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Research Article

Exploitation of Aspergillus fumigatus secondary metabolites and silver nanoparticle coated crude extract against pathogenic bacteria isolated from surgical site wound infection

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Abstract

Multi drug resistance is increasing day by day due to misuse of antibiotics. Fungi have the ability to produce potent metabolites. They have potential to combat bacterial pathogens. Synthesis of silver nanoparticle (AgNPs) has gained significant importance in recent years due to its simple, nontoxic, less time consuming and cost-effective nature. This study was focused on biosynthesis of AgNPs from the extract of *Aspergillus fumigatus* applying different analytical technique such as UV-visible spectroscopy, X-Ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) for characterization of AgNPs. The effect of synthesis AgNPs and crude extract noted against different bacterial pathogens. Maximum antibacterial activity were noticed against tested bacteria by both fungal crude extract and (AgNPs) Maximum antibacterial activity of Ethyl acetate crude extract at 50µl concentration (12mg/1ml DMSO) showed (17mm) zone of inhibition against *E.coli*. While minimum antibacterial activity of Ethyl crude extract at 50µl concentration was observed against *S.typhi* (16mm). Highest antibacterial activity of Ethyl acetate crude extract at 100µl was noted against *E.coli* which showed (21mm) zone of inhibition. While (24mm) zone of inhibition was observed against *S.typhi* at 100µl concentration Ethyl acetate crude extract and AgNPs. While (25mm) zone of inhibition was observed against *E.coli* at 100µl concentration Ethyl acetate crude extract and AgNPs respectively. Surface Plasmon Resonance (SPR) observed at 432 nm during UV-visible spectroscopy which confirmed the synthesis of AgNPs. The SEM micrograph demonstrated the spherical shape of AgNPs. FTIR analysis revealed the involvement of phenolic, carboxyl and hydroxyl groups in reduction of Ag^+ ions to form AgNPs while stabilization components of AgNPs were amide linkage and amino acid. The XRD peak gave information about the phase purity, size, internal crystalline structure and nature of the synthesized AgNPs. It was suggested that AgNPs synthesized from the extract of *Aspergillus fumigatus* could be a great importance in the pharmaceutical and medical fields. While, the combination of AgNPs and crude extract *Aspergillus fumigatus* enhances their antimicrobial effect which increase their importance in future studies

Keywords: fungal secondary metabolites, silver nanoparticles, crude extract, MDR pathogen bacteria, SEM, TEM, UV, FTIR, XRD.

INTRODUCTION

Nanotechnology is newly emerging field in the science, engineering and technology. The size of nanoparticles (Nps) ranges from 1 to 100 nanometer (Mansoori, 2005 #1;Almutairi, 2015 #74)(Mansoori *et al.*, 2005). Silver nanoparticles are considered the most important and reliable in all metal nanoparticles for its wide range application. AgNPs can be used for treating different bacterial and fungal infections and as a drug carrier along with its least cytotoxic effects (Prabhu *et al.*, 2012). Silver NPs are reported to have antibacterial activity against different human pathogens and even known to be effective against multi-drug resistant pathogens (Liu *et al.*, 2015). NPs have applications in drug delivery as well as in anti-cancer therapy (Rashmi *et al.*, 2005). AgNPs have been introduced into more than 200 consumer products including clothes, drugs and cosmetics (Dhuper *et al.*, 2012). Nanoparticle drug bearers can bypass the blood-brain barriers and the tight epithelial junction of the skin that ordinarily block delivery of drugs to the target site (Li *et al.*, 2011).Fungi is one of the largest groups of eukaryotic organisms that play a vital role in ecosystems. Fungi have many applications in industry, agriculture, medicine, and environment (Ramesh *et al.*, 2014). Fungal species are significant producers of antimicrobial agent but due to resistance, they required more attention and research for discovery of new antimicrobial agents and 20 most demandable and very successful drugs are extracted from fungi worldwide. The production of secondary metabolites such as antimicrobial agents is the most important property of fungi (Pinruan *et al.*, 2007).Some soil fungi can produce several novel secondary metabolites which have pharmaceutical, agricultural and industrial importance these metabolites are antibiotics, antiviral, anticancer and antioxidant compounds. These metabolites can be exploited for medical therapy and are used as therapeutic agents (Farjana *et al.*, 2014). This fungus synthesizes numerous secondary metabolites of pharmacological importance (Nierman *et al.*, 2005). *Aspergillus fumigatus* is fungal specie which belongs to the genus *Aspergillus. Aspergillus* genera also include *Aspergillus oryzae* & *Aspergillus nidulans*. There are 40 different species of fungi to produce secondary metabolites and these are called polyketide family (Bala *et al.*, 2013). *Aspergillus* species has potential to produce several varieties of secondary metabolites by optimization of growth factors or environmental condition like temperature, pH and time. These parameters can increase the productivity of secondary metabolites (Khan *et al.*, 2018). *Aspergillus fumigatus* can produce secondary metabolites like gliotoxin, helvoic acid and fumagillin (Pena *et al.*, 2010). Fumagillin is a large bio-molecule which has the ability to treat infections (Sethi *et al.*, 2013). Fumagillin antibiotic can be extracted from *A. fumigatus.* Many species of *Aspergillus* genus can produce antibacterial agents like aspergillic acid which is produced by *A. flavus*, penicillic acid produced by *A. ochraceus* and fumagillin which is produced by *A. fumigatus* (Khalil *et al.*, 2021).

