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Synthesis, Characterisation, Antibacterial and Cytotoxic Assay of Zinc Oxide (ZnO) Nanoparticles

Richa Yadav[1*], Madhurima Bandopadhyay[1], Aritra Saha[1] and Abul Kalam Azad Mandal[1]

[1]School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, 632014, India.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of the present experiment was to synthesise ZnO nanoparticles by the environmental friendly process of bioreduction of ZnO salt with *Camellia sinensis* crop shoot extract and post synthesis to characterise and check their antibacterial potentiality and cytotoxicity.
Place and Duration of Study: Fermentation technology lab, School of biosciences and technology, VIT University, Vellore between July 2013 to December 2013.
Methodology: Green tea leaf extract (5%, 10% and 15%) and ZnO salt solution (0.1 M and 0.01 M) were prepared in different concentrations and mixed in three volumetric ratios (1:1, 1:2 and 1:3) to make a total of 18 combinations. The mixtures were incubated in a rotary shaker for 24-48 h. The synthesised nanoparticles were characterised subsequently by UV-Vis absorption spectroscopy, atomic force microscopy (AFM), particle size analysis, Fourier transfer infrared (FTIR) spectroscopy and X-ray diffraction (XRD) analysis. Upon characterization antibacterial activity of the nanoparticles were tested on three gram positive (*Staphylococcus aureus*, *Salmonella* sp., *Bacillus* sp.) and two gram negative (*Escherichia coli*, *Pseudomonas* sp.) bacteria. Finally haemolytic activity of the ZnO nanoparticles were compared with bulk ZnO salt and plant extract on RBC.
Results: The 370 nm peak obtained in UV-Vis spectrophotometer confirmed about the synthesis in case of only 15% extract. The AFM and particle size analysis revealed size of the nanoparticles at

**Corresponding author: Email: y25richa@gmail.com*;

about 70 nm. FTIR analysis confirmed identical functional groups like tea leaf extract. Finally the crystalline nature of the nanoparticles were evident from XRD experiment. Antibacterial assay revealed inhibitory effect of the nanoparticles on all the five bacterial strains by producing prominent zone of inhibition. From the haemolytic assay the synthesised ZnO nanoparticles were found out to be biocompatible and non-toxic up to a conc. of 60 µg/ml.

Conclusion: The present experiment provided a simple, rapid and environmentally benign technique of synthesising spherical to elliptical ZnO nanoparticles having efficient antibacterial activity and non-toxicity which can be used for developing a successful drug delivery system.

Keywords: Zinc oxide nanoparticles; nanoparticles; antioxidant; anticancer; antimicrobial; tea.

1. INTRODUCTION

Nanotechnology today is a rapidly growing field of 21st century research [1]. Nanobiotechnology is a blend of biotechnology and nanotechnology, which has emerged to produce nanoparticles (Nps) having wide range of applicability. Nanoparticles may be defined as solid particles within a size range of 1 - 100 nanometers [2]. They exhibit new or improved physico-chemical properties, reactivity and toxicity based on specific characteristics such as size, distribution and morphology. Metallic nanoparticles comprise different functions that are not usually observed in its bulk phase [3,4]. They have been subjected to extensive study because of their several unusual catalytic, electronic, magnetic, antimicrobial and anti-inflammatory properties [5,6]. Among the variety of metal oxide nanoparticles, zinc oxide nanoparticles (ZnONps) are important because of their variety of uses in industrial sectors including environmental, synthetic textiles, food, packaging, medical care, healthcare, as well as construction and decoration. ZnONps have also been proved to be the potential agent for drug delivery [7], treatment of leukemia and carcinoma cancer cell [8]. Previously conventional production of ZnONps included various physical and chemical methods. Methods such as chemical precipitation [9,10], simple solution-based methods, electrochemical and photochemical reduction [11,12], solvothermal/hydrothermal [13], sol–gel techniques [14] are most widely used [15]. Although synthesis by chemical methods takes less time in comparison to other methods yet the presence of toxic chemicals on the nanoparticle surface may have detrimental effects in case of medicinal applications [16]. Recently synthesis of metallic nanoparticles by biological methods has proved to be eco-friendly and cost effective [17]. Production of nanoparticles by utilisation of plant extract are sometimes less expensive [18] compared to the microbial [19,20] and whole plant mediated [21,22] synthesis. So, here the synthesis of ZnONps have been carried out by the bioreduction of ZnO salt with *Camellia sinensis* crop shoot extract. Post synthesis, characterisation of the same has been carried out to check the size, crystallinity and the bonding pattern. Their potentiality to act against the microorganisms has also been tested over five bacteria. Finally the haemolytic activity and cytotoxicity of the nanoparticles have been investigated.

