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Research Article

Chemical fabrication of superparamagnetic nanoparticles against *Leishmania tropica*

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Keywords
Leishmania tropica, superparamagnetic iron oxide nanoparticles, SPIONs, co-precipitation method.

Abstract

Due to the diversity of effective applications of superparamagnetic iron oxide nanoparticles (SPIONs) in different bioapplications at a rate exponentially, the procedure of synthesis of magnetic nanoparticles is vital for producing small and stable particles and their successful applications. In this work, we report the synthesis of SPIONs using co-precipitation method based on sodium hydroxide (NaOH) mediated precipitation of Fe 3+ and Fe 2+ salts in an aqueous solution using Trisodium citrate acid as a surfactant within closed system under Nitrogen inert atmosphere. The resulting synthesized SPIONs were characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray powder diffraction (XRD), and vibrating sample magnetometer (VSM), and Zeta potential analysis (Zp). TEM and SEM images indicated that the particles are spherical shape of with a size of ≥ 8 nm. XRD pattern showed the presence of peaks corresponding to the phase of magnetite Fe_3O_4, while the VSM study demonstrated that superparamagnetic properties and the saturation magnetization was around 50 emu/g.

The leishmanicidal activity of SPIONs against the promastigote culture of *Leishmania tropica* was studied with three different concentrations of the nanoparticles *in vitro*. The results showed that the three concentrations of the nanoparticles could increase parasite mortality rate in time-dose dependent manner. The highest concentration of SPIONs (23.2µg/µl) was more effective compound for inhibiting the parasite growth with mortality rate of 70%.

This method has the potential to be a step-change for research into that produced SPIONs can be used as promising antiprotozoal agent.

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1. Introduction

The manufacture of Fe_3O_4 MNPs whose dimensions and magnetism are controllable has gained much attention since time due to its distinctive properties, making it a candidate for the use of a variety of diagnostics and treatments based on nanoparticles [1-2]. At present, Fe_3O_4 SPONs is widely accepted due to its

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chemical and physical properties such as surface chemistry, size distribution, magnetism, modulation, etc. Multifunctional iron oxide nanoparticles (IONPs) have developed as a encouraging material for a wide range of for such biological usages as the therapeutic drug and gene delivery, immunology, biotechnology, medicine, and engineering [3-4]. Leishmaniasis is one of the main tropical/sub-tropical diseases caused by an intracellular parasite of more than 50 species of protozoa parasites of the genus *Leishmania* [5]. The disease characterizes a severe global health burden that cause increased rate of mortality and morbidity in different area mainly in tropics and subtropics areas [6].

The remarkable impact of human protozoan infections has been increased by the absence of active and effective vaccines and drugs, for inhibition and treatment of these diseases. Unluckily, the utility of existing drugs is being increasingly threatened by growing of parasite drug resistance. Treatment and prevention have consequently been reliant on drugs, numerous of which have become inactive demanding the search for alternatives. The pioneering approaches to guarantee a supportable discovery of lead compounds.

Recently, many kinds of nanomaterials such as metal oxide nanoparticles [7-9], polymeric coated metallic nanoparticles [10-11] have been implicated in the pharmacological activities as possible pharmacological effects.

Considering the biological and antimicrobial activity of SPIONs, the purpose of this study is to synthesize iron oxide magnetic nanoparticles SPIONs and their anti-leishmanial effects in vitro against promastigotes of *L. tropica*.

