Original Article

In Vitro Study the Effects of Amphotericin B Silver Nanoparticles and Amphotericin B on the *Leishmania tropica*

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Abstract

Background: Iraq is an endemic country with a high frequency of both types of leishmaniasis, visceral and cutaneous, with several clinical manifestations. However, multidrug-resistant strains are serious problems for public health worldwide. So, the continuous development of antiparasitic agents has become of increasing importance for medicine. **Objectives:** This study aimed to estimate the inhibitory activity of amphotericin B silver nanoparticles against *Leishmania tropica* in comparison to that of amphotericin B *in vitro*. **Materials and Methods:** *Leishmania tropica* isolate samples provided by Al-Nahrain University's Biotechnology Research Center. The stock culture of *L. tropica* used in this study has been maintained in the laboratory by weekly serial subculturing in RPMI 1640 medium. **Results:** The present study revealed that the final test of transmission electron microscopy analysis revealed that the infection with *L. tropica* has more effects when treated (Amph-B-AgNPs), and the parasite was well distributed and most compounds were outside parasites compared with the parasite before treatment. When the concentration of Amph-B-AgNPs increased, the antiparasitic effect increased while the parasite's viability curve decreased. **Conclusions:** The study showed that the amphotericin B silver nanoparticles had the best therapeutic effects on *L. tropica* compared with amphotericin B alone.

Keywords: Amphotericin B, *leishmania tropica*, silver nanoparticles

Introduction

Infection with a unicellular parasite from the genus *Leishmania* results in the worldwide parasitic disease leishmaniasis.[1] Leishmaniasis is represented as one of the major vector-borne communicable diseases in the world. It is a zoonotic disease that is, caused by obligatory intracellular protozoa of the genus *Leishmania*.[2] The disease transmits to humans when humans, sand fly, and the reservoir host share the same environment.[3] The genus Phlebotomus is naturally responsible for the transmission of many protozoal parasites like *Leishmania.*[4] *Leishmania tropica* causes anthroponotic cutaneous leishmaniasis, but *L. major* causes the zoonotic cutaneous leishmaniasis.[5] In Iraq, one of the endemic diseases is cutaneous leishmaniasis (CL). It is seen as a global health problem and an uncontrolled disease, it is caused by the intracellular protozoa that belong to the *Leishmania* genus.[6] The most prevalent type of leishmaniasis is CL, often regarded locally as (tropical sore, oriental sore, and Baghdad sore), it is a social and public health issue in many developing nations. *Leishmania* major in desert regions and *L. tropica* are the main causes of the old world disease.[7]

Leishmaniasis is a vector-borne disease caused by *Leishmania* parasites, which cause a range of clinical manifestations in man. These are didactically classified into CL, the most common form of the disease, and visceral leishmaniasis (VL), the life-threatening form. There are so far no vaccines approved for humans. Conventional drugs pose limitations ranging from low efficacy and high cost to systemic toxicity. Low efficacy derives in part from difficult drug access to the parasites, which ride themselves inside macrophage phagosomes. This prompts to high dosage, with consequent increased

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DOI: 10.4103/MJBL.MJBL_971_23

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Submission: 12-Jul-2023 **Accepted:** 22-Aug-2023 **Published:** 30-Apr-2026

How to cite this article: Hassone WS, Faraj AA. *In vitro* study the effects of amphotericin B silver nanoparticles and amphotericin B on the *Leishmania tropica*. Med J Babylon 2026;23:230-5.

toxicity. Difficult intracellular drug access can be overcome with nanomedicines such as biocompatible lipid and polymeric nanoparticles that can be phagocytosed by the infected macrophages.[8] Nanoparticles promise an alternative approach to current antibiotics in the treatment of infectious diseases.[9] Silver nanoparticles (AgNPs) confer many advantages over other drugs. In the same concentrations which are low enough to make human cells susceptible, it is lethal for many pathogenic microorganisms without any side effects on humans.[10] Moreover, due to silver's high level of germicidal capacity and its distinctive physicochemical qualities, such as its small size (between 10 and 1000 nm), large surface area, electrical charge, and shape, various investigations have noted that silver has antileishmanial effects.[5] Although several drugs are used against *Leishmania* infection, they are associated with several adverse complications. Therefore, a new effective treatment needed to be found the effect of carbon nanotube nanoparticles (CNT NPs) on *Leishmania donovani* promastigotes was assessed. AgNPs have an important effect in stimulating the production of oxygen species. The objective of this study was to examine macrophage cytotoxicity upon exposure to *L. tropica* and AgNPs.[11]