2. MATERIALS AND METHODS

2.1 Materials

Zinc Oxide (ZnO, > 99.9% pure) was purchased from Sigma Aldrich, Germany. Ultrapure I M Tris-HCl and hydrochloric acid were purchased from Sigma Aldrich, Germany. Semi-fermented tea (*Camelia sinensis*) leaf was purchased from Twinings of London, Chennai. The antibiotics rifampicin (5 µg/disc), ciprofloxacin (5 µg/disc) and tetracycline (30 µg/disc) and nutrient agar media were purchased from Himedia, India. All reagents used were of analytical grade. All the bacterial strains viz. *Staphylococcus aureus*, *Salmonella* sp.*, Bacillus species, Escherichia coli* and *Pseudomonas* sp. were procured from the Institute of Microbial Technology (Chandigarh, India).

2.2 Synthesis of Zinc Oxide Nanoparticles

Green tea leaf was taken and washed thoroughly. It was then homogenized to make the powder which was later used to prepare the crop shoot extract by mixing with 100 ml of Milli-Q water in 250 ml conical flasks. Three different concentrations of leaf powder were taken into consideration, 5%, 10% and 15% (w/v). The extracts prepared were stored in different bottles and kept at 4℃ for further use. Simultaneously appropriate amount of ZnO powder were mixed with Milli-Q water to make concentrations of 0.1 M and 0.01 M. Proper mixing was not obtained

until the pH came to 1.3 by the addition of drops of HCl. The acidic pH might have helped in catalysing the solubility of crop shoot extract in the ZnO salt solution. Prepared crop shoot extract was mixed with ZnO salt solution in varying amount. The volume of salt added was kept constant whereas the same for shoot extract varied. So, by considering two concentrations of salt solutions (0.1 and 0.01 M), three different concentrations of shoot extract (5%, 10% and 15%) and three volumetric ratios of mixing (1:1, 1:2 and 1:3) were prepared. The mixtures were taken in a separate sealed teflon containers and kept in an incubator with vigorous shaking at 37℃ for overnight.

2.3 Characterization of Zinc Oxide Nanoparticles

The reduction of pure zinc ion (Zn^{2+}) into zinc (Zn^{0}) was monitored by measuring the UV-Vis spectrum of the reaction medium (samples). The formation of nanoparticles was primarily detected by recording the absorption spectra of the samples as the function of the reaction time at room temperature using UV–visible spectrophotometer (Lasany, Model: 1-2902) using a quartz cell (1 cm path) and in the range of 350-400 nm. The shape and structural configuration of the formed nanoparticles were well determined by AFM. About 100 µl of nanoparticle was dissolved in 1 ml of 70% ethanol and ultrasonicated for 30 seconds with a pulse rate of 4. The sample solution thus produced was used to prepare a smear on a slide and dried. The exact size of the nanoparticles were determined by the principle of dynamic light scattering in a particles size analyser (NanoBrook 90Plus). FTIR Siemen (IR Prestige 25) spectrometer with KBr beam splitter was used to ascertain the involvement of bioactive components in nanoparticles synthesis. The FTIR spectra of synthesized nanoparticles were recorded at a resolution of 4 cm^{-1} in the wave number region of 500-4000 cm^{-1}. The crystallinity and phases of the zinc nanoparticles were characterized by a X-Ray diffractometer (XRD-6000, Shimadzu) operated at 45 kV and with a 30 mA current with Cu Kα (λ = 0.1541 nm) radiation. Data was recorded for 2θ range of 10° to 80°with a step of 0.02°.

2.4 Biological Activity: *In vitro* Antimicrobial Activity

In vitro antimicrobial activity of the nanocolloids was screened against a total of five bacterial strains including three gram positive *Staphylococcus aureus*, *Salmonella* sp., *Bacillus* sp., and two gram negative *Escherichia coli*, *Pseudomonas* sp. Nutrient agar media was poured in molten form at an amount of 20 ml in each of the 10 sterile petri-plates. The plates were allowed to solidify and by the help of a tip, wells were prepared in 5 plates. In each of these plates three different amounts of nanoparticles 124 were used (10, 20 and 30 µg). In remaining 5 plates antibiotic impregnated disks viz; rifampicin (5 µg /disc), ciprofloxacin (5 µg/disc) and tetracycline (30 µg/disc) were used. The plates were incubated for 24-48 h at 37℃.

