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Functionalization with bio-molecules derived from oyster mushroom (*Pleurotus florida*) diminished the antibacterial potential of the mycogenic metal oxide nanoparticles (NPs)

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ABSTRACT

Mushrooms are mostly decomposers and possess the ability to digest food extracellularly by secreting specific enzymes. These enzymes and other bio-molecules present in the mycelium extracts of the *P. florida* have been used to produce zinc, copper and iron NPs extracellularly using metal salts as precursors. This work includes synthesis of metal oxide NPs by use of different concentrations (0.1 to 0.9 mM) of three metal salts *viz.,* zinc, copper and iron which were formed by incubating the salts with mycelium extracts of *P. florida* up to 96 hours under shaking conditions at 25±2°C in BOD incubator. The synthesis of the NPs was identified by performing the UV-Vis spectroscopy analysis at regular intervals to observe the time-dependent NPs synthesis. The visual color change in the reaction mixture was recorded and development of white, green and brown colors in zinc, copper and iron salt precursor containing sols respectively was recorded. The shape and formation of NPs was identified through TEM analysis. Incubation of the ferric chloride salt precursor with mycelial extract produced cubic shaped NPs. The antibacterial activity studies of the developed NPs using different concentrations (0.1 to 0.9 mM) along with the antibiotic standards (penicillin and gentamycin at the rate of 200 and 20 mg L^{-1} respectively) were performed against both gram positive and gram-negative bacteria. The results revealed formation of no inhibition zone by the biogenic NPs as compared to inhibition zones formed by the standard antibiotics (maximum 17 mm for *E. coli* in gentamycin).

Keywords: Antibacterial activity, nanoparticles, *Pleurotus florida*, transmission electron microscopy (TEM), UV-Vis spectroscopy

Mushroom fruit body represents soft, fleshy, spore-bearing basidiocarp which typically thrives above the ground on soil. These fruit bodies contain high amounts of amino acids, proteins, vitamins and different bioactive compounds with diverse biological activities (Chang, 1996). Further, these macrofungi have a predominant ecological role as decomposers and thus, their hyphae secrete certain proteins (enzymes) to digest food extracellularly. Apart from these enzymes a variety of other biomolecules are also produced by the mycelial network. These biomolecules and enzymes existing in the mycelial extracts of the mushroom can be used to generate different metal or metal oxide nanoparticles (NPs) using metal salts as precursors. Several published reports have given proof for the use of mushroom extract act as a protecting and reducing agent for the synthesis of gold/ silver and other types of NPs. Therefore, this green

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bio-synthetic approach involves the use of biological components to generate nano-scale structures or entities that possess the benefit of improving the nanomaterial biocompatibility (Xie *et al.,* 2007). Though biological systems other than fungi also possess the ability to synthesize and assemble a range of inorganic nanomaterials such as amorphous silica (diatoms), magnetite (magnetotactic bacteria), gypsum and calcium carbonate layers (S-layer bacteria) and minerals such as calcite into functional superstructures (Kroger *et al.,* 1999 and Lovley *et al.,* 1987). But the fungi can be excellent candidates, which utilize the biomolecules and the enzymatic process for the synthesis of metallic nanostructures (Ahmad *et al.,* 2002).

Pleurotus species (oyster mushrooms) belongs to a group of white rot fungi, found throughout the hard wood logs and stumps in the forest and cultivated all over the world mostly in Asia, Europe and America. Owing to their simple, higher biological efficiency and easy and low-cost production technology, these fungi are now being cultivated as edible mushrooms (Barh *et al.,* 2019). It is a saprophytic fungus, consumed as a delicacy and identified for its attractive flavor, excellent taste and ample nutrition. It is cultivated in Punjab, India during October to April under natural environmental conditions (Khanna and Kapoor, 2007).

