Research Article

Copper and Zinc Oxide Nanoparticles: Experimental Considerations in their Application as Antimicrobial against *Leptospira Hyos*

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Abstract

The antimicrobial action of copper and zinc oxide nanoparticles (NPs) synthesized by using neem leaf extract against *Leptospira hyos* was evaluated. The experiment involved cultivating *L. hyos* as a test bacterium in a medium supplemented with 8% serum and the application of different concentrations of the NPs as treatments. The synthesis of copper oxide nanoparticle was validated as shown by its absorption peak at a wavelength of 265 nm in UV-Visual spectroscopy while that of the zinc oxide nanoparticle registered an absorption peak at a wavelength of 270 nm. The efficiency of the copper and zinc nanoparticles as antimicrobials against *L. hyos* was related to the significant differences in the reduction of mean recovery counts expressed in McFarland units (MFUs) as an effect of NP concentration and duration of exposure time. Result of the study led in the definition of the minimum inhibitory concentration (MIC) of the copper and zinc oxide NPs at 10%. These data provide a basis in validating these nanoparticle preparations as potential agents for the control of leptospira infections in animal production settings.

Keywords: Copper nanoparticle; Fletcher's media; Good health and wellbeing (SDG3); Leptospirosis; Life on land

UV-Vis; Zinc oxide nanoparticle

1. Introduction

Leptospirosis is a fatal zoonotic disease that infects animals and humans caused by pathogenic helical-shaped spirochetes of the genus Leptospira [1]. The broad diversity of this organism made it possible for the pathogen to infect a wide range of hosts and thrive into a variety of reservoir hosts like rodents. Transmission of the disease is strongly driven by environmental factors such as poor sanitation, disasters and floods in urban settings [2].

In the field of veterinary practice, leptospirosis is one of the major concerns of farm animal practitioners as well as small or companion animal practitioners. Food producing farm animals like cattle, swine and does are relatively susceptible to infections with leptospira, resulting in production losses due to reduced milk production, reproductive failure, premature birth or stillbirth and even abortions [3,4]. Dogs are highly susceptible to leptospiral infections, notably stray and sheltered dogs which increase the risk of zoonotic transmission to people. The inadvertent adoption of chronically infected dogs from shelters reportedly exposes homeowners to a greater risk of acquiring the disease and would contribute to the spread of infection [5]. Leptospirosis reportedly causes a number of complications and damage to many body organs. Current treatment against the pathogen reportedly depends on the use of antibiotics like ampicillin and doxycycline. However, with the information on the progressive evolution of antimicrobial resistance in many microbial pathogens, effort directed on the search for an alternative control agent against leptospira will have to continue.

Diagnostic work on Leptospira is quite risky due to its zoonotic nature and its inability to grow in ordinary culture media. Interest in the application of nanoparticles in diagnostics and therapeutic applications is now gaining momentum in the medical and allied sciences [5]. Research work on Leptospira in this laboratory was extended toward the isolation and cultivation of the pathogen in an artificial medium used by other researchers [3] and explored the possibility of coming up with herbal-synthesized nanoparticles that were validated as antimicrobials against Leptospira. Basic technics in microbiology were floated up and applied in the evaluation and confirmation of the anti-leptospiral activity of the herbal-synthesized NPs.

2. Materials and Methods

2.1 Bacterial strains used in the study

Pure cultures of *Leptospira hyos* (Accession number MK629955) which was previously utilized in a study that evaluated its susceptibility to antibiotics [3] and disinfectants [6] were recovered and used for this experiment.

2.2 Synthesis of the candidate Copper oxide and Zinc oxide NPs in *Azadirachta indica* (Neem) Leaf Extract

Leaves of *A. indica* (Neem tree) were collected and washed thoroughly with distilled water before the leaves were allowed to dry under room temperature. The air-dried neem leaves were cut into small, fine pieces using a drum blender tumbling machine (India Mart). The leaves (250 gms) were placed in a beaker containing 1000 mL distilled water and boiled in a hot plate at 80°C for 10 to 15 minutes. The extract was allowed to cool before filtration then transferred into tubes, stored in the refrigerator and used in the synthesis of nanoparticles.

