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RESEARCH ARTICLE

The Characterization and Antileishmanial Evaluation on Leishmania Major with Chitosan/Zno Bio-Nanocomposite as Drug Delivery Systems

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ABSTRACT

Objective(s): Leishmaniasis is a global disease that poses a threat to human life and is associated with complications. Current medications have limitations due to serious side effects, costs and drug resistance. Nanotechnology has received increased attention in recent years, owing to its extensive range of applications in various fields including parasitology and its inherent therapeutic properties.

Objective: This study was designed to assess the effects of chitosan and chitosan-ZnO nanocomposite interventions on Leishmania major.

Methods: In this study, different concentrations of the nanocomposite were prepared (200, 100, 50 and 25 µg/mL), the parasite was cultured at 24, 48 and 72 h intervals and the viability of promastigotes and nanocomposite toxicity were evaluated by MTT assay. IC50 was determined by counting parasites. The inhibitory effect of the chitosan and nanocomposite were compared with standard drugs using different concentrations.

Results: The IC50 for nanocomposite after 72 hours were 50 and 10 µg/mL for promastigotes and amastigotes, respectively. In addition, 15% toxicity of nanocomposite on macrophage cells was found. The MTT assay showed 18.54 % promastigote viability after 72 h exposure to 200 µg/mL concentration of nanocomposite. Results showed significant differences between treatment groups as compared to control groups.

Conclusions: The above nanocomposites showed low toxicity and anti-leishmanial effects on both promastigote and amastigote forms. This study revealed anti-leishmanial activities of nanocomposites but further study is needed for in vivo evaluation of nanocomposites application for cutaneous leishmaniasis.

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INTRODUCTION

Leishmaniasis is one of the important health-threatening problems which affect people around the world, especially individuals in tropical and subtropical areas. The progress of disease might be due to the immune system and genetic makeup of the host. Glucantime, Pentostam and Amphotericin B are the first and second lines of the drugs that were used respectively but these drugs have many serious side effects. Due to the absence of desirable vaccines, medications and drug-resistance *Leishmania*, hence, many research has been designed to overcome the above problem. Novel therapy application especially nano-therapy is an important field and method that would solve the problem in this area [1-3].

During the last decade, nanotechnology-based drug delivery systems have been used to increase the performance of medicines in treating some diseases. Combined use of a nano-carrier system with the anti-leishmanial drugs is a new and hopeful approach as these nano-carriers can penetrate the macrophages and reach the parasite efficiently [4]. Some nano-carriers also enhance efficacy and reduce drug toxicity with sustained release of the drug. Nanotechnology is a valuable method that would provide treatment against various forms of leishmaniasis using targeted delivery [5,6]. The nanocomposite is a multiphase material where one of the phases has one, two or three dimensions of less than 100 nanometers (nm) or structures having nano-scale repeat distances between the different phases that make up the material. The idea behind Nanocomposite is to use building blocks with dimensions in nanometre range to design and create new materials with unprecedented flexibility and improvement in their physical properties. The material at nanoscale exhibits unique physicochemical properties, which are accredited to their ultra-small size, high surface to volume ratio, composition, presence of biochemical moieties on the surface (peripheral coatings or functional groups), hydrophilic or hydrophobic nature, physical appearance (shape or morphology) and aggregation [7]. As the particle size decreases, the interparticle forces such as van der Waals and electrostatic forces increase, and as a result, the size, shape, and biological properties of the nanoparticles change [8]. Metal oxide nanoparticles such as iron oxide, copper oxide, zinc oxide, etc, have different usage in the various sciences. Zinc is a good choice for biodegradable materials due to its higher degradability in vivo and biocompatibility for tissue regeneration and treatment. Zinc oxide (ZnO) is a non-organic, water-soluble white powdery compound and is also a safe biological complex. Zinc oxide nanoparticles (ZnO NP) are absorbed in various ways such as skin contact, inhalation and oral. ZnO NP is one of the five zinc compounds that are currently listed as generally recognized as safe by the U.S. Food and Drug Administration (FDA) [9,10]. This nanoparticle has an antibacterial effect on gram-positive and gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [11]. The antibacterial activity of ZnO is attributed to the generation of reactive oxygen species (ROS) on the surface of these oxides [12,13]. The present investigation was aimed to evaluate the antileishmanial activity of ZnO nanoparticles on *L. major* in vitro conditions.

