ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 13, Issue, 03, pp. 62004-62007, March, 2023
https://doi.org/10.37118/ijdr.26147.03.2023

RESEARCH ARTICLE OPEN ACCESS

GREEN SYNTHESIS OF COBALT FERRITE NANOPARTICLES WITH SICILIAN LEMON AND ITS ACTIVITY AGAINST THE PROLIFERATION OF TRYPANOSOMA CRUZI

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ARTICLE INFO

Article History:
Received 26th January, 2023
Received in revised form
14th February, 2023
Accepted 20th February, 2023
Published online 28th March, 2023

KeyWords:
Nanoparticles, Green Chemistry, Cobalt Ferrites, Trypanosoma cruzi.

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ABSTRACT

In the last decades, nanoparticles have been the subject of intense studies, since intrinsic characteristics, such as their size, morphology, physical and chemical properties, that allow the manipulation of functionalities and applications mainly in the biomedical area, whose research has demonstrated several advantages in pharmacodynamics and pharmacokinetics, demonstrating its ability in the drug delivery system. Besides, another important features in producing nanoparticles is related respect to the principles of green chemistry to its synthesis, thus resulting in reduction of the generation of harmful substances, mainly for the environment. In this work, green synthesis of a cobalt ferrite nanoparticle was performed using the Sicilian lemon and subsequently tested against *Trypanosoma cruzi* for its antiproliferative activity. Cobalt ferrite nanoparticles were synthesized by a sustainable method with the aid of Sicilian lemon (*Citrus limonia*) using water as the only solvent. The synthesized nanoparticles were characterized by X-ray diffractometry and scanning electron microscopy. The X-ray diffractometry confirmed that the nanoparticles crystallized in a $CoFe_2O_4$ pure phase. SEM images show an agglomeration of fine particles that, in concentrations of 20 and 50 µg/mL, were evaluated against *Trypanosoma cruzi*. The nanoparticles produced with the aid of Sicilian lemon showed antiproliferative activity against *T. cruzi* and by transmission electron microscopy was observed inside the parasite, thus resulting in its growth inhibition.

Citation: ***Bruna Lorena Silva Fagundes, Valeska Cardoso Oliveira, Pedro Eleutério dos Santos Neto, Monaliza Araújo dos Santos et al., 2023.*** "Green synthesis of cobalt ferrite nanoparticles with sicilian lemon and its activity against the proliferation of trypanosoma cruzi". *International Journal of Development Research*, 13, (03), 62004-62007.

INTRODUCTION

In the last decades, nanoparticles (NPs) have been the subject of intense studies, since intrinsic characteristics such as their size and morphology grant a range of physical and chemical properties (Bayda *et al*., 2019). Such characteristics allow the manipulation of functionalities and applications mainly in the biomedical area, whose research evidenced several advantages in the pharmacodynamics and pharmacokinetics proving the capacity in the system of drug release (Agnieszka *et al*., 2012). The objective of our work was to synthesize and manufacture NPs respecting the principles of green chemistry and reducing the generation of harmful substances (Verçoza *et al*., 2019).

The cobalt ferrite ($CoFe_2O_4$) NPs were synthesized with the aid of Sicilian lemon (*Citrus limonia*) and their magnetic properties and antiproliferative activity were studied to utilize the particles against a range of disease caused by several pathogens. The *Trypanosoma cruzi* was choose as a potential target, because it is the etiological agent of Chagas disease (Balouz *et al*., 2017; Pérez-Molina and Molina, 2018). This parasite is primarily transmitted by feces of triatomine bugs vector as *Triatoma infestans* or *Rhodnius prolixus*, oral transmission through contaminated food, and also by blood transfusion or organ transplantation (Junior *et al*,. 2017). This neglected disease affects millions of people in Brazil and Latin America (Kratz, 2019).

