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BIOSYNTHESIS OF SILVER AND ZINC OXIDE NANOPARTICLES AND EVALUATION OF THEIR *IN-VITRO* ANTI CANCER PROPERTY AGAINST ACUTE MYELOID LEUKEMIA (TPH1) CELLS

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ABSTRACT

In the present study, the yeast *Pichia fermentans* was isolated from fruit waste and identified and utilized for the biosynthesis of silver (Ag NPs) and Zinc oxide (ZnO NPs) nanoparticles. The synthesized nanoparticles were characterized by X-Ray Diffraction, Transmission Electron Microscopy, Energy Dispersive Spectroscopy and Fourier Transform Infrared Spectroscopy. The characterization study revealed the maximum size of Ag NPs was 98.6 nm and ZnO NPs was 140 nm. The nanoparticles were evaluated for their anticancer activity against Acute Myeloid Leukemia cells (THP1) cells. The concentration required to make 50% cell death was observed at 500µg/ml for the silver nanoparticles whereas ZnO NPs showed a proportionate increased cytotoxicity at higher concentration.

INTRODUCTION

Nanotechnology is an emerging, interdisciplinary area and Nanoparticles are being viewed as fundamental blocks of Nanotechnology. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. The most effectively studied nanoparticles today are those made from Noble metals and find vast applications in various fields ranging from medical to physical fields (Henley *et al*., 2006). The nanoparticles are significant because of the unique physicochemical characteristics of metal nanoparticles including catalytic activity, optical properties, electronic properties, antibacterial properties and magnetic properties (Catauro *et al*., 2005; Crabtree *et al*., 2006) and gaining the interest of scientists for their novel method of synthesis. Conventionally, nanomaterials are synthesized using either chemical or physical methods which include micelles sol process, chemical precipitation, mechanical shaking, hydrothermal method and chemical vapour deposition method (Taleb *et al*., 1997).

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Unfortunately many of these methods have several disadvantages such as high cost, consumption of high energy and chemical method of synthesis involving carcinogenic chemicals impart genotoxic effect in the medical applications (Leela *et al*., 2008). There has been a resurgence of interest in plants and plant derived products as a source of medicine in the last few decades. Microbial method of synthesis found to take place extracellularly would make the nanoparticles biocompatible and the reaction time has been reported to be very short compared to that of other methods. Among the nanoparticles, silver nanoparticles play a significant role in the field of biological system, living organism and medicine (Belly and Kydd, 1982). The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups (Bragg and Rainnie, 1974), although other target sites remain a possibility. Amino acids, such as cysteine, and other compounds containing thiol groups, such as sodium thioglycolate, neutralized the activity of silver against bacteria (Reynolds, 1963; Sondi and Sondi, 2004). Silver was also proposed to act by binding to key functional groups of enzymes. Silver ions cause the release of K^+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes, is an important target site for silver ions (Miller and McCallan, 1957; Fuhrmann and Rothstein, 1968).

In addition to their effects on bacterial enzymes, silver ions caused marked inhibition of bacterial growth and were deposited in the vacuole and cell wall as granules, they inhibited cell division and damaged the cell envelope and contents of bacteria (Brown and Smith, 1976). Silver is now extending its applications in cancer treatment as antitumor molecules, and many attempts turned up meaningful and positive (Lima *et al*., 2012). It was reported that AgNPs posses antitumor effects against the cervical carcinoma cells , embryo fibroblast 3T3 cell, lung cancer H1299 cells , breast cancer MCF-7 cells, and glioblastoma multiforme U-87 cells (Arokiyaraj *et al*.,2014). AgNPs could be delivered into the cell by the Trojan effect and inhibit the RNA polymerase activity and the gene transcription via a direct reciprocal interaction. The tumor cells were more sensitive to AgNPs damage than normal cells (Sriram, 2010). The particle size and surface features of AgNPs are very important for biomedical considerations. AgNPs with smaller particle size seemed to have a stronger penetration ability and greater toxicity for cancer cells (Li *et al*., 2012).

ZnO nanoparticles now have a wide range of applications in cancer therapy, biosensing, drug/gene delivery, nanomachines that can act as biological mimetic, biomaterials for tissue engineering, shape memory polymers such as molecular switches, etc. Owing to this wide application of ZnO nanoparticles, a variety of ZnO nanostructures have been synthesized, including nanoparticles, nanowires, nanorods, nanotubes, nanobelts and other complex morphologies (Vaseem *et al*., 2010). A ZnO nanoparticle, as a wide band-gap semiconductor, can readily absorb UV rays. Owing to this property, ZnO nanoparticles have a wide range of application, from electronic devices, cosmetics and facial products to biomedical application. ZnO nanoparticles are now being widely researched for their anticancerous properties. Some of the characteristic features of ZnO nanoparticles behind their surge in anticancer therapy are described below. ZnO nanoparticles show relatively high biocompatibility. Their bulkier form is generally recognized as safe (GRAS) by the FDA. Zinc is an important co-factor in various cellular mechanisms and plays an important role in maintaining cellular homeostasis; hence ZnO shows biocompatibility. The administered ZnO can be easily biodegraded or can take part in the active nutritional cycle of the body (Zhou, *et al*., 2006). ZnO nanoparticles have an inherent nature of showing selective cytotoxicity against cancerous cells in in vitro condition compared with other nanoparticles. They can be further surface engineered to show increased selective cytotoxicity (Hanley *et al*., 2008). In the present study, *Pichia fermentans* isolated from fruit waste was identified used for the biosynthesis of Silver nanoparticles (Ag NPs) and Zinc Oxide nanoparticles (Zn NPs) and evaluated their anti cancer activity against Acute Myeloid Leukemia (TPH1) cells .