On the other hand, Chitosan is a linear polysaccharide derived from the Chitin natural polymer.

Chitosan is obtained by deacetylation of chitin, which is an aminated polysaccharide found in the exoskeleton of arthropods and crustaceans. Chitin is a highly hydrogen-bonded semi-crystalline polymer, which makes it difficult to solubilize in most organic solvents. Chitin and chitosan have several biomedical applications. Chitosan nanocomposites are widely used in drug transmission, tissue engineering, antimicrobial, medical and cosmetic equipment's [14,15]. It is suggested that the antimicrobial activity of chitosan and its derivative biomaterials relies on numerous factors like the degree of deacetylation, molar weight, pH, the presence of metal cations, pKa and microorganisms species [16]. Chitosan, either alone or mixed with other polymers, active agents and metallic nanocomposites, has been extensively used in many biomedical applications, including in wound dressing as an antimicrobial agent [Biomaterials based on chitin and chitosan in wound dressing applications [17]. In recent years, several studies have been performed on the significant antimicrobial effects of nanoparticles, especially their anti-parasitic role with fewer side effects [18-21]. The findings show that nanoparticles such as silver, gold, chitosan and metal oxides, individually or in composite, have a lethal or inhibitory effect on protozoa and worms [22-25]. This study was designed to assess the effects of chitosan and chitosan-ZnO nanocomposite interventions on

Leishmania major (MRHO/IR/75/ER) *in vitro*.

MATERIALS AND METHODS

Materials

Zinc acetate tetrahydrate, potassium hydroxide, Acetic Aside and ethanol were obtained from Merck. Chitosan (average M_W = 45 *kDa* with a degree of acetylation >75%) was obtained from Sigma-Aldrich. Glucantime and Amphotericin B obtained from Sigma Co (USA) were applied as a standard control drug. Leishmania (MRHO/IR/75/ER), the Iranian standard strain was obtained from Tehran University of Medical Sciences. Batch manufacturing of parasites was taken in RPMI 1640 culture media was purchased from Gibco© (Ireland). Balb/c peritoneal macrophages (J774A.1 cell line) were obtained from the Tehran University of Medical Sciences

Synthesis of Zinc Oxide Nanoparticle

Zinc acetate (0.1 M) solution was prepared in ethanol with pH=5. This solution was stirred for 90 minutes in instruction to obtain a homogeneous Zinc salt mixture. Then, potassium hydroxide (0.1M) solution was obtained in ethanol with a pH =11. The KOH solution was added in zinc acetate solution dropwise under continuous stirring. The reaction was carried out at ambient temperature with a pH =7. The final solution was stirred for 2 hours using a magnetic stirrer and obtained a colloidal ZnO nanoparticle solution [26, 27].

Nano drug Synthesis

Chitosan (2% w/w) solution was prepared in Acetic Aside. Then, the chitosan solution was added to the colloidal solution of zinc oxide nanoparticles in a 1: 1 ratio for 15 min. After the physical and chemical interactions of chitosan and zinc oxide nanoparticles, the chitosan/zinc oxide Nanocomposite was formed [28-30].

Preparation of Standard Drugs

Glucantime and Amphotericin B were serially diluted with distilled water to 20 and 0.6 µg/mL, respectively. It should be explained that amphotericin B was directly toxic to the parasite at the amastigote stage and did not seem to depend upon macrophage activation for its antileishmanial activity. Therefore, amphotericin B was used only in the Promastigote stage (not in the amastigote stage). Each experiment was carried out in triplicates [31-34].