2.5 Haemolytic Assay

Haemolytic assay was performed by preparing about 3 ml of human blood (Blood+ 100 µL 25 mM EDTA) and diluting it upto 5 ml by addition of PBS (Phosphate buffer saline). After further centrifugation and repeated washing with saline, 100 µL of the washed RBC was taken and diluted upto 10 ml with saline. Different concentrations like 20 µg, 40 µg, 60 µg, 80 µg and 100 µg of ZnONps were prepared and added to 200 µL of final solution of RBCs taken in 24 well plate. In all the four rows the first well was the negative control.

3. RESULTS AND DISCUSSION

3.1 Characterisation of ZnO Nano-particles

The formation of a yellowish-white precipitate after the overnight incubation was the primary indicator of the probable synthesis of ZnONps by the reduction of Zn^{2+} metal ions by the organic reducible agents present within the plant material. During synthesis, the change in colour of the solution from brown to pale yellow was an indication that zinc oxide salt had been reduced. Aqueous tea leaf extract that contains phenolic compounds, proteins and carbohydrates simultaneously act as reducing agents for zinc ions. Complete reduction of zinc salt took about 24 h. The absorbance of reduced zinc colloidal was scanned in UV-VIS spectrophotometer from 300 to 600 nm. In the literature it is already reported that the optimum peak is of the range 350 nm-400 nm [23]. The surface plasmon resonance (SPR) band of zinc nanoparticles appeared at 370 nm due to presence of the components of the plant extract. This result confirmed that the salt used was reduced by the plant extract to form the Zinc oxide nanoparticles.