2. Material and methods

2.1. Chemical synthesis

Nano-sized crystallites of iron oxide were synthesized by cost effective co-precipitation method. 1.35 g of Iron (III) chloride hexahydrate (FeCl3. 6H2O) and 0.69 g of Iron (II) sulfate ($FeSO_4.7H_2O$) was mixed with 1.77 g of trisodium citrate and 1.2 g of NaOH. At the beginning, all materials were solved with 20 ml of deionized water. The reaction mixture and reaction temperature was slowly raised up to 80 °C under reflux and reacted for 60 min with continuous stirring, followed by the drop wise addition of iron salts solution in trisodium citrate and NaOH mixture. An immediate formation of black precipitate was observed and then thoroughly rinsed with ethanol and then distilled water to filter-off the impurities. Through each rinsing step, samples were separated from the supernatant using a permanent magnet

2.2. Characterization of SPIONs

The structural characterizations of the nanoparticles were examined with X-ray diffractometer system (XRD) with a CuKα radiation source generate of 40 kV 30 mA. The average hydrodynamic diameter and the zeta potential of the samples were determined using a Malvern Zetasizer ZS (Malvern Instruments Ltd., U.K.). While TEM and SEM techniques used to identified nanoparticle sizes, crystal structure, and morphology. Vibrating sample magnetometer (VSM) the magnetic properties characterization (VSM) was used for SPIONs in this work.

2.3. Preparation of the culture media

A Peptone yeast folic (PY) medium was prepared according to the method of Husain F. Hassan et al. [12] consisting of the following ingredients: The following materials were added for 100 milliliter of the media (NaCl (0.9 gm), Na2HPO4 (0.75 gm), yeast extract (0.25 gm), peptone (1 gm), folic acid (0.004 gm), and completed with water up to 100 ml). Then the mixture was homogenized by a water bath at 60 ° C for 10 minutes. Followed, the solution was filtered by filter paper and the pH is adjusted to 7.2 using 0.1 N HCL.

Then, the culture mediums were distributed in tubes (4.5 ml in each tube). The medium was autoclaved at 15 psi for 15 min and 0.03 ml of Gentamicin sulphate was added at 80 mg\ml to inhibit bacterial growth. Fresh urine was collected from a single adult male volunteer, cleared by centrifugation at 2000 g for 5 min and sterilised by passage through a 0.22 μm membrane filter. The pH value of urine was adjusted to 7.2 and 0.25 ml and added to the PY medium by sterile syringe filter.

Biphasic cultured *L. tropica* promastigotes were kindly provided by Prof. Hussain F. Hassan group (Department of Biology, Kirkuk University, Iraq). The parasite cells in cultures media were sediment by centrifugation at 4000 g for 10 min and washed twice with sterile Hanks' balanced salt solution (HBSS).

An aliquot (0.45ml) of 1 × 105 parasites/mL were inoculated into 4.5 mL of PY medium in each tubes. After incubation for 72 hours at 27 ° C for growth and reproduction to occur, media of experiment group cultures were supplemented with different concentrations of previously prepared SPIONs (23.2, 2.32, and 0.232) μg/μl. Py media was used as a control. The parasite cellular growth was controlled by estimation of the growth rate within the incubation time (72h). Cellular growth was estimated every 24 hours started from the second day (after 48hours) of addition SPIONs to the culture media by. The survived promastigotes were counted with haemocytometer under standard light microscopy. Cellular viability was evaluated for enumeration of stained and unstained cells by Trypan blue dye exclusion .

3. Results and discussion

3.1. Morphology, Structure, Size, Magnetization, and Colloidal Stability Analysis

The X-ray diffraction (XRD) potter of Fe_3O_4 MNPs were synthesized by the co-precipitation method with a CuKα radiation source generate of 40 kV 30 mA (Fig 1**).** It show that the character peaks Fe_3O_4 are at $2\Theta = 29.8^o$, 35.5^o , 42.9^o , 54.38^o , 56.96^o and 62.86^o, being similar to the previously reaches for Fe_3O_4 nanoparticles [70,71,72]. In the current study the formed nanoparticle were Fe_3O_4 with comparison with XRD delta sheet as show in (Fig 1).