There are several reports which depict the *Pleurotus* species based synthesis of nanomaterials including the *Pleurotus sajor caju* (Vigneshwaran *et al.,* 2007; Nithya and Ragunathan, 2009); *Coriolus versicolor* (Sanghi and Verma, 2009); *Agaricus bisporus* (Narasimha *et al.,* 2011); *Pleurotus* spp. (Mazumdar and Haloi, 2011), *Pleurotus florida* (Bhat *et al.,* 2011); *Pleurotus ostreatus* (Devika *et al.,* 2012; Ma *et al.,* 2013); *Pleurotus sanguineus*, *Schizophyllum commune*, *Lentinus sajor caju*, *Trametes feei*, *Trametes pocas* (Chan and Don, 2013), *Pleurotus sapidus* (Sarkar *et al.,* 2013), *Lentinus edodes* (Vetchinkina *et al.,* 2014), *Inonotus obliquus* (Nagajyothi *et al.,* 2014) *Ganoderma lucidum* (Karwa *et al.,* 2014) and *Pleurotus cornucopiae* (Owaid *et al.,* 2015). The study of fungal species as NP producers in myco-nanotechnology is relatively old now as the first study on myco-nanotechnology involved the use of fungus *Volvariella volvacea* for the biosynthesis of gold nanoparticles (Au NPs) (Philip, 2009).

The affinity of certain mushroom macromolecules towards specific metal element(s) can be useful to convert metal ions and oxygen into the solid nanoparticles. The nanoparticles (NPs) can be synthesized by incubation of soluble metal salts in mushroom extract such as salts of silver, gold and cadmium along with mushrooms to biosynthesize AgNPs, AuNPs and CdNPs respectively (Li *et al.,* 2011). Likewise, ferrous sulphate ($FeSO_4$) was used to form FeNPs by incubation of the precursor with *Pleurotus* spp. Similarly, zinc sulfide had been used to synthesize ZnNPs with *P. ostreatus* (Senapati and Sarkar, 2014). The biologically synthesized NPs exhibit several potential activities such as antimicrobial, antifungal, antioxidant and anti-inflammatory properties. Therefore, biological synthesis of metal/ metal oxide nanoparticles can be considered as an eco-friendly approach in comparison to physical and chemical methods (Tejeda *et al.,* 2009; Sharma *et al.,* 2009). Vigneshwaran *et al.* (2007) showed antibacterial activity of Silver-protein NPs produced by *P. sajor-caju* against *S. aureus* and *K. pneumonia*. Also, Saravananand Nanda (2010) recorded anti-fungal properties of *Aspergillus clavitus* derived NPs against methicillin-resistant *S. aureus* (MRSA) and methicillin- resistant *S. epidermidis* (MRSE).

This paper aims to represent the extracellular biogenic synthesis of zinc, copper and iron nanoparticles using different concentrations of their chloride and sulphate salts as precursors, i.e. Zinc sulphate ($ZnSO_4$), Zinc chloride ($ZnCl_2$), Copper sulphate ($CuSO_4$), Copper chloride ($CuCl_2$), Ferric sulphate ($FeSO_4$) and Ferric chloride ($FeCl_2$). The synthesized NPs were characterized through the Ultraviolet-visible spectroscopy and Transmission

electron microscopy techniques. The antibacterial activity of the generated NPs was examined against different pathogenic microorganism such as *Aeromonas*, *Bacillus*, *Salmonella*, *Staphylococcus*, *Escherichia coli*, *Yersinia* and *Listeria* sp.

MATERIAL AND METHODS

Media and reagents

Potato dextrose agar (PDA) and broth (PDB) were used for the growth and maintenance of the *Pleurotus florida* mycelia throughout the experiment. The antibacterial activity of the generated NPs was determined through well diffusion assay on nutrient agar media. The chloride and sulphate precursor salts for the three heavy metals (Zn, Cu and Fe) were of analytical grade. Stock solution of (1.0 M) these salts ($ZnSO_4$, $ZnCl_2$, $CuSO_4$, $CuCl_2$, $FeSO_4$ and $FeCl_2$) were prepared and stored @ 4°C for further research.