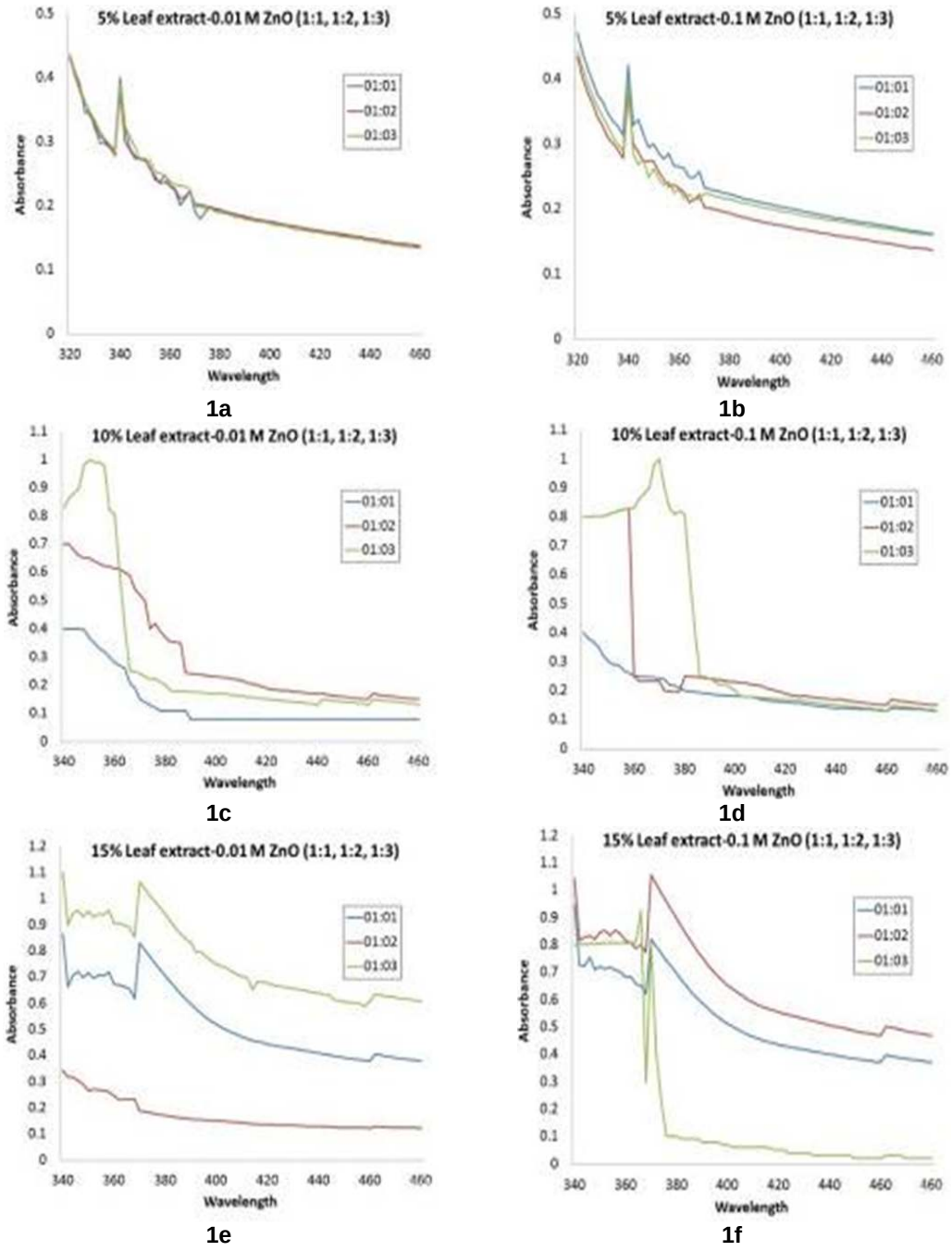


Fig. 1. a, b, c, d, e, f- UV-Spec analysis of Synthesis of ZnONps with various concentration of leaf extract. Presence of clear peak at about 370 nm is confirming the synthesis. 1(a) 5% leaf extract (LE). with 0.01 M ZnO in 1:1, 1:2 & 1:3 ratio. 1(b) 5% L.E. with 0.1 M ZnO in 3 ratios. 1(c) 10% L.E. with 0.01 M ZnO in 3 ratios. 1(d) 10% L.E. with 0.1 M ZnO in 3 ratios. 1(e) 15% L.E. with 0.01 M ZnO in 3 ratios. 1(f) 15% L.E. with 0.1 M ZnO in 3 ratios

Interestingly, in case of 5% and 10% leaf extract no characteristic peak has been obtained. Clear distinct peak can be only seen for 15% concentration at 370 nm. So by parameter specification we can conclude that 15% crop shoot extract of tea is necessary for ZnO nanoparticle synthesis (Fig. 1) above. The smear of the nanoparticle solution on the slide kept

under the Atomic force microscopy gave the size and shape of the formed nanoparticles. The image obtained in atomic force microscopy showed presence of nanoparticles of approximately size of 64 nm ± 10 (Fig. 2). The particle size analysis indicates the size distribution of nanoparticles by intensity (Fig. 3). Peak 1 has an intensity of 97.3% with maximum size of 87.26 nm which states that about 97.3% of the nanoparticles have size around 87.26 nm. Similarly Peak depicts about 2.7% of the nanoparticle having size around 2508 nm. Overall the average size of the synthesised ZnONps was obtained as 71.53 nm which is a fair enough value as it lies within the predefined range of nanoparticle size 1-100 nm. The results obtained by the FTIR analysis of the zinc nanoparticles were compared with the same done with the plant extract (Fig. 4). Several peaks of variable intensity were located at about 827,1037,1145,1240,1323,1402,1529,1631,1641 cm^{-1}. The common peaks were noted at 1402.25, 1631.78 and 1641.42 cm^{-1}. The peaks obtained by the analysis confirmed the presence of the functional groups. In the ZnO nanoparticle sample. The peaks that were common to the

FTIR spectra of the tea plant extract and the ZnONps depicted that these corresponding functional groups were from the plant source. These peaks were mostly due to the symmetric stretches of C-C=C. The peak 1641.42, 1631.78 revealed the presence of alkenes and aromatic compounds. The polysaccharides and flavones are believed to be the main groups responsible for the synthesis [15]. The initial peaks in the FTIR analysis of plant extract and ZnONps presumed to be due to the presence of biomolecules on the surface. The X-ray diffraction pattern (XRD) of the synthesized nanoparticles is evident from Fig. 5. Due to the occurrence of crystal symmetry and related face velocities the common crystal form of the produced ZnONps is hexagonal in shape. The Bragg reflections according to the sets of face centered cubic (FCC) lattice planes were observed. The peaks reflected the degree of crystallinity of the Zinc oxide nanoparticles. The XRD analysis confirmed that the nanoparticles synthesized using ZnO salt and tea extract were crystalline in nature.

3.2 Antibacterial Activity

The inhibitory response of the nanoparticles was observed against the test bacterial strains. The strains viz. *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas sp*., *Salmonella sp* and *Bacillus sp*. showed prominent zone of inhibition due to the nanoparticles (Figs. 6b, 6d, 6f, 6h, 6j) in comparison to the antibiotic disks (Figs. 6a, 6c, 6e, 6g, 6i) increasing in diameter with increasing concentration. Hence the bacterial strains where found to be sensitive towards the nanoparticles.