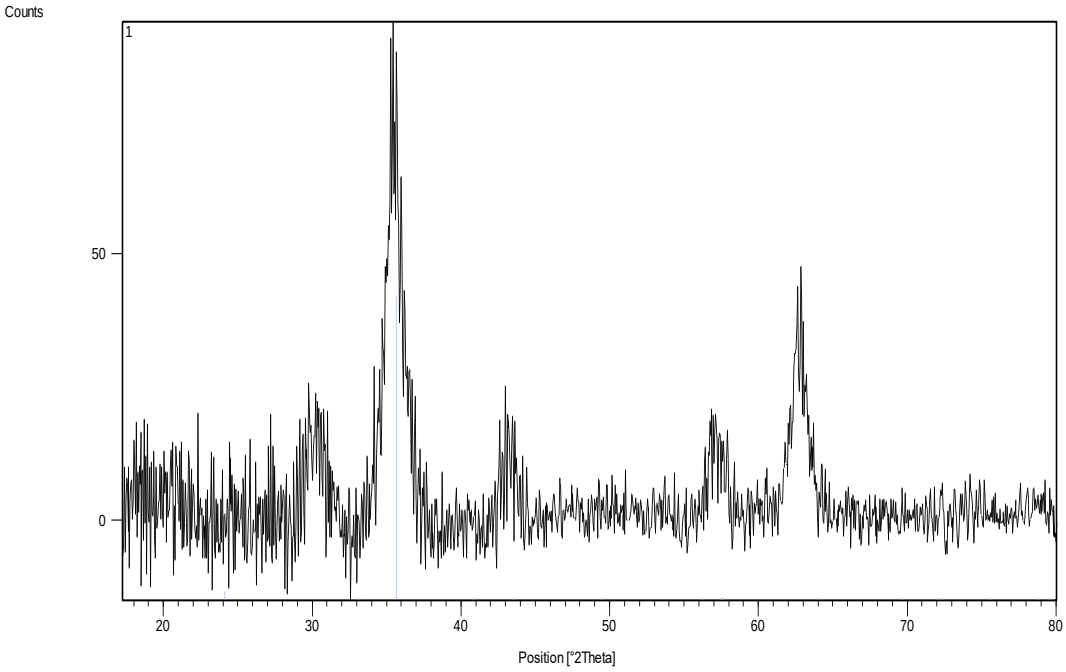

Fig.1. X-ray diffraction for Fe_3O_4 powders prepared at 80C°

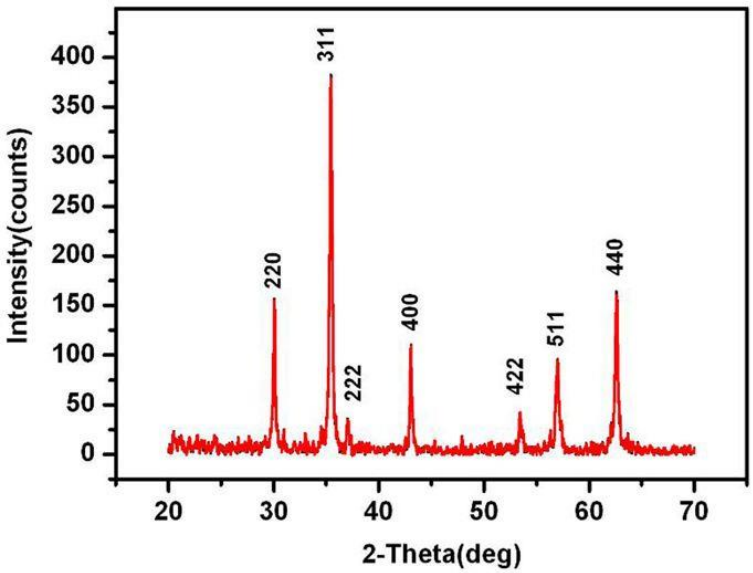

Fig .2. XRD Fe_3O_4 SPIONs delta sheet

3.2. Scanning Electron Microscope (SEM).

Figure (3) shows the scanning electron microscope (SEM) image Fe_3O_4 SPIONs which are prepared by the co-precipitation method at 80C°, indicate that the nanoparticle were spherical with diameter of about (23±4). The image of SEM were companied with others, it is in agreement with which obtund by [13].

