Macro and Micro-fungi mediated synthesis of Silver nanoparticles and its applications

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Abstract: ***Silver nanoparticles of size ranging from 1 ~ 100 nm are petite metallic colloidal particles, with its applications in diagnostics, biomarkers, imaging, cell labeling and drug delivery. Fungus-mediated synthesis of silver nanoparticles is an ecofriendly and green process with a comparatively simpler downstream processing. In the present study, the ability of macrofungi and pine stand soil fungi was evaluated for their ability to synthesize both extracellular as well as intracellular silver nanoparticles. When the macro and microfungi were challenged with 1 mM silver nitrate, colour change of the cell free filtrates indicated the formation of silver nanoparticles. The presence of silver nanoparticles was confirmed by Surface Plasmon Resonance absorption band in visible wavelength visualized every 24h upto 72h.Silver nanoparticles are known to possess a sharp peak in a range of 400-450 nm and peaks observed at 457nm, 403nm and 414nm by mushroom support their synthesis in comparison to that of 349nm by soil fungi. Transmission Electron Microscopic analysis of the silver nanoparticles revealed the nanorange, dimensions and structural conformation ofbio synthesized nanoparticles. Synergistic study of the synthesized nanoparticles revealed a significant antibacterial activity against four pathogens viz. MTCC 730 (Escherichia coli), MTCC 1925 (Streptococcus pyogenes), MTCC 96 (Staphylococcus aureus) and MTCC 430 (Bacillus cereus).Additionally, the silver nanoparticles inhibited the growth of the yeast pathogen MTCC 183 (Candida albicans) which showed synergistic enhancement in activity along with flucanazole. The fungal samples were analyzed for phytochemical constituents who led to reduction of silver nitrate into nanoparticles. The results obtained indicated that the experimental voucher fungus are more competent than soil fungi in synthesizing silver nanoparticles and can be used a potent natural antibacterial source for various pharmaceutical and textile applications.***

Keywords: Mushrooms, Nanoparticles, UV Spectra, TEM, Antimicrobial

1. Introduction

Nanotechnology, a new era of science and technology basically deals with the mater at their molecular level. The metal nanoparticles are widely been used in various biological allied fields as a source of catalysts, optoectronics, drug delivering unit, both as a chemical or biological sensor for its unique structural conformation, chemical composition and properties [1]. Because of its enormous importance, eco-friendly and cost-effective techniques are continuously getting explored for its synthesis. Among all the microbes, fungi possess higher tolerance and metal accumulating properties, which is an elemental trait for the synthesis of metal nanoparticles [2]. Northeastern paradox of India is one of the biodiversity hotspots with diverse ranges of flora and fauna has engrossed the researchers over the years, but only about half are explored for their applicability [3].The fungal system is also known as myco-nanofactories for its metal microbe interaction which have contributed to the process of bio-nanoparticles synthesis. Another benefit of considering fungal community in metal nanoparticles synthesis is its availability and ease to scale up. Fungi are known as secretors of various extracellular enzymes, thus making ease to use it for the synthesis of large scale metal nanoparticles [1,4].Filamentous fungal species *viz. Fusarium oxysporum*, *Verticillium* sp., *Aspergillus* sp., *Rhizopus* sp., *Penicillum fellutanum*, *P. brevicompactum*, *Alternaria alternata* are extensively explored for the synthesis of silver nanoparticles by either deposition of metal on the surface of cytoplasmic membrane or by synthesizing active extracellular enzymes [5,6]. But reports are scarce for the synthesis from macrofungi (Mushroom).

Mushrooms are rich in lectins, polysaccharides, phenols, polyphenols, terpenoids and are known to possess antioxidant, anti-inflammatory, antitumor, antiviral, antimicrobial, anti-cancerous activities and so on [7,8]. An extracellular synthesis of silver

nanoparticles has been reported with few edible straw mushrooms including *Volvariella* sp., *Agaricus bisporus*, *Pleurotus ostreatus* but the underlying mechanism for its synthesis is yet to be elucidated [5]. Although several enzymes, active components together determines the reduction of metal ion into nanoparticles, the identification of the potent active compound in a biomatrix is utmost important for the pilot production of the stable active metal nanomaterials.

Biologically synthesized nanoparticles are considered non xenobiotic that have shown wide range synergistic activity against the clinical microbes. The monovalent silver ion (Ag^+) get release in the microbial cell are known to suppress the respiratory enzymes and chain and thus inhibit the growth of the microbes apart from its antagonistic activities against the cellular membrane [9-11].

