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RESEARCH ARTICLES

Biosynthesis of silver nanoparticles using mushroom extract and its toxicity assessement in zebrafish embryos

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Abstract
The study undertakes the synthesis of "Silver Nanoparticles" (AgNPs) via biological approach in order to overcome the toxic effects caused by synthesis using conventional chemical methods by conducting in vivo toxicity in zebrafish embryos. Edible oyster mushroom *P. ostreatus* were utilized for synthesizing AgNPs in aqueous phase. 1 mM silver nitrate when combined with the prepared aqueous extract of *P. ostreatus,* causes reduction of silver nitrate to form nanoparticles of silver which were characterized using UV–VIS Spectroscopy, Scanning Electron Microscopy (SEM), and Fourier Transform Infra-Red study (FTIR) techniques. In vivo toxicity assessment was carried out as per OECD guidelines for Fish Embryo Toxicity (FET). Study showed dose-dependent toxicity to different concentrations of biosynthesized AgNPs on exposure to zebrafish embryos and LC50 value was determined to be 1.8 mg/L.

Keywords AgNP · Zebrafish · *P. ostreatus* · FET · SEM · FTIR

Abbreviations

AgNPs	Sliver nanopartilcles
SEM	Scanning electron microscopy
FTIR	Fourier transform infra-red study
ICP-OES	Inductively coupled plasma-optical emission spectrometry
OECD	Organization for Economic Co-operative Development
LC_{50}	Lethal concentration 50
$AgNO_3$	Silver nitrate
HNO_3	Nitric acid
EHS	Environmental and human safety
AR Grade	Analytical grade reagent
d.p.f	Days post fertilization
FET	Fish embryo toxicity

Introduction

Silver nanoparticles range between 1 and 100 nm in size and are preliminary derived from silver (Polte 2015). While often described as being 'silver' some are made up of a large percentage of silver oxide due to their large ratio of surface to bulk silver atoms. Their large surface area permits the coordination of a vast number of ligands. The properties of silver nanoparticles applicable to human treatments are under investigation in laboratory and animal studies, assessing potential efficacy, toxicity, and costs. The most common methods for nanoparticle synthesis fall under wet chemistry, or the nucleation of particles within a solution. When a silver ion complex, such as $AgNO_3$ or $AgClO_4$, is reduced to colloidal Ag in the presence of a reducing agent, nucleation occurs. Dissolved metallic silver ions bind together to form a stable surface when the concentration rises high enough.

Many metallic nanoparticles have been synthesized using plant extract, bacteria and fungi as well. Bacteria and fungi are easy to manage and can be genetically modified. So bacterial and fungal nanoparticle synthesis is a viable option. This opens the door to developing biomolecules that can produce AgNPs of various shapes and sizes in bulk, which is one of the most difficult challenges in nanoparticle synthesis (Ahmad 2003). Without the use of harmful reducing agents, these techniques have been found to successfully produce stable monodisperse nanoparticles. An enzymatic process is

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said to be responsible for the reduction of silver nanoparticles, and the silver nanoparticles produced are highly stable due to interactions with proteins excreted by the fungi.

The present study deals with green synthesis of Silver nanoparticles utilizing the oyster fungus, *Pleurotus ostreatus* (Basidiomycota), commonly known as edible oyster mushroom. *Pleurotus* species mushrooms have excellent antioxidant, antitumor and anti-inflammatory properties apart from being high in nutrients like zinc, iron, potassium, calcium, phosphorus, vitamin C, folic acid, niacin, vitamins B-1 and B-2. *Pleurotus* has also been reported with hematological, antiviral, antitumor, antibacterial, hypocholesterolic and immunomodulatory activities, and antioxidant properties. (Raman 2021) High amounts of lovastatin, an approved hypolipidemic drug, and pleuran, an immunomodulating polysaccharide, Anti-atherosclerotic, hypoglycemic, antioxidant, anticancer, and immunodulatory properties are all present (Chowdhury 2015). *P. ostreatus* is classified as a medicinal fungus because of its wide range of biological activities. *P. ostreatus* fruiting bodies and extracts have been used in the therapy of diseases, especially diabetes, arteriosclerosis and cancer (Piska 2017). It is also a potential source of active ingredients in cosmetics and topically applied preparations. Oyster mushrooms are one of the biggest types of edible mushrooms which are most common, versatile and easily available. The said study was carried out to research its high clinical significance and the chemical composition so that *P. ostreatus* may be considered as one of the suitable and potential reducing source of silver ions to produce silver nanoparticles.