Source of culture

For the biosynthesis of NPs, *P. florida* slant procured from the mushroom farm and bacterial cultures (*Aeromonas*, *Bacillus*, *Salmonella*, *Staphylococcus*, *Escherichia coli*, *Yersinia*, *Listeria* sps.) for antibacterial activity, were obtained from the microbiology department, Punjab Agricultural University (PAU) Ludhiana, Punjab, India. *P. florida* (fungus) and the bacterial culture were maintained on Potato dextrose agar (PDA) and nutrient broth (NB), respectively and preserved at 4°C till used. Master plate of *P. florida* prepared by transferring 6 mm bit from previously prepared culture plate and this master plate (Fig. 1) was used for the extraction procedure.

Extraction and reaction mixture prepared for biosynthesis NPs

The master plate of *P. florida* was prepared by subculturing 6 mm bit on PDA plate and incubated at 28°C for 10-15 days. About 200 ml PDB media in 500 ml Erlenmeyer flask was inoculated with the 6 mm bit from the master plate in Laminar air flow, and the inoculated contents were incubated at 25±2°C till the luxurious growth of the mycelia in the broth. After completion of incubation, the mycelial network was separated from the broth, washed thrice using autoclaved distilled water and filtered using Whatman filter paper 1. Then, 5 g of the harvested mycelium was weighed and re-suspended into 100 ml of the salt solution ($ZnSO_4$, $ZnCl_2$, $CuSO_4$, $CuCl_2$, $FeSO_4$ and $FeCl_2$) of varying concentrations of 0.1, 0.3, 0.5, 0.7 and 0.9 mM in 250 ml Erlenmeyer flask. Also, a positive (without salt) and negative control (without biomass) were maintained along with the test samples. The entire reaction mixture (sample and controls) was incubated in a BOD incubator, at 25±2°C for 96 hours under shaking conditions. The biosynthesized Zn, Cu and Fe NPs separated from mycelium and concentrated in hot oven at 45±2°C.

Uv-vis spectroscopy

The bio-transformed product was collected periodically in safety cabinet to perform UV-Vis spectroscopy to observe time-dependent formation of nanoparticles. After every 24 hours, about 2 ml aliquot was removed from each flask (salt + mycelium + water) containing precursor salts of varying concentrations (over 0.1 to 0.9 mM) regularly for 96 days and UV-Vis spectroscopy (UV-1800, SHIMADZU) analysis was performed for a range of 190 to 800 nm wavelengths with 2 nm resolution to observe the time dependent NPs production. Also, the color change in the reaction mixture with time was observed which became stable when the reaction was completed.

Transmission electron microscopy

Reaction mixture was filtered using Whatman filter paper 1 to separate the mycelium and organic content remnants and concentrated to perform TEM (Hitachi H-7650) @ 80kV acceleration voltagefor the observation of NPs biosynthesis. All the samples (0.9mM concentration) ofzinc (as zinc chloride and

zinc sulphate), copper (copper chloride and copper sulphate) and iron (ferric chloride and ferric sulphate) NPs were sonicated for 20 minutes in a bath sonicator (Model-Equitron)for even dispersion of the particles and about 10 µl of sample was casted on carbon/ formvar coated copper grid and air dried before imaging in TEM.

Antimicrobial activity

The antibacterial activity of the biosynthesized NPs was evaluated through well diffusion method (Kaur and Kalia, 2016) using gram positive (*Bacillus*, *Staphylococcus*, *Listeria*) and gram negative (*Aeromonas*, *Salmonella*, *Escherichia coli*, *Yersinia*,) bacteria. Respective bacterial cultures were grown overnight in nutrient broth (NB) under shaking condition (120 rpm) @ 37°C to attain the log phase growth of the bacterial cultures. The freshly prepared log phase culture (100 µl) was spread on the solidified nutrient agar (NA) containing petriplates with the help of spreader to prepare the lawn culture of the bacteria. Uniform well of 6 mm diameter were prepared on these NA containing petriplates with the help of a cork borer. About 1-2 drops of the molten NA was added to the well to avoid the leakage of sample from the base of the well. Approximately 50 µl of the biosynthesized NPs samples prepared using various concentrations of the precursor salts (0.1, 0.3, 0.5, 0.7 and 0.9 mM) along with penicillin and gentamycin (200 and 20 mg L^{-1} respectively) standards as control. All these plates were incubated in BOD incubator @ 37°C for 24 hrs to observe the zone of inhibition on the incubated plates.