Copper sulfate pentahydrate (0.06 grams) was dissolved in 250 mL neem leaf extract and the solution was mixed thoroughly with the use of a magnetic stirrer. A drop of

sodium hydroxide (NaOH) solution was added to optimize the pH to 7.2. The solution was left to stand overnight at room temperature to allow conversion and bio-reduction of the Copper salts to copper oxide NPs. Four (4) concentrations of the Copper oxide NP that included 90%, 75%, 50% and 10% were separately prepared for Uv-Visual absorption spectroscopy to validate the presence of the synthesized NPs as described [7].

Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$, 0.07 grams) was also dissolved in 250 mL neem leaf extract and a magnetic stirrer was also used to mix the solution. A drop of sodium hydroxide (NaOH) solution was added to optimize the pH to 7.2. The solution was left to stand overnight as in the above procedures to allow conversion and bio-reduction of the Zinc salts to Zinc oxide NPs. Four (4) concentrations of the Zinc oxide NP which included 90%, 75%, 50% and 10% were separately prepared for Uv-Vis absorption spectroscopy as described [8].

2.3 Cultivation of *Leptospira hyos*

A pure culture of *L. hyos* was grown in replicated tubes of Fletcher's medium supplemented with Sodium chloride (0.5 gm), Peptone (0.3 gm), Beef extract (0.2 gm), Agar (1.5 gms) in 100 mL Distilled water before adding rabbit serum (8%). The samples were incubated at 31ºC for 24 hours. When the growth of *L. hyos* in the culture medium was already indicated by the presence a dinger zone, the samples were collected and washed by centrifugation at 12,000 × g for 2 minutes. Samples of the sediments were re-suspended in 5 mL sterile deionized water (Thermo Fisher Scientific, USA) to monitor the cell density of the test pathogen using McFarland standards before the application of treatment. The bulk of the sample was used as the test organism for the evaluation of the antimicrobial activity of Copper oxide and zinc oxide NPs.

2.4 Evaluation on the susceptibility of *L. hyos* to Copper oxide and Zinc oxide NPs relative to the monitoring of recovery counts

The Copper oxide NP was separately dispensed in sterile tubes as 90% (T1), 75% (T2), 50% (T3) and 10% (T4) as treatments. From the Zinc oxide nanoparticle samples, concentrations at 90% (T5), 75% (T6); 50% (T7) and 10% (T8) were also separately prepared as treatments. A positive control included treatment with Doxycycline (T9). A negative control (T10) included Fletcher's medium containing *L. hyos* and a blank (T11) composed of Fletcher's medium only.

As *L. hyos* does not form colonies on the surface of a solid medium, fresh suspensions of the test pathogen (1 mL, with a density of 4.34 + 0.66 MacFarland units/MFU) were transferred in replicated tubes containing 1 mL Fletcher's medium. To each treatment of Copper oxide and Zinc oxide NP, 100 µL-volumes of *L. hyos* suspension was added in correspondingly to 3 replicated tubes with. The samples were incubated at 37ºC, and a provision was made wherein the test pathogen was collected after 30 minutes, 60 minutes and 24 hrs of interaction with the NPs. McFarland readings were taken to determine the cell density of *L. hyos* recovered after exposure to the different concentrations of NPs at indicated durations of interaction.

2.5 Statistical analysis

McFarland readings relative to the application of the different concentrations of Copper oxide and Zinc oxide NP were expressed as mean McFarland units (MFU) of 3 replicates. Differences in MFUs as an effect of the application of different concentrations of nanoparticles; and MFU data taken before and after 30 minutes, 60 minutes and after 24 hr application of NPs within treatments were statistically analyzed using Least Significant Diferrences (LSD) where p-values at 1% and

5% were considered significant.

3. Results

3.1 Characteristics of the synthesized Copper Oxide and Zinc Oxide NPs synthesized with neem leaf extract

The synthesis of the NPs was validated based on the results of Ultra-Violet Visible (UV-Vis) Absorption spectroscopy (Figure 1). The presence of absorbance peaks at a wavelength of 265 nm in UV-Vis Absorption Spectroscopy was demonstrated by the different concentrations of Copper oxide NP synthesized by neem leaf extract.