CHARACTERIZATION

Instruments

To perform Fourier, Transform Infrared spectroscopy (FTIR) a Thermo Nicolet NEXUS 870 FTIR from Nicolet Instrument Corp., the USA was used. The surface morphology of samples was investigated using a Field Emission Scanning Electron Microscope (FESEM Sigma, Zeiss Germany.

Parasite Culture

10% fetal bovine serum, 1% of pen/strep were added to the media. The culture was incubated at 18-24 °C. Cell passage was performed every 72 h and each test was accomplished in triplicate [31-34].

Promastigote and Amastigote Growth Inhibition Assay

The IC50 and IC90 for promastigotes of L.major, promastigotes in 106 cells/ml in different concentrations (200, 100, 50 and 25 µg/mL) of ZnO nanoparticles, chitosan and chitosan-ZnO nanocomposite were accomplished at 24, 48 and 72 h. The temperature was adjusted at 18-24 °C and the parasite numbers were counted using a light microscope daily. A medium containing promastigote was used as a negative control, while another medium including Glucantime or amphotericin B and promastigotes were considered as positive controls, hence, the IC50 and IC90 were determined. The values also were calculated using linear regression. Amastigote in logarithmic phase (106 cells/mL), macrophages and the test groups were added in chamber slide. The chamber containing macrophages was evaluated as a negative control and another filled with amastigote and Glucantime was considered as a positive control. The plates were incubated for 24, 48 and 72 h at 37 °C. The parasites that were not entered into the macrophage were washed by PBS. For amastigote growth inhibition analysis, 100 macrophages were found to be infected by the parasite. The ratio of 106 parasites/105 macrophages has been used in this analysis. The stain smears were used for amastigote's count in every 100 macrophages. [31, 32].

MTT Assay

The colourimetric analysis was performed to evaluate the antileishmanial function of the nanodrugs. Promastigotes in the logarithmic

growth phase (106 parasites/mL) with different concentrations of NPs were cultured in 96-well plates containing RPMI under aseptic conditions and were incubated for 24, 48 and 72 h at 18-24 ◦C in a CO2 incubator (5%). The positive controls were promastigotes along with Glucantime or amphotericin B, and the negative control included promastigotes culture. After removing as much of the supernatant as possible, remaining material was incubated for 4 h at the same conditions. For the formazan crystals solution, the dimethyl sulfoxide was added to wells in dark conditions in a shaker for 10-15 min. Formazan crystals were dissolved by the addition of dimethyl sulfoxide (DMSO) to the wells and mixed in the dark on a shaker for 10 -15 min. the absorbance is measured at OD=490 nm with a 96-well plate reader. Then, the percentage of viable cells in control and test wells is calculated using the corresponding formula [32-34]. Balb/c peritoneal macrophages were cultured at 105 cells per well in a plate before adding the prepared nanoparticles and nanocomposite concentrations. The analysis was done the evaluate nanoparticles and nanocomposite toxicity in macrophages. The MTT assay was done according to the kit instructions mentioned above. To determine relative absorption (SI: Stimulation Index), the optical density of each sample was calibrated against the background of absorption in the control well (medium-plus macrophage) [31-34].