In the present study, we aimed at comparative analysis of active phytoconstituents and silver nanoparticles produce by both pine stand soil fungi and macrofungi (mushrooms) habitats at high altitudes of eastern Himalayan range in terms of its stability, shape, size and its efficacy singly and in combination to that of commercial antibiotics against few potent clinical microbes.

2. Materials and Methods

2.1. Sampling

Samples of pine stand soil and macrofungi were collected from subtropical forest siteswithin NEHU, Shillong in mid-monsoon of 2016 and brought to the laboratory under sterile conditions.

2.2. Isolation and Characterization

The macrofungi were washed with luke warm water to remove the dirt and were kept overnight by gills upside down to obtain spore print. A part of the macrofungi were preserved in 4% of formaldehyde and kept in the in- house culture collection center of the parent university. Whereas the soil fungi were isolated from the pine stand soil by serial dilution. The isolated colonies were sub-cultured to obtained pure colonies in potato dextrose agar (PDA) and were maintained by slant culturing for its identification

The identification of the macrofungi and soil fungi was carried out both by microscopic and nuclear ITS molecular barcode (ITS1F 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4R 5'-TCCTCCGCTTATTGATATGC-3'gene sequence) [12].The chromas so obtained after molecular sequencing were aligned using ClustalW software and searched for it homologies by BLAST to create an evolutionary distance matrix using Molecular Evolutionary Genetics Analysis software (MEGA 6) software. The sequences were later submitted to NCBI to obtain the accession numbers.

2.3. Extraction of secondary metabolite

The fresh fruiting bodies of the mushroom were air dried and ground to fine powder using a domestic blender.1:10 ration of dried mushroom powder to methanolic solvent were mixed and were set asideat shaker incubator for next 24 h where the supernatant were filtered for further analysis [13]. The fresh pure mycelia of the soil fungi were subjected to potato dextrose broth (PDB) in 1000ml Erlenmeyer flask for 7-8 days at 28ºC. The incubated flasks after week incubation were filled with 300 ml of methanol and were allowed to stand for a day in a shaker incubator until the extracellular metabolite get extracted in the solvent. Later the methanolic extract was extracted with sterilized distill water to remove the unwanted debris [14].

2.4. Qualitative phytochemical analysis of fungal metabolites

The extracellular methanolic extract of both macrofungi and soil fungi was subjected to chemical analysis to determine the active constituents. A slight modification method of Okerulu and Ani (2001) was considered to analyse the presence of alkaloids, phenolics, terpenoid, steroid and flavonoid content in the metabolites [15].

2.5. Biosynthesis of silver nanoparticles (AgNPs)

The biomass from both the fungi was obtained for the synthesis of AgNPs. About 10g of the fresh fruiting bodies were air dried and powdered into fine particles by a domestic grinder. The mushroom powders were suspended in 100ml of sterile distilled water for 10mins at 55ºC in Erlenmeyer flask which were later filtered and treated with 1mM $AgNO_3$ [16].

Fresh mycelia from the soil fungi were inoculated in PDB for 6-7 days at 28ºC and were kept in an agitator at 120 rpm. The filtered biomass after 6-7 incubation were harvested in sterile distilled water to obtained cell free extract which were treated with 1mM $AgNO_3$ [17].

Both the treated filtered were kept undisturbed at room temperature in dark. Control containing cell free

extract without $AgNO_3$ treatment were run concurrently along with the experiments.

2.6. Characterization of silver nanoparticles (AgNPs)

After 8h of treatment, the preliminary detection of the formation of AgNPs was carried out by visual observation of colour change in the cell free extract. The AgNPs formed were later characterized by UV-visible spectroscopy (CARY-100 BIO UV-vis Spectrophotometer; Varian Inc., Palo Alto, CA, USA) based on the surface plasmon resonance (SPR) at a range of 325-550nmwith a resolution of 1 nm for an interval of 24 to 96 hrs [18].Transmission Electron Microscope and Selected Area Electron Diffraction (SAED) of the synthesized biomatrix nanoparticles were acquired withJEOL JSM 100CX TEM instrument (Jeol, Tokyo, Japan). Distributions of particle size and average lattice of the nanoparticles were analyzed by Image J software (National Institutes of Health, USA).