MATERIALS AND METHODS

Isolation of fungi from collected sample collection: Samples were collected from soil at various locations at Peshawar city in sterilized polyethylene bags by using sterile spatula. After collection of samples, they were screened for fungal isolation by serial dilution method. For serial dilution method ten test tubes and one conical flask along with 9ml distilled water for each sample and Potato Dextrose Agar media (PDA) were sterilized at 121°C for 15 minutes and at 15psi in autoclave.

Batch fermentation about 500ml of Potato dextrose broth media (PDB) media was prepared in 1000 ml Erlenmeyer flask in Abasyn University labs. Prepared media was sterilized at 121oC for 15 minutes at 15 psi. After sterilization 10 % inoculum was added to the prepared PDB media. Inoculated flask was incubated at 25oC for 2 weeks in a shaking orbital incubator set at 120 rpm.

Extraction of secondary metabolites when the fermentation process was completed, the media containing the fungus and metabolites was mixed homogenously in 10% ethyl acetate solution. **Solvent extraction technique** was used to extract secondary metabolites from the mixture using methanol and ethyl-acetate as extracting organic solvents. Equal volume of each solvent was added to the mixture and was mixed for 10 minutes and then was left steady for 5 minutes. After 5 minutes two clearly immiscible layers was formed in the container. The upper organic layer formed by the solvent was used separated from the lower layer as the lower layer was pellet of fungal crude extract containing our desired product. Rotary evaporator was used to evaporate the organic solvent and the remaining extract collected as desired secondary metabolite.

Biosynthesis of silver nanoparticles Total 10 grams of the fungal crude extract was mixed in 100ml double distilled water (ddH2O) at 25°C for 48 hours in a 250 ml flask with continuous agitation at 120 rpm. Cell filtrates was collected after incubation for 48 hours through Whatman filter paper. 1 mM of silver nitrate solution (AgNO3) was added to the filtrates in the flask and was incubated in a dark room for 24 hours at 28°C.

Characterization of mycosynthesized silver nanoparticles

Mycosynthesized AgNPs was characterized through Ultra Violet Vis-Spectroscopy, Transmission Electron Microscopy, Fourier Transform Infrared Spectroscopy and X-ray diffraction (XRD) spectrum according to the standard procedures (Madakka, et al.,2018). Synthesis of AgNPs was confirmed by UV-visible spectroscopy (Model

UV1900 UV-vis spectrophotometer Japan) in the bio lab pharma Peshawar, Pakistan. Characterization of AgNPs was carried out via SEM (Model; tabletop China), XRD (Model;JDX 3532 Japan) and FTIR (Model;IRAffinity-1S Shimadzu) and was performed at public health research lab (PHRL) Khyber Medical University Peshawar, Pakistan.