3.3 Haemolytic Activity

The experiment of haemolysis produced a significant result on the biocompatibility of Zinc oxide nanoparticles. According to Fig. 7, the composition of the first 5 wells of each row were

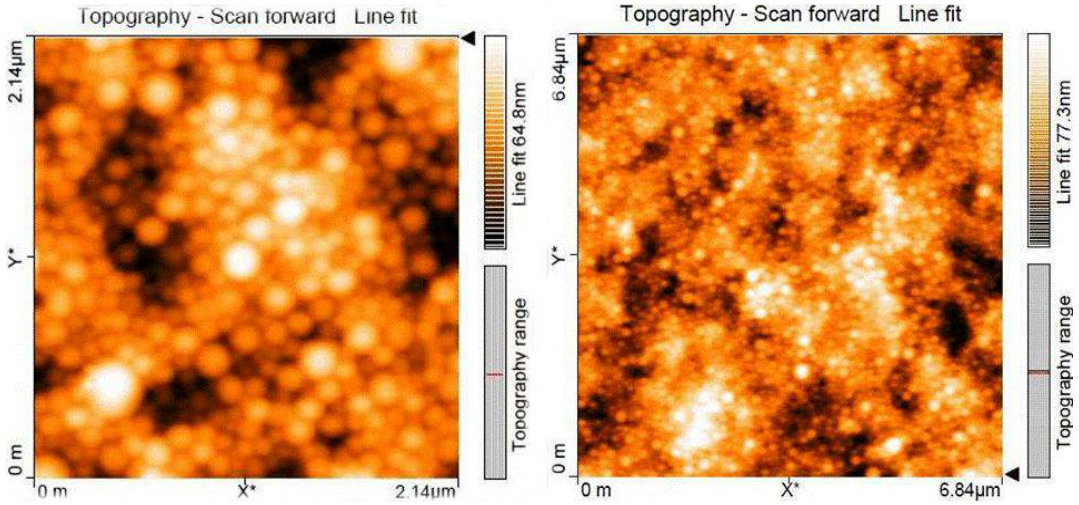


Fig. 2. The image of the atomic force microscopic analysis

as follows-1st row: RBC+Saline+0.01 M bulk ZnO, 2nd row: RBC+saline+0.1 M bulk ZnO, 3rd row: RBC+NaCl+plant extract, 4th row: RBC+NaCl+ZnONps. The 6th well of each row has negative control i.e, only RBC+Saline. Now as the picture depicts, it was observed that for 0.01 M and 0.1 M ZnO bulk materials in all the wells the RBCs got haemolysed within 10-15 minutes of incubation. In case of plant extract no haemolysis occurred. Interesting results were obtained for Zinc oxide nanoparticles. In the case of nanoparticles of amount 20 µg, 40 µg and 60 µg no haemolysis occurred but for 80 µg and 100 µg after almost half an hour of incubation RBC lysis occurred. So, minimum amount at which haemolysis occurred was 60 µg. Also nanoparticles took more time for haemolysis than bulk ZnO. Therefore it can be concluded that ZnONps are nontoxic to the RBC cells upto the amount of 60 µg implying MIC=60 µg.