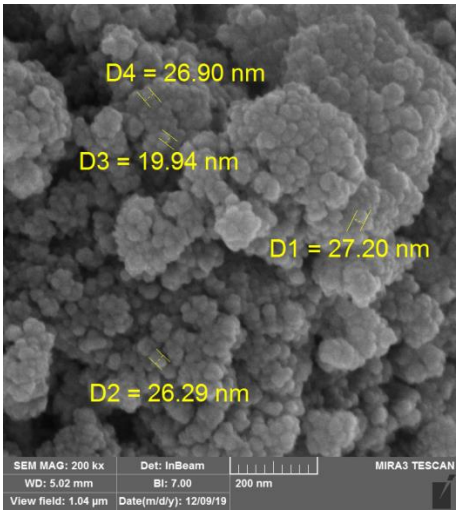

Fig.3. SEM electron microscopic photographs of Fe3O4 nanoparticles

3.3. Transmission Electron Microscopic (TEM).

In order to obtain the actual size of Fe_3O_4 SPIONs which are prepared by co-precipitation method was tested by TEM. Figure (4) shows the transmission electron microscopic (TEM) photographs of the Fe_3O_4 synthesized via co-precipitation method,, the actual size of Fe_3O_4 SPIONs with an average diameter of 5 nm. Our comparison to previous studies was identical with [14].

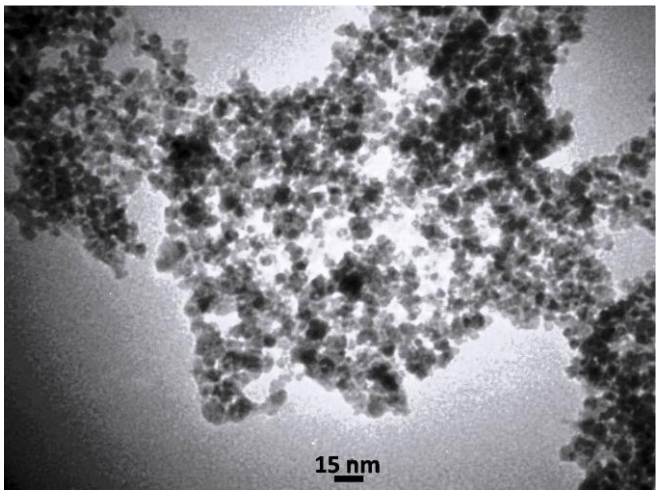

Fig .4. Transmission electron microscopic photographs of Fe3O4 nanoparticles

3.4. Magnetic Characterization (VSM) Analysis.

Vibrating sample magnetometer was used to find the magnetic properties of synthesized Fe3O4 SPIONs. Figure (5) shows the typical characteristics of superparamagnetic are observed showing almost immeasurable coercivity and remanence. The saturation magnetization at which is 51.21 emu/g, is significantly less than that of the bulk magnetization [15], which is MS(bulk) 92emu/g.