Surgical wound sample evaluation activity

Surgical wound samples were collected from the patient of different hospitals such as Lady Reading Hospital (LRH), Peshawar Pakistan using sterile cotton swabs. The samples were transported to the microbiology laboratory at Abasyn University Peshawar, for culturing and identification of bacteria.

RESULTS

Characterization of AgNPs Synthesized from the Extract of *A. fumigatus*.

5.3.1 UV-visible spectrophotometry

Change in color was observed from pale yellow to grayish after addition of fungal extract to silver nitrate solution. Strong peak specific for the production of AgNPs was observed at 432 nm as shown in Fig. 5.1

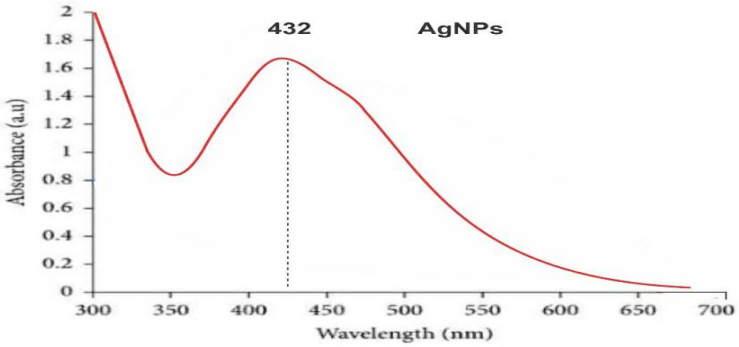

Fig. 5.1: UV-visible spectra of AgNPs synthesized through crude extract of *A. fumigatus* showing peak at 432 nm

5.3.2 SEM micrograph of synthesize AgNPs

SEM Analysis of AgNPs showed almost spherical structure. Nanoparticles were observed aggregates formed its indicated stabilization of the synthesized AgNPs. SEM micrographs showing the silver nanoparticles appeared as spherical shape and observed sized were range from 15 – 90 nm as shown in figure. Fig. 5.2

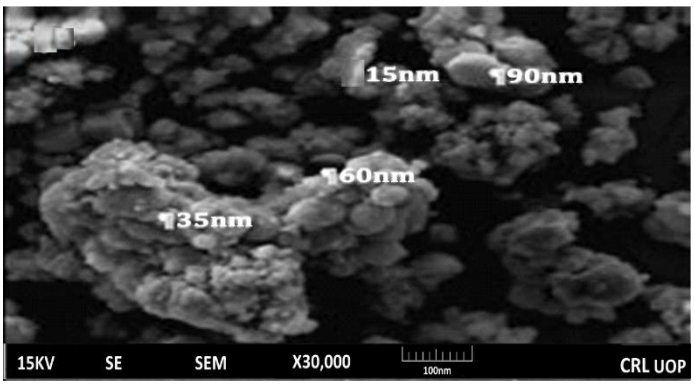

Fig. 5.2: SEM micrograph of synthesized AgNPs

5.3.3 TEM (Transmission Electron Microscopy)

TEM measurement was carried out to determine the morphology and size detail of the synthesized silver nanoparticles, size and shape of the nanoparticles were recorded. TEM micrographs show clearly split and spherical in shape with the size is more than 100 nm the structure of pentagonal bio prisms our result of TEM is 25 nm as shown in Fig. 5.3.