Materials and Methods

Samples collection and culturing of *L. tropica* in media (RPMI 1640)

Leishmania tropica isolate was obtained from Al-Nahrain University, Department of Biotechnology Research Center. The *L. tropica* stocks used in this study were maintained *in vitro* by weekly serial culture in RPMI 1640 medium. On the other hand, *L. tropica* promastigotes were cultured on RPMI 1640 medium, which was supplemented with 1% glutamine; 10% fetal calf serum, penicillin 1 U/100 mL, streptomycin 1 mg/100 mL then incubated at 28.[12]

Preparation of amphotericin B stock solution and silver nitrate

The amphotericin treatment was supplied by the Indian company Homdia as stock powder and prepared in the laboratory by dissolving 2 mg of amphotericin in 10 mL of DMSO solution. But silver nitrate has been supplied from Kosdaq, Busan and Seoul, South Korea, and prepared in the laboratory by dissolving 8.49 g of silver nitrate in 1 L of distilled water to obtain three concentrations of 25, 50, and 75 mM.[13]

Synthesis of amphotericin B–silver nanoparticles compound

Amphotericin B–silver nanoparticles compound was prepared by adding 1 mM $AgNO_3$ solution to 9 mL of the amphotericin B solution in the biological methods at room temperature. After that color change of the solution from colorless to dark brown indicated the silver generation of nanoparticles from the protein. Silver nitrate conversion to nano silver particles was found to be successful as suggested by the change in color of the solution to brown.[14]

Fourier-transformed infrared radiation (FTIR)

The filtrate containing AgNPs was analyzed with the Fourier-transform infrared spectrometer. The spectrum was recorded in attenuated total reflectance (ATR) mode with resolution 0.2 in the wavelength range of 400–4000 nm spectrometer. FTIR Micro-Spectroscopy is the most recent FTIR technique and combines a FTIR (Perkin Elmer, Hamburg, Germany).[15]

Scanning electron microscopy (SEM)

The morphological study of the amphotericin B silver nanoparticle was observed by scanning electron microscopy (SEM; H-7650, Hitachi, Hitachi, Japan), to produce images from the interaction of the electron beam with atoms at various depths within the sample the measurement size of the dry powder amphotericin B silver nanoparticles.[16]

Transmission electron microscope (TEM)

Transmission electron microscopy (TEM; JEOL-JEM-2100, Japan) was done as described by Sadowski *et al.*[17]

Determination of minimum inhibitory concentration (MIC) of amphotericin B and amphotericin B nanosilver

To determine the minimum inhibitory concentration of amphotericin B and amphotericin B nano silver against *L. tropica* use broth dilution method was done as described by Lovati *et al.*[18]

Ethical approval

Ethical Approval was granted through the local committee and used at the College of Veterinary Medicine within the University of Baghdad (number 577, date on March 12, 2023) before starting this study.

Result

Synthesis of silver nanoparticles

Add 10 mL of $AgNO_3$ at 50 mM solution to (90 mL) amphotericin B at a concentration of 2 mg/100 mL in a separate conical flask. The samples are put on a magnetic stirrer at room temperature for 2–3 h and inspected for color change. The color change of the solution will be checked every 24 h as shown in Figure 1. The solution was left in the conical flask and incubated at room temperature for another 48 h until the completion of the reaction.

Fourier-transformed-infrared radiation (FTIR)

The filtrate containing AgNPs was analyzed with the Fourier Transform Infrared spectrometer. The spectrum was recorded in ATR mode with resolution 0.2 in the

