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ORIGINAL ARTICLE

Biosynthesis of Antimicrobial Silver Nanoparticles against Gram-Negative and Gram-positive Bacteria by the Entophytic Fungus Aspergillus Fumigatus

SULEMAN KHAN[1], MAHDI H. ALSUGOOR[2], FARKAD BANTUN[3], SAAD ALGHAMDI[4], NAIF ALSUHAYMI[2], ANAS S.DABLOOL[5], ZAIN ALHINDI[4], MUHAMMAD SALMAN[8], KOMAL AMAN[1]

[1]*Department of Health and Biological Sciences Abasyn University Peshawar, Pakistan 25000*
[2]*Department of Emergency Medical Services, Faculty of Health Sciences, AlQunfudah, Umm Al-Qura University, Makkah 21912, Saudi Arabia*
[3]*Department of Microbiology, Faculty of Medicine, Umm Al-Qura University, Makkah 21912, Saudi Arabia*
[4]*Laboratory Medicine Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah 21912, Saudi Arabia*
[5]*Department of Public Health, Health Sciences College at Al-Leith, Umm Al-Qura University, Makkah 21912, Saudi Arabia*
[6]*Department of microbiology, faculty of veterinary Sciences Chulalongkorn University Bangkok, 10330,Thailand*
Corresponding author: Suleman Khan and Saad Alghamdi, Email: ssalghamdi@uqu.edu.sa

ABSTRACT

Multi drug resistance is increasing day by day due to misuse of antibiotics. Several potent metabolites are produced by fungi. Synthesis of silver nanoparticle (AgNPs) Due to its simple, harmless, time-saving, and cost-effective characteristics, it has acquired great popularity in recent years. A variety of analytical techniques were used to synthesize AgNPs from Aspergillus fumigatus extracts, including X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM). 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 (15mm) zone of inhibition against E.coli. While minimum antibacterial activity of Ethyl crude extract at 50µl concentration was observed against S.typhi (12mm). Highest antibacterial activity of Ethyl acetate crude extract at 100µl was noted against E.coli which showed (20mm) zone of inhibition. While (17mm) zone of inhibition was observed against S.typhi at 100µl concentration Ethyl acetate crude extract and AgNPs (25mm) zone of inhibition was observed against E.coli at 100µl concentration Ethyl acetate crude extract and AgNPs respectively. During UV-visible spectroscopy, surface Plasmon Resonance (SPR) was observed at 432 nm, which confirmed the synthesis of AgNPs. The SEM micrograph demonstrated the spherical shape of AgNPs. The results of FTIR research revealed that phenolic, carboxyl, and hydroxyl groups played a crucial role in the reduction of Ag+ ions into AgNPs, while amide linkage and amino acids stabilized AgNPs. AgNPs synthesized were XRD peaks that revealed phase purity, size, internal crystalline structure, and nature. In the pharmaceutical and medical fields, AgNPs synthesized from Aspergillus fumigatus extract could be of great importance. 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 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 are one of the most diverse families of eukaryotic creatures that contribute to the health of ecosystems. Fungi are used in a variety of industries, including agriculture, medicine, and the environment (Ramesh et al., 2014). Fungal species produce enormous amounts of antimicrobial agents, however owing to resistance, they require greater attention and research in order to identify new antimicrobial agents (Khan et al., 2018). Fungi are responsible for the extraction of 20 of the most popular and effective medications in the world. Fungi's most essential trait is their ability to produce secondary metabolites like antibacterial compounds. (Pinruan et al., 2007). Several novel secondary metabolites can be produced by soil fungi. Which have pharmaceutical, agricultural and industrial importance these metabolites are antibiotics, antiviral, anticancer and antioxidant compounds. (Farjana et al.,2014). This fungus synthesizes numerous secondary metabolites of pharmacological importance (Nierman et al., 2005). Aspergillus fumigatus is a fungus that belongs to the Aspergillus genus. Aspergillus oryzae and Aspergillus nidulans are two other Aspergillus genera.

There are 40 different species of fungi to produce secondary metabolites and these are called polyketide family (Bala et al., 2013). 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: To collect soil samples from different points in Peshawar city, sterilized polyethylene bags were used. The serial dilution method was used to confirm the fungal isolation after the samples were collected. For the serial dilution method, ten test tubes and one conical flask were sterilized at 121°C for 15 minutes at 15 psi with 9ml distilled water per sample and Potato Dextrose Agar medium (PDA).