The zebrafish (*Danio rerio*) is a freshwater fish that belongs to the minnow family (Cyprinidae) of the order Cypriniformes. The zebrafish is a vertebrate organism that was first used to research developmental biology and embryogenesis (Chan 1998; Parng 2007). Zebrafish have recently been used to investigate pathogenic mechanisms in a variety of circumstances, including cancer research and drug development. The main advantages of using wild-type zebrafish larvae for drug screening revolve around ethical considerations (three R's) and easy access and care of the animals (compared to genetically modified models). In terms of the various benefits provided by zebrafish, it is a one-of-a-kind model in terms of environmental and human safety (EHS). In the near future, the information gathered from nano EHS studies may be used to help manage the risk of nanomaterials and nanotechnology-related products. The data may also help us develop effective guidelines for protective measures, quality control, and design strategies for improving nanomaterials and reducing toxicity. Several specific protocols for toxicity screening have been used with this model organism. One of the most important events for researchers studying chemical/nanomaterial toxicity is zebrafish hatching. The lethal concentration (LC_{50}) of different chemicals in zebrafish embryos can be compared to the LC_{50} in mammals to see if zebrafish embryos can serve as a predictive model for mammalian toxicity.

For the present study we have used wild type zebrafish as they are easy to maintain and represent a suitable alternative to overcome the cost of the experiments for the purchase and maintenance of genetically modified zebrafish models. There are various studies existing, which demonstrate the successful application of wild-type zebrafish, including their larvae, to model various diseases and for toxicity studies.

Material and methods

Reducing source: In present study in order to reduce silver ions, commonly available fresh edible oyster mushroom, *Pleurotus ostreatus,* were used which were procured from "The Mushroom Company, Khar East, Mumbai".

Chemicals: Silver nitrate ($AgNO_3$) (AR Grade, Hi-Media).

Animals: Zebrafish of wildtype [*Danio rerio*; 4 to 6 months old] were housed at 5–6 adult fish per tank. Adult fishes obtained were held in the laboratory for at least 12 days before they are used for testing. They were held in potable water and under following conditions:

Light/dark cycle: 14–10 h photoperiod daily,
Temperature: 26.0–28.5 °C,
pH: 6.8–7.5,
Feeding: Dry flakes Twice a day and live feed of brine shrimp once a day,
Weight: Avg. 0.5–0.8 g,
Size: Avg. 1.5 to 2.0 cms,
Gender: BOTH sexes.

Fish breeding was carried out in-house to get embryo for the study. The study was duly cleared by the IAEC. For toxicity studies, 5 days post fertilization (d.p.f), larvae were used which were transferred to 2 L containers at a density of 150 larvae per container and were housed in a temperature-controlled environment.

Methodology

Part I: biosynthesis and characterization of AgNPs

10 gms of fresh edible oyster mushroom, *Pleurotus ostreatus*, were weighed and washed thoroughly with double distilled water. They were crushed finely and transferred to a beaker containing 100 mL of sterile distilled water (1:10) and allowed to soak for 10 min. The resultant mixture was boiled for 30 min and then filtered through Whatman filter paper no. 1. Resultant filtrate was then subjected to centrifugation at 10,000 rpm at 4 °C for 30 min. Supernatant collected was used as extract and stored at 4 °C for further

use.1 mM $AgNO_3$ was prepared freshly by adding 6.795 mg of silver nitrate (AR grade, Hi-Media) in 40 mL distilled water. Mixture was divided into two flasks 'A' (Test) containing 20 mL of Extract and 20 mL of 1 mM $AgNO_3$, and flask 'C'(Control) containing 20 mL of Extract and 20 mL of distilled water (Martínez-Flores et al. 2020). In addition, one more flask was prepared and labeled as flask 'B' (Blank) containing 20 mL of 1 mM $AgNO_3$ and 20 mL of distilled water. All the flasks were covered with silver foil and kept on hot plate with magnetic stirrer at 70 °C. Colour change was monitored.

