concentration and incubation time.

Table (1): Effect of Sb drug against viable cells *L.donovani* after 24, 48 and 72hr.

Concentration (µg/ml)	Time (hr)			L.S.D Value
	24	48	72	
25	0.600 ± 0.11	0.629 ± 0.12	0.628 ± 0.09	0.133 NS
50	0.59 ± 0.09	0.610 ± 0.09	0.600 ± 0.12	0.147 NS
100	0.571 ± 0.05	0.600 ± 0.07	0.590 ± 0.09	0.136 NS
200	0.540 ± 0.07	0.590 ± 0.07	0.570 ± 0.08	0.107 NS
300	0.50 ± 0.07	0.550 ± 0.09	0.530 ± 0.11	0.096 NS
Control	0.64 ± 0.07	0.640 ± 0.06	0.637 ± 0.08	0.138 NS
LS.D	0.193 NS	0.144 NS	0.162 NS	---
NS: Non-significant.				

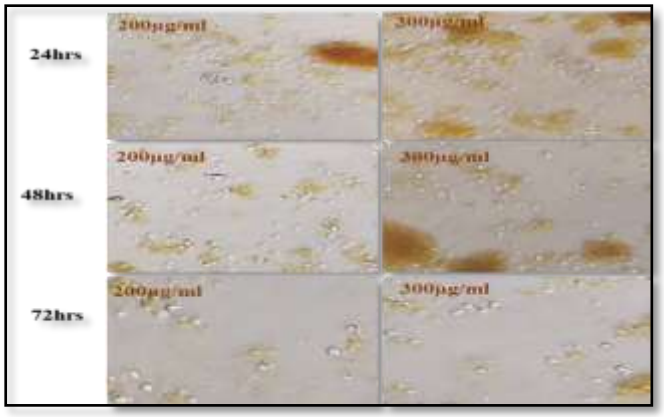

Figure (6): Cell viability of *L. donavani* treated with Ag_2O NPs after 24, 48 and 72 hours under inverted microscope.

Table (2): Effect of Ag_2O NPs against viable cells *L. donovani* after 24, 48 and 72hr.

Concentration (µg/ml)	Time (hr)			L.S.D Value
	24	48	72	
25	0.469 ± 0.08	0.506 ± 0.08	0.413 ± 0.07	0.114 NS
50	0.459 ± 0.03	0.458 ± 0.05	0.373 ± 0.07	0.152 NS
100	0.458 ± 0.07	0.448 ± 0.06	0.345 ± 0.06	0.139 NS
200	0.449 ± 0.08	0.423 ± 0.03	0.279 ± 0.07	0.147 *
300	0.390 ± 0.04	0.334 ± 0.04	0.155 ± 0.04	0.207 *
Control	0.64 ± 0.07	0.640 ± 0.06	0.637 ± 0.08	0.138 NS
L.S.D	0.206 *	0.137 *	0.172 *	---
* (P<0.05) , NS: Non-significant.				

Figure (7): Cell viability of *L. donavani* treated with MgO NPs after 24, 48 and 72 hours under inverted microscope.

Table (3): Effect of MgO NPs against viable cells *L. donovani* after 24, 48 and 72hr.

Concentration (µg/ml)	Time (hr)			L.S.D Value
	24	48	72	
25	0.550 ± 0.06	0.598 ± 0.14	0.474 ± 0.08	1.42 NS
50	0.540 ± 0.06	0.560 ± 0.10	0.447 ± 0.06	0.138 NS
100	0.530 ± 0.08	0.514 ± 0.14	0.430 ± 0.06	0.098 NS
200	0.528 ± 0.05	0.503 ± 0.08	0.403 ± 0.04	0.148 NS
300	0.492 ± 0.06	0.450 ± 0.07	0.349 ± 0.05	1.40 *
Control	0.64 ± 0.07	0.640 ± 0.06	0.637 ± 0.08	0.138 NS
LS.D	0.163 NS	0.155 NS	0.149 NS	---
* (P<0.05) , NS: Non-significant.				