2.7. Antimicrobial Susceptibility Assay

In vitro antimicrobial activities of the synthesized nanomaterials were carried out using disc diffusion method against fourbacterial clinical isolates viz. *Escherichia coli* (MTCC730), *Staphylococcus aureus* (MTCC96), *Bacillus cereus* (MTCC430), *Streptococcus pyogenes* MTCC1925) and a pathogenic yeast *Candida albicans* (MTCC183) [19]. The pathogenic isolated wererevived on Brain Heart Infusion (BHI) media (for bacteria) at 37°C for 18 to 24 h and PDB (for pathogenic yeast) at 28°C for 48 h which were further diluted to attain a turbidity of 0.4-0.5 McFarland standard. Antimicrobial assay of the synthesized AgNPs was assayed in Mueller Hinton Agar (MHA) plates using sterile susceptibility disc (Himedia, India) of diameter 6mm onto which 50µl of the AgNPswas separately loaded. The synergistic activity of the AgNPs was measured for its efficiency with standard commercial antibiotic Gentamicin (10µg) and Flucanozole (25µg) after 24 and 48h incubationat 37°C and 28°C for bacteria and fungi respectively. The zone of inhibition depicted by the synthesized AgNPs and AgNPs + Gentamicin, were measured individually.

2.8. Assessment of fold area increase

Synergistic activities of the synthesized AgNPs were analyzed by calculating the mean surface area of zone of inhibition by antibiotic and to that of AgNPs in combination with antibiotics. The fold increase area was determined by the following relation

$$(B^2 - A^2)/A^2$$

where A is zone of inhibition for antibiotic and B is AgNPs in combination with antibiotic.

3. Results and Discussions

3.1. Study area

The samples were collected from subtropical forest sites covering around 4.14km^2 within NEHU, Shillong (25º36’59.25”N, 91º53’51.17”E) with an elevation of 4346ft in mid-monsoon of 2016 (Figure1).

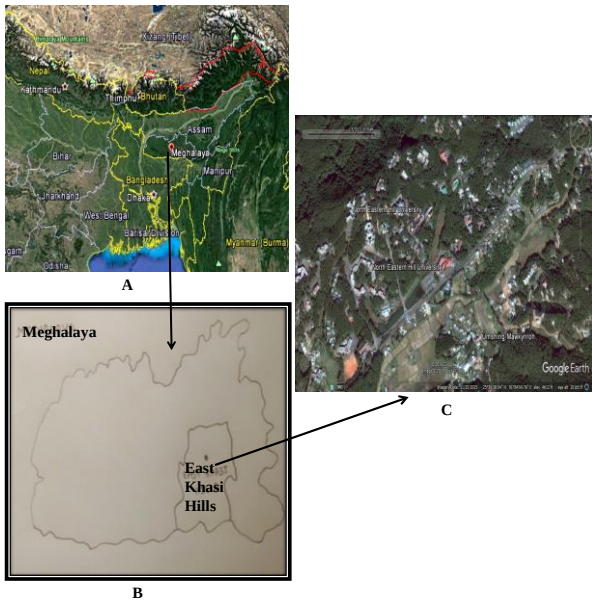


Figure1: GIS mapping of the study site.

3.2. Identification of fungal isolates

The morphology (physical/spore morphology) study revealed distinct characteristics which gave a tentative identification of the isolates as illustrated in TABLE 1. The nuclear ITS region of rRNA gene were sequenced and the chromas obtained were deposited to NCBI GenBank. Both the macrofungi and soil fungi were identified based on the closest homology of the ITS sequence (rRNA gene) by NCBI BLAST search and was aligned in ClustalW program to construct a phylogeny with bootstrap replica of 1000 using MEGA6 software (Figure2).

TABLE 1: Morphological and Microscopic observation of the fungi

Sample	Colony texture/ Cap and stalk (cm)	Appearance	Colour	Margin	Filament	Spore print (µm)
MS 1	Cap diameter- 7.3 Stalk length- 6.4	Soft, slimy, convex, presence of gills	White	Plain curvy to round	-	3.67
MS 2	Cap diameter- 26 Stalk length- 11	Leathery soft, umbrella shaped with wrinkled surface, presence of pores	White	Wavy	-	5.53
MS 3	Cap diameter-6.2 Stalk- Absent	Rough, wavy edges	Yellowish brown with white edges	Plain round	-	3.58
S1	Rough	Irregular	White	Entire	Aseptate	-
S2	Smooth	Circular	Yellowish white	Curled to wavy	Thin long aseptate	-
S3	Rough	Irregular	Creamish white	Entire	Septate	-