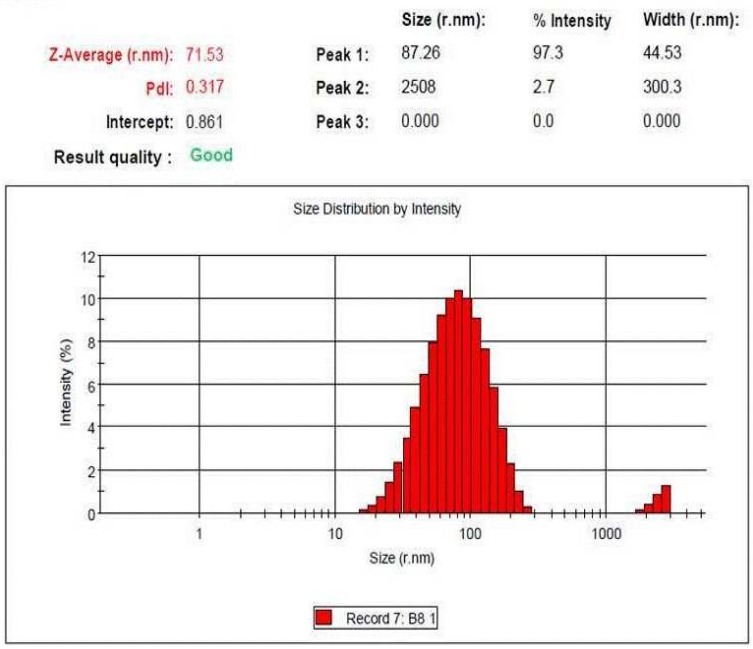
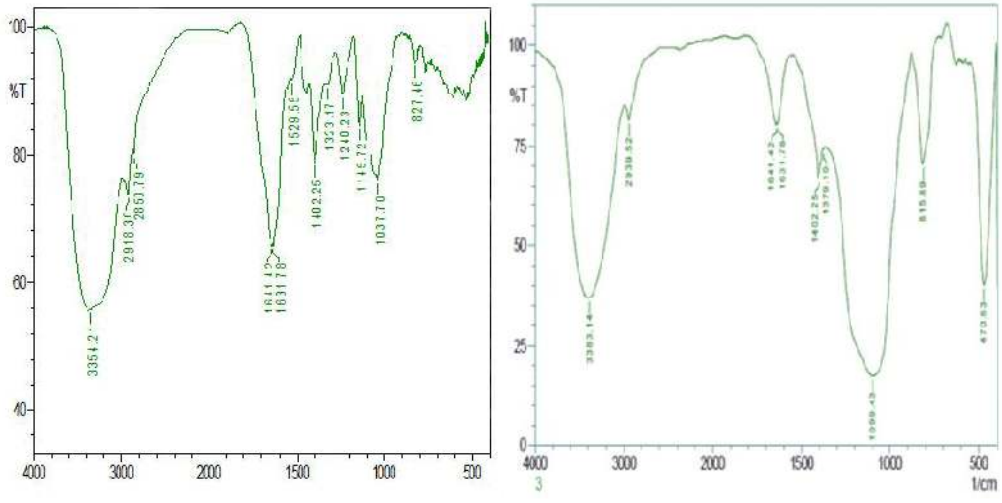


Fig. 3. Graph showing size distribution of nanoparticles with intensity



4a **4b**

Fig. 4. a, b- 4(a) FT-IR spectra of the biologically synthesized ZnONps. 4(b) FT-IR spectra of the plant extract of *Camelia sinensis*