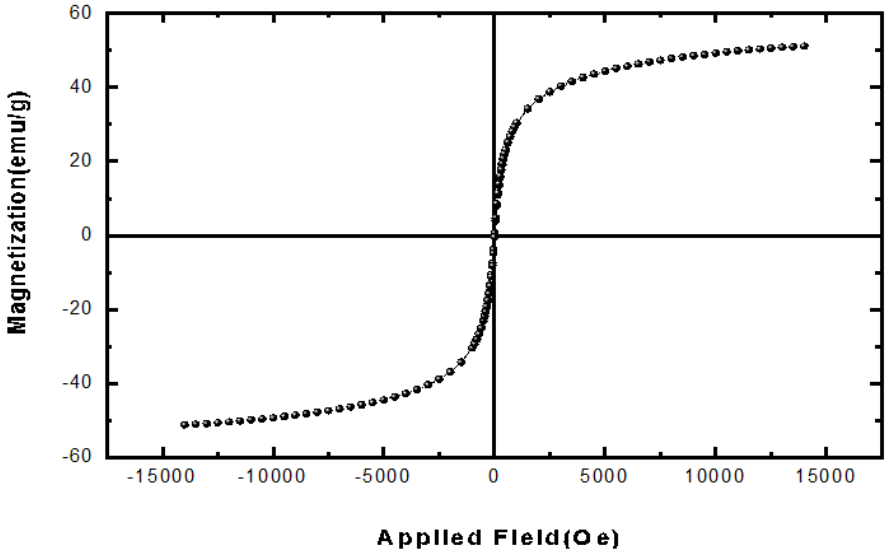

Fig .5. Magnetization versus applied field for nanoparticles at 303K. Experimental (solid rectangle) and calculated (solid line) data represent the best fit for Langevin function

3.5. Zeta Size (DLS) Analysis

DLS was used to determine the volume of hydrodynamic Fe3O4 SPIONs. Measurements in DLS show us that the size of complex nanoparticles is 216nm. The results are shown in Figure (6), the hydrodynamic volumes obtained are greater than those observed by TEM which reflect particles aggregation inside the liquid. TEM measurements do not supply information on particles hydrodynamic size. For this reason Dynamic Light Scattering (DLS) measurements have been made, where the volume-size and the number-size distributions have been obtained for SPIONs particles.

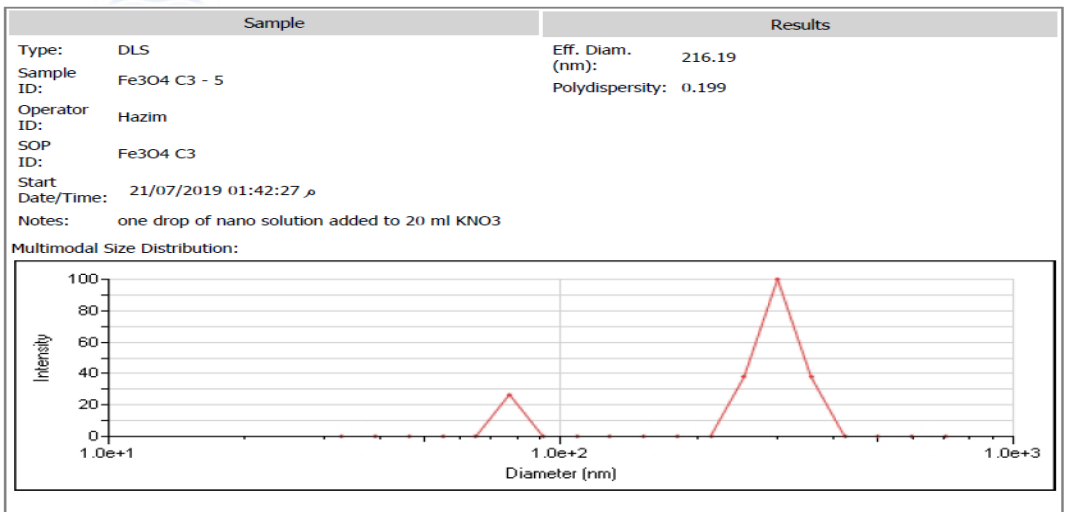

Fig .6. Zeta size of Fe3O4 SPIONs

3.6. Antileishmanial effect of Fe_3O_4 SPIONs

Leishmaniasis is considered an infectious and dangerous disease and it is the second most important protozoal disease, which is transmitted by several species

of sand flies, which belong to the genus Phlebotomus. The life cycle of Leishmania consists of two stages the intracellular amastigote in the mammalian host and the promastigote in the fly. In the intestine of the sand fly, motile promastigotes are found where they develop into a virulent infectious form through several morphological stages. During the bite of a sand fly, the metacyclic form of the parasite is transmitted to the host cells to transform to amastigotes after invading macrophages and multiply by simple division to invade other new macrophages [16].