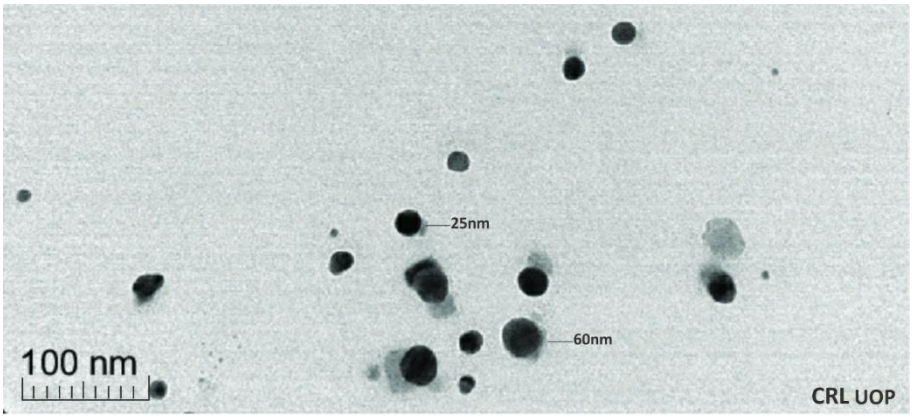

Fig. 5.3: TEM show scale 25 to 100 nm in size.

5.3.4 Characterization of XRD AgNPs Synthesized from the Extract of *A. fumigatus*

Analysis of XRD spectrum revealed that the sample was finely ground and homogenized material. The synthesized AgNPs were crystalline in nature. The four distinct diffraction peaks at 2θ values of 38.45, 46.35, 64.75 and 78.05 could be indexed to the (111), (200), (223) and (311) reflection planes of crystalline structure as shown in figure. Fig. 5.4

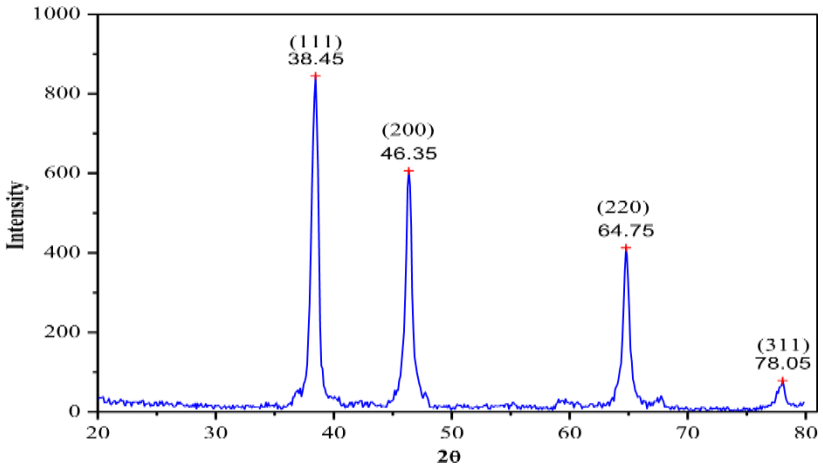

Fig. 5.4: XRD spectra displaying crystalline like nature of synthesized AgNPs

5.3.5 Fourier Transform Infrared Spectroscopy (FTIR) of fungal metabolite and synthesize nanoparticles

FTIR spectroscopy analysis was carried out to determine the possible interaction between silver and bioactive molecules which were responsible for the synthesis and stabilization of silver nanoparticles. FTIR analysis of crude extract of *A fumigatus* and synthesized AgNPs were performed and different peaks were observed. In the FTIR spectrum representing amines, carboxylic acids and alkenes, which played an important role in capping, stabilization and synthesis of AgNPs. In the current study. The peaks were in the range in 510, 775, 822, 916, 1005, 1050, 1100, 1210, 1230, 1280, 1500, 1535, 1570, 1692, 2620, 2845 and 3630 cm-1 showed different functional groups such as alcohol, alkanes, carboxylic acid or ester, amide, alkanes, aliphatic amines or phenol and amines respectively. After reaction with AgNO3 new peaks were observed at 1000, 1035, 1505, 1641, 2910, 3630, cm-1 indicating carboxylic, OH and amide groups of while alkenes, alkanes, alcohol phenol disappear after synthesized AgNPs within crude extract *A.fumigatus*, which played role in the synthesized of AgNPs as shown in Fig. 5.5