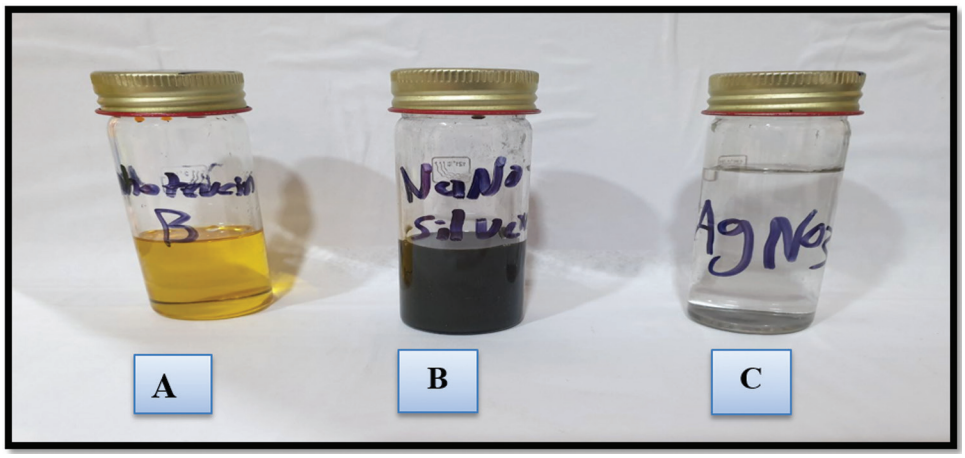

Figure 1: Preparation of silver nanoparticles: (A) amphotericin B, (B) amphotericin B silver nanoparticles, and (C) silver nitrate

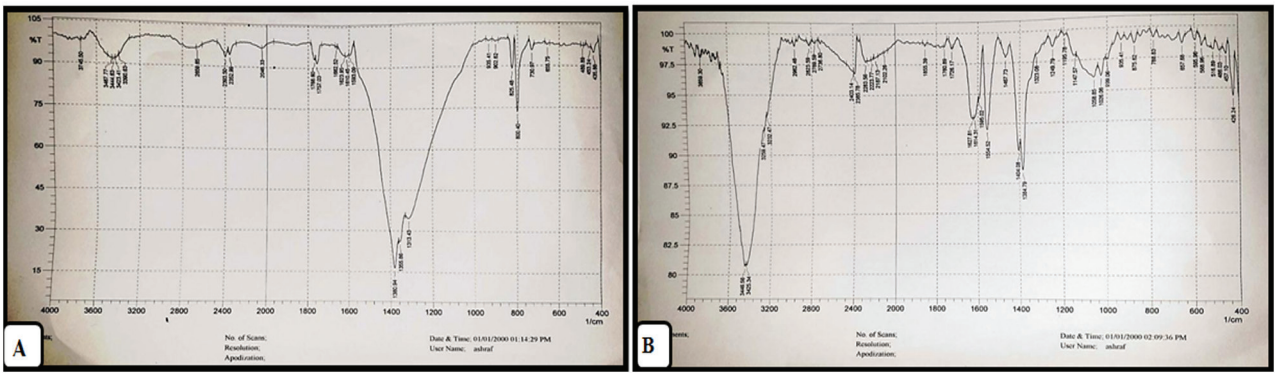

Figure 2: FTIR spectra: (A) silver nanoparticles and (B) amphotericin B silver nanoparticles

wavelength range of 400–4000 nm spectrometer as seen in Figure 2.

Scanning electron microscopy (SEM)

The results of SEM in the present study revealed that the amphotericin B silver nanoparticle crystallites appeared homogeneous surface morphology, with a wide size distribution of the particles from about 50–80 nm as seen in Figure 3.

In vitro the effects of Amph-B-AgNPs and amphotericin B on the *L. tropica*

In vitro the effects of Amph-B-AgNPs

The result showed in Figure 4A the *L. tropica* before treatment appeared normal promastigotes possess a single nucleus and are variable in length and shape (ellipsoid to slender). The most prominent features of promastigotes are the nucleus and the flagellum while; Amastigote of *Leishmania* are spherical to ovoid and have a regular arrangement of walls; after treatment with amphotericin B silver nanoparticles, the *L. tropica* swelled, irregular, and ruptured wall [Figure 4B].

The effects of amphotericin B on the L. tropica

Leishmania tropica before treatment with amphotericin B appeared rod in shape with regular parasite membrane [Figure 5A], while after treatment it appeared swelled and irregular with no clear contain [Figure 5B].