MATERIALS AND METHODS

Isolation and identification of Yeast

The fruit waste samples were collected from large fruit markets in Chennai, India. The samples were blended using sterile mortar and pestle. The blended fruit waste was washed with sterile distilled water and used as sample. The sample was serially diluted and plated on sterile Potato Dextrose Agar Medium (PDA) amended with Chloramphenicol. The plates were incubated at 30°C for 36 hours.

The colonies from the samples were subjected to simple staining using Crystal violet to reveal the morphology. The colonies that showed yeast morphology were purified using streak plate method and subcultured for further use. The yeast isolate selected for the study was primarily identified using cultural characters, microscopic and physiological characters. The identification of the isolate was confirmed by molecular identification using 18S ribosomal RNA coding gene sequencing. The obtained 18S Ribosomal sequence was subjected to BLAST (Basic Local Alignment Search Tools) at NCBI website for confirmation of the sequence and identification, and the sequence was submitted to GenBank/NCBI through sequin tool.

Biosynthesis of Nanoparticles

The yeast culture supernatant was prepared by subculturing yeast in sterile PDA broth. The culture was incubated at room temperature for 48 hours with mild agitation. The yeast cells were separated by centrifugation at 12000 rpm for 10 minutes. The supernatant was transferred to a fresh sterile tube and subjected to centrifugation twice for complete removal of biomass and debris. The cell free clear supernatant was used for biosynthesis of nanoparticles. Silver nanoparticles were prepared using silver nitrate ($AgNO_3$). The reaction mixture was prepared by mixing culture supernatant with 2mM silver nitrate solution in the ratio of 1:1. The reaction mixture was kept at room temperature with 100 rpm agitation for formation of silver nanoparticles. The reduced silver nanoparticles were separated by centrifugation at 1000rpm for 10 mins. The nanoparticles were washed twice with distilled water by centrifugation and dried at 60°C till it become dry crystals.
The zinc oxide nanoparticles were synthesized using 0.1 N Zinc nitrate solution in double distilled water and stirred well for complete dissolution of salt. The reaction mixture was prepared using 1% culture filtrate and zinc nitrate solution and appeared white in colour when kept at magnetic stirrer for 2 hours. The deposited nanoparticles were separated by centrifugation at 5000rpm for 5 minutes and washed twice using distilled water. The final product was dried at 60°C till it become powder.

Characterization of nanoparticles

The solid powder form of AgNPs and ZnO NPs were characterized by Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD). The analysis were carried out in SAIF, IIT Chennai, Department of Organic Chemistry, Department of Nanotechnology, University of Madras, Chennai.

X- Ray Diffraction (XRD)

The X-ray diffraction data was obtained in X-Pert Pro Diffractometer following step scan technique and with Cu-Ka radiation (1.500 A, 40 kV, 30 mA) in h–2h configuration. The dried metal nanoparticles were coated on to the glass substrate was analysed by X-ray diffractometer. The crystallite domain size was calculated using the Debye-Scherrer formula.

Fourier Transform Infra-red Spectroscopy (FTIR)

The nanoparticles were subjected to centrifugation thrice at 15,000 rpm for 20 min to remove biomass or compound that is not the capping ligand of the nanoparticles. The purified and

concentrated suspension was freeze dried to obtain dry powder. Finally, the dried nanoparticles were analysed by ALPHA FT-IR Spectrometer, Bruker, Germany for the detection of different functional groups in the range of of 4000 cm^{-1} to 500 cm^{-1}.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX)

The shape and size of nanoparticles were determined by Scanning Electron Microscopy (SEM) using the Quanta 200 FEG scanning electron microscope. The nanoparticles dissolved in water was placed on a copper grid and the images were obtained, The composition of nanoparticles was determined using the EDX coupled to the SEM.

Evaluation of Invitro anti-Cancer activity Property

The synthesized Ag NPs and ZnO NPs were evaluated for anticancer activity agains Acute Myeloid Leukemia cells (THP1) cells by MTT assay Acute Myeloid Leukemia cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/mL), Gentamycin (100µg/mL) and Amphotericin B (1mg/mL) obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over 24 hours before use. Cell viability was measured with the conventional MTT reduction assay. Acute Myeloid Leukemia cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 hours, in 200 µl of Rosewell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum. Then culture supernatant was removed and RPMI containing various concentrations of test sample was added and incubated for 48 hours. After treatment cells were incubated with MTT (10µl, 5mg/mL) at 37 C for 4 hours and then with Dimethyle sulfoxide (DMSO) at room temperature for 1 hour. The plates were read at 595nm on a scanning multi-well spectrophotometer.