RESULTS AND DISCUSSION

Appearance of reaction mixture under incubation

The reaction mixture containing salt + distilled water + fungal biomass initially (within 24 hrs) showed no visual change in the color. However, later the reaction mixture exhibited change in color gradually upto 4th day of incubation which stand still after reaction over. This visual change in color related with

Fig. 1. Prepared master plate of *P. florida* for the extraction procedure

the surface plasmon resonance (SPR), indicate that reaction occur for the biosynthesis of NPs. It had been observed that zinc chloride and zinc sulphate turned into white color similarly, copper salt either copper sulphate or copper chloride changed from blue to green

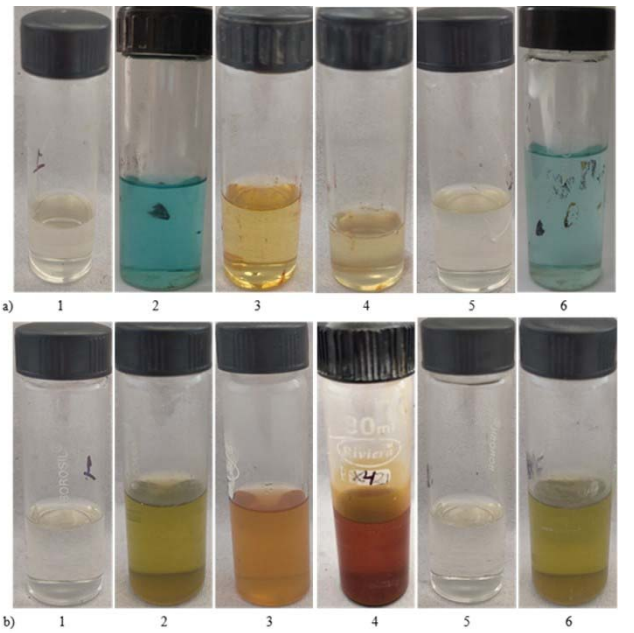

Fig. 2. Occurrences of visual color changed from initial (a: before incubation, 0hrs) and final (b: after incubation, 96hrs) reaction mixture indicated the biosynthesis of NPs from precursor salt**:** (1) Zinc sulphate (2) Copper sulphate (3) Ferric chloride (4) Ferric sulphate (5) Zinc chloride (6) Copper chloride, respectively

in color whereas ferric chloride changed its color from yellow to brown color reaction mixture (Fig. 2). By comparing the reaction mixture with their negative and positive controls, no such result found. Sunitha *et al.* (2013) observed similar change of color from pale yellow to brownish in colour for generation of the iron NPs after incubation of *F. oxysporum* with $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ salt for 120 hrs. In this study also, at the initial 24 hours stage no visual change was recorded.

UV-Vis spectroscopy of different concentrations of the formulations

By comparing all the concentrations of the precursor salts (0.1 to 0.9 mM) for the UV-vis spectra depicted the maximum absorption peak at 210 and 260 nm for the zinc sulphate and chloride precursor salt derived ZnONPs until the 4th day of incubation. It was observed that zinc chloride provided different peak i.e 330 nm (310 to 360 nm) for 0.1 mm concentration and 260 nm (between 240 to 280 nm,) for 0.9 mM concentration on the 4th day which can be attributed to reduction reaction that occurred as concentration increases, which is phenomenon of agglomeration. In case of iron NPs, the maximum absorption peak was recorded at 210 nm (with SPR band 190-220 nm) and 260 nm (with SPR band 240-300 nm) for both the iron chloride and iron sulphate precursor salt derived FeONPs respectively. Similarly, copper showed appearance of a strong SPR peak with distinct absorption peaks at 200 (range over 190-210), 220 nm (range from 210-230 nm) and 260 (range 240-280 nm) for copper sulphate and copper chloride precursors respectively, indicating the biosynthesis of the copper NPs of small size. In general zinc, iron and copper NPs display a surface plasmon peak within 200-300 nm (Kumar and Rani, 2013), 500-700 nm (Jia *et al.,* 2010) and 580-590 nm (Soomro *et al.,* 2013) respectively, so the absorption maxima for the biosynthesized NPs have shown a red shift for zinc, and blue shift for the iron and copper NPs.