Discussion

Characteristics of the nanoparticles were determined using the FTIR spectrum

The characteristics of the nanoparticles were determined using the FTIR spectrum, which was recorded to identify the possible interactions between silver and biologically active molecules involved in the synthesis of nanoparticles shows the FTIR spectra of amphotericin B and AgNPs samples within the range of 400–4000 cm^{-1}.

Accordingly, the majority of bands are shared in both spectra due to the similar structural components. Yet, there are some differences in the location of peaks due to their dissimilar symmetrical environments and the existing interactions. The observed peak in 3700–3390 cm^{-1} belongs to the hydroxyl functional group, which can result from phenolic groups and the absorbed moisture. Besides, the flexural vibration of the hydroxy group shows a peak in 1765.60–1757.03 cm^{-1}. A small shoulder is observed in both spectra in the range of 1757.03 cm^{-1}, which belongs to the C=O carbonyl group vibration. The observed vibrations in the range of 13,880.94, 1,355.86, and 13,131.43 cm^{-1} are caused by the stretching vibrations

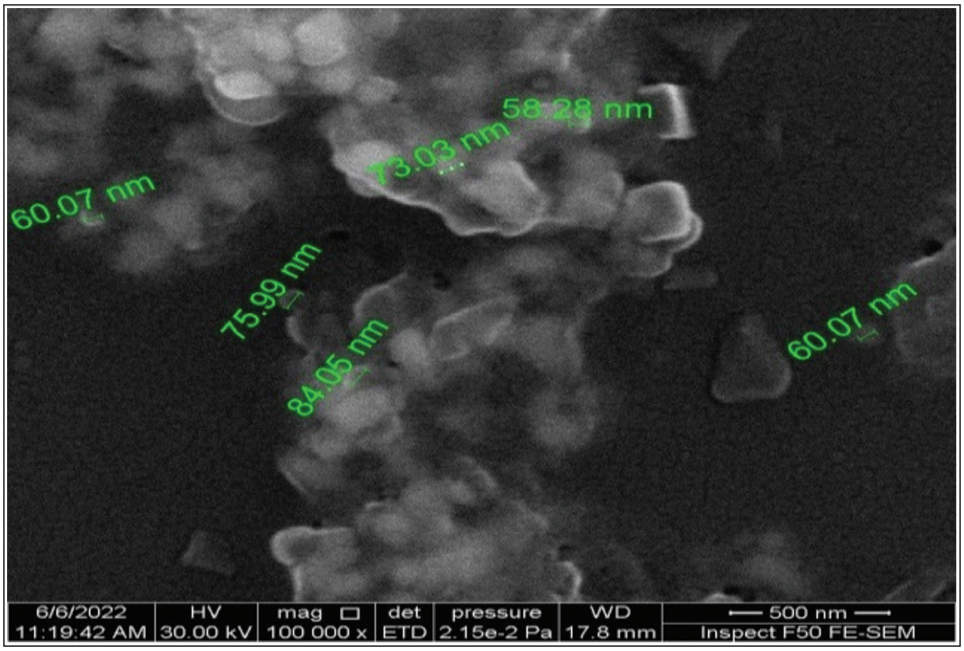

Figure 3: Scanning electron microscopy of amphotericin B silver nanoparticle shows crystallites appeared homogeneous surface morphology with a wide size distribution of the particles from about 50–80 nm

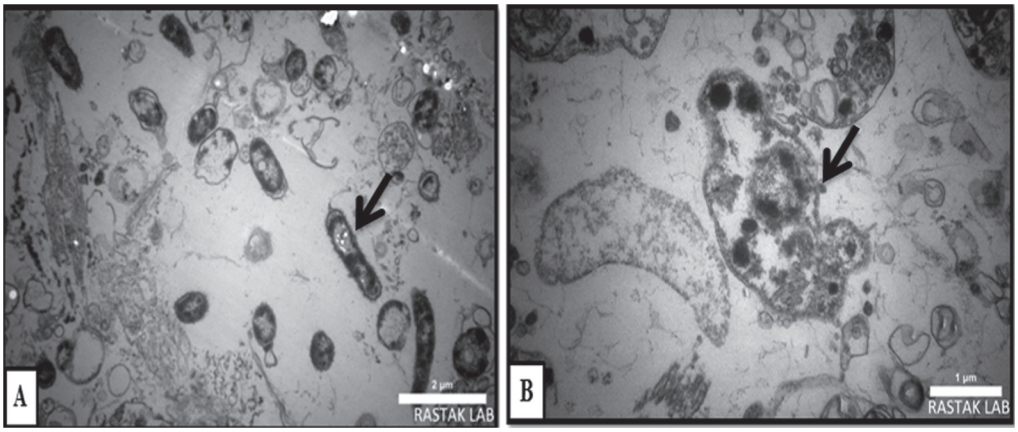

Figure 4: TEM of *L. tropica*: (A) before treatment with amphotericin B-AgNPs and (B) after treatment with amphotericin B-AgNPs