Cell viability (%) = (Average test OD/Control OD) x 100

RESULTS AND DISCUSSION

Yeast strain and Nanoparticle biosynthesis

The Yeast strain isolated from fruit sample waste showed creamy white mucoid colonies in PDA agar (Figure 1). The microscopic observation showed typical yeast cell structure with budding cells (Figure 2). The preliminary biochemical test results showed the fermention of Glucose and assimilation of D-Xylose, Succinate, Lactate and Citrate. The molecular identification through 18S ribosomal RNA coding gene sequencing results revealed that the obtained sequence had 649 base pairs (GenBank Accession KX943531). The BLAST results showed that the obtained gene sequence showed 100% identity with *Pichia fermentans* strain G4 (Genbank Accession KF156788) (Table.1). Besides, BLAST sequence alignment provided information that the obtained sequence is a partial sequence 18S ribosomal RNA gene containing internal transcribed spacer 1, 5.8S subunit gene, internal transcribed spacer 2, and part of 28S subunit coding gene. Hence, the yeast isolate was identified and confirmed as *Pichia fermentans*.

Figure 1. Yeast isolate in PDA plate

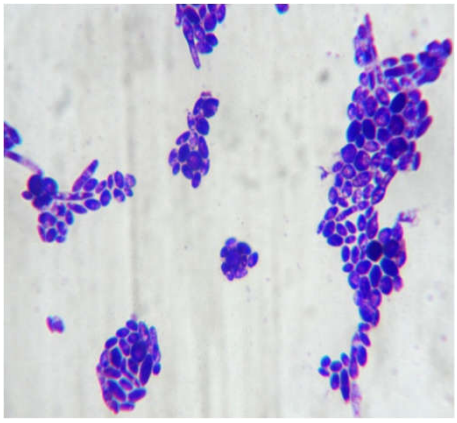
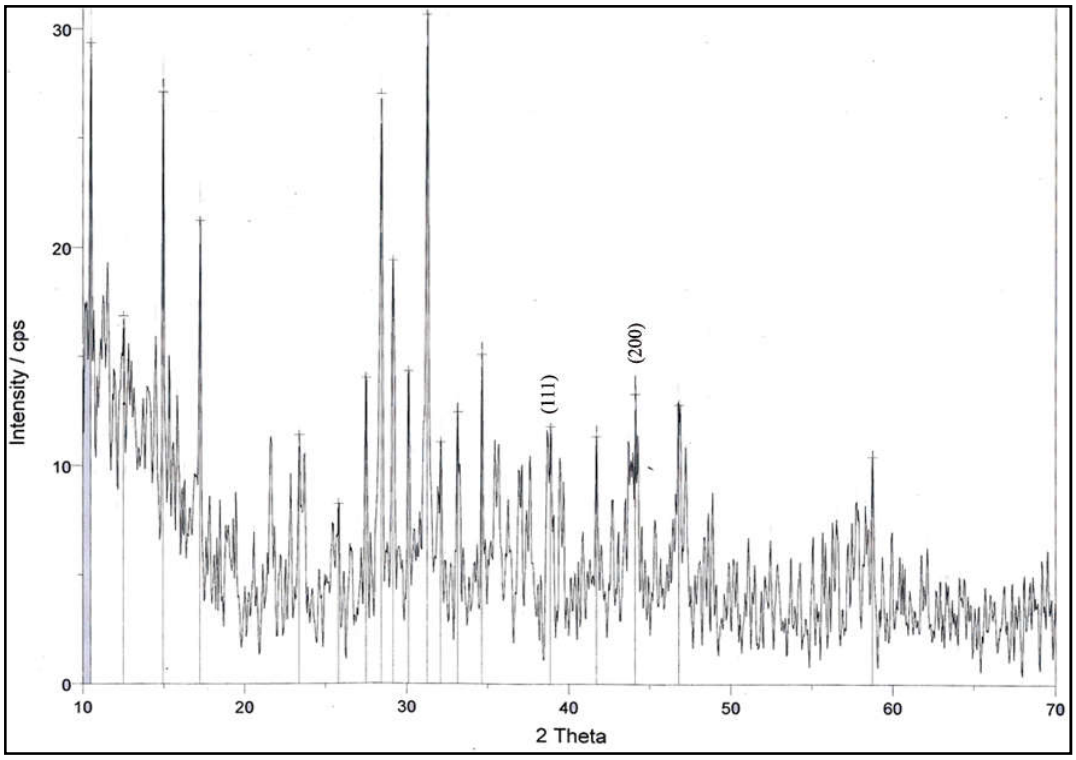