Statistical analysis

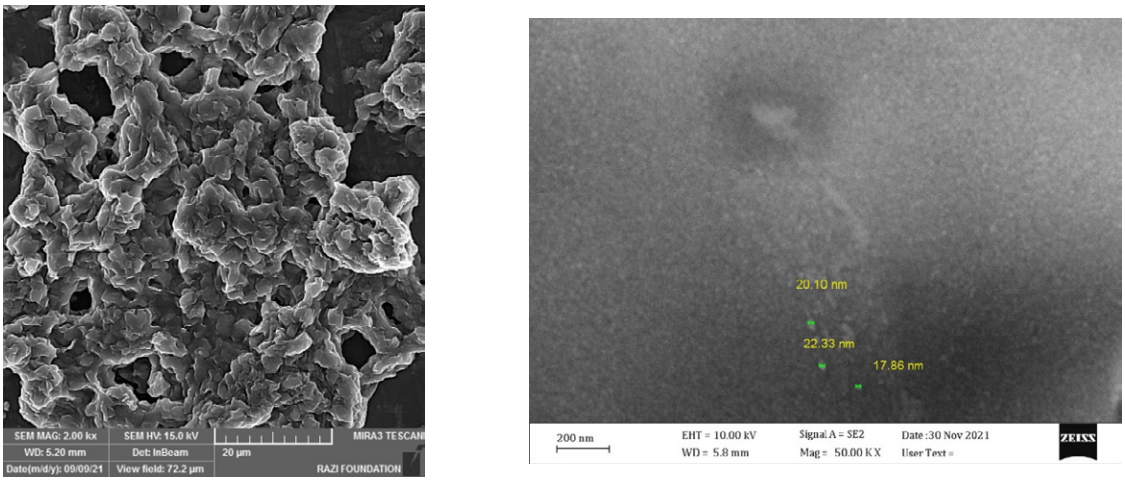
One-way analysis of variances, ANOVA and t-test, and the SPSS software V.21. used for statistical analysis. The $P < 0.05$ was considered a significant difference between groups. The results were acquired by averaging three separate tests. Each test was done thrice.

RESULTS

SEM Analysis

Fig 1A shows the micrographs of ZnO nanoparticles as observed by conventional FESEM. The structure of the nanopowders synthesized corresponds to the wurtzite (hexagonal form). In fact, from the samples obtained, in Fig. 1(A) it can be noted that the agglomeration of particles quickly takes place. These effects are due to the fact a great number of byproducts are present in the solution. But when the washing process does the size starts to be homogeneous. This behaviour can be due to a decrease in the by-product concentration as the washing process steps increase, and this provokes the reduction of the kinetic energy around the nanoparticles with the byproducts.

The surface of a drug delivery carrier is one of the most important factors determining the release behaviour of the drug. The interfacial interactions between chitosan and ZnONPs may change the surface morphology of the Chitosan-ZnO nanocomposite, which have a great influence



A **B**

Fig.1. FESEM images of A) ZnO nanoparticles and B) Chitosan-ZnO nanocomposite.