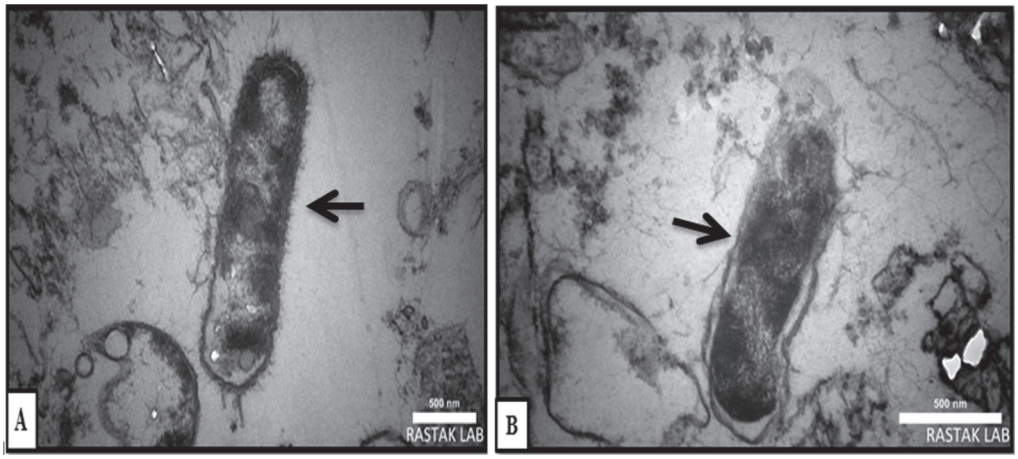

Figure 5: TEM of *L. tropica*: (A) before treatment with amphotericin B and (B) after treatment with amphotericin B

of C–C, C–N, and C–O. As shown in Figures 4 and 5A. These vibrations are displaced slightly and detectable in the AgNPs spectrum due to the interaction of the stabilizer with the silver particles. The oxygen-metal (silver in this sample) vibrations show peaks in the range of 400–800 cm^{-1}. Peaks in 2962.46–2823.59 cm^{-1} are caused by the stretching vibrations of the C–H hydrocarbon structure. The observed vibration at 2403.14–2385.78 cm^{-1} in the AgNPs sample may be attributed to CO_2 absorption during the FTIR sample tableting. As shown in Figures 4 and 5B, the peaks of phenolic, amine, and carbonyl groups are observed in the spectra of AgNPs, revealing the effect of these functional groups on the synthesis of AgNPs. This study was aimed at identifying the functional groups and organic compounds in the sample. Bioactive compounds (flavonoids, proteins, exopolysaccharides, polyphenols, and alkaloids) in amphotericin B can bind to AgNPs via multiple functional groups and, therefore, stabilize AgNPs.[19] Also, this was agreed upon by Ghatee *et al.*,[20] who studied the characterization of AgNPs synthesized using Viola tricolor aqueous extract as a reducing and capping agent.

A study by Xia *et al.*[21] showed the changes occurring to AMB after being sequentially mixed with PEG and ZnO, the FTIR spectra of ZnO, AMB, AMB-PEG as well as ZnO-AMB-PEG. The spectrum of pure AMB exhibited the typical reported bands for this molecule located at 3392, 2935, 1692, and 1559 cm^{-1} and assigned to the O–H, C–H, C=O, and C=C stretching vibrations, respectively.[22]