Figure 2. Microscopic view of yeast

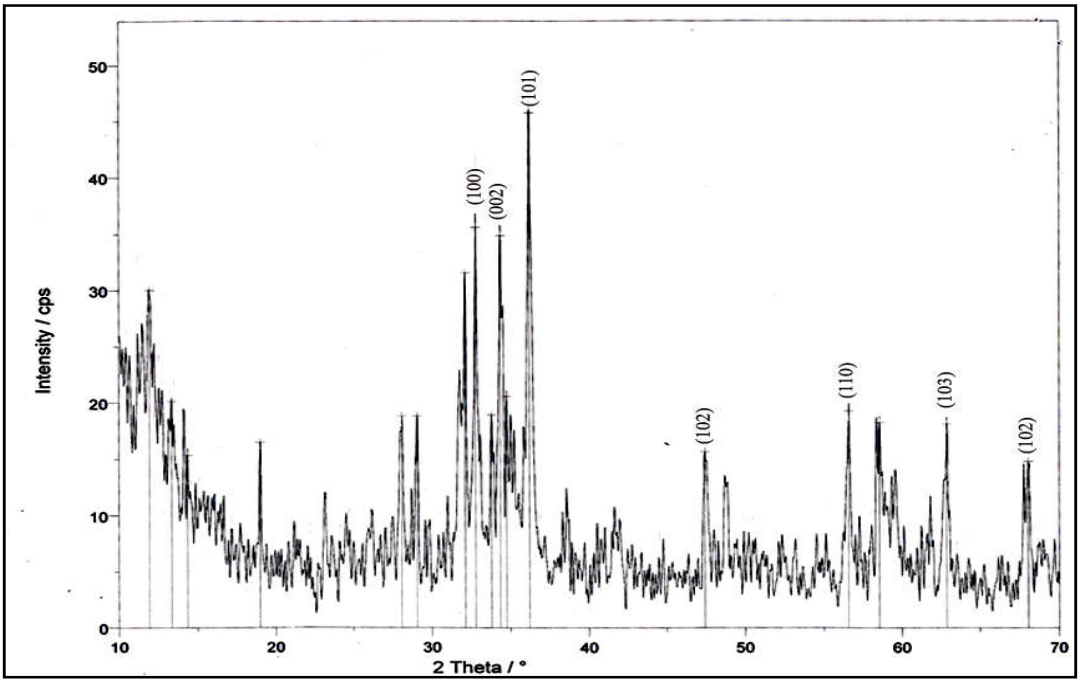
(A) Silver Nanoparticles

(B) Zinc Oxide Nanoparticles

Figure 3. Synthesized Nanoparticles



(A) Silver Nanoparticles



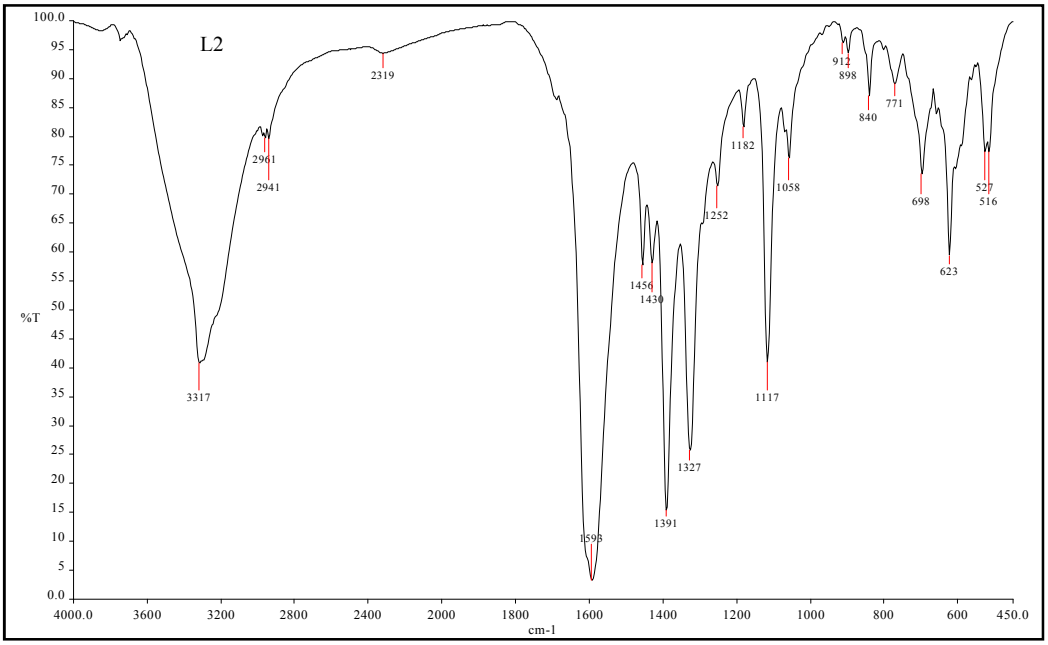
(B) Zinc Oxide Nanoparticles

Figure 4. XRD profile of Nanoparticles

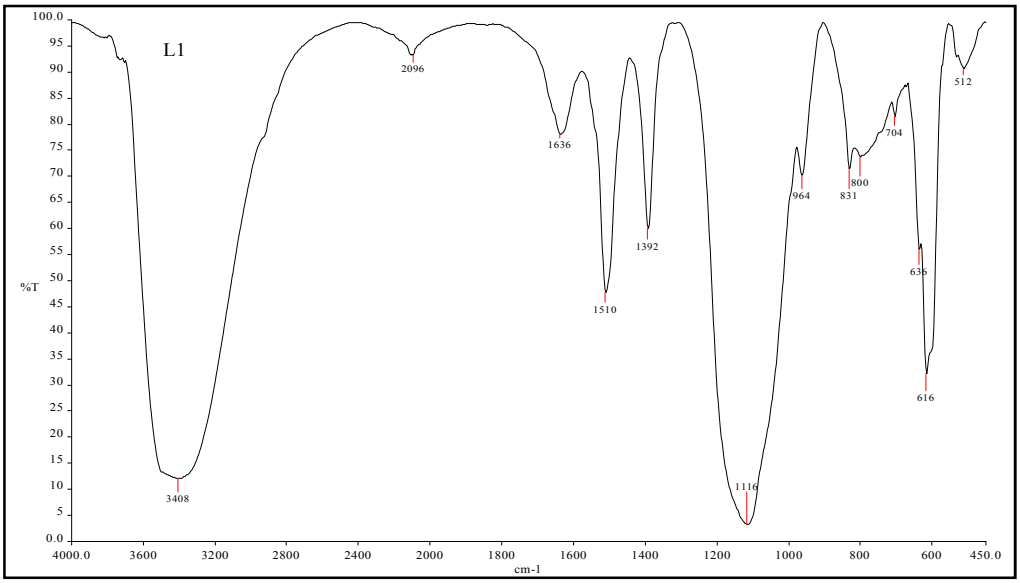
During the synthesis of silver nanoparticles, the reaction mixture appeared as blackish brown in colour and that turned to grayish deposits during reduction reaction. The separated pure silver nanoparticles appeared as glittering greyish crystalline powder in nature (Figure 3A). The biosynthesis of ZnO nanoparticles showed initially the formation of white haziness followed by white colour deposition after reduction into ZnO particles (Figure 3B). The purified and dried ZnO nanoparticles were white colour amorphous powder.

Characterization of nanoparticles

The XRD pattern of silver nanoparticles indicated the presence of three diffraction peaks, which agreed well with 111 and 200 diffractions of face centered cubic silver. The size of the nanoparticles was calculated by Debye-Scherer's formula and found as 80.3 nm (Figure 4A) (Nahee *et al*., 2011). The XRD profile of Zinc oxide nanoparticles agreed with 100, 002,101,102,110,103 and 112 peaks of ZnO nanoparticles with the particle size of 89.6 nm (Figure 4B) (Talam *et al*., 2012).