Batch fermentation A 1000 ml Erlenmeyer flask was prepared with 500 ml of Potato dextrose broth media (PDB) at Abasyn University labs. The sterilized media was sterilized at 121oC for 15 minutes at 15 pressure for 15 minutes. Following sterilization, 10% inoculum was added to the prepared PDB medium. Inoculated flasks were incubated for two weeks at 25°C, shaking at 120 rpm, in an orbital shaker incubator.

Secondary metabolite extraction when the fermentation process was completed. The fungus and metabolite-containing medium

was homogeneously mixed in a 10% ethyl acetate solution. **Solvent extraction technique** Methanol and ethyl-acetate were used as extracting organic solvents to extract secondary metabolites from the mixture. The mixture was then swirled for 10 minutes before keeping steady for 5 minutes. At 5 minutes, two distinct immiscible layers had formed in the container. The upper organic layer formed by the solvent was used separately from the bottom layer since the lower layer was a pellet of fungal crude extract carrying our desired product. A rotary evaporator was used to evaporate the organic solvent, as well as the residual extract were taken as the 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 gathered using sterile cotton swabs from patients at various hospitals, including Lady Reading Hospital (LRH) in Peshawar, Pakistan. The samples were taken to Abasyn University Peshawar's microbiology department for growing and identification of pathogenic bacteria.

RESULTS

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

5.3.1 UV-visible spectrophotometry: Following the addition of fungal extract to the silver nitrate solution, the colour changed from pale yellow to greyish. At 432 nm, there was a strong peak that was particular for AgNP synthesis as shown in Fig. 5.1

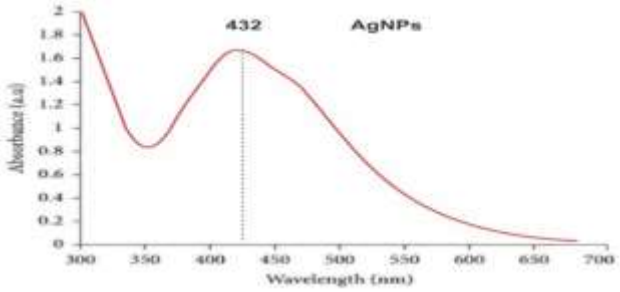

Fig. 5.1: UV-visible spectra of AgNPs synthesized through crude extract of A. fumigatus showing peak at 432 nm

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

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 could be indexed to the (110), (111), (200) and (211), (220), (311) reflection planes of crystalline structure as shown in figure. Fig. 5.4

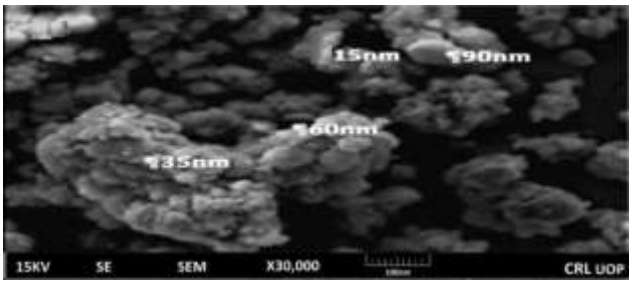

Fig. 5.2: SEM micrograph of synthesized AgNPs

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.

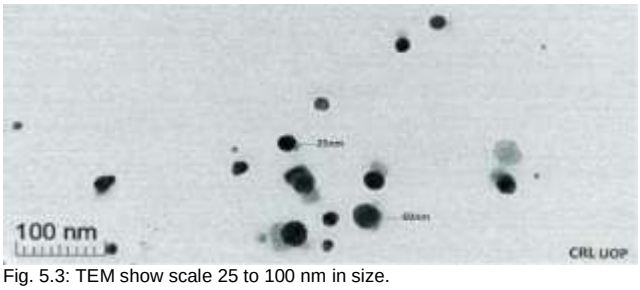

Fig. 5.3: TEM show scale 25 to 100 nm in size.