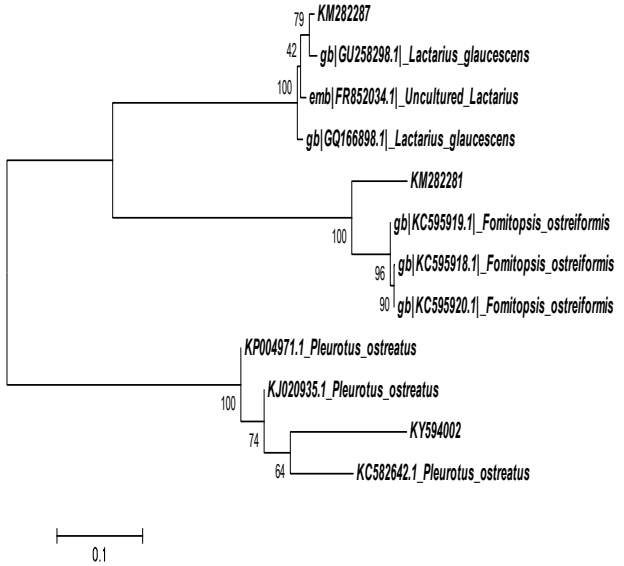


Figure2: Evolutionary positions of macrofungi with their related species based on rRNA-ITS sequence similarity.

They were identified to be *Pleurotus ostreatus* (KY594002), *Lactarius glaucescens* (KM282287) and *Formitopsis ostreiformis* (KM282281). Soil fungi *Trichoderma strigosellum* (KX831664), *Myrothecium verrucaria* (KY594003), *Penicillium striatisporum* (KY594004) were obtained from the pine stand soil (Figure3).

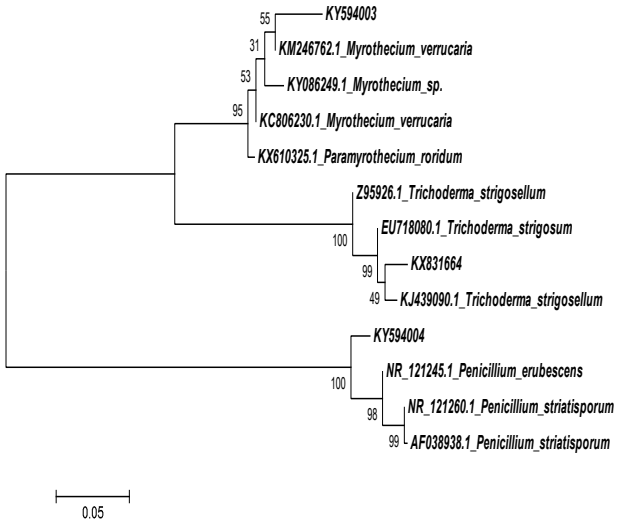


Figure3: Evolutionary positions of soil fungi with their related species based on rRNA-ITS sequence similarity.

3.3. Phytochemical analysis of the fungal metabolite

The comparative phytochemical profiles of the fungal metabolites are presented in TABLE 2. Presence of alkaloid was observed in all the soil fungi and macrofungi except in *Pleurotus ostreatus*. Terpinoid/steroid content are negligible in the mushroom extract however, the presence of steroid in *Penicillium striatisporum*is quite significant followed by the terpenoid content in *Myrothecium verrucaria.* Flavanoid in the group of polyphenols are known for various immunity promoting aspects including antioxidant, anti-inflammatory, anticancer and antimicrobial properties [20]. They exist widely in mushrooms which correlate with our study as the flavanoid content in macrofungi metabolite with significant high than that of soil fungal metabolites. As per the reported value 100g of dry mushroom weight have around 76-1000mg of flavonoid which correlates with our study [21.The mushroom metabolite display higher rate of antioxidant activity owning to the higher amount of combined polyphenolic compound and alkaloids [23, 24]. The phenolic compounds are known as aromatic metabolites that give colour, taste, odour along with certain health promoting aspects. The phenolic constituent in our study shows significant result in the mushroom metabolite than that of soil fungal metabolite owning to higher rate of antioxidant activities of the mushrooms as reported [13]. The aura of various phytocontituents in the metabolites may be the consequences of diverse

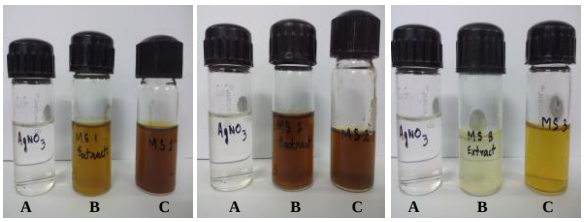
geographical habitats in which the soil nutrients and environmental or epigenetic factors have an important influence.The presence of these phytoconstitutent acts as an indicator for the particular species to ascertain them as a precursor for the advancement of various pharmaceutical products.