(A) Silver Nanoparticles



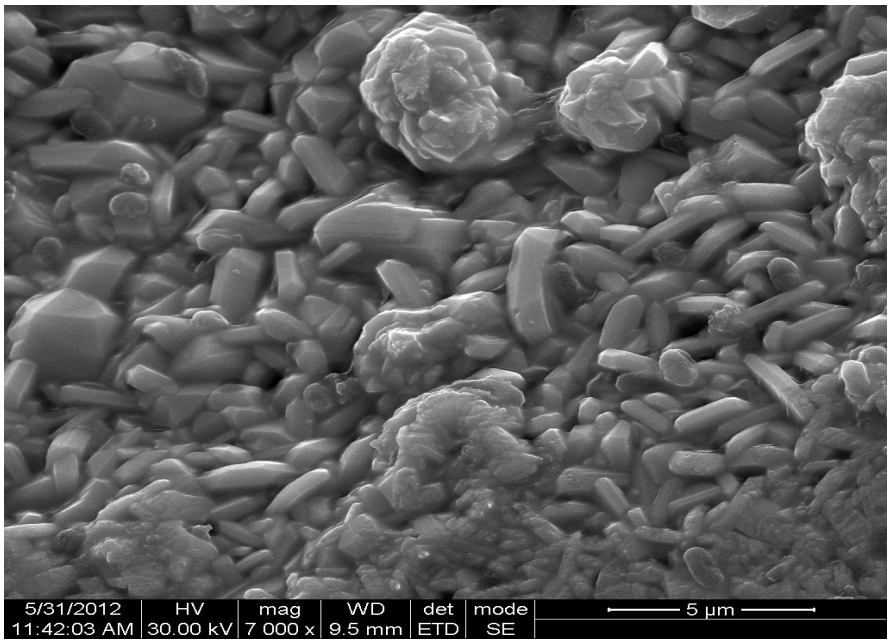
(B) Zinc Oxide Nanoparticles

Figure 5. FTIR profile of Nanoparticles

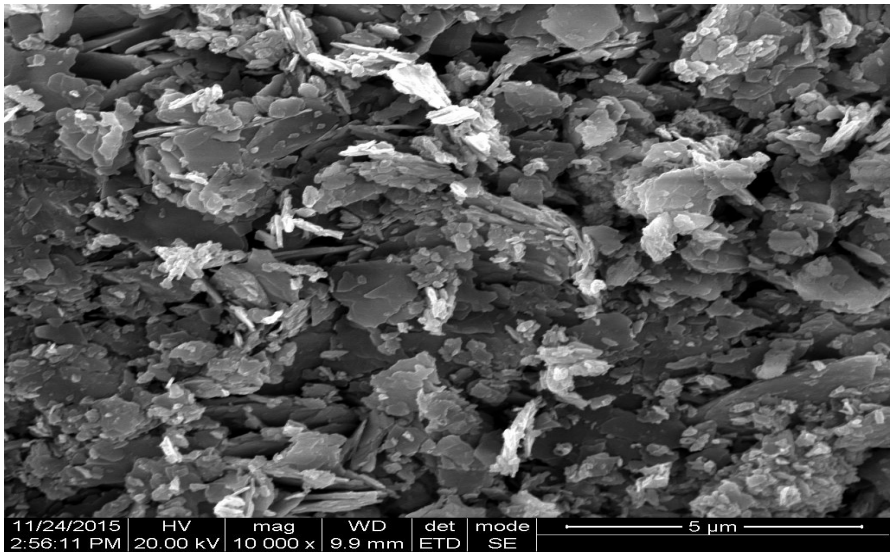
The FT-IR spectra peaks of silver nanopartiles was observed at 2961 cm $^{-1}$ corresponds to stretching vibration of –OH bond (Figure 5A). After the reduction with the $AgNO_3$, the shift in the peak at 516 cm $^{-1}$ towards lower frequency is attributed to the formation of nanoparticles. The stretching frequency of N–O is observed at above 1593 cm $^{-1}$. This was confirmed by the changes in the peak shift of silver nitrate and silver nanoparticles appeared in medium (Monali *et al*., 2009)**.** The FTIR spectrum of ZnO nano material showed the Zn –O absorption band at 512 cm $^{-1}$ (Figure 5B) The peaks at 1636 cm $^{-1}$ and 3408.20 cm^{-1} indicated the presence of –OH and C=O residues, probably due to atmospheric moisture and CO_2 - (Zak *et al*., 2011). The SEM analysis showed that the particles were rectangular and semispherical cuboidal shaped with size ranging from 53nm to 98.6nm. Some of them were appeared as single individual and some were aggregated (Figure 6A) (Monali *et al*., 2009). The shape of ZnO nanoparticles was found to be irregular, aggregated, with size ranging from 72.3 nm to 140nm (Figure 6B) (Wilhelmi *et al*., 2013). The EDAX results of silver nanoparticles showed the presence of sharp peaks formed between 3 to 3.5KeV indicateed the presence of silver in the sample (Figure 7A) (Manish *et al*., 2009). The EDAX profile of ZnO nanoparticles showed three different peaks at 1.1, 8.8 and 9.5, confirmed the presence of Zinc (Figure 7B). The peaks formed at 0.5Kev showed the presence of oxygen.

Anticancer activity

The cytotoxicity of the silver nanoparticles was evaluated in vitro against THP1 cells at different concentrations. The analysis showed that the cytotoxicity increased proportionately at higher concentration.