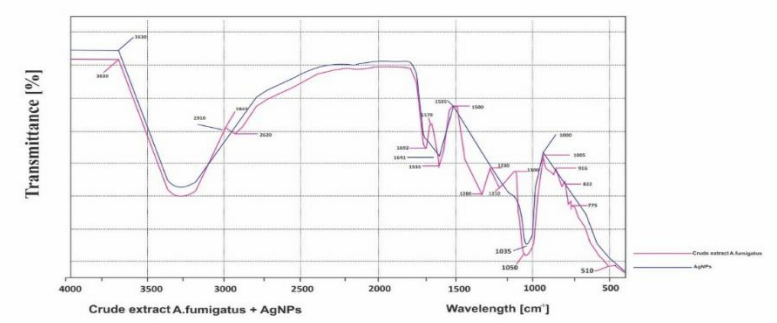

Fig. 5.5: FTIR analysis of crude extracts of *A. fumigatus* and synthesized AgNPs

Antimicrobial Activity of Fungal crude extract

Antimicrobial activity of extract were noted against different pathogenic (Clinical Isolates) and using agar well diffusion assay method and noted different zone of inhibition of each bacteria, wells were loaded with different concentration's 50 µL and 100 µL. Maximum zone of inhibition were reported at different concentration of different bacteria. Ethyl acetate crude extract at 50 µL concentration's 17 ± 0.15 mm, 18 ± 0.1 mm , 16 ± 0.4 mm, 19 ± 0.26 mm, 17 ± 0.30 mm,16 ± 0.36 mm, zone of inhibition against *E.coli sp, Pseudomonas, Klebsiella sp proteus sp, S,aureus, , and Salmonella sp*, respectively. While in 100 µL zone of inhibition was noted, 21 ± 0.15 mm, 22± 0.40 mm, 20 ± 0.35 mm, 21 ± 0.26 mm, 22 ± 0.45 mm 19 ± 1.10 mm zone of inhibition against *E.coli sp, Pseudomonas, Klebsiella sp proteus sp, S,aureus,* and *Salmonella sp*, respectively as shown in Fig. 5.6.

A

B

Fig 5.6. Antibacterial activity of Fungal crude extract of *Aspergillus fumigatus*.

Table 5.4: Antibacterial activity of Fungal crude extract of *Aspergillus fumigatus*

Bacterial Strains	Fungal Metabolites		Standard Deviation 50 µl	Standard Deviation 100 µl	Average 50 µl	Average 100 µl	Ciprofloxacin
	50 µl	100 µl					
E. coli	17 ±0.15	21	0.15mm	0.15 mm	17.3	17.3	23
Pseudomonas	18	22	0.1mm	0.40 mm	18.2	21.63	26
Klebsiella	16	20	0.4mm	0.35 mm	16.8	19.66	23
Proteus	19	21	0.26mm	0.26 mm	19.2	20.6	28