Part II: toxicity assessment in zebrafish embryos

For the toxicity assessment Fish Embryo Toxicity (FET) was performed. This assay was designed as per Organization for Economic Co-operative Development (OECD) guidelines test no. 236. wherein, a generation of Zebrafish embryos from the same clutch ($n = 10$ embryos) were exposed to different concentrations (0.1, 1.0, 5.0 and 10 mg/L each) of biologically synthesized AgNP starting at 4 hpf and monitored till 120 hpf. Control embryos were exposed to distilled water. The tests were carried out in triplicate from statistical point of view.

Results

Part A: biosynthesis of AgNPs

When 20 mL of 1 mM silver nitrate solution was added to 20 mL aqueous extract of *P.ostreatus* at 70 °C for 4 h resulted in reduction of silver ions. Initial stages of reduction of silver ions showed a color change from yellow to yellowish brown. This color change is the visual indication of the formation of silver nanoparticles. The color intensity increased with the time upon stirring. The colour change could be monitored at 2 h from light yellow to yellowish brown and finally dark brown, indicating the formation of silver nanoparticles. At 4 h the yellow-colored solution turns dark brown which could be because of the increased concentration of nanoparticles and also the particle size (Fig. 1).

AgNPs sample were analyzed at wavelength range 200–700 nm on Epoch Microplate Spectrophotometer. Absorbance peak at 400 nm was observed in test sample whereas no such peak was observed in control as well as in blank. Absorbance peak was due to formation of AgNPs. Peak width gives idea about polydispersity of AgNPs (Fig. 2).

AgNP sample was analyzed using ICP-OES (Avio 200). AgNP sample was digested with conc. HNO_3 in order to determine the concentration of silver, which was found to be 28.3 ppm indicating possible agglomeration of AgNPs which might have led to the formation of small aggregates.

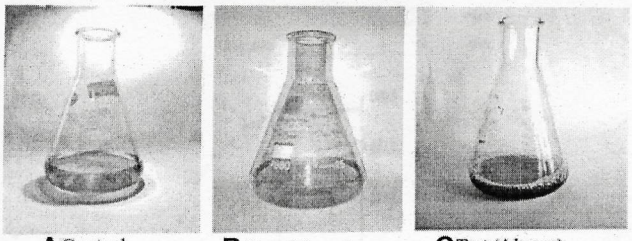


Fig. 1 Biosynthesis of AgNPs showing the colour difference in the **a** Control flask containing aqueous extract, **b**, **c** at 2 & 4 h after addition of 1 mM silver nitrate solution to the extract

Nova Nano SEM NPEP303, Scanning electron microscopy was employed for the surface morphology of biosynthesized nanoparticles. Scanning electron micrograph at 100,000 × and 200,000 × magnification of silver nanoparticles synthesized using *P.ostreatus* is presented in Fig. 3.

The surface morphology of most of the nanoparticles was found to be anisotropic in shape. SEM images clearly illustrated the polydispersed AgNPs nanoparticles in the range of 27–100 nm. Furthermore, SEM images revealed that most of the particles were spherical in shape. Few AgNP aggregates of size 97.16 nm to 100.4 nm were observed which may be due to agglomeration or improper capping (Chart 1).