(A) Silver Nanoparticles

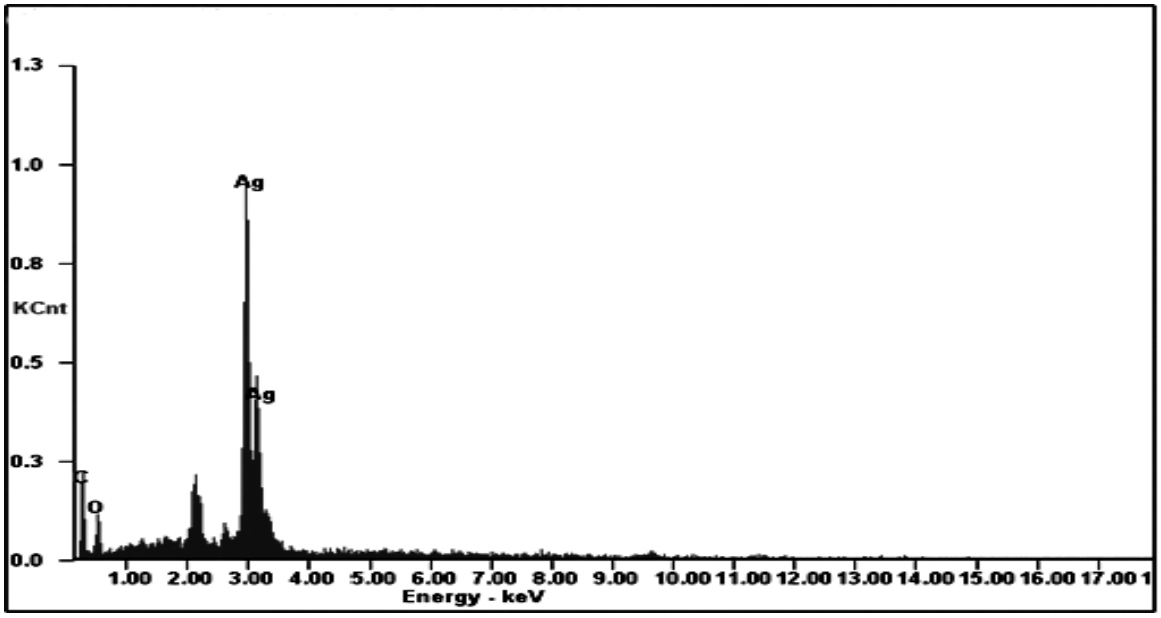


(B) Zinc Oxide Nanoparticles

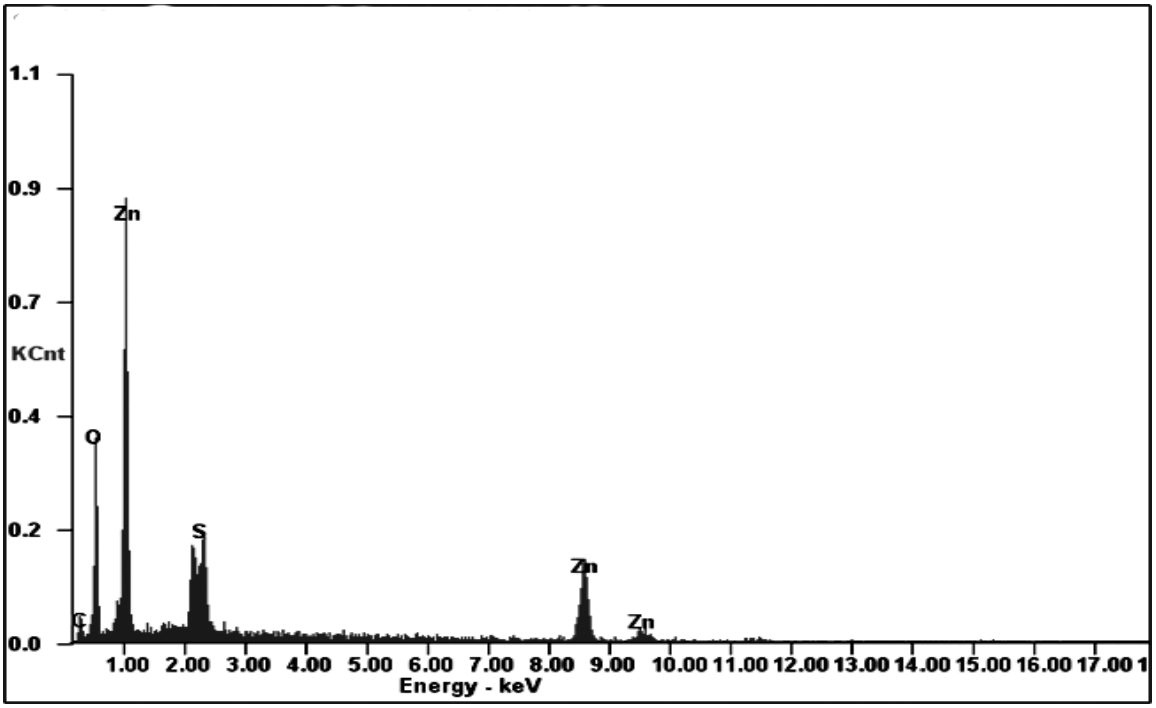
Figure 6. SEM analysis of Nanoparticles

Table 1. Blast analysis of *Pichia fermentans* 18S ribosomal RNA coding sequence

Sequences producing significant alignments						
Description	Max score	Total score	Query cover	E value	Ident	Accession
Pichia fermentans strain G4 18S ribosomal RNA gene, partial sequence	1275	1275	100%	0.0	100%	KF156788.1
Pichia fermentans isolate QY26 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	KU147482.1
Uncultured fungus clone D39 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	JN054689.1
Uncultured *Pichia* clone m10 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	HM151324.1
Uncultured *Pichia* clone m14 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	HM151322.1
Uncultured *Pichia* clone p5 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	HM151320.1
Uncultured *Pichia* clone P9 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	HM151317.1
Pichia fermentans strain ATCC 10651 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	GQ458040.1
Unclassified marine fungus clone JJ15_BASS small subunit ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	EU154982.1
Pichia fermentans strain NRRL Y-1619 18S ribosomal RNA gene, partial sequence	1149	1149	100%	0.0	97%	EF550372.1



(A) Silver Nanoparticles



(B) Zinc Oxide Nanoparticles

Figure 7. EDAX analysis of Nanoparticles