Multiple medical treatment options are used throughout the world for treatment of leishmaniasis such as pentamidine, antimonials, amphotericin B, and miltefosine. However, all these drugs have mild and severe side effects, including pancreatitis, anemia, cardiac toxicity, and kidney failure, complicating the treatment [17]. Additionally, the existing anti-leishmanial treatments are very expensive and the parasite has developed resistance against most of available drugs [18]. Therefore, there is an urgent need to develop and creating new agents and treatment strategies that can be biocompatible, economically efficient, and affordable for the poorest people.

Nanomedicine (the use of medical applications of nanotechnology for human benefit) is one of the interdisciplinary fields in this area that can contribute to the development of new drug agent and new strategies for different diseases treatment. Currently nanoparticles are employed in modern science, including metal oxides such as iron oxide, silver oxide, etc [19-27]. The reason that it is inexpensive, low toxic, and effective as a drug agent for treating various diseases. The objective of the current study was to evaluate the leishmanicidal efficiency of SPIONs against *L. tropica* (promastigote stage), the major epidemic of anthroponotic cutaneous leishmaniasis. For evaluation of antileishmanial efficacy of iron oxide nanoparticles, the viability of promastigotes was tested in both control and test groups (iron oxide nanoparticles concentrations) at various time intervals (48h, 72h, and 96h). The results indicated that the SPIONs had antileishmanial activity against the promastigotes of *L. tropica* based on a dose-dependent response (Table .1.).

Parasite growth kinetics were observed on average PY made at various time intervals(48h, 72h, 96h) under a light microscope at 400x magnification. Adding iron oxide nanoparticles to the media at a concentration of 23.3µg/µl remarkably increased the mortality rate of cells from $37x10^5$ to $9x10^5$ cells/ ml after 48 hours of incubation (Table 1.). However, after 72hours there is an increase in the cell density to about $37x10^5$ cells/ml before decreasing again to 33 $x10^5$ cells/ml after 96 hours. However the scenario of the other two concentrations (2.32µg/µl and 0.231µg/µl) of iron oxide nanoparticles was different, there is a consistent decrease in the number of promastigotes with the incubation time before they slightly increased over 96hours exposure to iron oxide nanoparticles. Beside this determination, there is a considerable decrease in promastigotes survival was observed in iron oxide nanoparticles treated groups as compare to without iron oxide nanoparticles exposed treated groups (control). This was clear from the rate of promastigotes growth inhibition based on a dose-dependent response, since

iron oxide treated groups showed high growth inhibition between 70%- 66% with different concentration compared with control group as it is shown in Fig(7).

Although the exact mechanism of iron oxide nanoparticles has not been fully understood, three possible approaches have been proposed to clarify the future for their promising anti-leishmanial activities. The parasite cell wall can be broken down by physical contact, oxidative stress with reactive oxygen species (ROS) and release of sustainable ions from particle surfaces [28-30]. The direct interaction between iron oxide nanoparticles and Leishmania cells may cause deformation and membrane damaging with subsequent changes in the membrane potential and permeability, leading to severe cytoplasm leakage and cell damage [31-33].The ROS generated inside the cell damages the cell membrane, DNA, and other vital proteins. Iron oxide ions from the nanoparticles can inhibit the enzymes by displacing important minerals such as sodium, potassium, calcium and magnesium, which contribute in the action of enzymes [34-37].

The nanoparticle acts more comparable to solid surface and they are more likely able to interact locally with lipid molecules or domains in the cell membrane. Therefore, once the nanoparticles are attached to the cell membrane, intermolecular forces between lipid molecules who control on the membrane's integrity or stability against physical damaging might be disrupted because of lipid destruction and local shape deformation of the lipid layers. Since the structural integrity of cell membranes is vital for cell viability and functions, thus the possible changes in the membrane configuration on the molecular level in turn leads to the cell death [38]. Thus, it is believed that iron oxide particles may use several mechanisms simultaneously to kill Leishmania cells. This makes it difficult for Leishmania and other pathogens to develop resistance to iron oxide nanoparticles [39].