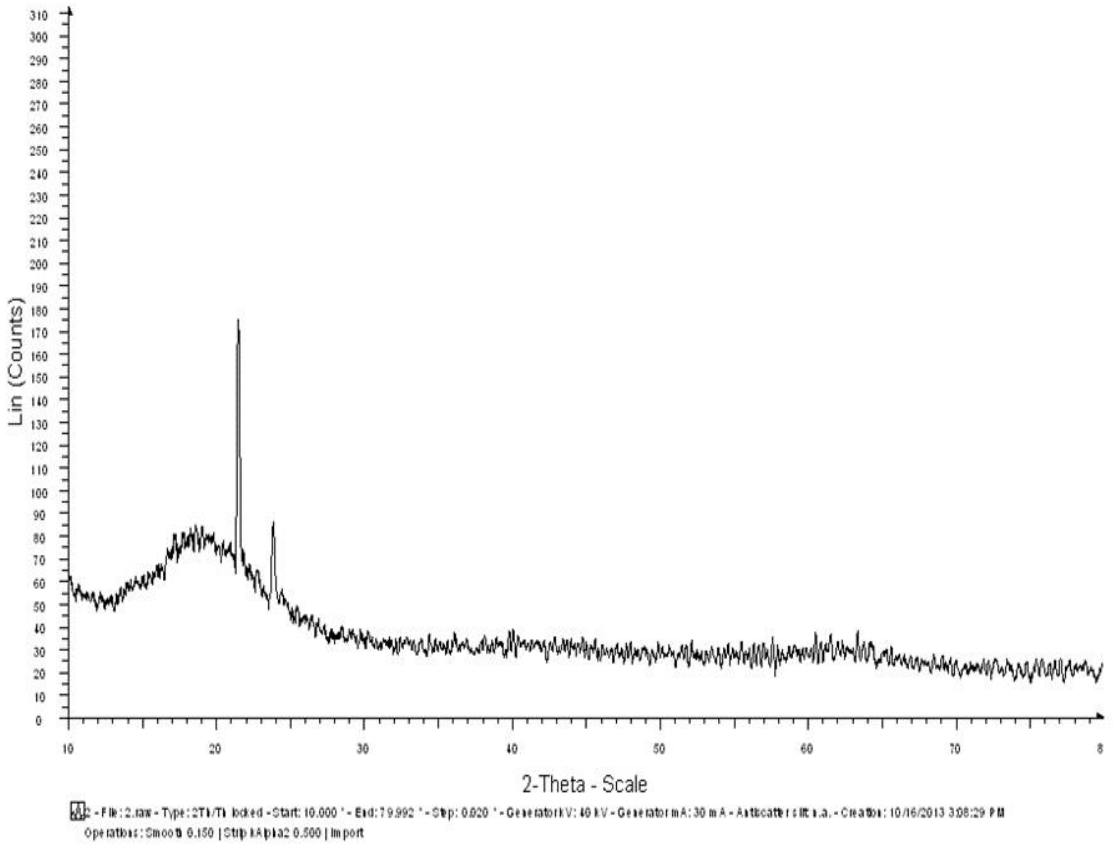
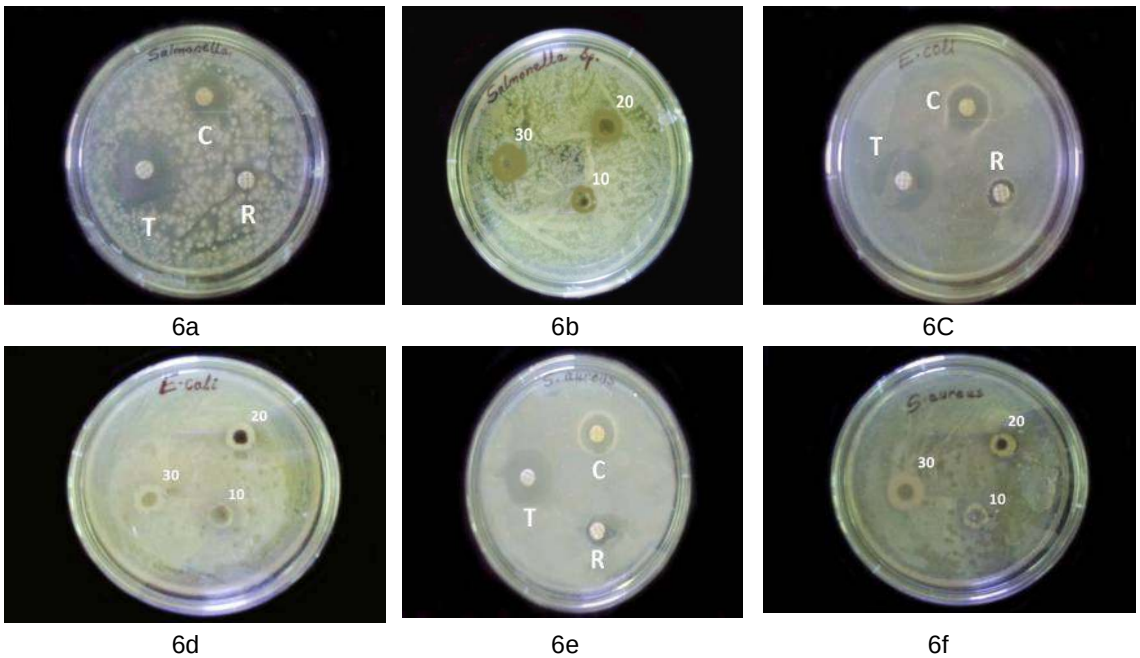
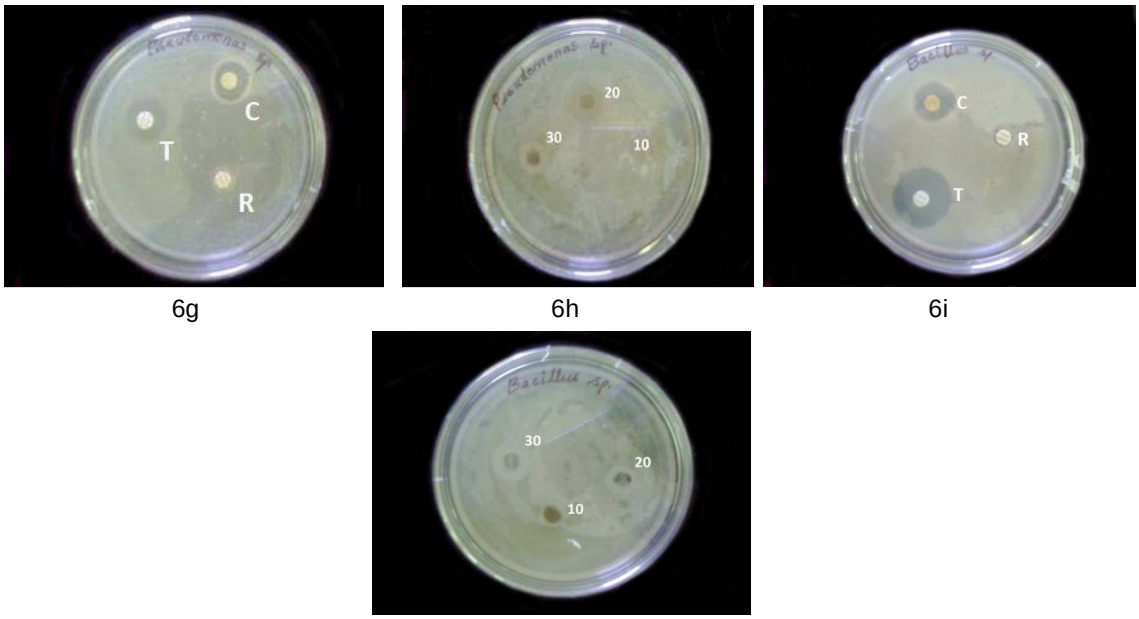