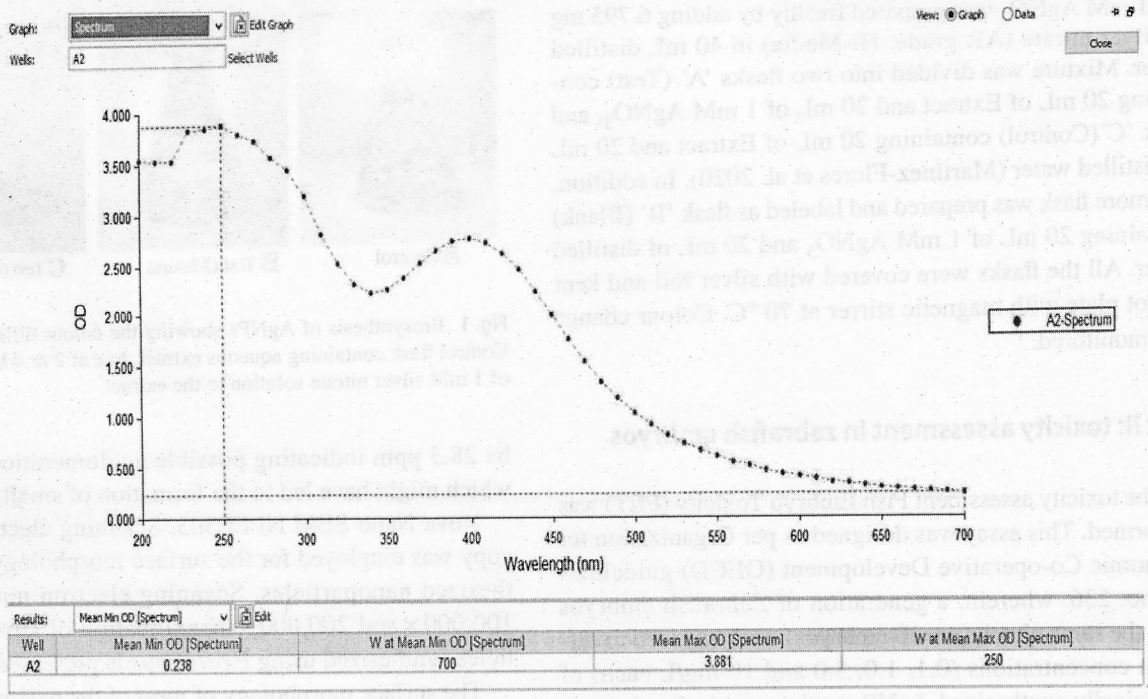
In order to analyze functional groups; AgNPs sample extract was digested with conc. HNO_3 and subjected to FTIR (Bruker; Alpha-2).

The sample extracts of AgNP were analyzed in the range of 4000–600 cm^{-1} showed peaks at wavelength 1851 & 1644 cm^{-1} (Fig. 4a) which matches with the peaks of Silver Standard (Fig. 4b) i.e. 1848 & 1643 cm^{-1}.

Other than these two peaks, the spectra of AgNP confirms the presence of O–H (H-bonded), C–H (Stretch), C≡C (Stretch), C=C (Stretch), C–O (Stretch), C–H (OOP Bend) & C≡N (Stretch). FTIR study suggests the presence of following functional groups (Table 1).

Part B: toxicity assessment in zebrafish embryos

Zebrafish embryos were monitored at 4 hpf, 8 hpf, 24 hpf, 48 hpf, 72 hpf, 96 hpf and 120 hpf on exposure of different concentrations of AgNPs. Normal growth was observed in 0.1 mg/L and 1.0 mg/L whereas embryos did not survive in 5.0 mg/L and 10 mg/L. as shown in Table 2. No delay in hatching was observed in 0.1 mg/L and 1.0 mg/L. However, there was a difference of 45 min approximately in hatching period between the above two concentrations. Manual stopwatch counting technique was used to study the heart rate and on an average 108 heart beats per minute was observed in 0.1 mg/L and 1.0 mg/L concentrations till



Well	Mean Min OD [Spectrum]	W at Mean Min OD [Spectrum]	Mean Max OD [Spectrum]	W at Mean Max OD [Spectrum]
A2	0.238	700	3.881	250