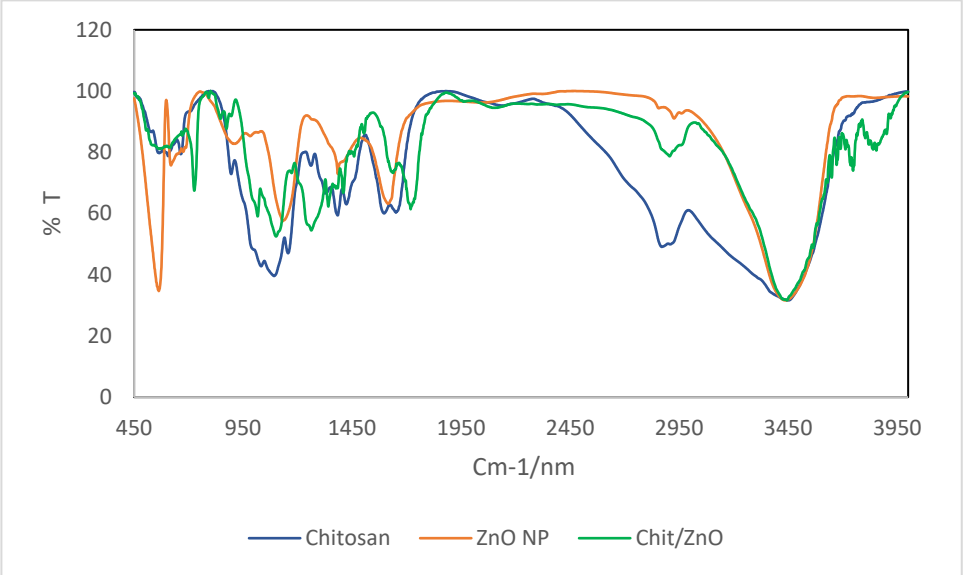


Fig.2. FTIR of samples

on drug release behaviours. Thus, the morphology of Chitosan-ZnO nanocomposite beads was also investigated. Generally, the nanocomposites were spherical with a diameter of about 21 nm and possessed a smooth surface. As it can be seen in Fig. 1B, the surface morphology of the chitosan-ZnO nanocomposites showed severe wrinkles and many cavities, which was caused by partial collapsing of the polymer network during drying. The porous structure could generate the capillary forces which facilitated the penetration of fluids into the beads and thereby the drug was easily released into the water.

FTIR Analysis

The chemical structure of nanoparticles and nanocomposite were characterized by FTIR. In the nanocomposite spectrum, due to the presence of ZnO, this peak has been transferred to a lower wavenumber of 3419 cm-1, which indicates the interactions between chitosan and ZnO. In the chitosan spectrum, the absorption peak 3427 cm-1 is related to the tensile vibrations of the NH2 and OH groups. Peaks cm-1 2925 and 2882 are related to asymmetric tensile vibrations of CH2 and CH3 chitosan polymer. Also, the peaks at 1647 and 1078 cm-1 are related to the bending vibrations of the NH2 group and the tensile CO, which in comparison with chitosan, the absorption peaks at 659 and 465 cm-1 due to the binding of the amide group and the tensile vibration of ZnO. (Fig.2.).

Nanocomposite Efficacy Evaluation by Parasite Count

The results of experiments showed a significant reduction in promastigotes and amastigotes inside macrophages count by nanocomposites as compared to control group (*P <0.05). In promastigotes, IC50 of nanocomposite was 90, 70 and 50 µg/mL, also IC90 of nanocomposite was 280,230,200 µg/mL after 24, 48 and 72 h, respectively. In promastigotes, IC50 of ZnO nanoparticles was 150, 90 and 90 µg/mL, also IC50 of chitosan was 210,80,50 µg/mL after 24, 48 and 72 h, respectively. In amastigotes inside macrophages, IC50 of nanocomposite was 100, 30 and 10 µg/mL, also IC90 of nanocomposite was estimated 400,300 and 270 µg/mL after 24, 48 and 72 h, respectively. In amastigotes inside macrophages, IC50 of ZnO nanoparticles was 200, 30 and 20 µg/mL, also IC50 of chitosan was 300,100,50 µg/mL after 24, 48 and 72 h, respectively (Figs. 3 and 4). Within 24 hours, the nanocomposite showed significant inhibitory effect on parasite growth at different concentrations. However, the longer the exposure time, the greater the effect of the drug, as compared to the standard drug, glucantime. Glucantime is described as the first line of leishmaniosis treatment, but its effect on amastigotes is greater than that of promastigotes.