Transmission electron microscopy

TEM was performed to reveal the porosity, shape and morphometry of myco-synthesized NPs from TEM micrograph (Fig. 3). TEM result showed that the myco-synthesized zinc oxide NPs obtained from zinc sulphate salt were of semi-spherical shape and uniform distribution. The size range of the ZnO NPs existed from 9.36 to 58.13 nm. Whereas, the zinc oxide NPs produced from zinc chloride precursor salt showed same results with a larger average size ranging from 21.27 to 118.36 nm. Therefore, homogeneous size distributions of the ZnO NPs were obtained by the incubation of precursor salts with the mycelium extracts. In case of copper oxide NPs, the copper sulphate derived NPs exhibited greater agglomeration compared to copper chloride derived NPs. In terms of morphological features, hexagonal and partially spherical shaped NPs were obtained on incubation of mycelial extracts with the copper sulphate precursor salt ranging from 12.82 to 48.86 nm. Whereas spherical, oval or partially spherical shaped NPs with average size of 22.55 to 60.09 nm were obtained with the copper chloride precursor salt. Similarly, incubation of the ferric chloride salt precursor with mycelial extracts produced cubic shaped NPs with average size of 11.90 to 167.63 nm. However, ferric sulphate salt precursor produced highly agglomerated spherical NPs of average range from 11.16 to 98.81 nm. The zinc sulphate and ferric chloride precursor NPs were poly dispersed whereas other precursor NPs were agglomerated. The mycogenically produced NPs with respective precursor salts showed similar results as depicted by the reports of the other researchers. Sunitha *et al.* (2013) synthesized biogenic iron NPs of spherical shape (5.8 nm) using *Fusarium oxysproum* mycelial extracts. The study showed similar results including formation of cubic mycogenic iron oxide NPs (range 60-70 nm) using *Aspergillus japonicas* in the presence of $K_4(Fe(CN)_6$, $K_3(Fe(CN)_6$ salt which revealed that there is no such significant difference

on the morphology of mycogenic NPs using different salt (Bhargava *et al.,* 2013). Kundu *et al.* (2014) have also reported the generation of ZnO NPs (100-200) by *Rhodococcus pyridinivorans* NT2 in the presence of $ZnSO_4$ salt which were moderately stable, hexagonal to roughly spherical in shape. Cuevas *et al.* (2014) reported formation of spherical copper NPs (5 to 20 nm) under alkaline conditions by *Stereum hirsutum* (a white-rot fungus) on incubation of 5.0 mM $CuCl_2$ salt with the fungal extracts.

Antibacterial activity of produced NPs

The myco-synthesized NPs were tested for antibacterial activity along with standard gentamycin and penicillin @ 20 and 200 mg L^{-1} respectively against negative and positive aforementioned bacterial cultures. The antibacterial activity of the NPs on the cultures was identified by the formation of zone of inhibition (clear area). The standard antibiotic solutions showed inhibition zone in case of gentamycin for gram

Table 1. Zone of inhibition against test human pathogenic microorganisms for standard specific antibiotics and fungal nanoparticles.

			Zone of inhibition (mm)						
Type of agent	**Type of precursor salt**	**Conc. Used (mM/ mg L^{-1})**	***Aeromonas***	***Bacillus***	***Salmonella***	***Staphylococcus***	***E.coli***	***Yersinia***	***Listeria***
Fungal NPs Zinc oxide nanoparticles	Zinc sulphate	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
	Zinc chloride	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
Iron oxide nanoparticles	Ferrous sulphate	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
	Ferrous chloride	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
Copper oxide nanoparticles	Copper sulphate	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
	Copper chloride	0.1	-	-	-	-	-	-	-
		0.3	-	-	-	-	-	-	-
		0.5	-	-	-	-	-	-	-
		0.7	-	-	-	-	-	-	-
		0.9	-	-	-	-	-	-	-
Reference standards	Gentamycin	20	13	-	16	-	17	-	-
	Penicillin G	200	-	-	-	15	-	-	-