Fig. 2 Absorbance peak of AgNP sample at 400 nm

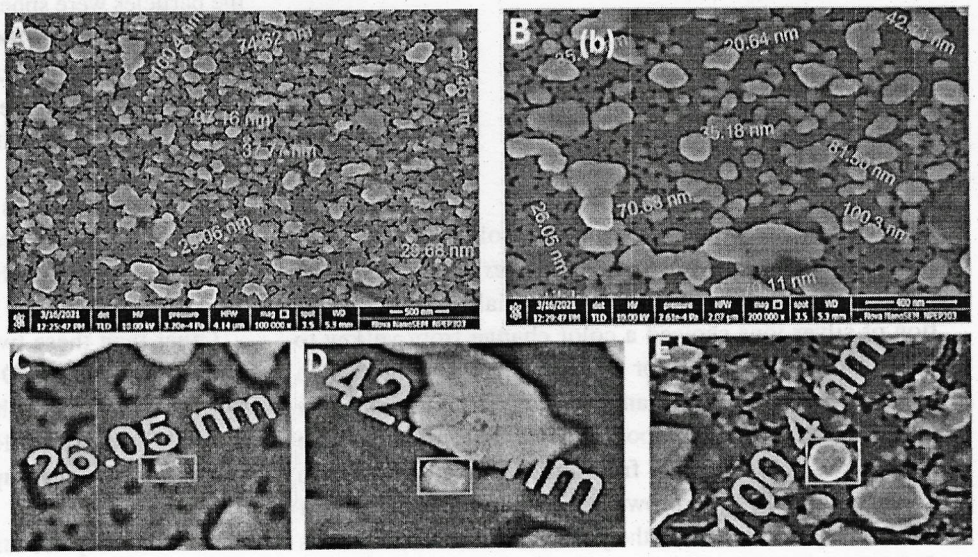


Fig. 3 **A**, **B** Scanning Electron Micrographs of Biosynthesized AgNPs; **C**, **D**, **E** Different sizes of AgNPs found (Magnification at 100,000X, (**b**) Magnification at 200,000X)

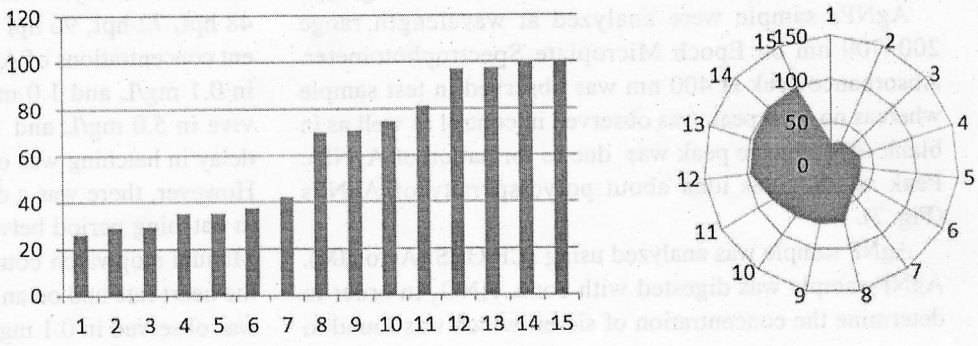


Chart 1 Size distribution of AgNPs in the range of 27–100 nm

Table 1 Functional group analysis of AGNP sample

Approx. wave number (cm^{-1})	Vibrational modes in IR region	Approx. wave number (cm^{-1})	Vibrational modes in IR region
3349	O–H (H-bonded)	1621	C=C (Stretch)
3268	O–H (H-bonded)	1141	C–O (Stretch)
3204	O–H (H-bonded)	1071	C–O (Stretch)
2875	C–H (Stretch)	977	C–H (Bend OOP)
2802	C–H (Stretch)	804	C–H (Bend OOP)
2258	C=C (Stretch)	771	C–H (Bend OOP)
2195	C=C (Stretch)	740	C–H (Bend OOP)
2109	C=C (Stretch)	673	C–H (Bend OOP)

2015). We have further confirmed the formation of AgNPs in the aqueous solution by using various analytical techniques like UV–vis spectroscopy, SEM, FTIR, ICP-OES. Similar to the various literatures, maximum absorbance was observed around 440 nm, which is characteristic of silver nanoparticles. Our results were in line with the study conducted by Irshad et al. (2020) on the fungal cell filtrate, which showed an absorbance peak at 440 nm which are specific for AgNPs. For the synthesis of AgNPs, it is hypothesized that the silver ions are required for the NADH-dependent nitrate reductase enzyme for their reduction. Al Bahrani et al. (2017) suggested that silver metal ions were reduced to AgNPs by active molecules found in the *P. ostreatus* basidiocarp extract. Our study also showed intense absorption peaks in the 400–470 nm range, which are common absorption bands

Table 2 FET assay results show a dose dependent response with decreased viability with increase in the concentration of the extract where LC50 value is 1.8 mg/L with SD of 0.34