MATERIALS AND METHODS

Synthesis and purification of green nanoparticles: The Sicilian lemon was purchased at a local market and 50 mL of its filtered juice was used in green synthesis. For the syntheses, 3.4 mmol of cobalt nitrate and 5.2 mmol of iron nitrate were used together with 50 mL of Sicilian lemon juice and 200 mL of water. The solution was heated under magnetic stirring for 15 h through the Sol-Gel process (Hench and West ,1990). Subsequently, the sample was submitted to the oven (80°C) for 24 h and transferred to a muffle for heat treatment at 300° C for 1 h. The formation of black solids in the sample was observed. The sample was centrifuged (3000 rpm/20 min), the precipitated collected and oven dried at 80 °C for 24 h.

X-Ray Diffraction (XRD): The crystallinity of the obtained ferrites was evaluated by the X-ray diffraction (XRD) technique, using a PANalytical X'Pert Pro diffractometer located at the Centro Brasileiro de Pesquisas Físicas (CBPF), equipped with a monochromator and a rotating sample mounting, operating at 40 kV and 40 mA. The measurement was performed in a 2θ range of 5°-80°, with step of 0.05°/s, with Cu-Kα radiation. The diffractograms were analyzed and compared to the JCPDS (Joint Committee on Powder Diffraction Standards) database.

Scanning Electron Microscopy (SEM): Scanning electron microscopy was used to study and analyze the size of Cobalt ferrite nanoparticles produced. For this, a small amount of powder was sprinkled under a carbon tape mounted on a stub. Microscopy images were taken at the Instituto Nacional de Metrologia, Qualidade e Tecnologia (Inmetro) in the Magellan 400 microscope, using a voltage of 10 kV with a current of 0.4 nA.

***Trypanosoma cruzi* proliferation assay:** The protozoa Y strain of *Trypanosoma cruzi* epimastigotes were maintained in LIT culture medium (Liver in fusion tryptose) supplemented with 10% fetal bovine serum (FBS). Cultures for the treatment were prepared when the epimastigotes were in the exponential phase (48 h), with a minimum concentration of $1x10^7$ parasites/mL. At this time of growth, the nanoparticles were added in the concentrations of 1, 10, 20 and 50 µg/mL. As the Cobalt ferrite nanoparticles were diluted in DMSO, vehicle control was made adding 25 uL of DMSO, which correspond to 2,5 % of the total culture volume. Cultures cells were maintained at a constant temperature of 28 ºC. To ascertain the proliferation rate an aliquot of 0.01 mL of the cultures was taken and diluted in 4% formaldehyde during 8 days. The evaluation was made using the Neubauer chamber counting the number of parasites in a contrast phase microscopy.

Transmission electron microscopy in scanning electron microscopy (STEM-IN-SEM): In order to evaluate the possible ultrastructural damage caused by nanoparticles in the epmastigote forms of *Trypanosoma cruzi*, control and treated cells were washed in PBS pH 7.2 and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2. Then, the cells were washed twice in 0.1 M sodium cacodylate buffer pH 7.2 and post-fixed in a mixture of 1% osmium tetroxide, 1.25% potassium ferrocyanide, 5 mM $CaCl_2$ in cacodylate buffer 0.1 M for 30 min at room temperature in the dark. Finally, the cells were washed, dehydrated in increasing concentrations of acetone and incorporated in Epon. Ultrathin sections were obtained using a PT-PC PowerTome ultramicrotome (RMC Boeckeler, USA), stained with uranyl acetate and lead citrate, and observed using a TESCAN VEGA 3 LMU operating in Scanning-transmission electron microscopy mode at 30 kV.

STATISTICAL ANALYSIS

Statistical analysis was done using GraphPad Prism by one-way analysis of variance (ANOVA). Results were considered statistically significant at $P \leq 0.05$ (*).