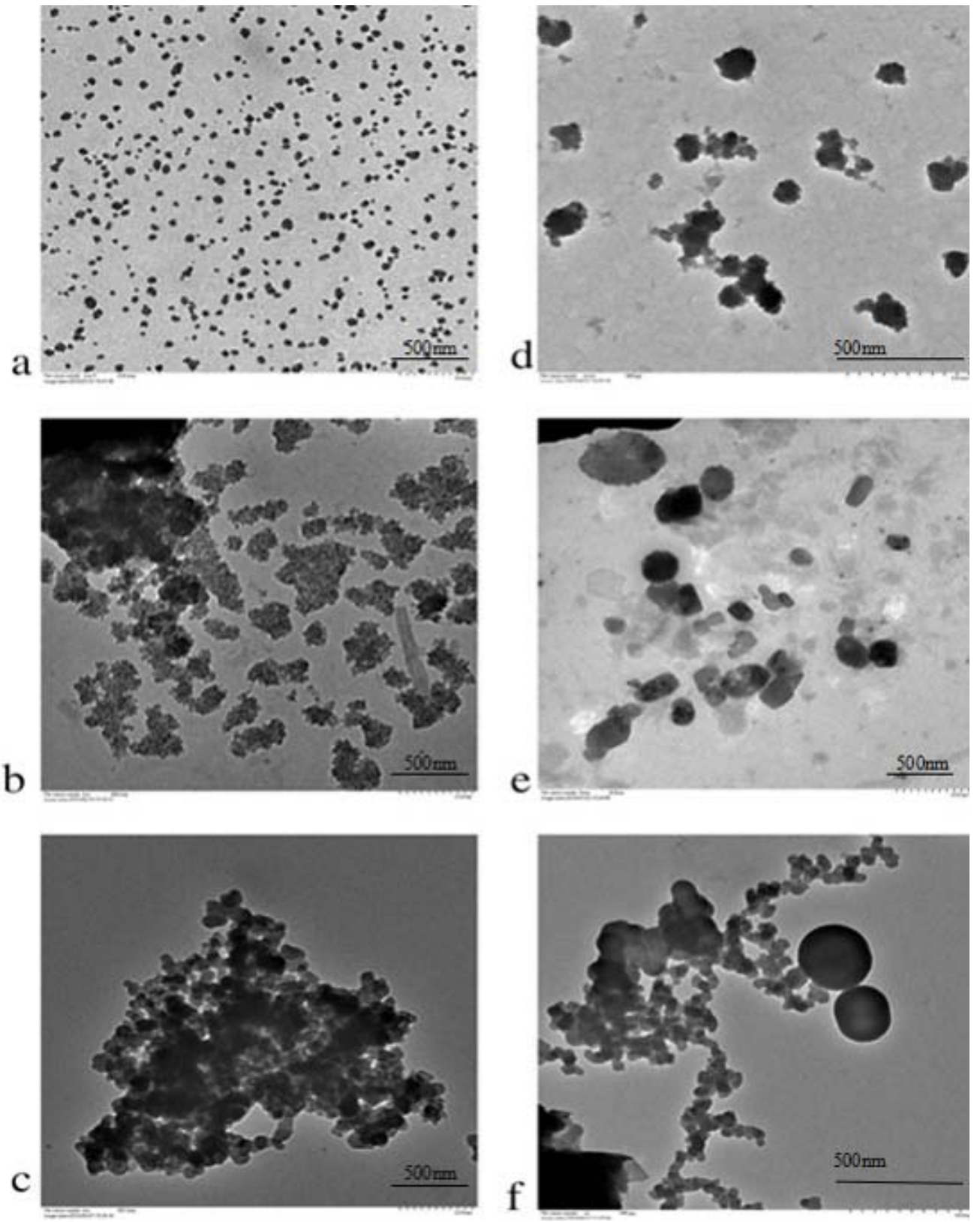

Fig. 3: TEM of the biosynthesized NPs generated by incubating the *P. florida* mycelial extracts with different precursor salts a) zinc sulphate b) ferric sulphate c) copper sulphate d) zinc chloride e) ferric chloride f) copper chloride