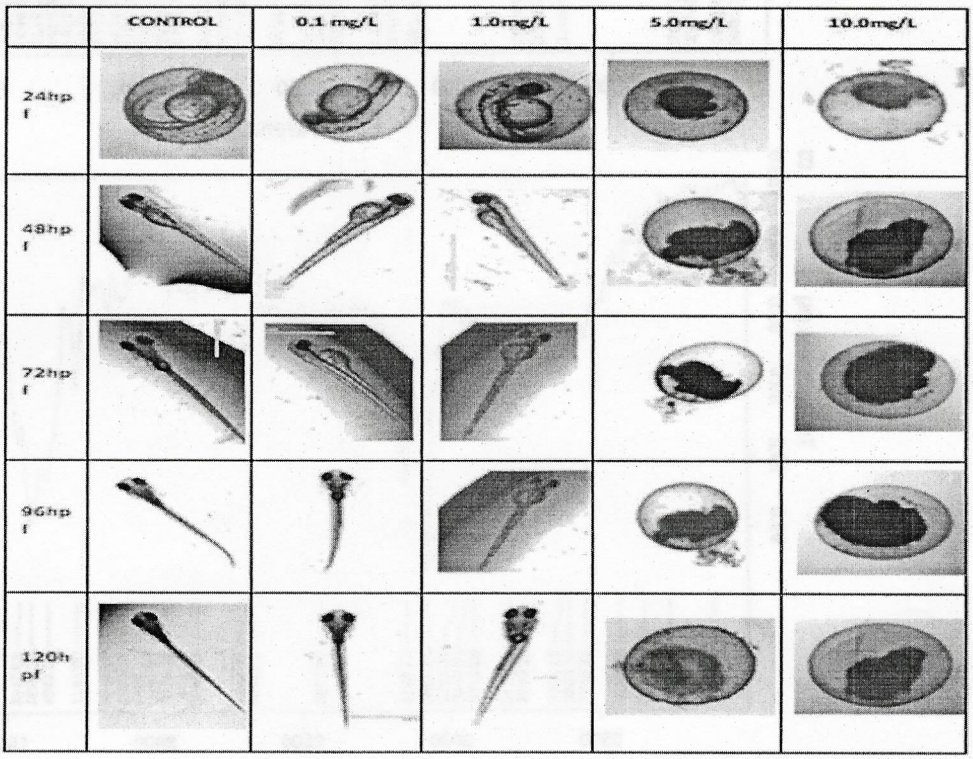


Table 3 Statistics of toxicity assessment showing dose dependent response to increase in the concentration of the extract when compared to the control

	% Viability		Heart rate (Beats per Minutes)		Hatching period (Hours)	
	Average (%)	Average standard deviation	Average	Average standard deviation	Average	Average standard deviation
Control	100	0	108.2	0.3	24.2	0.4
0.1	80	0.8	108.6	1.15	24.8 h	0.42
1.0	56.6	0.4	128.8	0.4	30.2 h	4.24
5.0	0	0	–	–	–	–
10.0	0	0	–	–	–	–