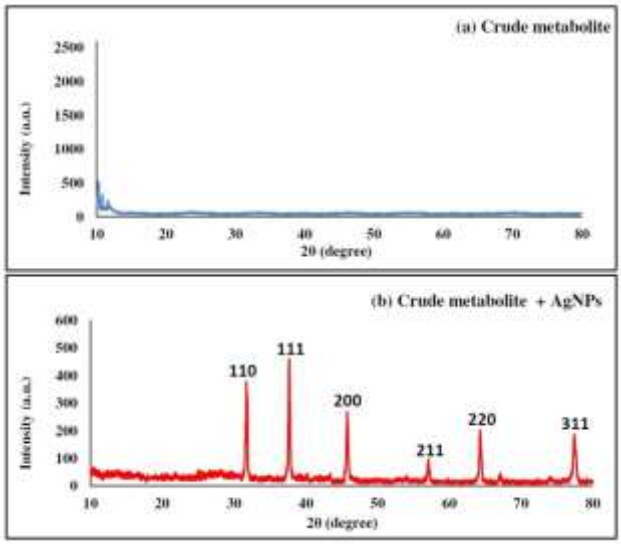

Fig. 5.4: XRD spectra displaying crystalline like nature of synthesized AgNPs

5.3.5 Fourier Transform Infrared Spectroscopy (FTIR) of fungal metabolite and synthesize nanoparticles: The interaction of silver with bioactive molecules responsible for the creation and stability of silver nanoparticles was investigated using FTIR spectroscopy. Different peaks were identified in the FTIR examination of crude A fumigatus extract and manufactured AgNPs. Amines, carboxylic acids, and alkenes are all represented

in the FTIR spectrum. Which helped with AgNP capping, stability, and synthesis. The peaks in the current study were in the range between 1040, 1245, 1545, 1640, 1720, 1900, 2200, 3000, 2900, and 3300 cm-1. Alcohol, alkanes, carboxyl group or ether, amine groups, alkanes, aromatic amines or phenol, and amines were among the functional groups displayed. New peaks were found after reaction with AgNO3, showing 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

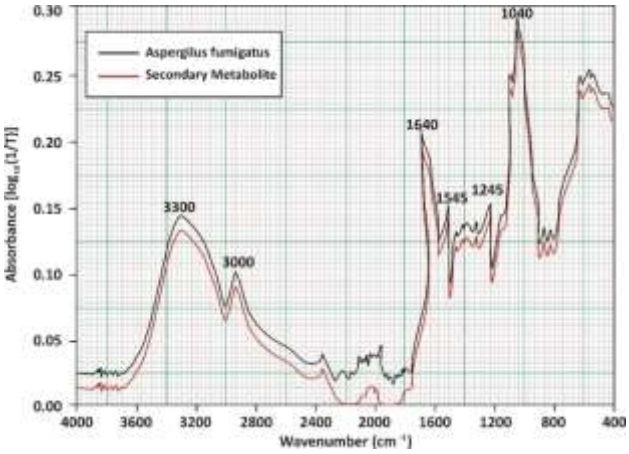

Fig. 5.5: FTIR analysis of crude extracts of A. fumigatus and synthesized AgNPs

Antimicrobial Activity of Fungal crude extract: Using the agar well diffusion assay method, the antimicrobial activity of the extract was evaluated against a variety of bacterial (clinical isolates) bacteria, showing various zones of inhibition for each bacteria. Wells were filled with different concentrations of 50 µL and 100 µL. At various concentrations of bacteria, the maximum zone of inhibition was reported. At various concentrations of bacteria, the maximum zone of inhibition was reported. crude ethyl acetate extract at 50 µL concentration's zone of inhibition against E.coli sp, 15 ± 0.15 mm Pseudomonas, 18 ± 0.1 mm , S,aureus, 16 ± 0.4 mm, and Salmonella sp, 16 ± 0.36 mm respectively. While in 100 µL zone of inhibition was noted, zone of inhibition against E.coli sp, 20 ± 0.15 mm Pseudomonas, 21 ± 0.15 mm S,aureus, 23 ± 0.15 mm and Salmonella sp, 17 ± 0.15 mm respectively as shown in tab 5.4.

Antibacterial activity of synthesized AgNPs: Antimicrobial activity of AgNPs was observed against various bacteria utilizing an agar well diffusion assay in which wells were loaded with various concentrations of AgNPs in 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 zone of inhibition against E.coli sp, 22 ± 0.36 mm, Pseudomonas, 21 ± 0.36 mm, , S,aureus, 21 ± 0.36 mm,, and Salmonella sp, 19 ± 0.36 mm, respectively. While in 100 µL zone of inhibition was noted 25 ± 0.75 mm, 24 ± 0.75 mm, 25 ±. 0.45 mm, 22 ± 0.98 mm, zone of inhibition against E.coli sp, P,aeruginosa, S,aureus, and Salmonella thyphi sp, respectively as shown in table 5.5

Table 1:

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	15	20	0.16mm	0.16mm	17.4	17.4	24
Pseudomonas	17	21	0.2mm	0.41mm	18.3	21.53	25
S. aureus	16	23	0.30mm	0.46mm	17.7	22.3	28
Salmonella	12	17	0.37mm	1.11 mm	15.9	18.34	23

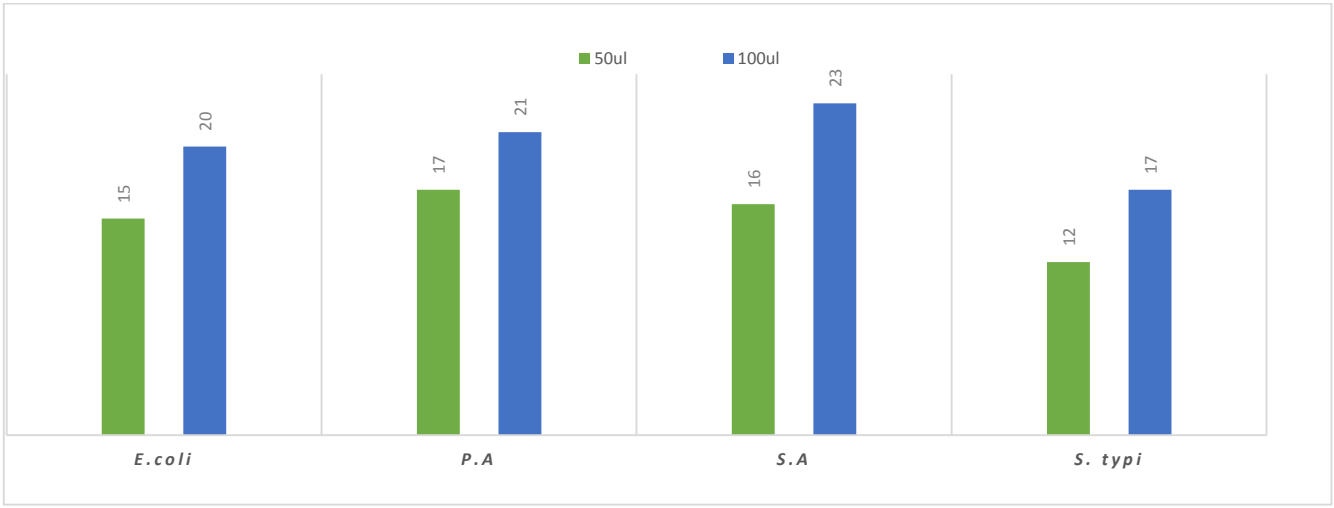

Fig 5.7: Key: E.coli (Escherichia coli), P.A (Pseudomonas aruginosa), mS.A (Staphylococcus aureus), S.typi (Salmonella typhi).

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	22	25	0.36mm	0.75 mm	22.7	23.9	22
Pseudomonas	21	24	0.88mm	0.75mm	22.6	23.1	24
S. aureus	21	25	0.56mm	0.80 mm	21.5	25.5	25
Salmonella	19	22	0.55mm	0.98 mm	21.5	23.5	24