S. aureus	17	22	0.30mm	0.45 mm	17.6	22.4	29
Salmonella	16	19	0.36mm	1.10 mm	15.7	18.33	21

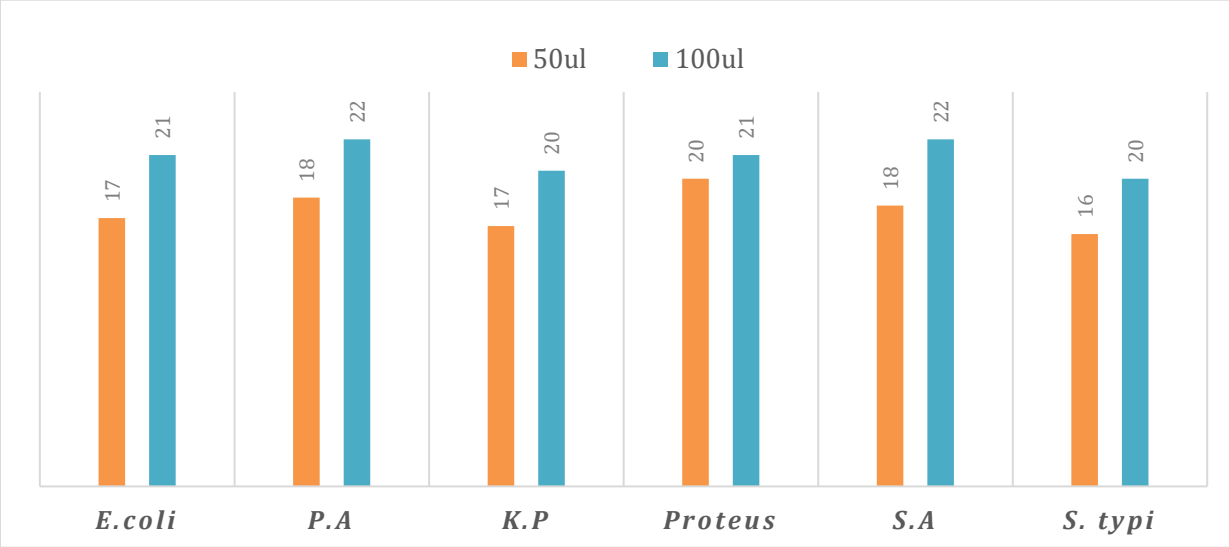

Fig 5.7. Key: E.coli (Escherichia coli), P.A (Pseudomonas aruginosa), K.P (Klebsiella pneumonia), Proteus, S.A (Staphylococcus aureus), S.typi (Salmonella typhi).

Antibacterial activity of synthesized AgNPs

Antimicrobial activity of synthesized AgNPs were noted against different bacteria and using agar well diffusion assay method and noted different zone of inhibition of each bacteria, wells were loaded with different concentration's 50 µL and 100 µL. Maximum zone of inhibition were reported at different concentration of different bacteria. Synthesized AgNPs activity at 50 µL concentration's 23 ± 0.36 mm, 22 ± 0.88 mm , 22 ± 0.47 mm, 24 ± 0.60 mm, 22 ± 0.56 mm, 22 ± 0.55 mm, zone of inhibition against *E.coli sp, Pseudomonas, Klebsiella sp proteus sp, S,aureus,* , and *Salmonella sp*, respectively. While in 100 µL zone of inhibition was noted 25 ± 0.75 mm, 23 ± 0.75 mm, 24 ±. 0.45 mm, 27 ± 0.98 mm, 26 ± 0.80 mm and 24 ± 0.98 mm zone of inhibition against *E.coli sp, Proteus sp, P,aeruginosa, S,aureus, Klebsiella sp,* and *Salmonella sp,* respectively as shown in table 5.5.

Table 5.5: Antibacterial activity of synthesized AgNPs

Bacterial Strains	**Synthesized silver nanoparticles**		**Standard Deviation 50 µl**	**Standard Deviation 100 µl**	**Average 50 µl**	**Average 100 µl**	**Ciprofloxacin**
	50 µl	**100 µl**					
E. coli	23	25	0.36mm	0.75 mm	22.7	23.9	22
Pseudomonas	22	23	0.88mm	0.75mm	22.6	23.1	24
Klebsiella	22	24	0.47mm	0.45 mm	22.3	24.3	23
Proteus	24	27	0.60mm	0.98 mm	23.4	26.3	25
S. aureus	22	26	0.56mm	0.80 mm	21.5	25.5	25
Salmonella	22	24	0.55mm	0.98 mm	21.5	23.5	24