The concentration required to produce 50% cell death was 500µg/ml for the silver nanoparticles (Figure 8A). There are several investigations on cytotoxicity made against fibro sarcoma wehi 164, rat liver derived cell line (BRL 3A) etc. The Fibro sarcoma wehi 164 cell line showed the cytotoxicity at 2.6 µg/ml silver nanoparticles and decreased cell proliferation by 50% (Hussain *et al*., 2005) whereas in rat liver derived cell line the cells exposed to metal nanoparticles showed a decrease in size, an irregular shape and a significant dose-dependent impairment of the mitochondrial function (Mona *et al*., 2009) . The cytotoxicity in various cultures such as a monolayer cell culture, a tissue explant culture model and a mouse expurgated wound model was also studied. The results showed that Aquacel Ag and Contreet Foam presented the most significant cytotoxic effects in keratinocyte and fibroblast cultures. Recently Acticoat was used in post- cardiac surgery mediastinitis using a recently introduced silver releasing dressing claiming prompt antibacterial activity (Burd *et al*., 2007). The cytotoxicity of the zinc oxide nanoparticles demonstrated a considerable cytotoxicity against the cells. The analysis showed that a proportionate cytotoxicity increased at higher concentration (Figure 8B). Supportively, several investigation on cytotoxicity studies were reported in Human cell lines (U251 and IMR-90), lung tissue and on liver enzymes in male rat. In human cell lines (U251 and IMR-90) the experiments demonstrated that the ZnO nanoparticle capped with fluorescein isothiocyanate exhibited more cytotoxic characteristic as compared to ZnO nanoparticle capped with starch. The study also revealed that the toxicity of ZnO nanoparticle were size and dose dependant (Chue and