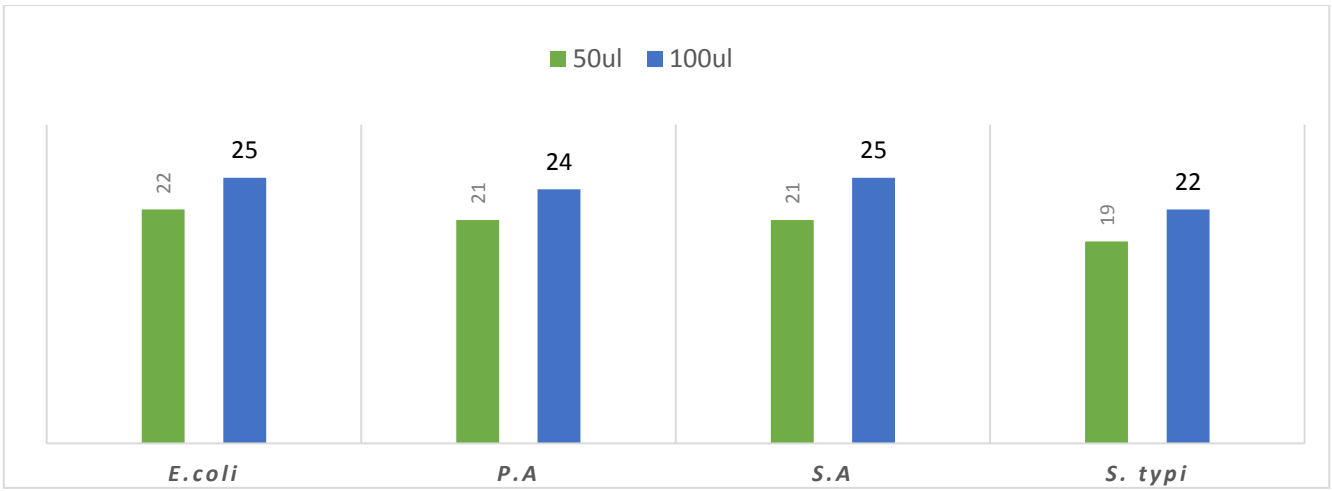

Fig 5.7: Key: E.coli (Escherichia coli), P.A (Pseudomonas aruginosa), S.A (Staphylococcus aureus), S.typi (Salmonella typhi).

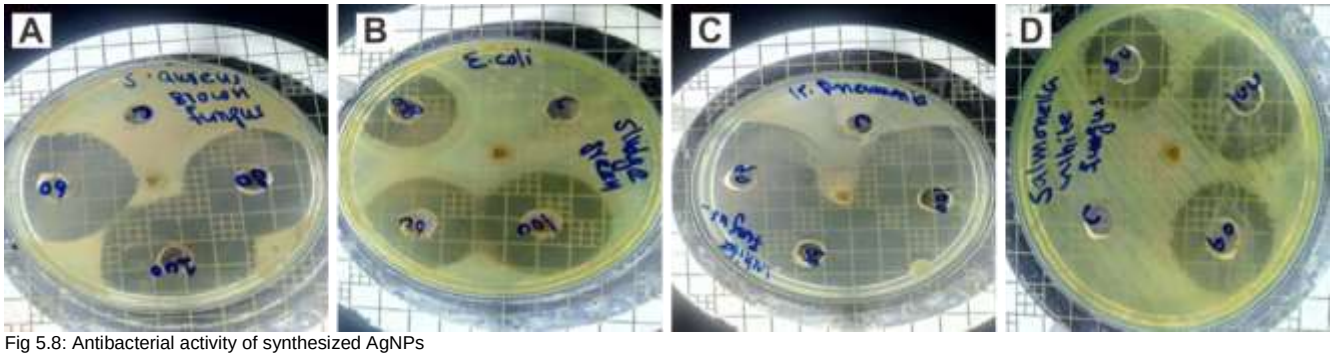

Fig 5.8: Antibacterial activity of synthesized AgNPs

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 nanoparticles were generated from A. fumigatus extract, and the silver nanoparticles were analyzed using a variety of analytical techniques, including UV-visible spectroscopy, FTIR, SEM, TEM, and XRD. According to UV-visible spectroscopy, the sample absorbed energy at 432 nm, which is a representative peak value of AgNPs. Furthermore, it was proven that strong absorption AgNPs are dependent on the concentration of Ag+ NPs in A. fumigatus extract.

The overall topology of the AgNPs was determined using the SEM method. The mono distributed and spheroidal topology of the AgNPs synthesized using A. fumigatus extract was confirmed by SEM micrographs taken at 30,000X lenses, which showed particle sizes in the range of 15-100 nm.

The chemical contents of A. fumigatus extract worked as a strong reducing agent, resulting in the creation of spherical shaped AgNPs, according to the TEM results. The size nanoparticles generated in the range of 25 to 60 nm were analyzed using TEM. Furthermore, XRD was a quick analytical technique that was largely utilized to determine the phase of a crystalline material. Different peaks in the FTIR spectrum representing amines, carboxylic acids, and alkanes that carried out the capping, stabilization, and synthesis of AgNPs were observed in the FTIR spectrum representing amines, carboxylic acids, and alkanes that carried out the capping, stabilization, and synthesis of AgNPs were observed in the FTIR spectrum representing amines, carboxylic acids, and alkanes that carried out the capping, stabilization. The antibacterial efficacy of fungal crude metabolites and mycosynthesized silver nanoparticles at two concentrations, 50 µL and 100 µL, was assessed against all identified pathogens in the current study. Against every isolated surgical site wound infection, the crude metabolite ethyl acetate extract showed substantial inhibition zones ranging from 16 mm to 27 mm. When we increased the amounts of crude metabolites used against these isolated pathogens, we saw a significant rise in the inhibitory zones.

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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