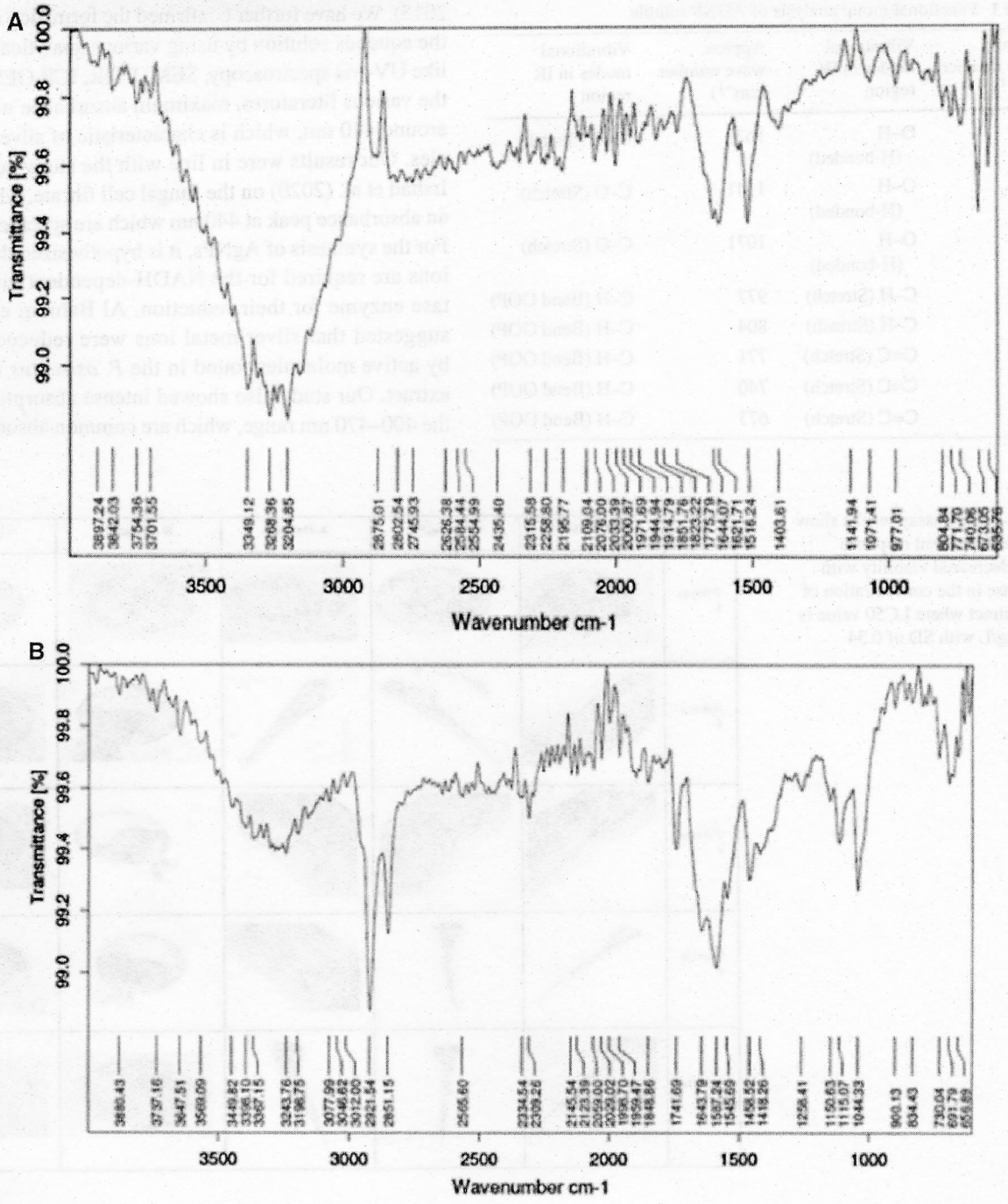


Fig. 4 FT-IR Spectra of AgNP (**a**), Silver Std (**b**)

48 hpf, which falls in the normal range as seen in the control group. However, increase in heart rate was observed at 96 hpf and 120 hpf. in the 0.1 mg/L and 1.0 mg/L groups. A statistics of toxicity assessment is presented in Table 3. Toxicological studies in zebrafish embryos carried out using different parameters revealed that AgNPs concentration up to 1.0 mg/L does not leads to any kind physical deformities and the LC_{50} value was found to be 1.8 mg/L with a standard deviation of 0.34 (Chart 2).

Discussion

Nanoparticles synthesis began once 1 mM silver nitrate solution was added the aqueous extract of *P.ostreatus*. The gradual color change was observed in the solution from yellow to yellow–brown to dark brown indicating the formation of silver nanoparticles as shown in Fig. 1. This color changing property is unique to noble metals due to the surface plasmon vibration which is an an optical property (Ibrahim