negative bacteria (*Aeromonas*, *Salmonella*, *Escherichia coli*, *Yersinia*) with 17, 16, 14 and 15 mm zone of inhibition (ZOI) against *E. coli*, *Salmonella,Aeromonas* and *Yersinia.* Whereas ZOI for the penicillin for the gram-positive bacteria (*Bacillus*, *Staphylococcus*, *Listeria*) exhibited a 15 mm inhibition zone for the *Staphylococcus* bacterial culture only (Table 1). But the myco-synthesized NPs provided no inhibition zone within the concentration range of 0.1 to 0.9 mM against any pathogenic bacteria (Fig. 4). It means these NPs can be utilized for other purposes such as bio-fortification. The produced biogenic NPs may require further higher concentrations for causing inhibition of the bacterial cultures. However, the antibacterial activity in the given concentration range was not observed for both the positive and negative controls. Kaur and Kalia (2016) have reported the antibacterial activity of the fungus derived ZnO NPs on different gram negative and gram positive bacteria. They have also observed the higher antibacterial activity against pathogenic bacteria such as gentamycin for *E. coli* and *S aureus* inhibition zone 19 and 21 mm respectively. In case of *P. florida* synthesized NPs from $ZnCl_2$ and $ZnSO_4$ salts no inhibition zone were observed for a concentration ranging from 2, 5, 8 and 10 ppm except for $ZnSO_4$ which showed inhibition for *S. aureus* @ 8 and 10 ppm concentration.

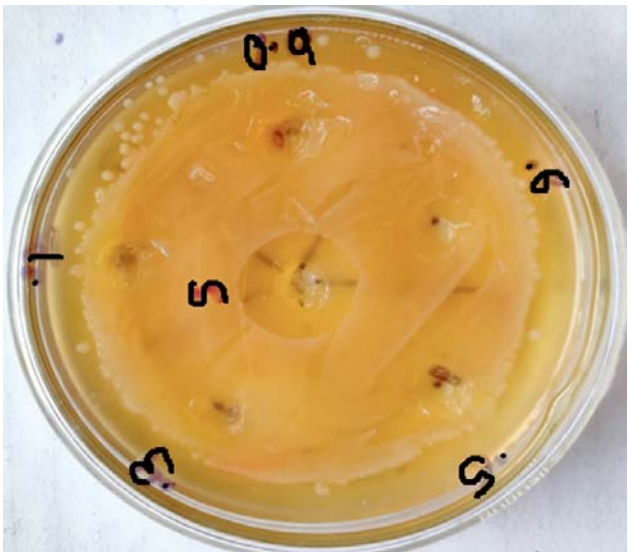

Fig. 4. Antibacterial activity of the biosynthesized NPs and gentamycin as standards (in center) against *E. coli*

CONCLUSION

Green synthesis of NPs using different salt precursors is a useful technique as it leads to synthesis of NPs which have improved eco-safety or lower harm to the environment and the living beings. This research utilized the extracellular biomolecules of the *P. florida* for the biosynthesis of NPs. Although the biogenic NPs exhibited similar capabilities and properties as other NPs produced from conventional methods, the microbial synthesized zinc (partial spherical), iron (cuboid) and copper (spherical) NPs were of different size, shape and properties. Moreover, the precise position of the SPR band also differed which depended on the size, shape and other properties of biogenic NPs. The produced mycogenic metal oxide NPs had diminished antibacterial activity (in 0.1 to 0.9 mM conc. range) which may find applications in bio-fortification and other similar uses. The biogenic NPs produced were of improved cost-effectiveness and better yield therefore, these biogenic NPs are beneficial for innovative fields such as biofortification of the mushrooms.

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