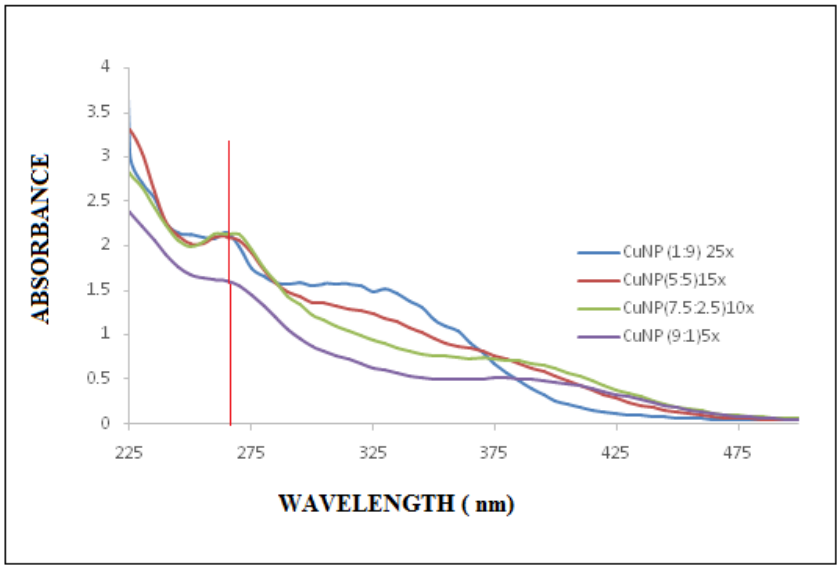

Figure 1: Uv-Vis absorption spectroscopy data of the Copper oxide nanoparticle. Copper oxide nanoparticle samples in different concentrations (90%, Violet; 75%, Green; 50%, Red; and 10%, Blue).

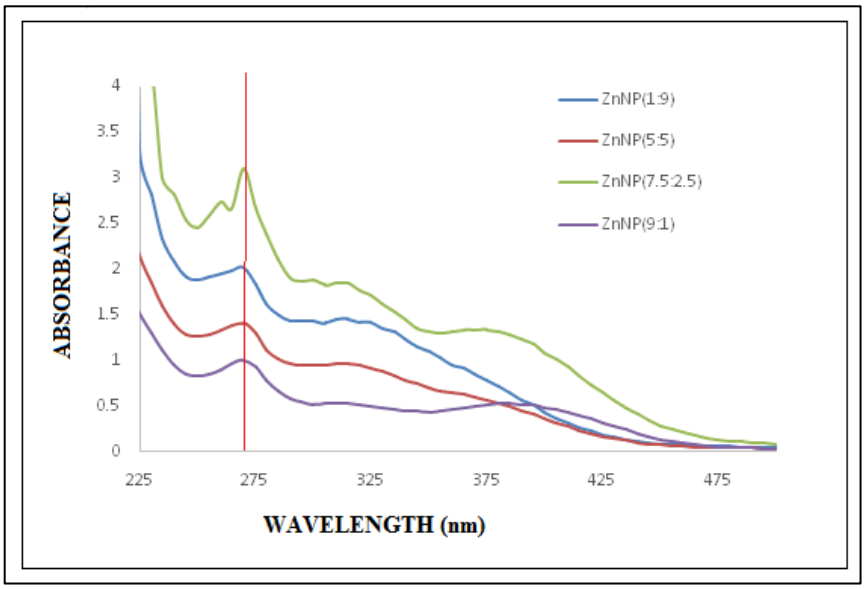

Figure 2: Uv-Vis absorption spectroscopy of the Zinc oxide nanoparticle. Zinc oxide nanoparticle samples in different concentrations (90%, Violet; 75%, Green; 50%, Red; and 10%, Blue).

The presence of absorbance peaks at a wavelength of 270 nm in UV-Vis Absorption Spectroscopy was demonstrated in Figure 2. This wavelength was demonstrated by the different concentrations of Zinc oxide NP synthesized with neem leaf extract.

3.2 *In-vitro* susceptibility of *Leptospira hyos* to Copper Oxide and Zinc Oxide NPs and relative reduction of microbial counts

The study evaluated the antimicrobial effect of the Copper and Zinc oxide NPs synthesized with neem leaf extract against *Leptospira hyos*. Table 1 provides the data on the cell density (expressed in McFarland units/MFU) of the test pathogen *L. hyos* recovered after exposure to the 2 NP preparations applied at different concentrations.