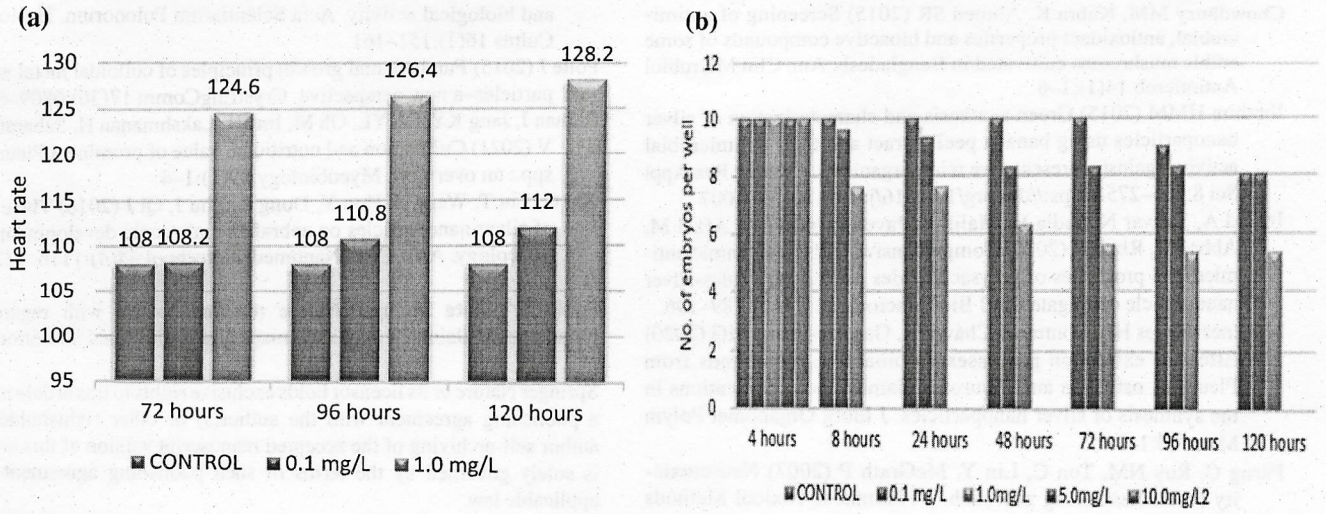


Chart 2 **a** Graphical representation of the Heart rate at 72, 96 &120 hpf where increase in heart rate was observed at 96 hpf and 120 hpf. in the 0.1 mg/L and 1.0 mg/L groups. **b** Average viability in all the exposure group showing dose dependent viability on exposure to various concentrations of AgNPs

of spherical AgNPs due to their surface plasmon resonance, confirming the formation of AgNPs. FTIR measurements were carried out to identify the major functional groups in the aqueous extract and their possible involvement in the synthesis and stabilization of silver nanoparticles. In addition, we also carried out the *invivo* toxicity of the synthesise AgNPs using zebrafish as a model organism. Xia et al. (2016) found that when zebrafish embryos treated with different concentrations of nano-Ag, with respect to morphological characteristics (mortality, deformity rate, and heartbeat) they too found a dose-dependent increase in mortality and hatching delay which was also evident in our study. The safety limit for the concentration of AgNPs as reported should be less than 5 mg/L, whereas our studies found the LC50 to be 1.8 mg/L which falls within the prescribed range.

Conclusions

In conclusion we found that *P. ostreatus* has potential to reduce metallic silver ions to form corresponding spherical silver nanoparticles showing spectral absorption peak at 400 nm which was investigated and confirmed by FTIR and SEM. AgNPs of size 27–100 nm was observed. FTIR analysis suggested that the reduction was due to unsaturated aliphatic compounds and the phenolic compounds present in *P. ostreatus*. The presence of amide linkages suggests proteins which might act as a capping agent thereby maintaining stability of nanoparticles. Toxicological studies carried out using zebrafish embryos evaluated various developmental parameters revealed that AgNPs concentration up to 1.0 mg/L do not cause any kind physical deformities and the LC_{50} value was found to be 1.8 mg/L with a standard deviation of 0.34. Here we can say that even though toxicity of silver nanoparticles is a major issue, biomolecular capping using green synthesis process reduces the toxicity. Furthermore, histopathology studies on adult fish may help understanding if there is any kind of tissue specific damage on accumulation of AgNPs. Dose-dependent toxicological study is suggested in adult zebrafish and higher order organisms. Here we demonstrated the effective synthesis of silver nanoparticles using biological method and use of zebrafish as a fast and efficient model organism to study toxicity.

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Declarations

Conflict of interest The authors have no conflicts of interest with regards to this manuscript.

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