TABLE **2**: Phytochemical screening of the fungi

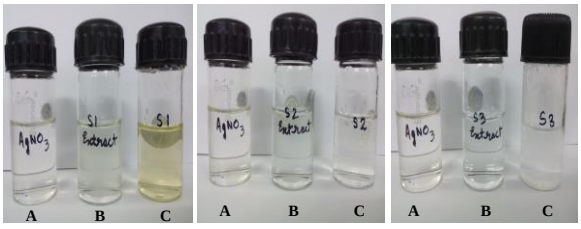
Sample	Alkaloids	Phenols	Terpinoid	Steroid	Flavanoid
P. ostreatus (MS1)	Negative(-)	Positive(++)	Negative(-)	Negative(-)	Positive(+++)
L. glaucescens (MS2)	Positive(+)	Positive(+++)	Negative(-)	Negative(-)	Positive(++)
F. ostreiformis (MS3)	Positive(+)	Positive(+)	Negative(-)	Negative(-)	Positive(+)
M. verrucaria (S1)	Positive(+)	Negative(-)	Positive(+)	Negative(-)	Positive(+)
T. strigosellum (S2)	Positive(+)	Positive(+)	Negative(-)	Positive(+++)	Positive(+)
P. striatisporum(S3)	Positive(+)	Positive(+)	Negative(-)	Positive(++)	Positive(++)

3.4 Synthesis of AgNPs and its Characterization

The change in colour after 24h of treatment of the cell free extract indicates the primary visual formation of AgNPs (Figure4) [19].



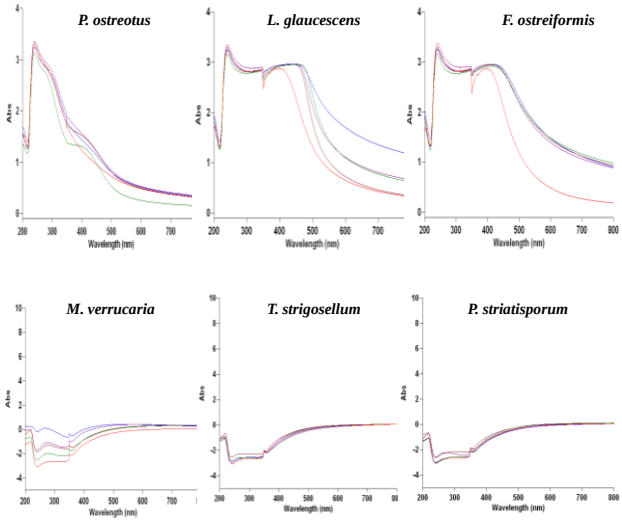
AgNPs synthesis by macrofungi using A-Silver nitrate (1mM) , B - Mushroom extract, C-Mushroom extract + Silver nitrate solution (1mM)



AgNPs synthesis by soil fungi using A-Silver nitrate (1mM) , B- soil fungal extract, C- Soil fungal extract + Silver nitrate solution (1mM)

Figure4: Digital images of the synthesized AgNPs with different metabolites.

The confirmatory test for the biosynthesized nanomaterial from fungal matrix was further characterized UV-visible spectra at a range of 200-800nm at a regular interval of 24-96h (Figure5).