On the other hand, iron oxide nanoparticles have been increasingly used in biomedical applications for drug and gene delivery, MRI, and hyperthermia because of their least toxic effect shown on human tissue and body, which has attracted researchers to explore this system for maximum biomedical [40]. Therefore, these properties make iron oxide nanoparticles a antileishmanial drug candidate for treating Leishmania parasite or even delivering antileishmanial drugs as they proved there efficacy for different parasite drug and gene delivery [41] .

Treatment efficacy of iron oxide nanoparticles in this study depends on the concentration, three different concentrations were tested, and 23.2 µg/µl was found to be more efficient for inhibiting the parasite growth compared to the other tested concentrations (2.32, 0.232) µg/µl. The results were analyzed by amount of viable cells after particles treatment that indicate SPIONs may exerted a toxic effect on Leishmanial cells, causing suppression of activity and inhibiting their spread.

4. Conclusions

In this report, SIONPs were synthesized via a co-precipitation method were mainly spherical and homogeneous with an average size of about 5 nm. SPIONs exhibited potent antileishmanial activity against promastigote culture of *L. tropica* species on *in vitro* model. In addition, further clinical studies are required to evaluate exact effect of SPIONs on other *Leishmania* species in animal models as a new therapeutic agent against leishmaniasis.

Table.1. Cultivation of *L. tropica* Promastigotes in Different concentration of Fe_3O_4/NPs

Time (hour)	No of promastigotes in iron oxide nanoparticles concentrations			
	Control	(23.2 µg/µl)	(2.32 µg/µl)	(0.232 µg/µl)
0	$34x10^5$	$37x10^5$	$32x10^5$	$42x10^5$
48	$70x10^5$	$9x10^5$	$20\ x10^5$	$27x10^5$
72	$90x10^5$	$37x10^5$	$19x10^5$	$23x10^5$
96	$109x10^5$	$33x10^5$	$34x10^5$	$37x10^5$
%Growth	100	30	31	34
%Inhibition	0	70	69	66

Initial inoculation, $1x10^5$ promastigotes

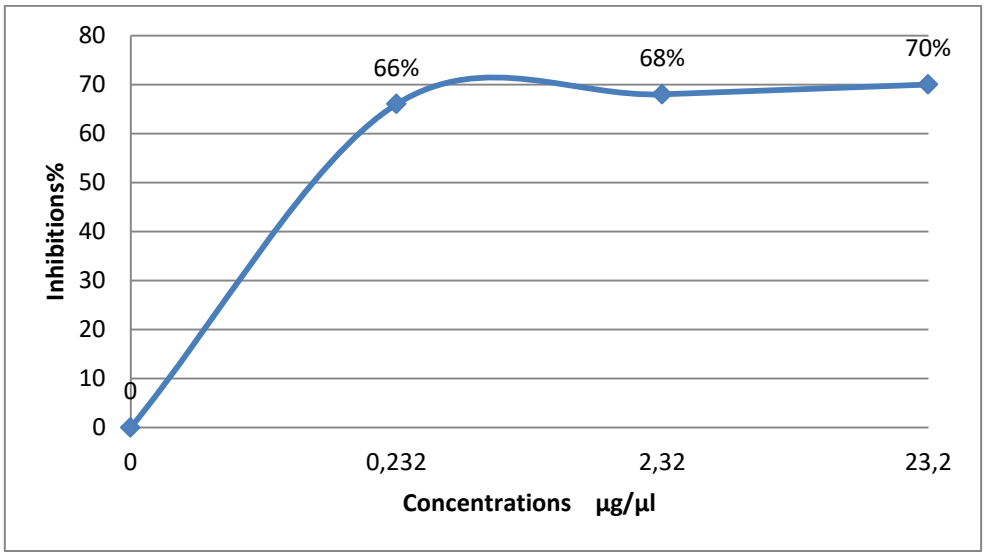

Fig.7. The effects of iron oxide nanoparticles concentrations on L. tropica promastigotes growth

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