The AMB-PEG composite preserved the characteristic peaks of pure PEG. However, after conjugation with PEG polymer chains, the three bands located at 3,392, 11,692, and 1,559 cm^{-1} corresponding to the O–H, C=O, and C=C stretching vibrations of AMB were either broadened or vanished. These observations indicated the formation of a single compound from AMB and PEG polymer because of PEG polymer encapsulation. On the other hand, after mixing with ZnO, all the specific bands associated with AMB disappeared, and the FTIR spectrum was fairly similar to that of pure ZnO, implying that the PEG-encapsulated AMB was further capped by ZnO nanoparticles, as confirmed by the SEM analysis.[23]

Scanning electron microscopy (SEM)

The results agreed with Mohammadi *et al.*,[23] who found amphotericin B-loaded polymeric nanoparticles. SEM was utilized to determine the size and morphology of the blank and drug-loaded nanoparticles. The SEM image shows that the prepared nanoparticles were spherical in shape and spatially separated, which confirmed the absence of aggregation. The nanoparticles were uniform in size and shape. This gave a preliminary result about the broadness of the particle size distribution (low polydispersity), which was in excellent agreement with previous studies.[9]

In vitro the effects of amphotericin B silver nanoparticles on the *L. tropica*

The findings were agreed with Tiuman *et al.*,[24] who investigated transmission electron microscopy and showed a loss of membrane integrity associated with *Leishmania amazonensis* with amphotericin B exposure. The accumulation of lipophilic compounds in the cytoplasmic membrane and membrane constituents of microorganisms has considerable effects on the loss of cellular integrity and inhibition of respiratory cellular activity in mitochondria.[25] This interaction with cell membranes eventually leads to cell death.

Whereas the results showed interaction with cell membranes and cytoplasmic vacuolization, leading when treatment with amphotericin B-AgNPs in agreement with Zein *et al.*[26] That used biosynthesized AgNPs using an aqueous extract of Eucalyptus camaldulensis leaves (AEECL) and revealed The highest concentration of AgNPs (3.75 µg/mL) showed the biggest suppressive effect on parasites, whereby it inhibited the growth of parasites by 90%. The IC50 was calculated using linear regression for a curve that obtained the effect of different concentrations of AgNPs on *L. tropica* promastigotes' viability IC50 values were found to be 1.7 and 1.8 µg/mL for 24 and 48 h, respectively, as well as the results were agreed with Mohammadi *et al.*[23] that used synthesis silver (Ag) nanoparticles (NPs), ginger extract against *L. major* promastigotes. Most of the reports suggest that the antileishmanial properties of AgNPs could be attributed to the slow release of silver ions from the nanoparticles' surface, which destroy the surface of the cell then penetrate the cytoplasm and bind with the target sites. Furthermore, AgNPs can produce reactive oxygen species. It is commonly known that *Leishmania* is highly sensitive to these oxygen species, and the drug, which could generate ROS, will be an efficient antileishmanial agent.

However, some studies used different agents but gave the same results as Tiuman *et al.*[24] revealed parthenolide had antileishmanial effects against axenic and intracellular amastigotes of *L. amazonensis* presenting electron microscopic studies revealed extensive cytoplasmic vacuolization, leading to the examination of the possibility that parthenolide induces autophagic cell death. Autophagy cell death is a process that is, thought to occur in all eukaryotes and is characterized by an accumulation of autophagic vacuoles. This mechanism occurs for energy production for survival when cells recycle their cytoplasmic contents during periods of environmental stress or certain stages of development. A double-membrane vesicle called the autophagosome forms in

the cytosol, engulfing organelles and bulk cytoplasm. Subsequently, these vesicles fuse with lysosomes, where their contents are degraded and recycled.[19]

In addition, another study found morphological alterations of *Leishmania infantum* chagasi promastigotes treated with 25 µg/mL usnic acid were observed by transmission electron microscopy. The compound caused changes in the cytoplasm density, cell swelling, and loss of cell polarity. Changes in mitochondrial morphology were characterized by marked swelling. We also noticed an increased number of intracellular vacuoles. Blebs were observed in the plasma membrane and detached from the membrane. It was also observed that the membrane was separated from the cytoplasm and there was a higher accumulation of fat compared to controls.[7]

Conclusions

According to the data, the present study concluded that amphotericin B silver nanoparticles had the best effects on *L. tropica* compared with amphotericin B alone.

Acknowledgments

The authors are thankful to the staff of the Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Iraq for their support and for providing the facilities during the sample processing.

Authors' contribution

All authors contributed equally to this work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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