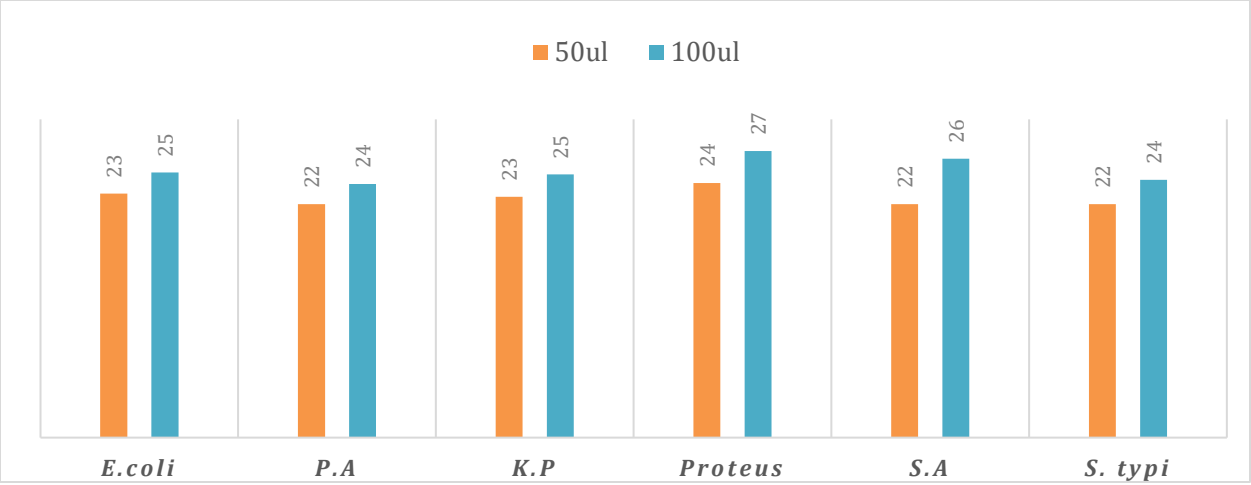

Fig 5.7. Key: *E.coli* (*Escherichia coli*), *P.A* (*Pseudomonas aruginosa*), *K.P* (*Klebsiella pneumonia*), *Proteus*, *S.A* (*Staphylococcus aureus*), *S.typi* (*Salmonella typhi*).