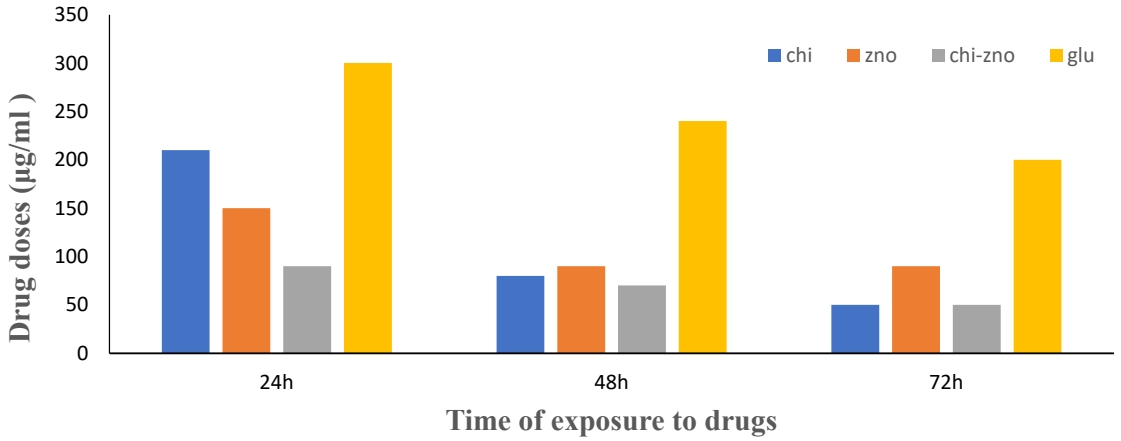


Fig.3. IC50 of promastigotes after addition of various concentrations of nanomaterial and drug to the test and control groups in incubation stages.

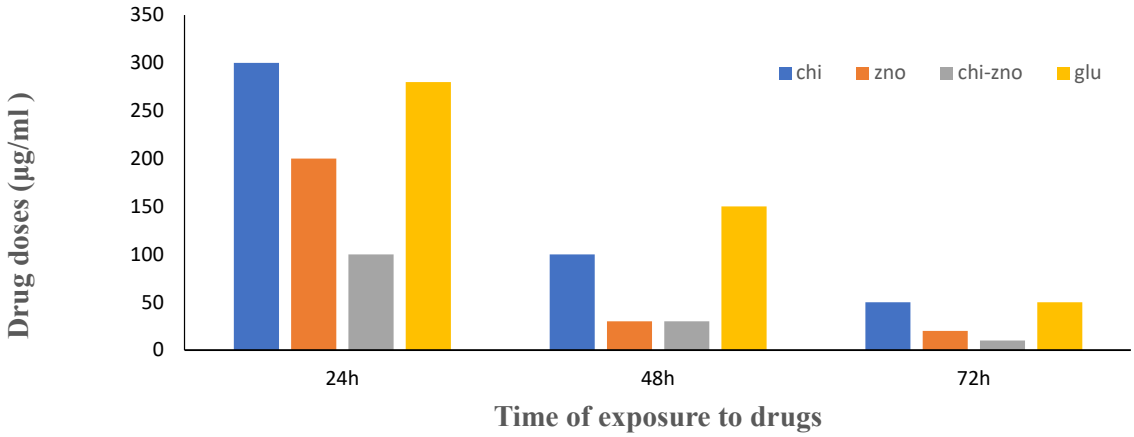


Fig.4. IC50 of amastigotes inside macrophages after addition of various concentrations to the test and control groups in incubation stages.

In promastigotes, IC50 of glucantime was 300,240 and 200 but about amastigotes inside macrophages , IC50 of glucantime was 280,150 and 50 after 24, 48 and 72 h, respectively. It should be noted that, glucantime had no significant effect on inhibiting the growth of promastigotes *in vitro* and to achieve the desired IC50 and IC90, much higher concentrations is required, [21-22].

MTT Assays for Promastigotes Evaluation

The promastigotes exposed to the various concentrations of chitosan-nanosheet, ZnO nanoparticles, chitosan-ZnO nanocomposite, glucantime and Amphotericin B. Within 72 hrs Chotosan-ZnO nanocomposite exhibited the lowest survival rate compared to control groups (18.54 %) at 200 µg/mL concentration. All the results of the treatment groups were significantly different from the control group (*P <0.05) (Table 1). The percentage of promastigotes survival showed the drug treatment dose and exposure time-dependent. By increasing the dose of the drugs and the duration of drug exposure, a decrease in the number of parasites was observed. *In vitro*, amphotericin B along with glucantime was used as a positive control due to the lesser effect of glucantime alone on promastigotes. Amphotericin B is not the first treatment choice because of its high cost and serious side effects, except in cases of drug resistance [35,36].

Table 1. Percentage of promastigotes survival after the addition of different concentrations of nanomaterials and standard drugs to the test and control groups in incubation times. *P<0.05 significant difference. (Values were expressed as mean ± SD.).