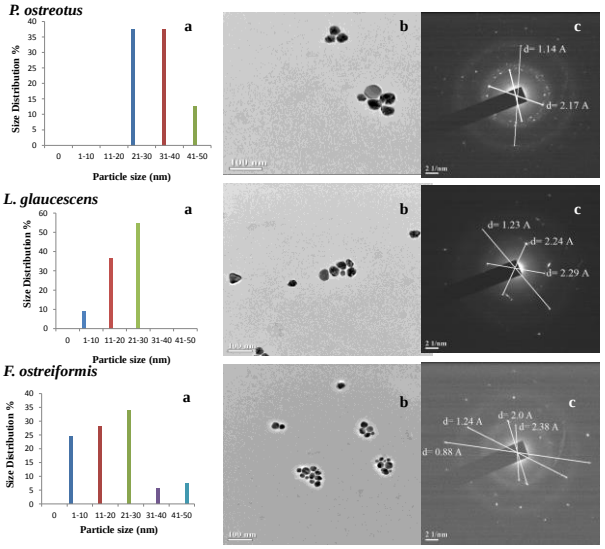
Graphs indication; Red- 24h, Blue-48h, Purple-72h, Brown – 96h, Green- 144h

Figure5: UV-Vis spectra showing different absorbance at different time interval

Fungal cell free extract was treated with 1mM concentration of silver nitrate solution. A sharp peak at around 420nm indicates the formation of silver nanoparticles which supports our findings where a peak was observed at a range of 420-450nm in nanoparticles synthesized from macrofungi indicates its formation. Time correlation was observed in the synthesized nanomaterial from macrofungi which seems to be almost stable after 72h of treatment. The initial changes in color was observed in the treatment of cell free extract of soil fungi metabolite but a proper peak was not observed while viewing under UV spectra and was almost stable after 24h which indicates the cell free macrofungal extract being more potent for synthesis of AgNPs. A size distribution histogram indicates the clusters of synthesized nanoparticles with varied diameters [Figure6 (a)-7(a)]. The absorbance at 210nm indicates the presence of protein in the fungal filtrate and at 280 nm indicates the presence of tryptophan, tyrosine and phenylalanine residues in the protein which suggest the possible oxidation reduction conversion of silver ion into nanoparticles [17].

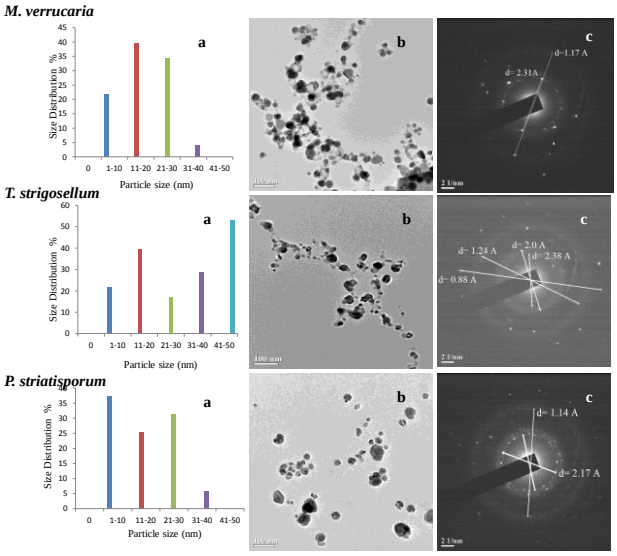
Further confirmation on the shape and size of the biomaterials was viewed in Transmission Electron Microscope (TEM). Shape, measurements and lattice diameter based on TEM images, reveals the final confirmatory formation of AgNPs with morphology approximately spherical for all synthesized AgNPs with uniform distribution in the synthesized

nanomaterials from macrofungi. However, a significant agglomeration could be seen in the TEM micrographs of AgNPs synthesized from soil fungi. The data obtained was analysed for the size of the synthesized AgNPs which ranges from 18nm to 25nm in diameter, with an average size of 21.5 ± 2.4 nm [Figure6 (b)-7(b)].The selected area electron diffraction (SAED) pattern indicates the crystalline biomatrix synthesized. The crystalinity nature of the synthesized nanoparticles was further investigated for the presence of lattice fringes with an average d-spacing value range from 2.01-2.3 Å. The ring shaped SAED pattern obtained in the study gives a confirmatory reduction of the silver ion into silver nanoparticles and indicates the nanoparticles to be crystalline in nature [Figure6(c)-7(c)].



Histogram (a) depict size distribution, micrograph (b) depict TEM analysis and SAED (c) depict the crystalline structure of silver nanoparticles synthesized from macrofungi

Figure6 (a): Histogram with size distribution of the synthesized AgNPs, **(b)**: TEM analysis of the synthesized AgNPs, **(c):** SAED electron pattern of the synthesized AgNPs from macrofungi.