Fig. 5. XRD peaks of the synthesised ZnONps



6a 6b 6C

6d 6e 6f



6g 6h 6i

6j

Fig. 6. a, b, c, d, e, f, g, h, i, j- Images of antibacterial assay with five different bacterial species. 6(a) *Salmonella sp.* with antibiotic disks, 6(b) *Salmonella sp*. with nanoparticles, 6(c) *Escherichia coli* with antibiotic disks, 6(d) *Escherichia coli* with nanoparticles, 6(e) *Staphylococcus aureus* with antibiotic disks, 6(f) *Staphylococcus aureus* with nanoparticles, 6(g) *Pseudomonus sp.* with antibiotic disks, 6(h)*Pseudomonus sp.* with nanoparticles, 6(i) *Bacillus sp.* with antibiotic disks, 6(j) *Bacillus sp.* with nanoparticles. T, C and R stands for the antibiotics tetracyclin, ciprofloxacin and rifampicin respectively. 10, 20 and 30 stands for 10 µg, 20 µg and 30 µg respectively

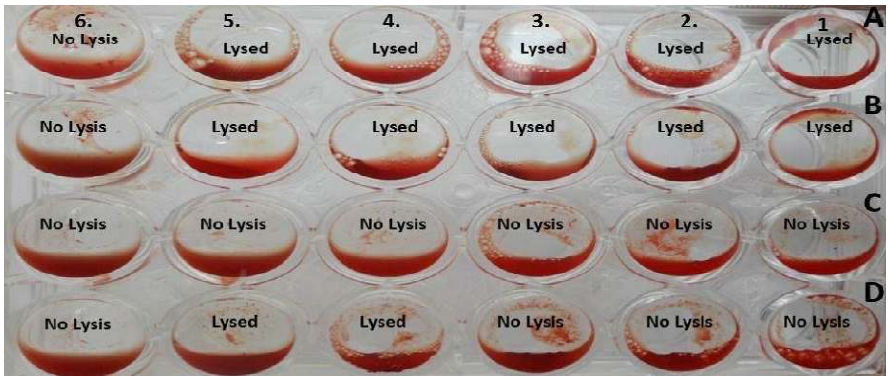


Fig. 7. Test for the haemolytic activity of ZnONps

4. CONCLUSION

In summary we described a simple, rapid, cost effective and environmentally benign technique for the synthesis of ZnONps by the biological reduction of *Camelia sinensis* leaf extract. Plant extract of 15% concentration and salt of 0.01 M taken in ratio of 1:3 produced the nanoparticles in the optimum range. The entire solution that was prepared for synthesis of nanoparticles was kept in dark at room temperature with continuous shaking until the synthesized precipitate was visible at the bottom of the solution. Various physico-chemical characterization technique also

reveals the crystalline nature and different chemical bonds present within the synthesised particles of size 71 nm. Our work can be further improvised by the inclusion of parameter optimisation like pH, temperature, time of contact between plant extract and the metal salt etc. The synthesised particles also bear antimicrobial property. Finally the haemolytic assay revealed a minimum inhibitory concentration of 60 µg/ml which can be used for devising a successful drug delivery system.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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