On the first 30 minutes of pathogen and Copper Oxide NP interaction, data in Table 1 show a comparably higher mean recovery counts of *L. hyos* associated with the application of 50 and 10% Copper Oxide NP. The application of 90 and 75% Copper Oxide NP produced lower recovery counts of *L. hyos* that were comparable but were significantly different from those counts derived with the application of 50 and 10% concentrations. The susceptibility of *L. hyos* to Zinc Oxide NP was marked by comparably higher mean recovery counts of *L. hyos* relative to the application of 50% and 10% Zinc Oxide NP. Lower mean recovery counts of *L. hyos* were obtained as a result of the application of 90% and 75% Zinc Oxide NP concentrations, where treatment with 75% Zinc Oxide NP contributed to significantly higher recovery count compared with the lower count obtained with the application of 90% Zinc Oxide NP.

Data on the mean recovery counts taken after 1 hr exposure of *L. hyos* to Copper Oxide NP show comparably higher *L. hyos* recovery counts in response to the application of 50 and 10% concentrations. A significantly lower recovery count was obtained with the application of 75% Copper Oxide NP while the lowest count was associated with the application of 90% Copper Oxide NP concentration. The mean recovery count of *L. hyos* taken after 1 hr exposure to Zinc Oxide NP was significantly higher with the application of 10% concentration compared to the lower mean recovery counts obtained with the application of 50, 75 and 90% Zinc Oxide NP.

It was demonstrated that after a 24-hr interaction time of the test pathogen with the Copper Oxide NP, comparably higher recovery counts were obtained with the application of 50 and 10% Copper Oxide NP. Lower counts were exhibited relative to the application of 75% and 90% Copper Oxide NP, where application of the 75% preparation contributed to significantly higher recovery count compared to the lower count derived as an effect of the 90% preparation. The response of the test pathogen to Zinc Oxide NP after 24-hrs of interaction was shown by significantly higher mean recovery count relative to the application of 10% Zinc Oxide NP compared to a moderately lower recovery count obtained with the application of 50% preparation. Lower recovery counts of *L. hyos* which were statistically different were obtained as a result of the application of 75 and 90% Zinc Oxide NP preparations.

Values represent the mean (± Standard Deviation, 3 replicates) cell density of *L. hyos* expressed as McFarland units (MFUs) before the application of the different treatments with Copper and Zinc oxide NPs and recovery counts also expressed as MFUs post-application with the different treatments. MFU of 0.5 is equivalent to 1.5×10^8 CFU/ml; 1.0 MFU/3.0×10^8 CFU/ml; 2.0 MFU/6.0×10^8 CFU/ml; 3.0 MFU/9.0×10^8 CFU/ml; 4.0 MFU/12.0×10^8 CFU/ml; 5.0 MFU/15×10^8 CFU/ml; 6.0 MFU/$18.0 \times$

10^8 CFU/ml; 7.0 MFU/21.0 × 10^8 CFU/ml; 8.0 MFU/24.0 × 10^8 CFU/ml; 9.0 MFU/27.0 × 10^8 CFU/ml; and 10.0 MFU/30.0 × 10^8 CFU/ml. $^{a, b, c, d}$(significant differences in cell density as an effect of the application of the different concentrations of CuO-based NPs (T1 to T4) separate from ZnO-based NPs (T5 to T8) at indicated durations of interaction (P<0.01). $^{w, x, y, z}$(significant differences in cell density as an effect of the duration of interaction between the NPs and the test pathogen (P<0.01).

Treatments	Description of treatment	Duration of Application			
		Before application	30 min post-application	1 hr post-application	24 hr post-application
T1	90% CuO	4.34 (0.66)^{w}	2.66 (0.47)^{bc,x}	2.66 (0.12)^{d,xy}	2 (0.47)^{c,z}
T2	75% CuO		3.00 (0.00)^{b,x}	2.99 (0.16)^{c,xy}	2.33 (0.82)^{b,z}
T3	50% CuO		4 (0.82)^{ab,x}	3.66 (0.20)^{ab,xy}	3.33 (0.94)^{a,z}
T4	10% CuO		4.3 (0.47)^{a,x}	3.86 (0.16)^{a,xy}	3.33 (0.47)a,z
T5	90% ZnO		3.36 (0.47)^{d,x}	2.19 (0.09)^{d,y}	2.15 (0.16)d^{,yz}
T6	75% ZnO		4 (0.00)^{c,x}	2.24 (0.04)^{c,y}	2.2 (0.16)^{c,yz}
T7	50% ZnO		4.33 (0.47)^{ab,x}	3.33 (0.02)^{b,y}	3.2 (0.47)^{b,yz}
T8	10% ZnO		4.33 (0.94)^{a,x}	3.66 (0.02)^{a,y}	3.33 (0.82)^{a,yz}
T9	Doxycycline (25 µg/mL, Positive control)		3.33 (0.47)^{x}	2.49 (0.02)^{y}	1.25 (0.94)^{z}
T10	Negative control (L. hyos suspension in Fletcher's medium)		4.33 (0.47)^{w}	4.29 (0.47)^{w}	4.28 (0.47)^{w}
T11	Fletcher's medium only	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