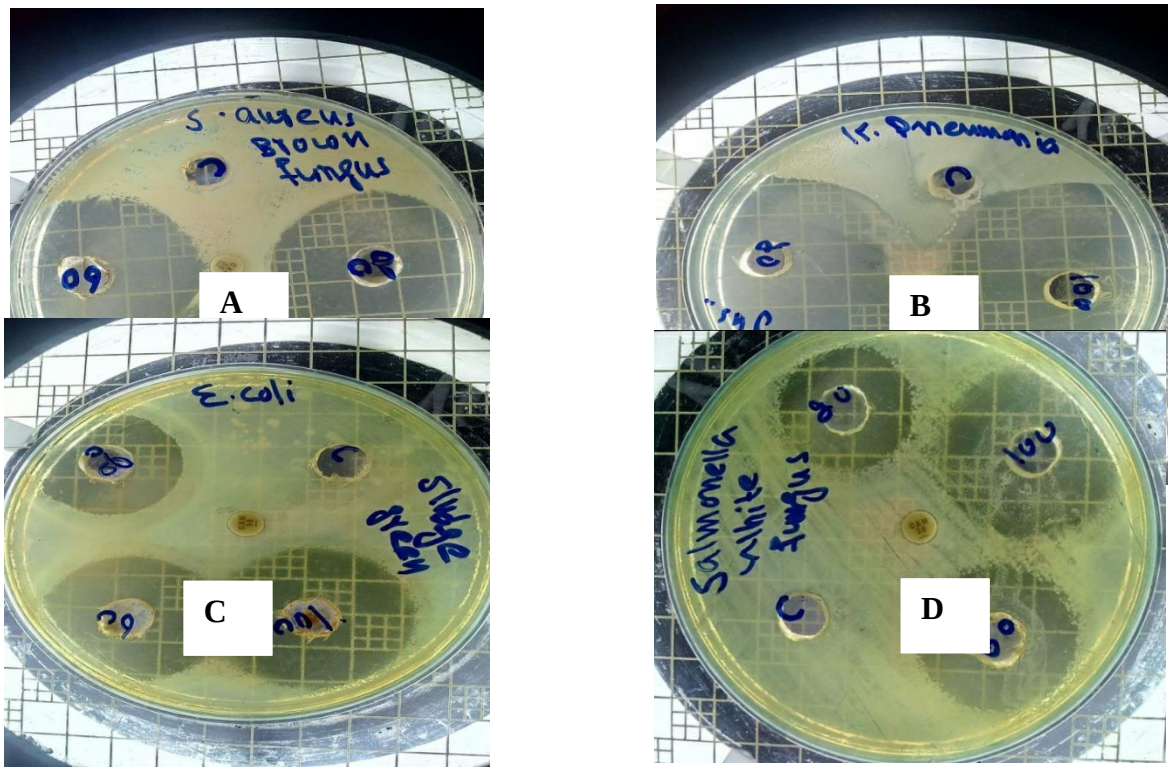

Fig 5.8. Antibacterial activity of synthesized AgNPs

DISCUSSION

Nanoparticle can be defined as a particle with a size of 100 nm or below. Nanoparticles act as a medium and carriers for antibiotics and natural antimicrobial complexes for the development of environment friendly, non-toxic, nanoparticles microorganisms are very important for the synthesis of nanoparticles. The synthesis of nanoparticles through microorganisms increases the biological applications of nanoparticles. Silver nanoparticles are considered the most important and reliable in all metal nanoparticles. AgNPs have wide range application in treating different bacterial and fungal infections and as a drug carrier along with its least cytotoxic.

In the present study, various fungal species were isolated from the soil in which *Aspergillus sp*, was selected for the production of secondary metabolites *Aspergillus sp*. was identified by morphological and microscopic characteristics. Isolated *Aspergillus sp*. was subjected for fermentation process to produce different mycochemicals. After fermentation, a solvent such as ethyl- acetate was used to extract metabolites from the fermentation media. Various mycochemicals such as steroids, terpenoids, alkaloids, flavonoids and tannins were detected in the ethyl- acetate extract.

In the current research work, silver nano-particles were prepared from the extract of *A. fumigatus* and then different analytical techniques such UV-visible spectroscopy, FTIR, SEM, TEM and XRD were used for the characterization of silver nanoparticle. From UV-visible spectroscopy, it was observed that sample had absorbed energy at 432 nm which was a characteristic peak value of AgNPs. Additionally, and also confirmed that high absorbance AgNPs depend on Ag+ NPs concentration with respect to extract of *A. fumigatus*.

SEM technique was used for finding overall topology of the AgNPs. The SEM micrograph of the synthesized AgNPs using extract of *A. fumigatus* confirmed that the mono dispersed and spheroidal topology of the AgNPs showing particle size in the range of 15-100 nm in size and observed at 30,000X lenses.

The SEM result indicated that the chemical constituents of *A. fumigatus* extract acted is a strong reducing agent and resulted in the formation of spherical shaped AgNPs. TEM the result of analysis of the size nanoparticles prepared in 25 to 60 nm.

Moreover, XRD was a rapid analytical technique primarily used for phase identification of a crystalline material. FTIR spectroscopy was used to measure the absorption of infrared radiations by AgNPs as a function of wavelength and different peak were observed in FTIR spectrum representing amines, carboxylic acids and alkanes that carried out the capping, stabilization and synthesis of AgNPs.

In the present research work antibacterial activity of fungal crude metabolites and mycosynthesized silver nanoparticles in two different concentration i.e. 50 µL and 100 µL were evaluated against all isolated pathogens. Ethyl-acetate extract of crude metabolites produces significant inhibition zones ranging from 16 mm to 27 mm towards all the isolated surgical site wound infection. Significant increase in the inhibition zones were also noted when we increased the concentrations of crude metabolites against these isolated pathogens.

Conclusions

Based on the findings following conclusion were made:

- Different phytochemicals including tannins, terpenoids and flavonoids were present in crude ethyl acetate extract.
- *A. fumigatus* extract has the potential to reduce silver synthesized of AgNPs at room temperature.
- The UV-visible spectroscopy confirmed the syntheses of AgNPs at 432 nm absorbance.
- The SEM micrograph reported the nanoscale size of synthesized AgNPs.
- FTIR analysis revealed that phenolic, carboxyl and hydroxyl groups of *A. fumigatus* extract were involved in reduction of silver while stabilization component of AgNPs were amide linkage amino acid.
- According to XRD result synthesized AgNPs were crystalline in nature.
- TEM showed of AgNPs spherical shape.

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