The IC50 of Sb drug, Ag_2O NPs and MgO NPs against *L. donavani* promastigote (24, 48, 72) hours using ALAMAR stain

The IC50 was calculated along the three times of follow up and was demonstrated a time-dependent inhibition of the parasite growth in which the IC50 value was measured after 24, 48 and 72 hours for Sb, Ag_2O NPs and MgO NPs. The concentration that the number of parasite by half (IC50) for Ag_2O was 2.404 after 72 hours only, and doesn't revealed any value in 24 hour , 48 hour. In comparison to Sb drug and MgO NPs which doesn't revealed the IC50 in all used concentration and periods. Figure (8 a-c) .Figure (9 a-c) and Figure (10 a-c).

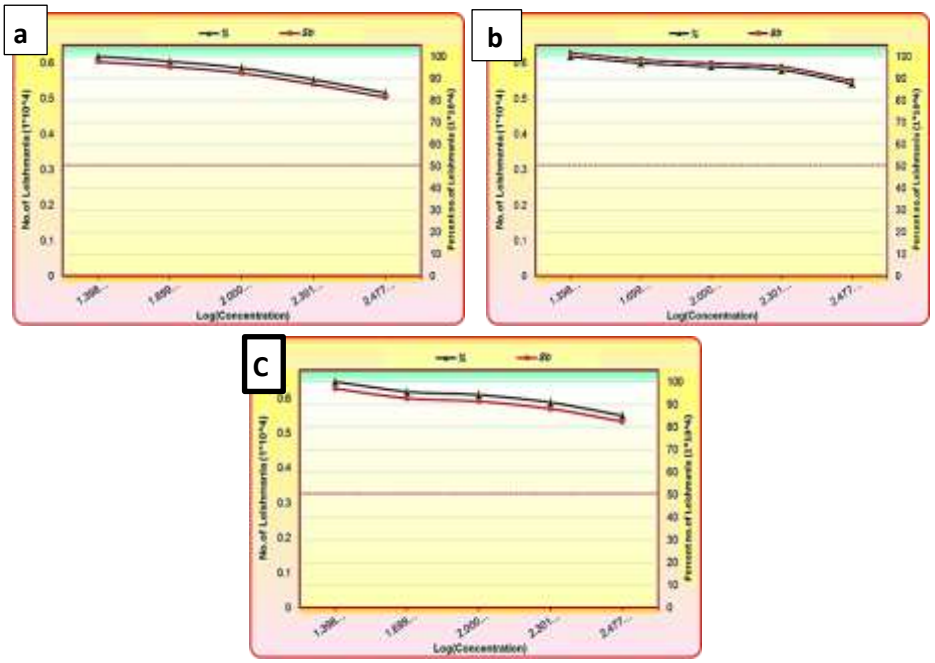

Figure (8): Inhibitory concentration (IC50) of Sb on *L. donovani* , a: after24 hours, b:after 48 hours, c: after 72 hours