Table 1: Cell density (expressed in MFUs) of recovered *L. hyos* after exposure to the different concentrations of Copper Oxide and Zinc Oxide NPs at indicated periods of observation.

The effect of exposure time on the effectiveness of Copper Oxide NP as an antimicrobial against *L. hyos* was considered. The application of the different concentrations of Copper Oxide NP contributed to the significant decline of recovery counts of *L. hyos* across the 3-time intervals described (first 30-min, after 1-hr and after 24 hrs). This trend was different with the application of the different treatments of Zinc Oxide NP where significant differences in recovery counts were only noted on the first 30-min and after 1-hr exposure time within each treatment, indicative of a short-lived antimicrobial function Zinc Oxide NP.

Results of the study show that the 2 NP preparations differ in terms of the duration of their antimicrobial effect on *L. hyos*. Data also defined 10% Copper oxide NP and 10% Zinc oxide NP as the minimum inhibitory concentrations (MICs) of the preparations when used as antimicrobials against *L. hyos*.

4. Discussion

The study describes a herbal-based synthesis of Copper Oxide and Zinc Oxide nanoparticles characterized by recent methods of analytical devices. As the synthesis of nanoparticles that uses plant extracts is not popularly undertaken and there is limited information in terms of the characteristics of nanoparticles in studies presently conducted, effort was made to make a comparison with the data generated by other scientists. The UV-VIS spectrophotometry is reportedly used for measuring the wavelength and intensity of ultraviolet and visible light absorbed by a sample [9]. This method provides information on the structure of a compound as it determines the type of chromophore, conjugated double

bond and auxochrome of an organic compound based on the measurement of wavelength and intensity of ultraviolet and visible light absorbed by the sample. The absorption of radiation causes the promotion of electrons from the ground state to the excited state in functional groups called chromophore and the absorption data generated by UV-VIS spectrophotometry in the form of transmittance or absorbance can be read as the UV-VIS spectrum. Electron excitation that takes place in UV-VIS spectrophotometry is reportedly recorded in a spectrum expressed as wavelength and absorbance according to the type of electrons present in the analyzed molecule.

A study on copper oxide nanoparticle was reportedly applied against Pseudomonas aeroginosa and Escherichia coli and this reportedly had an absorbance peak at a wavelength around 258 nm [10]. In another study on copper oxide nanoparticle synthesized with the use of Enicostemma axillare leaf extract, the NP had an absorbance peak at a wavelength of 264 nm in UV-VIS spectrum [7]. The data generated in this study corroborates the results of others and confirm the efficiency of the protocols applied in the synthesis of the copper oxide nanoparticle. The result of this experiment closely matches the findings of a previous study on zinc oxide nanoparticle synthesized through wet chemical method [8] and used against S. aureus and E. coli where the zinc oxide NP was characterized with an absorbance peak at 278 nm wavelength.

The availability of NPs for further testing explains that a herbal synthesizer is an ideal bio-reducing agent as it can produce a clean product with an environmentally safe procedure in the synthesis of NPs. The nanoparticles produced were evaluated to validate their value as antimicrobials against *Leptospira hyos* in vitro. The effectiveness of the copper and zinc nanoparticles as an antimicrobial to the test bacterium was generally related to the significant reduction of the test organism in reaction with the nanoparticles. It has to be emphasized that the significant reduction of L. hyos relative to copper and zinc oxide NP application which were noted within the first hour of interaction may define the action of these NPS as candidate antimicrobials or disinfectants for *L. hyos* and not directly as antibiotic agents which may require long term studies to address parameters on therapeutic indices. The inability of Leptospira species to form colonies on the surface of a medium required the need to monitor the cell density of the recovered test organism through McFarland units (MFUs) after interaction with the NPs. Microbiologists and diagnosticians consider turbidity as a visual measure of cell density of a bacterial sample in suspension with the assumption that a more turbid sample relatively possesses a higher number of cells within the culture [11]. Concurrent to these observations are the relative effect of NP concentration and duration of exposure time on antimicrobial action to *L. hyos*.