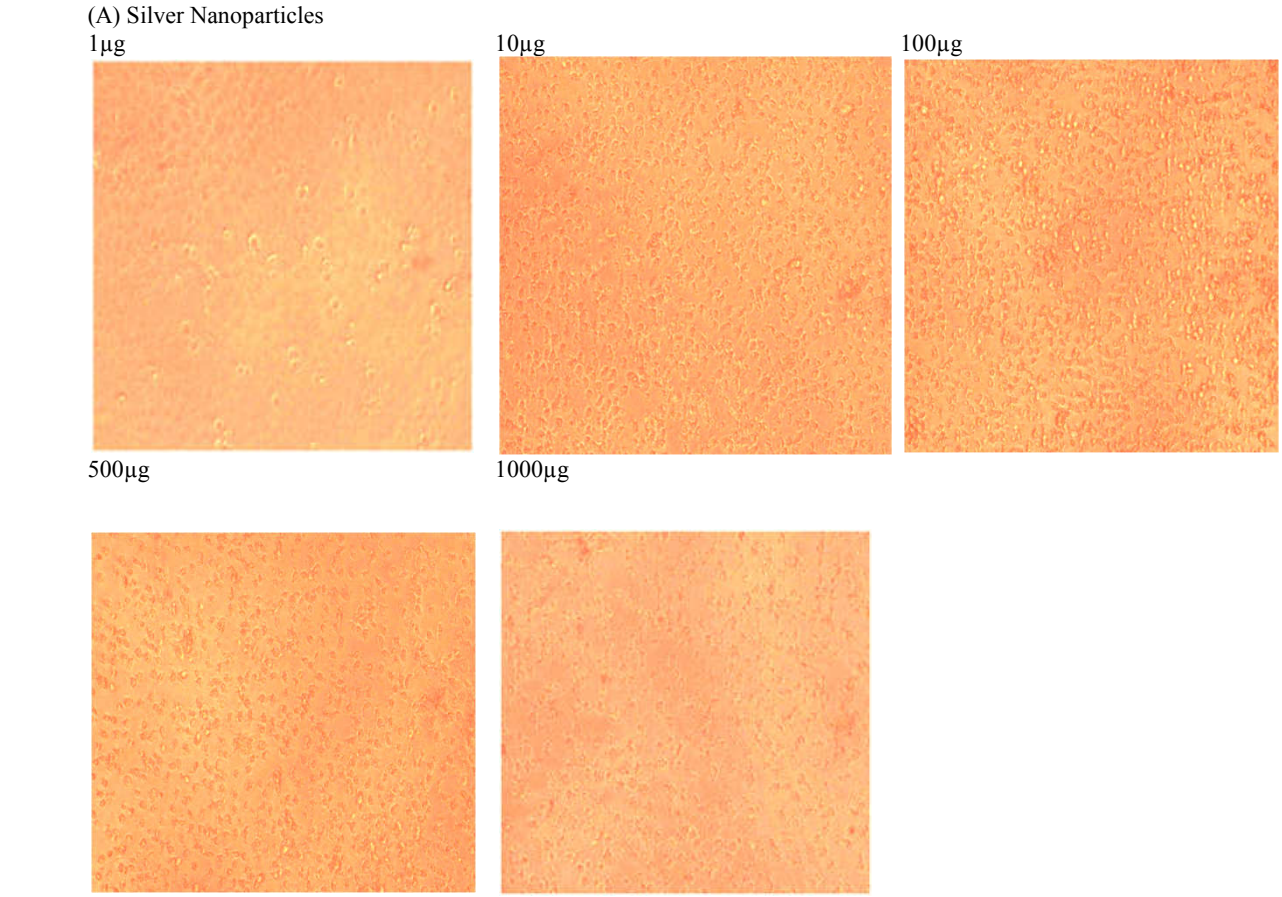


Figure 8. Anticancer Property of nanoparticles against TPH1 cells at various concentrations

Valiyaveettil, 2012). The results of the cytotoxicity in lung tissue showed that increasing doses of ZnO nanoparticles could cause significant damages to the lung tissue and emphasizes that exposure to high concentration of ZnO could cause irreversible damages to different organs including lung and threaten the human health (Ataollah *et al*., 2013).

Conclusion

The silver and Zinc oxide nanoparticles were biosynthesized from *Pichia fermentans*, the yeast isolated from fruit waste. The study on anticancer property of these nanoparticles against the tested cell line showed promising results and proved the earlier reports on anticancer property of silver and zinc oxide nanoparticles. As both the nanoparticles are eco-friendly, safe and used in pharmaceutical and other applications they could be utilized in developing therapeutic measures against dreadful disease like cancer.

Acknowledgement

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