Material	Time (h)	Survival at 25 (µg/mL), %	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)
Chitosan	24	86.32± 3.03 %	84.66±3.01	79.33±2.98	73.24±1.99
	48	54.65±2.99 %	57.32±2.86	44.48±3.11	33.95±2.01
	72	35.21±3.21 %	31.37±3.11	26.19±3.14	19.72±2.80
ZnO	24	81.11±3.78	50.98±1.59	72.88±3.90	63.02±2.99
	48	56±2.76	55.01±1.90	47.78±2.89	37.11±3.89
	72	50.98±1.59	45.60±2.83	40.90±1.80	31.09±2.84
Chi-ZnO	24	74.22±2.68	66.28±4.98	59.68±3.90	52.51±4.56
	48	45.89±2.82	39.64±3.81	33.89±3.80	31.43±2.70
	72	32.15±3.55	27.45±3.21	25.41±3.76	18.54±2.59
Glucantime	24	92±2.25	86.22±3.33	83.68±3.22	81.24±1.36
	48	79.57±3.22	76.33±3.54	72.75±2.22	69.32±2.21
	72	72.15±3.59	69.66±2.45	65.49±4.03	57.76±1.36
Amphotericin B	24	17.33±3.33	16.12±2.37	15.69±4.44	15.01±3.66
	48	14.02±4.22	14.01±1.39	13.68±2.23	12.49±3.22
	72	12.94±4.04	12.04±1.11	11.89±2.77	11.03±3.66
Negative control	24	100±2.42	100±4.11	100±2.44	100±3.61
	48	100±1.48	100±4.03	100±3.89	100±2.99
	72	99±1.99	99±2.89	99±3.21	99±4.44

Toxicity Assessment by MTT Tests

The toxicity of different concentrations of nanoparticles was evaluated on macrophage cells). Results showed 15%, 15% and 20% toxicity for the chitosan nano-sheet after 24, 48 and 72 h in 200 µg/mL concentration, respectively. On the other hand, ZnO nanoparticles showed 25%, 25%, 30% toxicity and 20%, 20%, 25% toxicity of chitosan-ZnO nanocomposite on macrophage cells after 24, 48 and 72 h in 200 µg/mL concentration, respectively. Thus, results showed the lowest toxicity for the chitosan nano-sheet group as opposed to other materials (15% at 24 hrs.), with significant differences as compared to the controls. Also, the toxicity of chitosan nano-sheet was lower than standard drug (glucantime) on macrophages, but with no significant differences (P>0.05). The toxicity of the above materials depended on the concentration and treatment time. The toxicity of ZnO nanocomposite on macrophages was slightly higher than chitosan, although this difference was not statistically significant (P>0.05). It could be due to the presence of the Zn ion and the induction of its toxic effect on the macrophages. Excessive Zn entry may cause tissue accumulation and impairment of iron and copper absorption and other adverse effects. These results were the average of at least 3 separate experiments [37].

DISCUSSION

Recently, brilliant advances have been made in the treatment of parasitic diseases by synthesizing new drugs using nanotechnology. Researcher in this area have been tried to develop nanomaterials with the most bioavailability efficacy, minimum toxicity, and low side effect and with minimum cost. NPs can be used directly or in composite, with routine drugs used to treat parasites. The present study was designed to evaluate the effectiveness of nanomaterials compared with ordinary standard drugs in a concentration-dependent manner against L. major. The high concentration of the nanomaterials led to protozoan elimination observed over the time in this study. However, in comparison between test groups and control groups, results showed the nanocomposite was much effective than other groups. The encouraging outcomes of applications of anti-parasites nano drugs properties, World Health Organization (WHO) and many pioneering researchers have focused on creating new effective nano-drugs. The admissible results of the use of anti-parasitic nano-drug compounds have encouraged the World Health Organization (WHO) and a number of eminent researchers to develop new, effective and efficient nano-medicines with acceptable results in the diagnosis and treatment of parasitic diseases