Synthesis of nanoparticles for antimicrobial testing is quite an interesting field for research, and many seek to find explanations to the mechanisms of their antimicrobial function. A study on copper oxide nanoparticles [12] described the adherence of NP to the cell wall of Gram-negative bacteria, exert an effect on the protein structures of the cell membrane and cause denaturation of intracellular proteins. These effects may probably support the reported bactericidal action of copper oxide (CuO) NPs on oral bacteria like Streptococcus mutans and Lactobacillus acidophilus which made the NP a candidate control agent for the prevention of dental caries and infections [13]. Another study dealt on the antimicrobial activity of copper oxide NP against Escherichia coli and Escherichia faecalis [14]. The bactericidal effect of copper oxide NP was related to the killing of a wide range of bacterial pathogens involved in hospital-acquired

infections. A wide range of Gram-positive bacteria like the methycillin-resistant S. aureus and Gram-negative bacteria were also found to be susceptible to the destructive action of copper oxide NP [15]. Researchers claim that copper NPs release Cu+ ions that cause local pH and conductivity changes in the bacterial cell membrane and because of the very small particle size of Cu+; these can gain entry into the bacterial cell and disrupt enzyme function that leads to bacterial death [15].

Other studies that define the efficacy of copper oxide NP was the effective elimination of Bacillus cereus after its synthesis in Momordica charantia (Bitter melon) [16]. One antibacterial activity of copper oxide NP commonly described [17] was its involvement in the treatment of E. coli infection. It was claimed that the NP mediated an increase or overproduction of cellular reactive oxygen species (ROS) level by 2-folds, resulting to lipid peroxidation, protein oxidation and DNA degradation which finally kill bacterial cells.

Studies also described zinc oxide NP as antibacterial agent against Gram-negative bacteria like *E. coli*, *Salmonella typhimurium* and *Klebsiella pneumonia* [18]. Reports describe the bactericidal action of zinc oxide NP against *Campylobacter jejuni* as a result of the adherence of NP to the cell surface which changes the structure of *C. jejuni* cells resulting to the formation of irregular cell surfaces, membrane blebbing and increase in membrane permeability [19].

A solid antimicrobial effect against *S. aureus* and *S. typhimurium* has reportedly been linked to zinc oxide NP [20]. Another report describes the antimicrobial activity of zinc oxide nanoparticles against food-borne pathogen *Salmonella typhimurium* and *Staphylococcus aureus* [21]. The antimicrobial activity has been associated with morphological changes in bacterial cells after treatment with NPs which caused pitting and deformation of treated bacterial cells. These changes reportedly resulted in simultaneous growth reduction of the test bacteria that eventually lead to cell death and decomposition. The nature of zinc oxide NP makes it a good and ideal agent when used as a preservative and packaging material for food products. Zinc oxide NPs reportedly demonstrate good antibacterial activity against carbapenem-resistant *A. baumanii*. The antibacterial activity of zinc oxide NP reportedly involves the production of reactive oxygen intermediates which elevate membrane lipid peroxidation that causes membrane leakage of reducing sugars, DNA and proteins that ultimately reduces cell viability [22].

5. Conclusion

Two preparations of herbal-synthesized NPs that were characterized using contemporary methods of spectroscopy were evaluated in terms of their antimicrobial action against *L. hyos*. Data that demonstrated significant reduction of mean recovery counts expressed as MFUs relative to the application of different concentrations the NPs; significant reduction of recovery count as effects of exposure time to the NPs; and the identification of 10% as MICs of the 2 NPs provide a basis in considering these nanoparticles as components of anti-leptospiral and disinfectants agents. Experiments are presently undertaken to explore the therapeutic value of the NPs for veterinary applications, paying special attention to parameters such as safe doses, lethal doses and other therapeutic indices to merit their applications in veterinary practice while monitoring their effects in the environment.

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Conflict of Interest

No conflict of interest relative to the submission of this paper for publication exists among the authors.

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