RESULTS

Nowadays, nanomedicine is a special part of medicine where several areas are interconnected as nanotechnology, pharmaceutical sciences and medicine with the objective of producing new treatment alternatives such as the use of nanoparticles that may be important tools in therapeutics as in the drug delivery system for several diseases (Bobo *et al.*, 2016). The nanoparticles produced in this work have a low cost, are easy to prepare and less aggressive to the environment providing a promising alternative of their use in possible treatments (Verçoza *et al.*, 2019; Romero and Morilla, 2010). The synthesis process of this work was performed using the sol-gel technique that refers to any route of materials synthesis that, at a given moment, a transition from the sol system to a gel system occurs. In this case, the colloidal solution (sol) acts as the precursor for an integrated network (gel) of discrete particles or polymers network (precursors normally used are metal alkoxides and metal salts such as chlorides, nitrates and acetates). The objective of the synthesis was the search for superparamagnetic nanoparticles whose property is directly linked to the particle size. The XRD analysis confirmed the crystalline character of the synthesized samples (Figure 1) and the $CoFe_2O_4$ pure phase was verified by Rietveld refinement (Rodriguez-Carvajal, 1993). The crystallite size of 20.76 nm was calculated using the well know Scherrer equation with the (311) reflection (most intense peak).

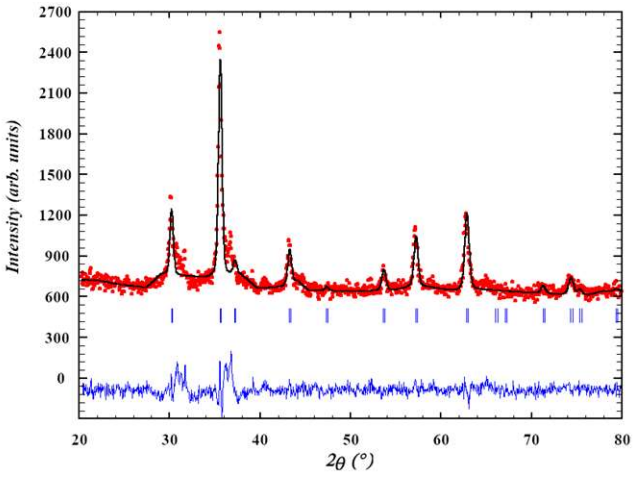

Figure 1. X-ray diffractogram of the nanoparticles synthesized with the aid of Sicilian lemon

Scanning electron microscopy (SEM) was used to estimate the size and characterize the morphology of the prepared nanoparticles (Nadaroglu *et al*., 2017). The SEM analysis showed a broad nanoparticles size distribution, in a range of approximately 6 to 30 nm, which sometimes can form aggregates as observed in Figure 2.

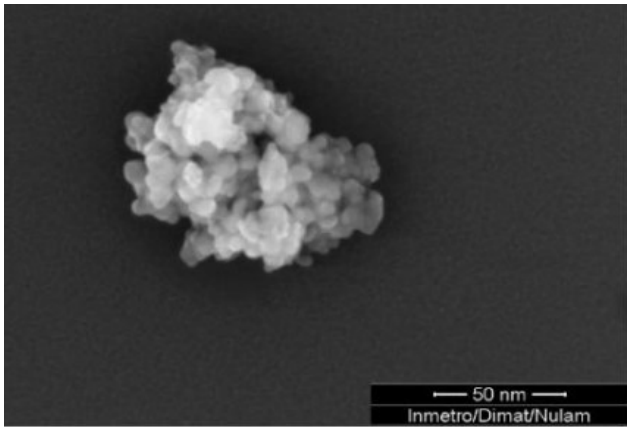

Figure 2. SEM Image of a nanoparticles aggregate

Despite the development of new strategies to control Chagas disease, effective and less toxic drugs are not available yet. Current available drugs do not always eliminate the parasite and frequently cause severe adverse reactions, which prevent the continuation of treatment. Furthermore, vaccines to prevent infection have not been successful so far. Thus, the molecular detailing of parasite development, including within its passage through the invertebrate host, may shed light on novel strategies to block *T. cruzi* transmission. The lemon ferrite NPs were tested for the proliferation activity of *Trypanosoma cruzi* epimastigotes at various concentrations (1-50 µg/mL). Lemon ferrite nanoparticles presented great results and, at concentrations of 20 and 50 µg/mL, demonstrated to have antiproliferative activity in *T. cruzi* (Figure 3).