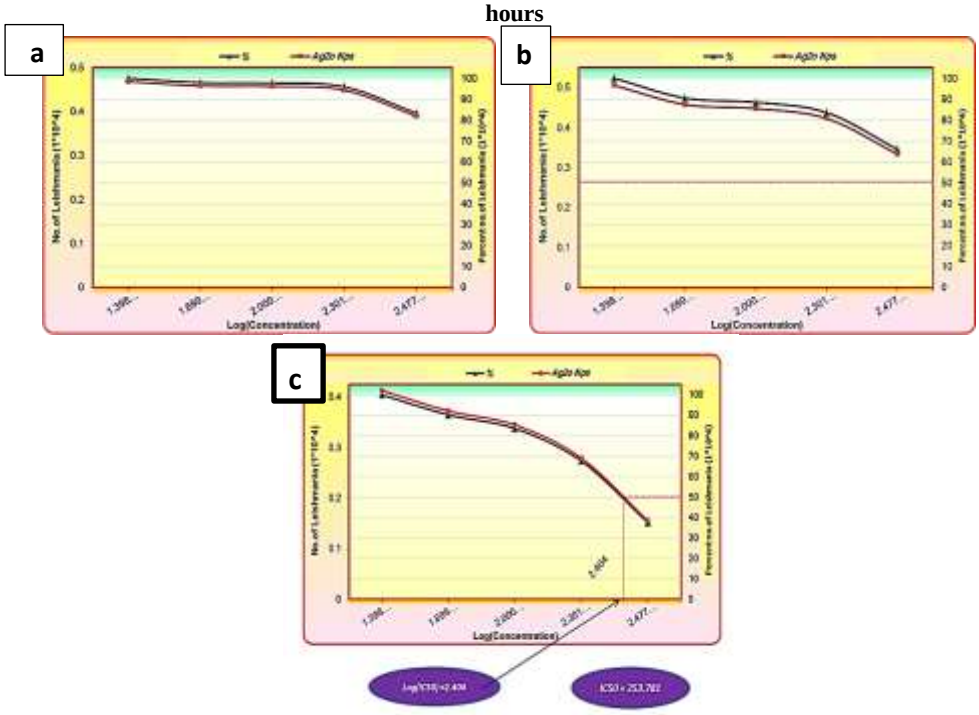

Figure (9): Inhibitory concentration (IC50) of Ag_2O NPs on *L. donovani* a: after24 hours, b: after 48 hours, c: after 72 hours

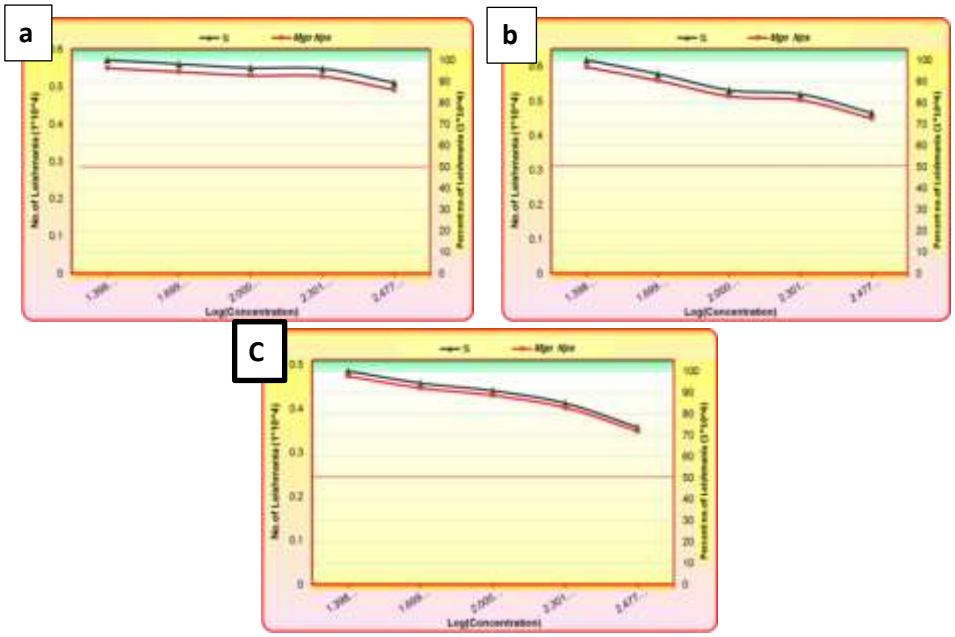

Figure (10): Inhibitory concentration (IC50) of MgO NPs on L.donovani , a: after24 hours, b:after 48 hours, c: after 72 hours