[38]. Innovation in this research is the synthesis of chitosan-nanosheet for the first time with a simple method instead of spherical chitosan, which is used in most investigations. Based on the literature reviews, no studies have been published on the effects of chitosan nano-sheet, and Chitosan-ZnO nanocomposite against L. major in vitro. The effects of antihelminth NPs including silver, chitosan were evaluated against helminth as Echinococcus multilocularis, Trichinella spiralis, Fasciola. The effects of anti-protozoa NPs including Silver, chitosan, and curcumin, copper, gold, selenium, curcumin, titanium was tested against protozoa such as Plasmodium, Toxoplasma gondii, Cryptosporidium parvum, Leishmania, E. histolytica, Giardia lamblia. Metal NPs such as CuO, TiO2 and Ag2O showed significant antibacterial and anti-parasitic effects [13]. In this survey, comparison of number of parasites in the control groups to the test groups at different time intervals showed that the effect of nanoparticles on parasites depends on dose and the length of exposure time. The results have demonstrated a direct relationship between the chitosan nano-sheet and nano-composites concentration in the inhibition rate of cultured parasites. In vitro and in vivo studies on human cell lines not only deny any toxic effects of chitosan, but confirm biocompatibility, biodegradability and mucosal adhesion and bioactivity. The FDA has confirmed the therapeutic effect of chitosan. Previous research has been conducted to confirm the stronger biological effect of synthetic chitosan than chitosan extracted from the fungus on Giardia and Trichomonas vaginalis in a dose-dependent manner. [39,40]. Research has shown that curcumin as a strong drug, combined with chitosan-tripolyphosphate nanoparticles has greater activity to eliminate Staphylococcus aureus and Pseudomonas aeruginosa infections than curcumin alone in infected sensitive female Balb / c [40,41]. The researchers reported that chitosan–tripolyphosphate conjugated chloroquine nanoparticles had in vivo anti-malarial efficacy against rodent parasites. Various concentrations of antimony sulfide nanoparticles (NPs) showed a great antileishmanial efficiency against L.major, with the greatest efficiency in vitro and in vivo experiments [42,43]. The previous study showed that the highest activity of the NPs against L. major was seen for Ag-NPs, followed by Au-NPs, TiO2-NPs, ZnO-NPs, and MgO-NPs also reported that the NPs included cytotoxicity on macrophages. Jebali et al. reported that the use of metal oxide NPs for the treatment of CL might include positive or negative outcomes. Elmi et al reported that chitosan nanoparticles were able to significantly remove P. falciparum, G. lamblia and T. vaginalis in comparison with the control group in vitro. The United States Food and Drug Administration has approved Zno as a safe material and is one of the 5 zinc compounds that are recognized to be safe. It is reported antiparasitic, antibacterial and antifungal effects of ZnO NPs with induction apoptosis, generation of hydroxyl ions and ROS. The previous data indicated that ZNPs showed anticoccidial and antioxidant properties. Delavari et al. showed that ZnO-NPs at concentrations of 120 µg/mL included anti-leishmanial effects against L. major amastigotes in vitro [44 – 47].

CONCLUSION

Based on mentioned results and consistent with several supportive studies, significant differences has been found in ZnO-Chitosan composite to eleminate L.major compared to the control group in vitro. Our results reinforce the importance of extending in vitro results to in vivo experimental plans and suggest that more attention needs to be given along with extensive studies with enormous sample size. In order to have more effective and efficient nanopharmaceutical compounds of nano composite and to limit the use of chemical drugs that cause drug resistance and have multiple side effects, more comprehensive and extensive studies should be performed.

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CONFLICT OF INTEREST STATEMENT

The authors of the present work declare no conflict of interest, financial or otherwise.

ETHICAL APPROVAL

This study was approved by the Ethics Committee of Iran University of Medical Sciences following Declaration guidelines with approval Code No: IR.IUMS. REC.1399.385.

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