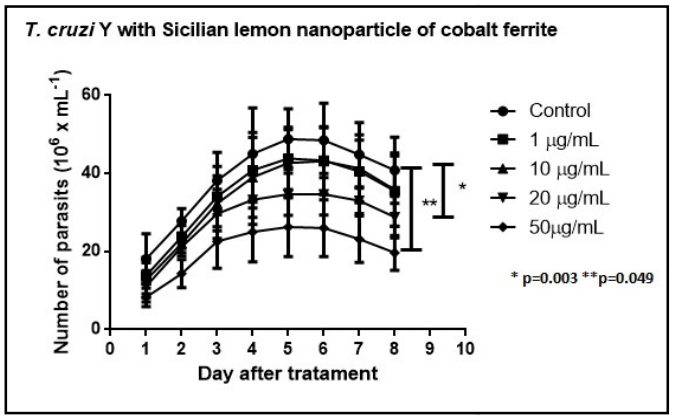

Figure 3. Treatment of *Trypanosoma cruzi* epimastigotes with different concentrations of Cobalt nanoparticles. The graphic shows all groups evaluated: Control parasites in the absence of nanoparticles (●) or parasites in the presence of 1 µg/mL (■) , 10 µg/mL (▲), 20 µg/mL (▼) or 50 µg/mL (◆) of Cobalt Ferrite nanoparticles. Proliferation rate (number of cells per milliliter in suspension) was measured. By increasing nanoparticle concentration in *T. cruzi* culture, its proliferation reduced. For the concentrations of 20 µg/mL and 50 µg/mL, the parasite growth significant inhibited. The bar represents means ± S.E.M., n = 3, using two-way ANOVA. * and ** Significantly different, ratio 20 µg/mL and 50 µg/mL to control (p <0.05 and p <0.01)

Observing the results, we can verify that the antiproliferative activity in *T. cruzi* may be better related to the geometric shapes obtained by the green synthesis of the nanoparticles than the size of the same; besides, the aggregation factor does not seem to have influenced its biological activity. To determine if the nanoparticles added to the culture medium were internalized by the parasites and to evaluate the possible ultrastructural damage caused by the treatment, TEM-IN-SEM modality of electron microscopy was employed. From the observation of Figure 4, it is possible to notice the presence of electrondense aggregates (indicated by an arrow) randomly distributed by the cytosol of the parasite. These electrondense aggregates were always surrounded by membrane. Due to the dense nature of nanoparticles and because they are composed of elements with large atomic rays, we believe that these aggregates are the internalized nanoparticles. These results indicate that *T. cruzi* epimastigotes are able to internalize the synthesized material. Finally, ultrastructural damage could not be clearly seen.

DISCUSSION

Looking at the results, we can see that the antiproliferative activity of the Cobalt NPs against *T. cruzi* epimastigotes may be better related to its geometric shape than to the size. The NPs aggregation appears not to influence the biological activity in *T. cruzi*, since it was able to internalize the lemon cobalt ferrites. This shows that the obtained NPS can be used as a potential tool for Chagas disease treatment. The use of nanosystems in the release of drugs is of great value in the treatment of several diseases, among which we can mention Chagas disease, as in these systems the cells are capable of internalized nanoparticles, given their nanometric size and their physical and chemical characteristics, with this, being able to carry with it a small and effective volume of drug (Islan *et al.* 2017;Verçoza *et al*., 2019; Look *et al*., 2010**)**.