Discussion

The antiparasitic action of metal oxide NPs includes the creation of adequately a lot of ROS to defeat the protection frameworks of the parasite(17). The generation of ROS is started by initiated by metal ions released from the NPs (18). ROS creation is thought to be the principal mechanism underlying the antimicrobial action of metal oxide NPs (10). ROS comprise superoxide anions (O^{2-}), hydroxyl radicals (OH.), hydrogen peroxide (H_2O_2) (19). Ag_2O NPs and MgO NPs dissolve and release metal ions (Ag^+, Mg^+) both in the medium surrounding the bacteria and in the cytoplasm(20). Thus, after endocytic uptake of a NP into the bacteria, a certain quantity of metal ions is released into the cytoplasm(21). Metal ions can also easily diffuse through the cell wall of the bacterium. These two processes result in the generation of ROS inside the cell (22). When ROS production overwhelms the cellular antioxidant defense system, oxidative stress will result(23). This is associated with damage of many key biomolecules inside a microorganism, including carbohydrates, proteins, lipids, genetic materials(20). Oxidative stress can also lead to depletion of reduced glutathione (24, 25), a compound which has an important role in scavenging and detoxifying ROS molecules (26) (The amount of ROS produced is controlled by the physicochemical properties of NPs, including their surface area, diffusibility, and electrophilic nature (15). The ROS produced lead to the disruption of the cell membrane, loss of permeability, damage to proteins and DNA, and damage to enzymes (27).

Ag_2O NPs is metal oxide nanoparticle; it have a great antimicrobial action (28). The Ag_2O NPs might be proved as a novel antibiotic (29), it can damaged the DNA of *E. coli* and it can terminate the cell cycle at the G2/M phase due to the DNA damage. The mechanism of this action is through oxidative stress (30-32). Magnesium oxide NPs are very stable, biocompatible and are very efficient antibacterial agents(33). MgO NPs have very effective bactericidal properties; they can bring about complete eradication of the pathogenic microbes (34). MgO NPs damage the cell membrane and cause lipid peroxidation, leading to the leakage of intracellular contents (35).

Conclusion

Our data determine the superiority of Ag_2O NPs and MgO NPs over standard in vitro pentostam that's it. Provides the possibility of using these Ag_2O NPs and MgO NPs as a drug target for candidates with better antileishmanial efficacy. In addition, its production is favorable compared to pentostam with Low cost manufacturing. We need further studies to develop Ag_2O NPs and MgO NPs as an antileishmanic drug and to discover its oral administration capabilities.

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دراسة مختبرية لسمية الجسيمات النانوية Ag_2O و MgO و عقار البنتوستام وتأثيره على الليشمانيا دونوفاني

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الخلاصة

الخلفية: الليشمانيا وهو عدوى طفيليه تسبب مرضًا شديدًا للإنسان. و تستخدم مركبات الأنتيمون خماسي التكافؤ (Sb Drug) كأدوية مضادة لليشمانيا ولكنها مرتبطة تسبب العديد من المضاعفات الضارة.

الهدف: لذلك، لا تزال هناك حاجة إلى الالتزام لإيجاد علاج جديد وناجح.

المواد والطرق : في هذه الدراسة ، تم تقييم تأثير جزيئات Ag_2O و MgO النانوية بمتوسط حجم نانوي للجسيمات (12-28) نانومتر و (7-12) نانومتر على التوالي على الليشمانيا. نسبة الفعاليه الحيويه من بروماستيجوتات بعد إضافة تراكيز مختلفة من Ag_2O و MgO الجسيمات النانوية وأدوية Sb (25 ، 50 ، 100 ، 200 و 300 ميكروغرام / مل) إلى زرع الطفيليات ، تم تقييمها باستخدام صبغة ALAMAR اللونية في (24,48,72) ساعة .

النتائج: تم قياس تركيز عدد الطفيليات بمقدار النصف (IC_{50}) بعد 24 و 48 و 72 ساعة لـ Sb و Ag_2O NPs و MgO NPs. بالنسبة لـ Ag_2O NPs ، كان IC_{50} 2.404 بعد 72 ساعة فقط ، ولا يكشف عن أي قيمة خلال 24 ساعة و 48 ساعة ، مقارنة بعقار Sb و MgO NPs الذي لا يكشف عن قيمة IC_{50} في جميع فترات التركيز والفترات المستخدمة.

استنتاج:
اعتمادا على النتائج المختبرية للبنتوستام والمواد النانوية ، تحدد بياناتنا تفوق المواد النانوية و يمكن استخدامهم كهدف دوائي ضد الليشمانيا دونفاني.

الكلمات المفتاحية : الليشمانيا ، اوكسيد الفضة واوكسيد المغنيسيوم النانوي، صبغة الالمار، التثبيط النصفي .