Histogram (a) depict size distribution, micrograph (b) depict TEM analysis and SAED (c) depict the crystalline structure of silver nanoparticles synthesized from soil fungi

Figure7 (a): Histogram with size distribution of the synthesized AgNPs, **(b)**: TEM analysis of the synthesized AgNPs, **(c)**: SAED electron pattern of the synthesized AgNPs from soil fungi.

3.5 Antibacterial activity of AgNPs in plate assay

Silver metal is known for its broad spectrum antimicrobial activities. Combine effect of the synthesized nanoparticles along with the commercial antibiotics results in enhance synergistic effects against pathogenic microbes. The antibacterial efficacy of the biosynthesized AgNPs were assayed against four clinical isolates of bacteria viz. *Streptococcus pyogenes* (MTCC1925), *Staphylococcus aureus* (MTCC96), *Bacillus cereus* (MTCC430), *Escherichia coli* (MTCC730) and a fungal pathogen, *Candida albicans* (MTCC183). The observed zone of inhibition by the synthesized AgNPs along with its synergistic effect with commercial antibiotics suggests its bactericidal and fungicidal properties. The zone of inhibition was found to be more efficient for gram negative *E. coli* and pathogenic fungus *C. albican*s than other indicator strains. The AgNPs synthesised from macrofungal metabolites *F.ostreiformis* showed highest efficiency with zone of inhibition(13mm0 followed by 11mm and 9mm by *P. ostreatus* and *L. glaucescens* respectively against *E. coli*. Moreover the same showed its potency against the pathogenic fungi *C. albicans* to be 11, 12 and 9mm respectively.

The synergistic activity of the synthesized AgNPs with the commercial antibiotic, Gentamicin and Flucanazole was evaluated by the increase in the fold

area where a significant activity, 71.7% efficiency was shown by the AgNPs synthesized by *F. ostreiformis* against *E. coli.* The AgNPs synthesized from soil fungi also showed fold area enhancement was viewed in association with Gentamicin against *B. cereus* with around 85% increase by AgNPs synthesized from *P. striatisporum* which supports the findings as reported by Devi *et al*., 2014 [3].The fold area increases depicts the potency of the synthesized bio-nanomaterials to enhance the antimicrobial activity with the commercial antibiotics as observed in our study in TABLE 3.

TABLE 3: Antimicrobial and synergistic activity of the synthesized AgNPs against clinical pathogens

Clinical Pathogens	AgNPs (Zone of Inhibition in mm)						Antibiotic [Gentamicin (G); Flucanozole (F)]	AgNPs + Antibiotic (Zone of Inhibition in mm)						Fold area increase (%)					
	MS1	MS2	MS3	S1	S2	S3		MS1	MS2	MS3	S1	S2	S3	MS1	MS2	MS3	S1	S2	S3
Streptococcus pyogenes	-	-	-	>10	-	-	G-29.5	32	30	32	30	31	32	17.6	3.4	17.6	3.4	10.4	17.6
Staphylococcus aureus	>10	-	-	>10	-	>10	G-34	37	36	35	37	34	34	18.4	12.1	5.9	18.4	-	-
Bacillus cereus	12	11	13	>10	-	>10	G-14.4	18	16	17	18	18	15	56	23.4	39.3	56	56	85
Escherichia coli	-	>10	>10	-	-	>10	G-14.5	17	15	19	15	16	16	37.4	7.1	71.7	7.1	21.7	21.7
Candida albicans	>10	>10	>10	>10	-	>10	F-27	34	29	28	30	27	27	58.5	15.3	7.5	23.4	-	-

4. Conclusion

This study compares the active potent silver nanoparticles synthesis from both macrofungi and *Pinus* stand soil fungi from the habitat of high altitude temperate to cold climatic zone of the Eastern Himalayan biodiversity hotspot region. The AgNPs synthesized from mushrooms reveals more potentiality in terms of their antimicrobial activity against few clinical pathogens than that of the pine stand soil fungi. The enhanced synergistic activities could be the result of high phenolic and flavonoid constituents which are known to retard the growth of microbes. Even though the size of the soil fungi and macrofungi synthesized nanoparticles are almost likely to be consistent which ranges from 18-25nm, but enhanced antimicrobial activities are seen by the mushroom synthesized nanoparticles either singly or in combination with the commonly used commercial antibiotics. This study is the first report on the comparative analysis of bioactive nanoparticles from soil fungi and macrofungi from the microhabitats of Northeast India which provides vista for their further exploration as effective candidates for pharmaceutical usages.

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