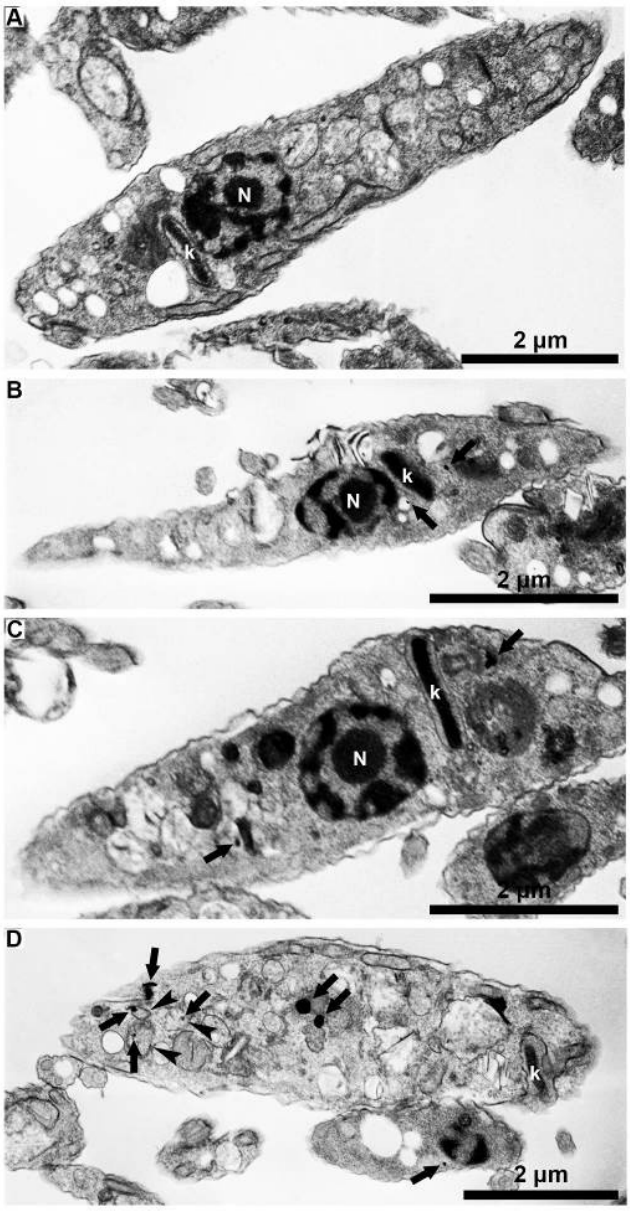

Figure 4. Internalization of Cobalt Ferrite nanoparticles by *Trypanosoma cruzi*. The epimastigote forms in the log phase were replicated in LIT medium, supplemented with 10% FBS in the absence (control, image A) or in the presence of 10 µg/mL (images B, C and D) of Cobalt Ferrite NPs. On the fifth day after the peal, the parasites were prepared for TEM-IN-SEM analysis according to Gross, et al., 2014. To locate the nucleus, we use the letter N; for the kinetoplast, the letter K. The arrows point to the presence of electrondense bodies distribuited throughout to the cytosol. In panel D, it is possible to observe that the nanoparticles appear surrounded by a membrane (arrowhead).

The ability to transpose of the cell membrane, coupled with the controlled release of the therapeutic molecule, decreases the drug's plasma concentrations and consequently reduces its toxicity, which in some cases may allow to have new alternatives that should be less toxic for the mammalian cells carrying the amastigote form (Branquinho *et al*., 2014). In the context of Chagas disease, where there is a large group of infected patients and only two drugs available (Nifurtimox and Benznidazole) that are clinically prescribed, where both have serious problems of toxicity and mutagenicity (Castro *et al*., 2006), the use of systems based on nanoparticles associated or not with drug delivery are a promising alternative treatment for this disease.

CONCLUSION

The importance of nanomedicine is to advance in new drugs, therapies or even in new technologies and pharmaceutical formulations. In addition, green synthesis associated to low cost methodologies, resulting in new nanomaterials without production of toxic waste could be an alternative in the development of new drugs and technology linked with the field of nanomedicine in various types of diseases.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) no Programa Redes de Pesquisa em Nanotecnologia no Estado do Rio de Janeiro (E-26/010.000981/2019) and Conselho Nacional de Desenvolvimento Científico e Tecnológica (CNPq). The authors acknowledge the INMETRO Divisão de Materiais, and Laboratório de Cristalografia e Difração facilities at Centro Brasileiro de Pesquisas Físicas.

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